Conservation of the critically endangered frog *Telmatobufo bullocki* in fragmented temperate forests of Chile

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Conservation Biology

at Massey University, Albany, New Zealand

Virginia Moreno-Puig

March 2015

Abstract

Amphibians are currently facing several threats and are suffering severe population declines and extinction worldwide. *Telmatobufo bullocki* (Anura: Calyptocephalellidae) is one of the rarest and most endangered amphibian species in Chile's temperate forests. It is the fifth most evolutionarily distinct and globally endangered (EDGE) amphibian in the world, and one of the world's top 100 priority species for conservation (Zoological Society of London, 2011). This stream-breeding frog is micro-endemic to the coastal Nahuelbuta mountain range in central-south Chile (37°-38°50' S), a hot-spot for conservation. This area has suffered severe loss and fragmentation of native forest, which has been replaced by extensive commercial plantations of exotic pines and eucalyptus. Despite its potential detrimental effects, the impact of native forest loss on this species has not been studied before. Furthermore, few historical observations exist, and the ecology and behaviour of the species is poorly known. In addition, current status and location of extant populations are uncertain, which makes conservation and targeted habitat protection difficult.

Through the use of different approaches and modern conservation tools this thesis aims to make a significant contribution to the conservation of *T. bullocki* and its habitat. Historical and new locations were surveyed to identify extant populations. A distribution modeling approach (i.e. Maxent) was used to infer the species' distribution within Nahuelbuta, generate a predictive habitat suitability map, identify important environmental associations, and assess the impact of main environmental threats (i.e. native forest loss, climate change). Field-based research (e.g. surveys, radio-tracking) was done to extend the

ecological and behavioural knowledge of the species (e.g. movement patterns and habitat use), and identify critical aquatic and terrestrial habitat for protection (i.e. core habitat). Mitochondrial and specifically developed microsatellite genetic markers were used to measure levels of intra-specific genetic variability, define genetic population structure and connectivity, infer evolutionary history (phylogeography), estimate effective population size and detect demographic changes (e.g. bottlenecks). Finally, a landscape genetics approach was used to relate landscape characteristics to contemporary patterns of gene flow, and identify important landscape features facilitating (i.e. corridors) or hindering (i.e. barriers) genetic connectivity between populations.

Telmatobufo bullocki was found in nine basins within Nahuelbuta, including historic and new locations. Presence of *T. bullocki* was positively related to the amount of native forests in the landscape. However, some populations persist in areas dominated by exotic plantations. Some frogs were found living under mature pine plantation adjacent to native forest, but no frogs were found in core plantation areas. *T. bullocki* makes extensive use of terrestrial habitat adjacent to breeding streams during the post-breeding season, moving up to 500 m away from streams. A core terrestrial habitat of at least 220 m from streams is proposed for the protection of populations. Population genetics and phylogeography revealed significant population structure. The northernmost and disjunct population of Chivilingo is geographically and genetically isolated from all other sampled populations and was identified as a separate evolutionary significant unit (ESU). The population of Los Lleulles was also identified as a separate management unit, while the remaining populations were grouped into two clusters forming a larger and more connected meta-population. Connectivity within groups was high, suggesting individuals are able to

disperse between neighbouring basins. Levels of genetic diversity were not homogeneous, and were lowest at Los Lleulles and highest at Caramávida. Results suggest disjunct populations are at highest risk and should be prioritised for restoration and habitat protection, while management of meta-populations should aim at maintaining and improving connectivity among basins. Landscape genetic results identified streams and riparian habitat as dispersal pathways, and least-cost-path analysis was used to identify a potential connectivity network.

Acknowledgements

I would like to thank my main supervisor Professor Dianne H. Brunton (Institute of Natural and Mathematical Sciences, Albany Campus, Massey University, Auckland, New Zealand) and co-supervisor Professor Phil Bishop (Department of Zoology, University of Otago, Dunedin, New Zealand) for their knowledgeable guidance and advice, continuous support, and encouragement throughout this thesis.

A very special thanks to the institutions that provided financial support for this research: The Mohamed bin Zayed Species Conservation Fund, European Association of Zoos and Aquaria Amphibian Conservation Fund (EAZA ACP), Rufford Small Grants for Conservation (The Rufford Foundation), Auckland Zoo Conservation Fund, and INMS (Massey University Auckland). I acknowledge CONICYT (BecasChile Doctoral Scholarship), Sir Neil Waters Scholarship (Massey University), and Massey University Doctoral Completion Bursary for financial support towards my studies at Massey University.

Thanks to Prof. Paul Rainey (NZIAS Massey University), Dr. Heather Hendrickson (NZIAS Massey University), and NZIAS Massey University Auckland for providing laboratory space for the genetics components of the thesis, and to Yeserin Yildirim for technical guidance with laboratory techniques. Thanks to Dr. Shauna Baillie for advice on the population genetics components, and Dr. Claudio Correa (Universidad de Concepción, Chile) for input on mitochondrial DNA analysis and for providing tissue and DNA samples. Dr. Matthew Perrott from the Institute of Vet, Animal & Biomedical Sciences, Massey University

prepared histological slides for skeletochronology. Thanks to Helen Díaz-Páez for her input and support on the initial stages of the project.

Thanks to Felipe Rabanal for inviting me to a fieldtrip to Butamalal, where I first met *T. bullocki*, and which turned out to be the starting point of this work. A very special thanks to all the people who enthusiastically volunteered to assist during long nights of fieldwork: Claudio Aguayo, Bárbara Alvarez, Sergio Araya, Camila Castro, Patrich Cerpa, Andrés Charrier, Estefanía Cifuentes, Claudio Correa, Martín Daiber, Colomba Elton, Victor Hugo Ernst, Edgardo Flores, Macarena Moreno, Bernardo Segura. Thanks to Tomás Elgueta for his time, enthusiasm, ingenuity, and for his documentary work. To the Aguayo and Corvalán families for providing accommodation during fieldwork.

Thanks to Forestal Arauco for providing radio-transmitters and access permits. To Patricio Viluñir (Forestal Arauco) for looking after my safety in the field and coordinating gate openings, to Raúl Briones (Bioforest) for collaboration and data access. Thanks to CMPC for access permits. To Zoológico Nacional de Chile and Danté Fenolio for allowing to film and collect DNA samples from captive *T. bullocki* and *T. australis*, and Osvaldo Cabeza (zookeper) for assisting. Fieldwork and DNA collection was conducted under SAG research permits nº 6977/2011, 5604/2012, 6514/2013 and CONAF authorisation 13-2011 IX.

Thanks to all the people who supported me during the past four years, my partner, my family and friends.

Table of Contents

Abstract ..... **iii**

Acknowledgements ..... **vii**

Table of Contents ..... **ix**

List of Figures ..... **xv**

List of Tables ..... **xxi**

List of Acronyms and Abbreviations ..... **xxiii**

Chapter 1: General Introduction **1**

1.2 Conservation biology and thesis scope 1

1.3 Background 3

1.1.1 Global amphibian declines 3

1.1.2 *Telmatobufo bullocki* 5

General description 5

Evolutionary history 7

Historical distribution 7

Conservation status 9

Natural history 11

1.1.3 Nahuelbuta Range 12

Description 12

Human settlement and land use change 16

Forestry industry 16

The potential impact of plantations on biodiversity 19

1.4 Thesis aim, specific objectives, and chapter outline 23

Chapter 2: A distribution model for *Telmatobufo bullocki*, and the impact of native forest loss and climate change **27**

2.1 Introduction 27

2.2 Methods 31

2.2.1 Study area 31

2.2.2 Amphibian surveys and data collection 33

2.2.3 Environmental variables 34
2.2.4 Maxent modeling 38
2.2.5 Model evaluation and selection 38
2.2.6 Model projection 39
2.3 Results 41
2.3.1 Presence points 41
2.3.2 Model evaluation and selection 44
2.3.3 Relative influence of environmental variables 45
2.3.4 Response curves 46
2.3.5 Suitable habitat 48
2.3.6 Model projections 49
2.4 Discussion 51
2.4.1 Relative influence of environmental variables 52
2.4.2 Projections 56
2.4.3 Limitations 59
2.4.4 Implications for conservation and management 60
Chapter 3: Movement patterns and habitat use 63
3.1 Introduction 63
3.1.1 Amphibian migrations and movements 63
3.1.2 *Telmatobufo bullocki* 64
3.2 Methods 65
3.2.1 Movement patterns and terrestrial habitat use 65
Study area 65
Frog sampling 66
Frog and environmental measurements 68
Fluorescent powder tracking 69
Radiotracking 70
3.2.2 Aquatic habitat 72
Sites 72
Larval sampling 72
Stream habitat characterisation 74
3.2.3 Data analysis 75
3.3 Results 75
3.3.1 Terrestrial captures 75

3.3.2 Terrestrial habitat use....76
Terrestrial microhabitat....79
3.3.3 Movement patterns....82
Tracking....82
Patterns of activity and distance moved....82
Direction of movement....84
3.3.4 Aquatic habitat use....85
Characterisation of *T. bullocki* breeding streams....85
Eggs....86
Tadpoles....87
Adults....90
3.4 Discussion....91
Chapter 4: Genetic diversity and spatial structure of populations....99
4.1 Introduction....99
4.1.1 *Telmatobufo bullocki*....103
4.2 Materials and methods....104
4.2.1 Sample collection and pooling....104
4.2.2 DNA extraction sequencing and genotyping....107
4.2.3 Genetic analysis....108
Microsatellite loci....108
Population structure and differentiation....109
Bottlenecks....110
Effective population size N_e....112
Mitochondrial DNA....113
Phylogeography....113
4.2.4 Historical demography....115
Mismatch distribution....115
Bayesian Skyline Plots....116
4.3 Results....116
4.3.1 Microsatellite loci....116
Population structure....117
Genetic diversity....118
Pairwise differentiation....120
Bottlenecks and N_e....121

Contemporary effective population size 124
4.3.2 Mitochondrial DNA 125
Diversity 125
Phylogeography 126
Mismatch distributions 129
Skyline plots, N_e 130
4.4 Discussion 133
4.4.1 Genetic diversity 133
4.4.2 Phylogeography and population structure 135
4.4.3 Historical demography and bottlenecks 138
4.4.4 Effective population sizes 140
4.4.5 Implications for conservation and management 141
In-situ management 141
Ex-situ conservation 143
4.4.6 Future directions 144
Chapter 5: Landscape genetics and dispersal pathways 147
5.1 Introduction 147
5.1.1 Habitat fragmentation and extinction 147
5.1.2 Landscape genetics 148
5.1.3 *Telmatobufo bullocki* and fragmentation 150
5.2 Methods 152
5.2.1 Study area 152
5.2.2 DNA sampling and genotyping 153
5.2.3 Genetic analysis 154
5.2.4 Landscape analysis 155
Resistance surfaces 156
5.3 Results 162
5.3.1 Genotyping 162
5.3.2 Landscape genetic analysis 163
Univariate analysis 163
Multivariate analysis 166
Dispersal network 166
5.4 Discussion 171
5.4.1 Effect of landscape features on *T. bullocki* functional connectivity 171

Streams 171
Slope and elevation 172
Effect of exotic plantations on functional connectivity 174
5.4.2 Dispersal network and implications for management 176
Chapter 6: General Discussion 179
6.1 Main findings 179
6.1.1 *T. bullocki* distribution and populations 179
6.1.2 Habitat use 182
6.1.3 Movements, behaviour, and detectability 183
6.1.4 Population genetic structure and genetic diversity 185
6.1.5 Main threats 185
6.2 Other threats 188
6.3 What can be done to protect *T. bullocki* populations and habitat? 191
6.3.1 Management 191
6.3.2 Other recommendations 192
6.3.3 Future research 193
Ecological studies 194
Management 196
6.4 Conclusion 198
References 201
Appendix A. Historical records for *Telmatobufo bullocki* from 1931 to 2006. 225
Appendix B. List of presence records 231
Appendix C. Stream surveys and tadpole relative abundance in 18 sites in Nahuelbuta. 233
Appendix D. Sexual size dimorphism in *T. bullocki* 235
Appendix E. Observations on *T. bullocki* growth and development. 237
Appendix F. Development of microsatellite markers for the critically endangered frog *Telmatobufo bullocki* and cross-species amplification in two related species. 243
Appendix G. Additional tables and graphs for Chapter 4 247
G. 1 Bayesian MSVAR run parameters 247
G. 2 Hardy-Weinberg Equilibrium test 248

G. 3 Evanno method to detect number of genetic clusters (*K*) 249
G. 4 Genetic diversity and *G*-statistics for 14 microsatellite loci 252
G. 5 Pairwise Jost D_{st} distances between basins and groups.....253
G. 6 Heterozygosity excess test results for the detection of genetic bottlenecks 254
G. 7 Molecular Phylogenetic analysis by Maximum Likelihood method 255
G. 8 Mismatch distribution: Test of spatial expansion 256
G. 9 Parameters of demographic expansion and neutrality tests calculated using the CR sequence.....258

Appendix H. Additional figures for Chapter 5. 259

Appendix I. Management guidelines for *T. bullocki* populations. 261

List of Figures

Figure 1.1. The critically endangered amphibian *Telmatobufo bullocki,* the focus of this thesis. Juvenile in native forest in Butamalal, Nahuelbuta Range, Chile (photo: Andrés Charrier). ..2

Figure 1.2. *T. bullocki* A) tadpole (size 75 mm total length, photo: Bernardo Segura), B) juvenile (size 39 mm SVL, photo: Andrés Charrier), C) adult female (size 82 mm SVL, photo: Tomás Elgueta). 6

Figure 1.3. Remaining *T. bullocki* native breeding habitat in upper Butamalal River (left). Exotic plantations have replaced native forest, and aggregate extraction has degraded *T. bullocki* habitat in the lower parts of the Butamalal Valley (right). 10

Figure 1.4. Location of Nahuelbuta mountain range in central-south Chile, showing historical *T. bullocki* distribution: 1) Lota 2) Ramadillas, 3) Caramávida, 4) Rucapehuén, 5) La Cueva, 6) Cabrerías, Butamalal, 7) San Ernesto, Elicura, 8) Vanerías, Nahuelbuta National Park 9) Cabrerías, Nahuelbuta National Park, 10) Vegas Blancas, 11) Vegas de Rucapillán, 12) Los Lleulles (locations obtained from the maps in: Formas et al 2001, Cei 1962, Péfaur 1971).13

Figure 1.5. Climograph for Contulmo weather station (38˚ 00' S, 73˚ 13' W), data downloaded from the national hydrometric and climatic database, *Dirección General de Aguas*, *Ministerio de Obras Píblicas* (http://snia.dga.cl/BNAConsultas/reportes).....15

Figure 1.6. Historical climatic records for Contulmo between 1987 and 2012, showing a positive trend in monthly temperature (left) and a negative trend in precipitation (right). Data for Contulmo station downloaded from the national hydrometric and climatic database, *Dirección General de Aguas*, *Ministerio de Obras Píblicas* (http://snia.dga.cl/BNAConsultas/reportes). 15

Figure 1.7. Land cover of Nahuelbuta (data reclassified from CONAF (2008)).....18

Figure 1.8. Edge between pine plantation (left) and native forest (right) in Caramávida, one of the historical *T. bullocki* locations (photo: Bernardo Segura). 20

Figure 1.9. Unsustainable forestry practices in Nahuelbuta, Chile. The large size of clear-cuts is exemplified with the satellite image of a clear-cut area of approximate 3,000 ha (left), while the poor riparian protection is exemplified in the right (Google Earth 2015 Digital Globe). 21

Figure 2.1. Location of the study area: Nahuelbuta Range (grey area) in central south Chile, between Regions VIII (Región del Biobío) and IX (Región de la Araucanía).....32

Figure 2.2. Diagram showing three different landscape scales considered in the study (L = large, M = medium, S = small). Dark grey represents native forest, light grey represents exotic plantation, and white represents non-forested areas. The percentage of native forest and exotic plantation was calculated for each scale using a moving window analysis. 37

Figure 2.3. Map of Nahuelbuta showing the 25 *T. bullocki* presence points used for modeling (black triangles), historical records (yellow dots) and areas where *T. bullocki* was not detected during this study (red crosses). Main basins with *T. bullocki* presence are labeled: 1) Chivilingo, 2) Ramadillas, 3) Caramavida, 4) Cayucupil, 5) Butamalal, 6) Huilquehue, 7) Provoque, 8) Calebu, 9) Los Lleulles. 42

Figure 2.4. Marginal response curves of the predicted probability of *T. bullocki* presence for the four predictor variables that most contributed to the best model. The curves show the mean response of the 10 replicate Maxent runs (red) and the mean ± one standard deviation (blue). 47

Figure 2.5. Marginal response curves for the percentage of native forest in A) small landscape, and B) medium landscape. The curves show the mean response of the 10 replicate Maxent runs (red) and the mean ± one standard deviation (blue). 48

Figure 2.6. Logistic output for the predicted *Telmatobufo bullocki* distribution in the Nahuelbuta Range for past (under native forest hypothesis), present and future conditions (year 2050 under RCP4.5 scenario). Black dots are the presence points used for Maxent modeling. .. 50

Figure 2.7. Map showing the predicted distribution of *T. bullocki* suitable habitat in the Nahuelbuta Range in present and future conditions under climate change scenario (RCP4.5, 2050). Only suitable areas (MTP probability of occurrence ≧ 0.46) are shown. Nahuelbuta National Park is shown in pink, and training presence points in black. 51

Figure 3.1. El Natri (front) includes areas of native forest, pine plantations, and open grass riparian areas. 67

Figure 3.2. Adult *T. bullocki* with radio-transmitter belt attached. 71

Figure 3.3. Diagram showing the VES surveying technique (left) and the stream measurements (right). The VES technique consisted of two observers moving upstream in parallel, visually scanning the streambed for tadpoles (using a viewing window and diving torch). Stream measurements included several wetted width measurements spaced through the sampling area (T1 - T4) and several depth measurements at each transect (crosses over T1 -T4) spaced approximately 1 m apart. 75

Figure 3.4. Map of the study area showing land cover type, surveyed areas, *T. bullocki* points and Provoque stream.....78

Figure 3.5. Frequency histograms of distance to stream according to sex (left) and age (right) for the full sample (*N* = 60).....79

Figure 3.6. Frequency histogram for distance to stream by month of capture for the full sample (*N* = 60).....79

Figure 3.7. Pine needle microhabitat: diurnal refuge (left, arrow points to hiding frog), and active frog in plantation area during the night (right).....81

Figure 3.8. Active adult frogs in the night.....81

Figure 3.9. Inactive *T. bullocki* inside burrows (left) and active frog at the entrace of burrow (right). 81

Figure 3.10. Frequency histogram of distance moved per night of activity. Includes all movements recorded from males, females and juveniles (total movements *N* = 32), using both radiotracking and fluorescent powders. 83

Figure 3.11. (A) Direction of movements (*N* = 31). The arrow is the mean weighted vector (mean direction weighted by distance moved). (B) Direction to the stream at the beginning of each movement, black line is the average with 95% CI. 84

Figure 3.12. Egg cluster of approximately 200 eggs (A), and detail of eggs (B), bar size 1 cm.....87

Figure 3.13. Histograms showing tadpole size (snout to vent length, mm) each month (October *N* = 39; November *N* = 21; December *N* = 33). 89

Figure 3.14. Two tadpoles at different stages found simultaneously (December 2013). 90

Figure 3.15. Aquatic microhabitat used by adult *T. bullocki*: frogs were found submerged hiding under big rocks and boulders. 91

Figure 4.1. Map of genetic sampling locations (black triangles) of *T. bullocki* in the Nahuelbuta Range. Samples collected in the same sub-basin were pooled together for population analysis and assigned the following codes CH = Chivilingo, CA = Caramávida, CY = Cayucupil, BU = Butamalal, HU = Huilquehue, PR = Provoque, CL = Calebu. Main towns and cities are shown as reference..... 106

Figure 4.2. Hierarchical clustering of individuals and populations given in STRUCTURE when *K* = 2 and *K* = 4. Each vertical bar represents an individual's probability of population origin, with each colour representing a different population. The original sampling population is shown with the two-letter code assigned..... 118

Figure 4.3. M-ratio simulations for the four populations or groups of populations (A = CH; B = CA, CY, BU; C = HU, PR, CL; D = LL). In each case, the calculated M-ratio is shown (dashed line) and the simulated critical values (M_c) for each model (i.e. SMM, TPM 95%, TPM 90%) are shown as a function of pre-bottleneck theta. Evidence for bottleneck is significant for models that are above the dashed line. 122

Figure 4.4. Marginal probability distributions for log(N_0) and log(N_1) in the left, and log(T) in the right for all runs including all groups. Posterior distributions were generated in MSVAR and kernel densities calculated in TRACER. 123

Figure 4.5. Gene tree constructed from 10,000 posterior trees output by BEAST (left). Branch labels correspond to posterior probabilities (only probabilities > 0.5 are shown), and scale is substitutions per site. The colours correspond to the geographic group of origin as shown in the map (right). 128

Figure 4.6. TCS network of concatenated mtDNA haplotypes. Population of origin is represented by colour. The size of each circle is relative to the number of individuals sharing the same haplotype. Black dots represent inferred missing haplotypes (nodes). Hatch marks represent the number of substitutions occurring on that branch. Shading represents clustering of individuals according to STRUCTURE results. 129

Figure 4.7. Observed mismatch distribution is shown (solid black line), along with expectations under the demographic expansion model (black dashed), and its confidence intervals (colour dashed). 131

Figure 4.8 Bayesian Skyline Plots (BSP) show the median of the population size (solid black), expressed as effective population size per generation time (log transformed), with 95% HPD (blue lines). *X*-axis is time expressed in units of substitutions/site. 132

Figure 5.1. Study area in the Nahuelbuta Range, Chile (red shaded area in the left). Yellow dots in the left represent all recent *T. bullocki* sightings. Yellow dots in the right represent sampling locations included in this study. 153

Figure 5.2. Resistance surfaces for each variable used for modeling. A) Streams: Proximity to streams as a power function with exponent 0.2. B) Slope: percentage rise in slope transformed with a power function with exponent 0.2. C) Elevation: categorical with low resistance between 50 and 1050 m a.s.l and high resistance for all other elevations. D) Land cover: categorical with native forest with low resistance and all other types with high resistance. Black dots represent the 21 sampling locations. 164

Figure 5.3. Map showing the least cost paths (LCP) connecting *T. bullocki* sampling locations and the land-use classification based on CONAF (2008). LCP shown represent potential historical dispersal routes under the best-supported model of landscape resistance. The paths follow stream and riparian habitat. 169

Figure 5.4. Map showing the dispersal network (least-cost corridors). Corridors include routes that are up to 10% more costly than the LCP. The colour represents the relative amount of paths that go through that corridor (path density). Warmer colours represent higher gene flow and connectivity. 170

List of Tables

Table 2.1. Summary of environmental variables selected for species distribution modeling.....37

Table 2.2. Descriptive statistics for environmental variables, based on 70 presence points in the Nahuelbuta Range.....43

Table 2.3. Mean area under the receiver operator curve (AUC) for training and test data for the four competing models (10 replicate runs).....45

Table 2.4. Percent contribution and permutation importance of all the variables included in the best Maxent model.....46

Table 3.1. Descriptive statistics for occurrence distances (m) to Provoque stream for each age and sex class.....77

Table 3.2. Physical measurements and physicochemical parameters of water for streams with confirmed *T. bullocki* presence (*N* = 15).....85

Table 4.1. Population name, code, geographical coordinates (UTM, zone 18S, WGS84), and sample sizes for the different markers used in this study (msat = microsatellites, COI = Cytochrome oxidase subunit I, CR = control region, COI+CR = concatenated sequence). 105

Table 4.2. Genetic diversity indices for each basin and group. Left: Molecular diversity for the 1265 bp concatenated (COI+CR) mtDNA sequence: number of samples *N*, number of segregating sites *S*, number of haplotypes H_N, haplotype diversity H_D, nucleotide diversity π, average number of nucleotide differences *k*, and haplotype richness after rarefaction to the smallest sample size H_R. Right: Genetic diversity at 14 microsatellite loci: Number of individuals *N*, average number of alleles N_A, number of private alleles N_{PA}, allelic richness A_R and private allelic richness PA_R, average observed H_o and expected H_E heterozygosity, average inbreeding coefficient F_{IS}. Red = low, yellow = medium, and green = high relative diversity. ns = non significant..... 119

Table 4.3. Pairwise F_{ST} between populations above diagonal (Weir and Cockerham 1984), R_{ST} (Slatkin 1995) below diagonal. Shading is used to highlight populations grouped together according to STRUCTURE results. 120

Table 4.4. Pairwise F_{ST} between groups above diagonal R_{ST} below diagonal..... 121

Table 4.5. Estimated parameters given by the combination of all runs in MSVAR for each group. for current effective population size N_0, ancient effective population size N_1, and time since population change T on a log scale. 123

Table 4.6. Estimated effective population size (N_e) and 95% confidence intervals using the linkage disequilibrium (LD) method excluding rare alleles (Waples and Do 2010), and approximate Bayesian computation (ABC) of Tallmon et al. (2008). The cut-off value (P_{crit}) to exclude rare alleles was calculated for each sample size (S). 125

Table 4.7. Diversity indices for the two mtDNA markers used. Sample size N, segregating sites S, number of haplotype H_N, haplotype diversity H_D, nucleotide diversity π, average number of nucleotide differences k. 126

Table 5.1. Land cover classes and resistance values assigned in each resistance hypothesis. A small value of 1 represents lower landscape resistance to movement (i.e. corridor), while the highest value 10 represents high landscape resistance to movement (i.e. barrier). 160

Table 5.2. Mantel r and P-values (10000 restricted permutations) for the correlation between pairwise genetic distance and LCP distance for 113 individuals across each resistance surface for the four variables considered. Models are ranked with the highest correlation on top (grey shaded rows). The top model for each variable was used in subsequent causal modeling approaches. 165

Table 5.3. Results of Mantel and partial Mantel tests for the null model (Euclidean) and the 16 landscape resistance models tested, ranked according to their Mantel r value. Variables included in the models are proximity to streams (St), slope (Sl), elevation (E), and land cover (LC). Mantel tests were performed between the pairwise genetic distance matrix (a_r) between 113 individuals and the Euclidean and least cost path distance (LCP) matrices. Models that meet the causal modeling statistical expectations are highlighted. 167

Table 5.4. Results of the second step of causal modeling. The top model identified in Table 5.3 (St) is tested against the other models also supported. 168

List of Acronyms and Abbreviations

Acronym	Meaning
AIC	Akaike Information Criterion
Bd	*Batrachochytrium dendrobatidis*
BI	Bayesian Inference
BIC	Bayesian Information Criterion
BSP	Bayesian Skyline Plot
BU	Butamalal
CA	Caramávida
CH	Chivilingo
CL	Calebu
COI	Cytochrome c oxidase subunit 1
CONAF	Corporacion Nacional Forestal de Chile
CR	Critically endangered (IUCN threat category)
CY	Cayucupil
CWD	Coarse woody debris
DD	Data Deficient (IUCN threat category)
DO	Dissolved oxygen
E	Elevation
EDGE	Evolutionarily distinct and globally endangered
EM	Expectation maximization algorithm
ESS	Effective sample size

ESU	Evolutionarily significant unit
FSC	Forest Stewardship Council
HCVA	High Conservation Value Area
HKY+I	Hasegawa, Kishino and Yano model of DNA substitution with invariable sites
HPD	Highest posterior density
HSI	Habitat suitability index
HU	Huilquehue
HWE	Hardy-Weinberg equilibrium
IAM	Infinite Allele Model
IBD	Isolation by distance
IBR	Isolation by resistance
IUCN	International Union for Conservation of Nature
LC	Land cover
LCP	Least-cost path
LL	Los Lleulles
ML	Maximum likelihood
MW	Mega Watt
mya	Million years ago
Ne	Effective population size
NR	Nahuelbuta Range
PCR	Polymerase chain reaction
PR	Provoque
SC	Specific conductance

SD	Standard deviation
SE	Standard error
Sl	Slope
SSD	Sum of square deviations
St	Proximity to stream
SVL	Snout-to-vent length
TMRCA	Time to most recent common ancestor
VES	Visual encounter surveying
VIE	Visible implant elastomer

Chapter 1: General Introduction

1.2 Conservation biology and thesis scope

The ultimate objective in conservation biology is to provide principles and tools to avoid the degradation and extinction of biological diversity (Soulé 1985). It does so through the application of basic biological knowledge (e.g. ecology, physiology, genetics, evolution) to the long-term conservation of ecosystems, communities, species, populations, and genes. Conservation biology is a relatively recent interdisciplinary field that rose naturally as a response to the increased impact of human activities, and the realisation that biological resources were being rapidly degraded and lost (Van Dyke 2003). Because resources for conservation are limited, an important aspect of conservation has been to identify and prioritise those systems or species that are more highly threatened (e.g. International Union for Conservation of Nature (IUCN) Red List of Threatened Species). The identification and protection of endangered species has become a central focus in conservation biology and an important part of current efforts to sustain the Earth's natural diversity.

The scope of this thesis encompasses the conservation biology of a rare and critically endangered (IUCN) frog *Telmatobufo bullocki* (Schmidt 1952), endemic to the Nahuelbuta Range in central-south Chile (Figure 1.1). Some basic, but critical conservation research questions underpin this work: What is the current distribution and conservation status of

the species, its populations, and habitat? What are the main threats? What could be done to improve the conservation status of *T. bullocki* and secure its long-term persistence? Due to the high level of impact throughout the species' range, I focus specifically on the loss of native forest, and its on-going replacement by exotic monoculture forestry plantations. What are the consequences of this dramatic habitat change? Are frogs using plantations as habitat? Are plantations fragmenting *T. bullocki* populations (i.e. Are plantations acting as barriers to dispersal)? How can *T. bullocki* be protected from the negative effects of plantations? Despite the potential negative effects on native biodiversity, the impacts of native forest loss, fragmentation, and exotic plantations on *T. bullocki* have not previously been studied.

Figure 1.1. The critically endangered amphibian *Telmatobufo bullocki,* the focus of this thesis. Juvenile in native forest in Butamalal, Nahuelbuta Range, Chile (photo: Andrés Charrier).

These research questions were addressed by looking at different patterns and processes, at different scales, and by using a suite of conservation tools. First, historical and new locations were surveyed to identify extant populations. A distribution modeling approach (i.e. Maxent) was used to infer the species distribution within Nahuelbuta (predictive habitat suitability map), identify important environmental associations, and assess the impact of main environmental threats (i.e. native forest loss, climate change). Then, fieldwork based research (e.g. radio-tracking) was done to extend the ecological and behavioural knowledge of the species (e.g. movement patterns and habitat use), and identify critical habitat for protection (i.e. core habitat). In the second half of the thesis, mitochondrial and microsatellite genetic markers were used in a conservation genetics approach. For this, microsatellite markers were specifically developed in the laboratory. Genetic markers were used to measure levels of intra-specific genetic variability, define genetic population structure and connectivity, infer evolutionary history (phylogeography), estimate effective population size and detect demographic changes (e.g. bottlenecks). Finally, a landscape genetics approach was used to relate landscape characteristics to contemporary patterns of gene flow, and identify important landscape features facilitating (i.e. corridors) or hindering (i.e. barriers) genetic connectivity between populations.

1.3 Background

1.1.1 Global amphibian declines

Amphibians are experiencing population declines and extinctions worldwide (Beebee and Griffiths 2005). Although such events are widespread among species, amphibians stand out due to their high number and proportion of threatened species; nearly one third of the

6,300 known species are threatened by extinction (Stuart et al. 2004, Wake and Vredenburg 2008). Factors associated with amphibian declines include: habitat loss and fragmentation, degradation and environmental contamination, overexploitation, increased UV radiation, global climate change, disease, and competition and predation by introduced species (Beebee and Griffiths 2005). More recently, the infectious disease chytridiomycosis caused by the chytrid fungus *Batrachochytrium dendrobatidis* has been identified as a major driver of amphibian declines, particularly in cool-climate mountain stream-breeding amphibians (Lips et al. 2006, Pounds et al. 2006).

There are 60 species (14 genera and 7 families) of native amphibians in Chile (Frost 2014). Although the number of amphibian species is lower compared to other countries of the region, Chile stands out for its high level of endemism with nearly 60% of amphibian species classified as endemic. The highest diversity and endemism of amphibians is concentrated in the temperate forests of central-south Chile. Nearly 50% of Chilean amphibian species are now in one of the IUCN threat categories, and 30% are data deficient (Díaz-Páez et al. 2008). Noticeably, 17% of amphibian species in Chile are critically endangered, the highest threat classification before extinction (IUCN 2011). Among threats identified as having caused the decline of amphibian populations in Chile are the destruction and modification of habitat, drainage and drying of wetlands, sedimentation as a result of deforestation, contamination of rivers, use for food, indiscriminate capture for pets, and the introduction of exotic species (Ibarra-Vidal 1989, Ortiz et al. 2010).

Despite all of the above, little research has been done on Chilean amphibian populations, furthermore little is known about most aspects of the ecology of threatened amphibians

(Ortiz and Díaz-Páez 2006, Méndez and Correa Q. 2008, Vidal Maldonado and Labra Lillo 2008). Studies explicitly investigating the factors affecting the conservation of amphibians in Chile and their declines are scarce (Ortiz et al. 2010), and there is an urgent need to study the basic ecology and behaviour of threatened amphibian species in Chile in order to aid in their conservation planning and management (Díaz-Páez and Ortiz 2003, Ortiz and Díaz-Páez 2006). In particular, the impact of human-induced change on amphibian populations needs to be assessed.

1.1.2 *Telmatobufo bullocki*

General description

Telmatobufo bullocki (Schmidt 1952) is an anuran frog belonging to the ancient Calyptocephalellidae family (Frost 2014). The species was first collected by Dr. Dillman Bullock in 1931, but it was only in 1952 that the species was formally described by Dr. Karl P. Schmidt from the Chicago Natural History Museum (Bullock 1954). Based on the preserved animal, Schmidt described *T. bullocki* as a; *"stocky toad-like frog; with relatively long limbs; glandular dorsal skin; high, rounded parotoid glands; fingers free without expansions at their tips; toes broadly webbed; well developed gland on forearm; general colour mottled greyish brown, with darker spots corresponding to the elevated glands"* (Schmidt 1952). Dr. Bullock added the distinctive yellow spot over the upper eyelids as a characteristic of the species (Bullock 1954). Two colouration patterns have been observed; dark brown with yellow reticulate around dorsal glands, and lighter brown with no yellow reticulate around glands (Figure 1.2). Adults are relatively big measuring 61-83 mm snout to vent length (Formas et al. 2001). During the breeding season (spring-summer) adult

males develop secondary sex characteristics: nuptial excrescences (under their jaw and in the thumbs) and enlarged forelimb muscles (Péfaur 1971). The free-swimming tadpole (Figure 1.2) is especially adapted to fast-flowing streams with a depressed body, a sucker-like mouth, and a strong muscular tail (72 mm total length, Gosner stage 41, Formas 1988, Formas et al. 2001).

Figure 1.2. *T. bullocki* A) tadpole (size 75 mm total length, photo: Bernardo Segura), B) juvenile (size 39 mm SVL, photo: Andrés Charrier), C) adult female (size 82 mm SVL, photo: Tomás Elgueta).

Evolutionary history

Telmatobufo bullocki is considered among the most evolutionarily distinct species in Chile, and one of the few representatives of an old lineage. The Calyptocephalellidae is one of the earliest families of the Neobatrachia anurans to diverge; its origins have been traced back to the Early Cretaceous, over 100 million years ago (Pyron and Wiens 2011). Studies on the evolutionary history of the *Telmatobufo* genus (currently with four species: *T. australis*, *T. bullocki*, *T. ignotus*, *T. venustus*) suggest divergence from its sister taxon *Calyptocephalella* (one extant species *C. gayi*), occurred around 35 million years ago during the Lower Oligocene (Nuñez and Formas 2000). Divergence times of *T. bullocki* from its sister taxon *T. australis* have been estimated between 23.5 and 20.5 mya, during the Miocene (Formas et al. 2001). This unique evolutionary history is considered an important part of amphibian phylogenetic diversity, and preserving it is a priority for conservation (Isaac et al. 2012).

Historical distribution

Since its description, *T. bullocki* has remained one of the most elusive amphibian species in Chilean temperate forests. During his excursions to the Nahuelbuta Range, on the road that connects the city of Angol and Nahuelbuta National Park, Dr. Bullock only encountered *T. bullocki* on three occasions (Bullock 1954). In 1960 a fourth specimen was found in Estero Cabrerías, Alto de Nahuelbuta (Colección Donoso-Barros) and, for the first time, a map with the collection points (Vegas Blancas, Estero Cabrerías or Butamalal[1]) was

[1] The location is given as "Estero Cabrerías", but the point in the map corresponds to what is best known today as Butamalal River. The official topographic map from *Instituto Geográfico Militar de Chile* uses both names "*Estero Cabrerías o Butamalal*".

provided in the book *Batracios de Chile* (Cei 1962). Here, J. M. Cei described the species as "*very interesting*" and "*among the most rare in the world*", featuring the illustration of *T. bullocki* on the book cover, contributing to the almost mythological nature of the species. Later in 1971, new points within Nahuelbuta (Vanerías inside Nahuelbuta National Park, and Estero Los Lleulles) were added to the species distribution and an updated map with four points was provided in Péfaur (1971). However, in this new map the location Cabrerías is placed inside Nahuelbuta National Park, where there is a second stream with the same name, creating confusion on which Cabrerías the historic sighting occurred. In this thesis both locations were considered (Figure 1.4).

Since Péfaur's (1971) publication, new locations have been added (Lota, Ramadillas, Caramávida, Rucapehuén, La Cueva) and an updated distribution map is given in Formas et al. (2001) that includes nine locations within Nahuelbuta (for a full list of all historical records and the sources see Appendix A). These new observations of the species allowed significant advances on the knowledge of the species biology such as the description of the karyotype (Venegas S. 1975), description of the tadpole (Formas 1988), and the study of phylogenetic relationships of the genus (Formas and Espinoza 1975, Nuñez and Formas 2000, Formas et al. 2001). The distribution was extended in 2003 with the sighting of the species in Quirihue, 150 km north of Nahuelbuta (Escobar et al. 2005), and Los Queules National Reserve 30 km northwest from Quirihue, in 2006 (Donoso et al. 2010). However the specimen of Los Queules National Reserve has been recently described as the new *Telmatobufo ignotus*, suggesting the species in Quirihue might be more closely related to *T. ignotus* than *T. bullocki* due to geographic distance (Cuevas 2010). Therefore, in this

thesis *T. bullocki* historical distribution is considered to be restricted to Nahuelbuta (Figure 1.4).

Conservation status

Although *T. bullocki* historically has been considered a rare species, the paucity of sightings (despite several surveys) sparked serious concerns about the conservation status of the species. In 2004 it was moved from data deficient (DD) to critically endangered (CR) by the IUCN Red List assessment, "*because its area of occupancy is probably less than 500 km^2, with all individuals in fewer than five locations, and there is a continuing decline in the extent and quality of its habitat in Arauco Province, Chile*". The assessors state that *T. bullocki* populations are "*extremely rare; extensive fieldwork by several herpetologists within the range of this species from 1992-2002 turned up only a single adult (in 2002)*". In spite of this, in 2008 the Chilean national classification system (*Reglamento de Clasificación de Especies, RCE*) listed the species as vulnerable (V) and rare (R). This discrepancy is presumably due to the incorporation in the RCE assessment of a new locality outside Nahuelbuta[2] that significantly increased the species' extent of occurrence. More recently, the Zoological Society of London (ZSL) positioned *T. bullocki* as the fifth most evolutionarily distinct and globally endangered (EDGE) amphibian (ZSL 2011), and one of the world's top 100 most threatened species in need of urgent conservation action (Baillie and Butcher 2012). Despite of the global importance and high levels of threat (Figure 1.3), no studies had been conducted on the current distribution or status of populations, and all threat

[2] Quirihue (Escobar et al 2005), possibly *T. ignotus* (Cuevas 2010).

assessments remained based on the few historical observations (Veloso et al. 2008, Sánchez P. et al. 2010, ZSL 2011).

Figure 1.3. Remaining *T. bullocki* native breeding habitat in upper Butamalal River (left). Exotic plantations have replaced native forest, and aggregate extraction has degraded *T. bullocki* habitat in the lower parts of the Butamalal Valley (right).

Historical observations of species sightings are clearly insufficient for modern conservation management. Current, precise and accurate, knowledge of species distribution is among the most basic and important kinds of information needed in conservation: we need to know where the species is to be able to protect it. As mentioned above, *T. bullocki* observation records are relatively old, and were provided with little accuracy in the literature (i.e. pre-GPS). Therefore, all conservation assessments to date have highlighted the need to identify remaining *T. bullocki* populations and assess their current status (Veloso et al. 2008, ZSL 2011). Consequently, updating the current *T. bullocki* distribution, including re-visiting historical populations to assess their persistence were main objectives in this thesis.

Natural history

The historical observations and previous work describe a close association between *T. bullocki* and fast-flowing streams. Tadpoles show typical adaptations to fast-flowing water (Formas 1988), while adults exhibit broadly webbed toes (Schmidt 1952). Furthermore, the secondary sex characteristics (nuptial excrescences) developed by males during the breeding season (August-October, Péfaur 1971), suggests amplexus occurs in the streams (Duellman and Trueb 1986, Wells 2010). Despite the clear association of this species with streams, the stomach content of the paratype specimen included a beetle larva, fragments of cockroaches, a spider, and miscellaneous insect remains, with a considerable mass of plant material, suggestive of terrestrial feeding habits (Schmidt 1952). Most individuals were found under logs or rocks close to streams, and under rocks in streams (Appendix A). Tadpoles were observed in streams during December and January (Formas et al. 2001). The *T. bulllocki* breeding call has not been described, and its vocalisations remain unknown[3], except for a weak *"bip-bip"* when handled (Péfaur 1971). The species is nocturnal.

Understanding a species' habitat and microhabitat use and needs is essential for the protection of populations. Habitat protection is among the most basic and straightforward conservation actions; however, if we don't know what constitutes habitat for a species there is little we can do to protect it. Although it is clear that *T. bullocki* need streams for reproduction, there is little detail on when aquatic habitat is used. Furthermore, the extent of terrestrial habitat use by *T. bullocki* remains unclear. Therefore, one of the objectives of

[3] Observations on captive individuals suggest they have a territorial underwater call (Osvaldo Cabeza, National Zoo keeper, pers. comm.)

this thesis was to describe terrestrial and aquatic habitat use in order to aid management for habitat protection.

1.1.3 Nahuelbuta Range

Description

The Nahuelbuta mountain range is a well-defined orographic feature located in central-south Chile (Figure 1.4). This section of Chile's Coast Range extends for 190 km (north-south) between the Biobío river (37º S) and the Imperial river (38º 50' S), reaching a maximum elevation of 1,533 m (Mardones 2005). It has an ancient geological history dating from the Paleozoic era (Mardones 2005). It incorporates parts of Arauco and Malleco provinces in the VIII (Biobío) and IX (Araucanía) Regions respectively.

Nahuelbuta represents the northern limit of Chile's temperate forest (Valdivian Ecoregion). The vegetation is rich, as it transitions from Maulino forest in the north (deciduous *Nothofagus* forest), to evergreen Valdivian forest in the south (*Eucryphia cordifolia*, *Aextoxicon punctatum* and *Laureliopsis philipiana*), and finally to conifer and tundra (*Araucaria araucana* and *Nothofagus pumilio*) above 1,000 m (Villagrán and Armesto 2005). This latitudinal and altitudinal distribution of the different phytogeographic elements is the result of a long history of dramatic climate changes (Villagrán 2001). During the successive glaciations of the Quaternary, Nahuelbuta remained ice-free serving as glacial refugia for many species (Villagrán 2001, Villagrán and Armesto 2005). Consequently, Nahuelbuta is characterised by a high biodiversity of narrow-range endemic species including plants, insects and herpetofauna (Smith-Ramírez 2004). Fifteen

amphibian species are present, including five micro-endemic species. Due to the high level of endemism and biodiversity, and the high levels of threat, Nahuelbuta has been identified as one of the highest priority areas for conservation within Chilean temperate forests (Wolodarsky-Franke & Díaz Herrera 2011).

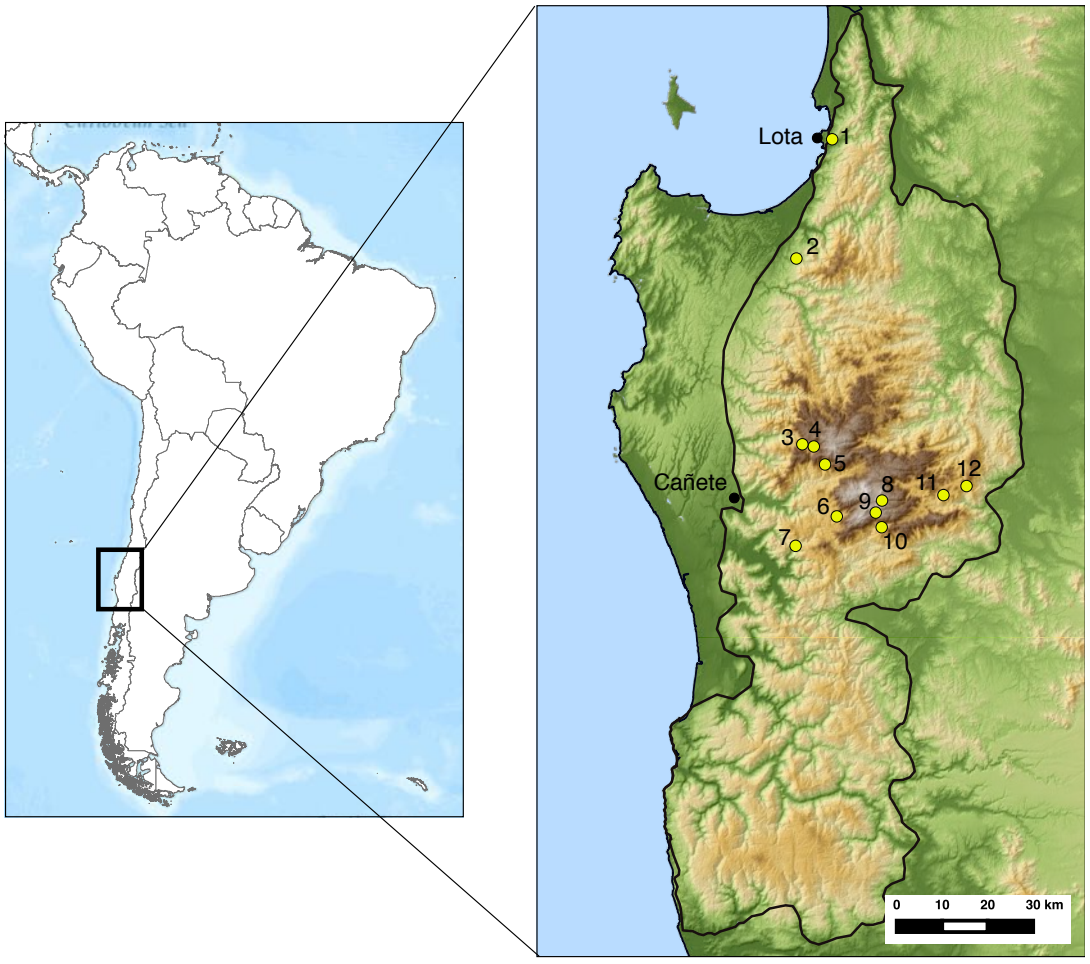


Figure 1.4. Location of Nahuelbuta mountain range in central-south Chile, showing historical *T. bullocki* distribution: 1) Lota 2) Ramadillas, 3) Caramávida, 4) Rucapehuén, 5) La Cueva, 6) Cabrerías, Butamalal, 7) San Ernesto, Elicura, 8) Vanerías, Nahuelbuta National Park 9) Cabrerías, Nahuelbuta National Park, 10) Vegas Blancas, 11) Vegas de Rucapillán, 12) Los Lleulles (locations obtained from the maps in: Formas et al 2001, Cei 1962, Péfaur 1971).

The climate of Nahuelbuta ranges from mediterranean in the north to temperate oceanic in the south, with a latitudinal and altitudinal gradient in precipitation and temperature. Nevertheless, the climatic pattern is generally characterised by relatively hot and dry summers, and wet and cool winters (Figure 1.5). However, as is occurring globally, the climate in central-south Chile is changing (Marquet et al. 2010). Historical temperature and precipitation records for Contulmo (38˚ S 73˚13' W), from 1987 to 2012 show a trend of increase in temperature of 0.05 ˚C per year, and a decrease in precipitation of 1.2 mm per year (Figure 1.6). Furthermore, models of future climatic conditions for the Nahuelbuta Range predict that annual precipitation will decrease between 14% and 25% by the end of the century (2080), while mean annual temperatures will rise between 1.5 °C and 2.5 °C depending on different greenhouse gas emission scenarios (Girvetz et al. 2009). Despite the potential threat of climate change on amphibians (Corn 2005, McMenamin et al. 2008), this has only been assessed for a few Chilean species (Marquet et al. 2010), and due to a lack of data this threat is yet to be evaluated for *T. bullocki*. However, results for the closely related *T. australis* suggest a high impact of climate change on *Telmatobufo* species (Marquet et al. 2010). Assessing the potential impact of climate change on the distribution of *T. bullocki* is one of the main objectives of the next chapter (Chapter 2).

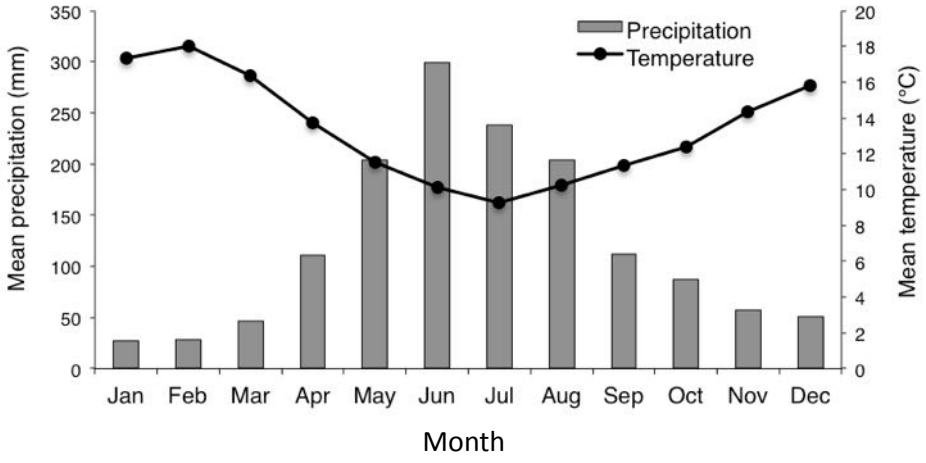


Figure 1.5. Climograph for Contulmo weather station (38˚ 00' S, 73˚ 13' W), data downloaded from the national hydrometric and climatic database, *Dirección General de Aguas*, *Ministerio de Obras Píblicas* (http://snia.dga.cl/BNAConsultas/reportes).

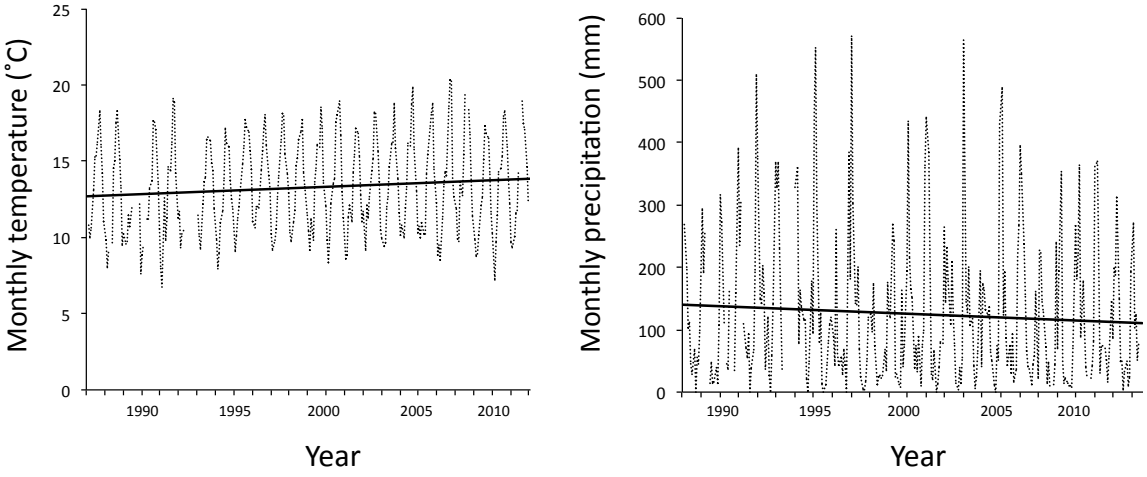


Figure 1.6. Historical climatic records for Contulmo between 1987 and 2012, showing a positive trend in monthly temperature (left) and a negative trend in precipitation (right). Data for Contulmo station downloaded from the national hydrometric and climatic database, *Dirección General de Aguas*, *Ministerio de Obras Píblicas* (http://snia.dga.cl/BNAConsultas/reportes).

Human settlement and land use change

Nahuelbuta has a long history of human settlement. The native Mapuche Lafkenche occupied these territories for thousands of years before Spanish colonisation. Mapuche settlements were small and scattered, and relied mainly on hunting, gathering, and small-scale subsistence agriculture. Significant landscape change only started with the arrival of the Spanish conquistadores, approximately 450 years ago (Rosenblitt B. and Nazer A. n.d.). Along with the Spanish colonisation, a process of degradation of native forest started, through extensive harvesting and clearing of areas for agriculture through fire (Echeverria et al. 2006). By the XIX century, many areas of Nahuelbuta, particularly lower altitudes, had been cleared to supply the increasing human population and exporting demands of cereals (Cisternas et al. 1999). Despite this, most of the native forest remained in high elevations and in steep areas that were unsuitable for agriculture. Due to the increasing degradation of native forest, and thanks to the initial petition made by Dr. Dillman Bullock, the Nahuelbuta National Park (6,832 ha) was created in 1939. In addition, 82 ha of native forest were protected near Contulmo in 1941 (*Monumento Natural Contulmo*). These are the only two state protected areas in Nahuelbuta, and together they cover approximately 1% of this region.

Forestry industry

In the early 1960's commercial pine plantations started to expand with the development of the Chilean forestry industry. The excellent growing conditions for fast growing exotic species, and the state subsidies introduced in 1974 (*Decreto Ley 701 de Fomento Forestal*) saw a massive expansion of exotic monocultures of *Pinus radiata* and *Eucalyptus globulus*,

used mainly for the growing timber and pulp demand (Estades and Escobar 2005, Echeverria et al. 2006). Plantations were mostly established in previously degraded lands; however, it is estimated that 10-20% have directly replaced native forests (Estades and Escobar 2005). Based on the available native forest survey (CONAF 2008), 50-70% of Nahuelbuta is covered by plantations, depending on the exact delineation of Nahuelbuta (Ortiz and Ibarra-Vidal 2005, Wolodarsky-Franke and Díaz Herrera 2011). Regardless of the exact proportion, it is clear that Nahuelbuta is dominated by this land use type, and what once was continuous native forest has become a collection of fragments in a plantation matrix (Figure 1.7).

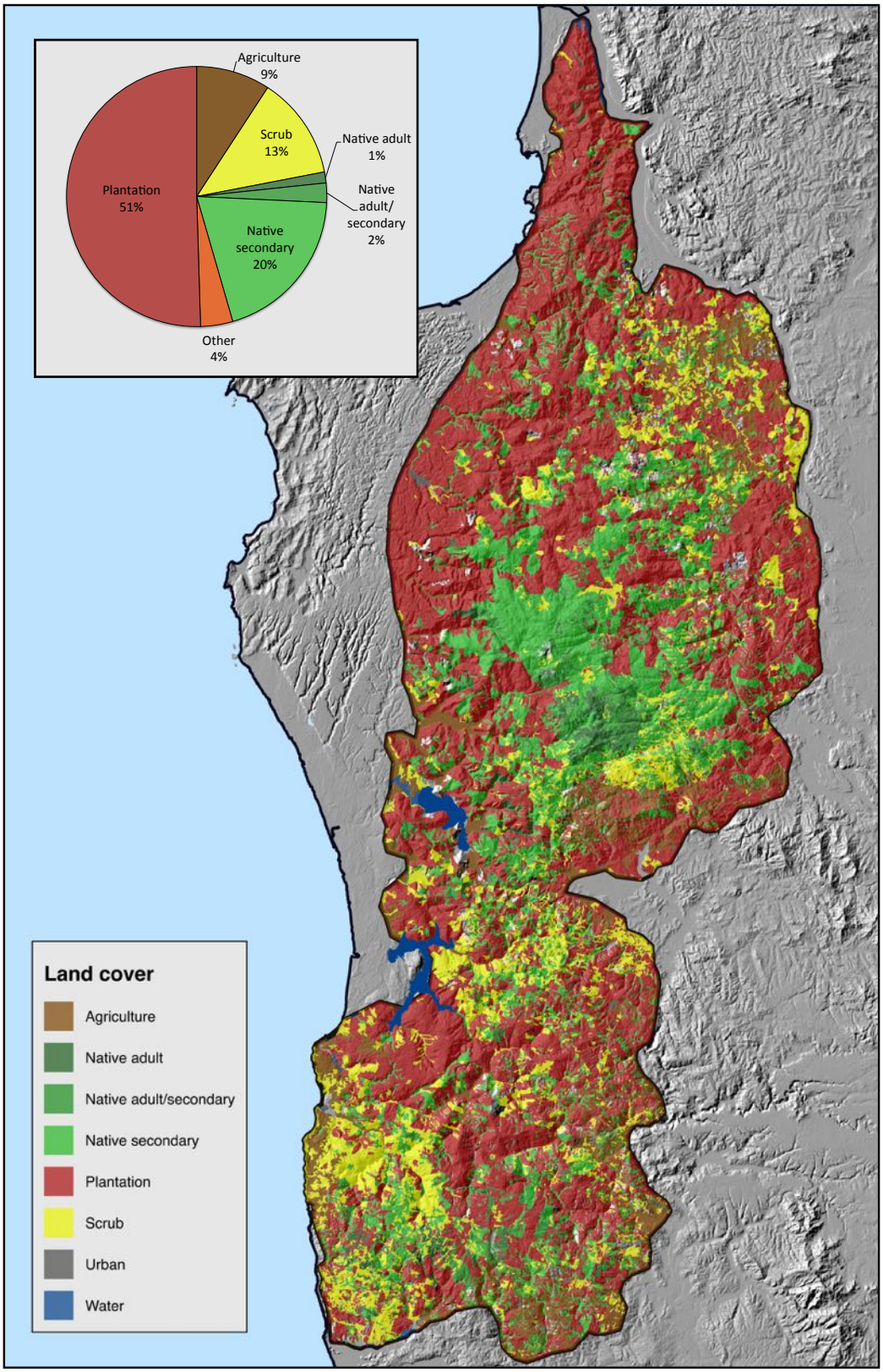


Figure 1.7. Land cover of Nahuelbuta (data reclassified from CONAF (2008)).

The potential impact of plantations on biodiversity

Although the forestry industry is generally considered sustainable, based on its renewable nature, exotic monoculture plantations can have several negative effects on the environment and native biodiversity. Both *P. radiata* and *E. globulus* can have significant effects on the ecosystem, as they change the composition, structure and complexity of the landscape (Figure 1.8). Plantations can change water quantity and quality in the catchments (Huber et al. 2010). For example, in a study conducted in Chile's coastal range, Little et al. (2009) found a significant decrease in stream flow, particularly summer runoff, as a response to the increase of *P. radiata* at the expense of native forests between 1975-2000. In addition, plantations have been found to alter soil hydrological properties and sediment transport (Oyarzún et al. 2011), which can cause cascading effects such as changes in macroinvertabrate fauna (Mancilla et al. 2009). *E. globulus* can also release toxic allelochemicals that may influence the composition and structure of the understory. Additionally, leaf litter leachates can result in deoxygenated and more acidic water, with increased phenolic contents and conductivity, which can in turn affect the viability and ecology of macroinvertebrate fauna (Molina et al. 1991, Canhoto and Laranjeira 2007).

The intensive management of forestry plantations and forestry operations can have a significant impact on amphibian species. Clear-cutting of forests has been found to have a strong negative effect on forest amphibian richness and abundance, as this process changes the structure of the landscape, exposing the floor to direct sunlight and winds, leading to higher temperatures and drier conditions (Petranka et al. 1993, Semlitsch et al. 2009). Clear-cuts have been related to decreased movements and dispersal of amphibians,

affecting connectivity and increasing population isolation (Johnston and Frid 2002, Popescu and Hunter 2010). Also, the increased sediment load and siltation of streams, associated to canopy removal and clear-cuts, can have significant negative effects on stream breeding amphibians, as fine sediment fills interstitial spaces that are used for oviposition and refugia (Corn and Bury 1989, Wahbe and Bunnell 2001, Stoddard and Hayes 2005, Semlitsch et al. 2009).

Figure 1.8. Edge between pine plantation (left) and native forest (right) in Caramávida, one of the historical *T. bullocki* locations (photo: Bernardo Segura).

These threats are enhanced by the unsustainable management of plantations that has characterised forestry in Chile (Estades and Escobar 2005, Frêne Conget and Núñez Ávila

2008). Poor regulations on forestry management have led to practices that pose a serious threat to biodiversity, including amphibians. For example, Figure 1.9 highlights two unsustainable management practices of plantations in Nahuelbuta: 1) large areas of clear-cuts and 2) lack of stream riparian protection. The synergistic combination of such practices could have an important and non-reversible effect on *T. bullocki* populations and habitat, and therefore are regarded as main threats for this species throughout this thesis.

Figure 1.9. Unsustainable forestry practices in Nahuelbuta, Chile. The large size of clear-cuts is exemplified with the satellite image of a clear-cut area of approximate 3,000 ha (left), while the poor riparian protection is exemplified in the right (Google Earth 2015 Digital Globe).

Nevertheless, plantation ecosystems in Chile are not a biological desert, and several native species, including birds and insects, can use mature plantations to some degree (Grez et al. 2006). However, the use of plantations by amphibian species in Chile remains poorly studied. Furthermore, the effect of plantations can be expected to be different for different species, even within the same group. For example, pine plantations have been

found to reduce usable habitat for frogs in Australia; however, two out of eight Australian species previously found in this habitat were still present in plantations (Parris and Lindenmayer 2004). This highlights the need to study the effects of plantations on a species-specific basis, as not all species will be affected in the same way. Rabanal and Alarcón (2010) reported the presence of *Alsodes vanzolinii* a critically endangered species of Nahuelbuta, in an area covered by plantations in central-south Chile, while Escobar et al. (2005) observed *Telmatobufo bullocki* in plantations 90 m from native forest[4]. These observations suggest that endangered native amphibians could be using plantations to some degree and this could have important conservation and management implications.

Moreover, the recent adoption of Forest Stewardship Council (FSC) principles and criteria (FSC 2012) by some of the main forestry companies operating in Nahuelbuta has opened an opportunity for the development of more sustainable practices, and the protection of endangered species present in plantation areas. The identification and protection of High Conservation Value Areas (HCVA), has added over 23,000 ha of protected native forest. It is within this context of opportunity for conservation that this thesis aims to provide basic knowledge and scientific-based management advice that could help in the conservation of *T. bullocki* and its remaining habitat.

[4] Although Escobar et al. (2005) identified this specimen as *T. bullocki*, its identity remains unclear due to the more recent description of *Telmatobufo ignotus* north of Biobío River.

1.4 Thesis aim, specific objectives, and chapter outline

The central aim of this thesis is to make a significant contribution to the conservation biology of *Telmatobufo bullocki*. In order to achieve this, several objectives were set. These objectives were organised into four Chapters, each addressing some of the research questions with different approaches and methodologies. Nevertheless, all chapters are closely interconnected. Each chapter is written as a stand-alone paper, containing its own introduction, methods, results and discussion. Therefore, some overlap and repetition is likely to occur, particularly in the introductions. Although a significant part of this thesis, the development of microsatellite markers is not included as a separate chapter in the main body of the thesis. Due to the more technical nature of the work, and because it is already published (Moreno-Puig et al. 2014), this was included as an appendix and cited as published work.

The general topic and specific objectives addressed in each chapter are:

Chapter 2: DISTRIBUTION

1) Assess current distribution, re-survey historic locations and add new locations

2) Model distribution and create a predictive habitat suitability map

3) Assess the impact of native forest loss and climate change on *T. bullocki* distribution

Chapter 3: HABITAT USE AND BEHAVIOUR

1) Describe terrestrial and aquatic habitat use (habitat and microhabitat)

2) Study movement patterns

3) Define core habitat for protection

Chapter 4: CONSERVATION GENETICS

1) Assess current levels and patterns of population genetic diversity

2) Estimate effective population size (N_e) and detect historical and recent changes in N_e

3) Assess genetic population structure and functional connectivity

4) Describe phylogeographic patterns

5) Define management units

Chapter 5: LANDSCAPE GENETICS

1) Assess the relative influence of different landscape features on gene flow

2) Identify dispersal corridors

In the final chapter (Chapter 6) the main findings and their significance are highlighted, management recommendations summarised, and future directions suggested.

One of the main hypothesis tested in this thesis is that native forest loss and replacement by forestry plantations in Nahuelbuta has a negative impact on *T. bullocki* populations. More specific hypotheses will be tested in each chapter. In Chapter 2, the main hypothesis is that *T. bullocki* distribution in Nahuelbuta is closely tied to native forest, and therefore recent native forest loss is a main threat that has reduced *T. bullocki* distribution and contributed to its poor conservation status. A second hypothesis in this chapter is that climate change is also an important current threat, and that future climatic conditions will further reduce *T. bullocki* distribution and increase its exctinction risk. Chapter 3 is more

descriptive and less hypothesis-driven; however, an implicit hypothesis is that *T. bullocki* has complex habitat requirements. In Chapter 4, the hypothesis that *T. bullocki* populations are genetically structured will be tested. In Chapter 5 the main hypothesis is that landscape features affect genetic connectivity between populations/individuals, and several more specific hypotheses will be tested regarding the relative effects of different landscape features (e.g. land cover, proximity to streams) on gene flow.

Chapter 2:

A distribution model for *Telmatobufo bullocki*, and the impact of native forest loss and climate change

2.1 Introduction

Knowledge of a species' distribution is critical for the conservation and management of threatened species (Guisan and Zimmermann 2000). However, detailed distribution maps are lacking in most cases, particularly for rare and endangered species. Surveying extended areas for cryptic species is time consuming and expensive, and might not be feasible in areas with difficult access or when resources are limited. Recently, species distribution models (SDM) have been widely used in such cases as a cost-effective alternative. SDM describe empirical correlations between species distribution (response variable) and environmental (predictor) variables (Elith and Leathwick 2009). They can be used to predict the occurrence of a species for locations where survey data are lacking, yielding a predictive map (Franklin 2009 p. 13). SDM can also be projected into hypothetical environmental conditions in order to infer the distribution of the species at another point in time (past or present). These projections can give valuable insight on how environmental changes (natural or human-induced) affect the species.

Species distribution models and predictive distribution maps are useful for many aspects of resource management and conservation planning including biodiversity assessment,

reserve design, habitat management and restoration, population, community and ecosystem modeling, ecological restoration, invasive species risk assessment, and predicting the effects of climate change on species and ecosystems (Franklin 2009 p. 12). Several SDM approaches exist, all of which require at least some knowledge on the species distribution (i.e. presence points) and data on environmental variables that are likely to be important for habitat suitability. In this Chapter, maximum entropy (Maxent) distribution modeling (Phillips et al. 2006) was used to: 1) build a predictive distribution map for *Telmatobufo bullocki* (Schmidt 1952) in the Nahuelbuta Range, and 2) investigate how main threats (native forest loss and climate change) might affect the species distribution. The habitat requirements for *T. bullocki* and the potential impact of recent environmental change on the species have not been assessed.

Telmatobufo bullocki (Anura, Calyptocephalellidae) is a critically endangered species (Veloso et al. 2008), which has been identified as one of the most threatened amphibians globally (ZSL 2011). It is currently known from a few locations within the coastal Nahuelbuta Range (NR), in central-south Chile; however, most records are old and were given with little accuracy in the literature (Chapter 1, Appendix A). Therefore, in this Chapter, historical locations were re-visited and several new locations surveyed in order to assess their current status, which has been identified as one of the main conservation priorities for this species (Veloso et al. 2008, ZSL 2011).

During the last few decades, native forests within the NR have been extensively replaced by commercial plantations of exotic pines (*Pinus radiata*) and eucalyptus (*Eucalyptus globulus*), leaving a highly fragmented landscape. Current plantation management allows

extensive and periodic clear-cuts with minimal protection of stream habitat, which makes forestry a major threat for amphibian populations. Habitat loss and fragmentation have been identified as main drivers for amphibian declines worldwide (Collins and Storfer 2003, Stuart et al. 2004), and are considered the greatest threats faced by most amphibian species in the Nahuelbuta Range, including *T. bullocki* (Ortiz et al. 2010, Fenolio et al. 2013). Despite its potential high impact on the species conservation, and the urgent need for management considerations, the effects of native forest loss and its replacement by exotic plantations on *T. bullocki* populations have not been previously studied.

Although habitat loss represents the primary immediate threat to *T. bullocki*, there is increasing evidence that climate change may also be contributing to amphibian population declines through both direct and indirect effects (Blaustein et al. 2010). Unusual weather conditions including increased temperatures, unusual frosts, dry winters, extended droughts, and changes in precipitation patterns have been linked to amphibian population declines (McMenamin et al. 2008, Blaustein et al. 2012, Walls et al. 2013). Models of future climatic conditions for the Nahuelbuta Range predict that annual precipitation will decrease between 14% and 25% by the end of the century (2080), while mean annual temperatures will rise between 1.5 °C and 2.5 °C depending on different emission scenarios (Girvetz et al. 2009). In addition, more extreme weather events such as flooding and drought are also expected, particularly associated with El Niño and La Niña anomalies (CONAMA 2008).

T. bullocki breeds in permanent fast flowing streams and needs both aquatic and terrestrial habitat to complete its life cycle (Chapter 3). This complex life cycle, along with

its physiological characteristics and behaviour makes *T. bullocki* potentially vulnerable to climate change. Investigating if and how global climate change is likely to affect the species distribution in the future will be a valuable tool in the long-term conservation planning of the species; to date this has not been undertaken.

In this Chapter, maximum entropy modeling (Maxent) was used to understand the relationship between *T. bullocki* and environmental variables (e.g. climatic, topographic), and to predict the species distribution throughout the Nahuelbuta Range. Maxent modeling is based on presence-only data, and therefore has been more commonly used when absence data is either lacking (e.g. museum records) or unreliable (e.g. low species' detection probability) (Elith et al. 2011). Maxent has been shown to outperform other modeling approaches, particularly for small sample sizes (Hernandez et al. 2006), thus it has been frequently used for modeling rare and endangered species distributions (Hernandez et al. 2006, Pearson et al. 2007). Moreover, Maxent models can be projected into both past and future environmental scenarios, and are therefore a valuable tool for assessing the impacts of environmental change (e.g. climate change, habitat loss). For example, Maxent has been used to infer past, present and future environmental suitability for *Leiopelma hochstetteri*, an endangered New Zealand frog (Fouquet et al. 2010), to predict the potential distribution of the American bullfrog, a problematic invasive species (Ficetola et al. 2007b), and assess extinction risk in cloud forest fragments under climate change and habitat loss (Ponce-Reyes et al. 2013). In this study, the Maxent model was projected into hypothetical past and future environmental conditions to assess the impacts of two of the main human-induced threats on amphibians: habitat loss and climate change.

2.2 Methods

2.2.1 Study area

The Nahuelbuta Range (NR) is a section of the Chilean coastal range (Figure 2.1). It extends for 190 km (north-south), between the Biobío River (37º S) and the Imperial River (38º 50' S), reaching a maximum elevation of 1,533 m above sea level (Mardones 2005). Its natural vegetation is rich in endemic species, as it includes components from the Maulino forest in the north, changing to Valdivian temperate rain forest towards the south (Villagrán and Armesto 2005). There is also a shift in vegetation according to elevation, with marked changes in composition particularly above 1,000 m. The climate of the region ranges from Mediterranean in the north to temperate oceanic in the south, with a latitudinal gradient in precipitation and temperature. Nevertheless, the climatic pattern is generally characterised by relatively hot and dry summers, and wet and cool winters. The highest areas of the NR (above 1,000 m) are covered by snow during the coldest months. The coastal location and high elevation of the NR creates a rain shadow effect on the Eastern slopes, where precipitation is consistently lower. The terrain is rugged with a complex and extensive stream and river network. This highly heterogeneous climatic and orographic pattern of the NR translates into a rich and unique biodiversity with several micro-endemic plants and animals, thus being recognised as a priority hot-spot for conservation (Myers et al. 2000, Wolodarsky-Franke and Díaz Herrera 2011).

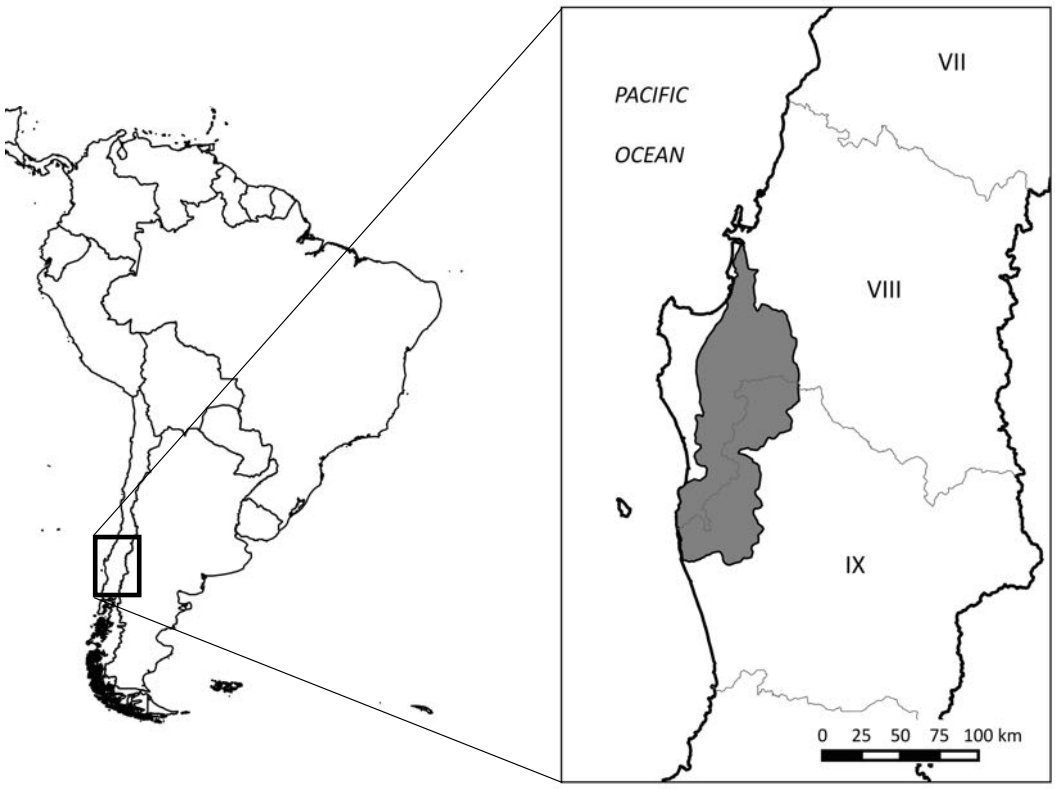


Figure 2.1. Location of the study area: Nahuelbuta Range (grey area) in central south Chile, between Regions VIII (Región del Biobío) and IX (Región de la Araucanía).

Despite its great biodiversity value, the area has suffered a dramatic and on-going replacement of its original native forest for agriculture, and more recently for extensive monoculture plantations (Cisternas et al. 1999, Wolodarsky-Franke and Díaz Herrera 2011). This change has been particularly dramatic at northern latitudes and lower altitudes. Two state-protected areas exist; Nahuelbuta National Park (6,832 ha) located at the highest point of the NR (above 1,000 m) and the 82 ha Contulmo Natural Monument. Some private areas owned by forestry companies have been protected under Forest Stewardship Council (FSC) principles and criteria, including the largest continuous

fragment of native forest of over 23,755 ha (Quebrada de Caramávida), adjacent to the National Park.

2.2.2 Amphibian surveys and data collection

Literature reviews and museum collection catalogues were used to compile all recorded sightings of the species (listed in Appendix A). Historical places with geographic reference were re-surveyed to assess the status of these known populations. In addition, 72 sites within Nahuelbuta were surveyed from 2011 to 2014 in search of new presence records. Some of these survey sites were near historical sites and/or near other survey sites. Survey sites were selected to include a gradient of native forest cover from mostly native to mostly plantation, and were constrained by accessibility. Clear-cuts, young plantations, and open areas (agriculture, urban) were not surveyed. To increase detectability, surveys were conducted mainly during the night, when frogs (larvae and adults) are expected to be more active. All sites were visited during Spring-Summer, and both streams and terrestrial habitat was searched. For stream habitat surveys, two observers moved upstream from a random start point thoroughly sweeping the area visually looking for tadpoles and adults with the aid of torches and a small underwater viewing window (see Chapter 3 for details). Terrestrial habitat adjacent to streams (up to 600 m away from streams on some locations depending on steepness and accesibility) was surveyed at night with torches using a visual encounter surveying (VES) technique (Crump et al. 1994). Most sites were surveyed once (for at least two observer-hours), but some sites were surveyed on more than one occasion, particularly when presence was expected (i.e. historical locations). Because of this uneven sampling, and the low detection probability of *T. bullocki* (Chapter 3), the analyses of presence/absence data are vulnerable to false absence records. To avoid this

uncertainty, presence-only methods were used, based only on recent and confirmed records.

2.2.3 Environmental variables

Several relevant environmental variables were initially considered as potential predictor variables for *T. bullocki* distribution (Table 2.2). Nineteen bioclimatic variables (Bio1 to Bio19) derived from temperature and precipitation data were downloaded from the WorldClim database, with a 30 arc second (≈ 1 km) resolution (Hijmans et al. 2005). These bioclimatic variables have been widely used in niche studies and species distribution modeling, but they are often highly correlated. In order to remove highly correlated bioclimatic variables, and reduce the final number of bioclimatic variables included, 10,000 random points were sampled from the study area and values from each variable were extracted. A pairwise correlation analysis (Pearson's *r* correlation, $\alpha = 0.01$, 2-tailed) was performed, and only the most ecologically meaningful variable was retained for each group of highly correlated variables ($r > 0.75$). After this, only four non-correlated variables were kept for further modeling: annual mean temperature (Bio1), mean diurnal temperature range (Bio2), annual precipitation (Bio12), and precipitation of driest quarter (Bio17).

Three topographic variables were considered as potentially important for *T. bullocki* distribution: elevation, slope, and topographic ruggedness index (TRI). The digital elevation model (DEM) used was the shuttle radar topography mission (SRTM) data available at a resolution of 87 m x 87 m pixel size. Slope is expected to be important for *T. bullocki* as they rely on high-gradient streams for reproduction; extended flat areas are therefore

possibly unsuitable for the species. Slope was calculated relative to the 8 surrounding pixel values. The TRI summarises the change in elevation at a local scale and is used as a measurement of terrain heterogeneity as described by Riley et al. (1999). Increased terrain heterogeneity is expected to create diverse refuges and microhabitat conditions, which are essential for amphibians. Slope and TRI were derived from the DEM using Raster Terrain Analysis tools in QGIS 2.4. A correlation analysis showed that TRI and slope were highly correlated ($r = 0.94$), thus only elevation and TRI were included in the final model.

To represent native forest loss at a landscape scale, land cover data were used to calculate landscape composition. Land cover data were obtained from the national land use database (CONAF 2008) reclassified into three classes (native forest, exotic plantation, non-forested), and rasterized to match the DEM resolution. The percentage of native forest cover (%NF) and percentage of exotic plantation cover (%P) in the landscape was calculated using neighbourhood analysis (moving window) in QGIS 2.4. Because %NF and %P showed a significant negative correlation ($r = -0.95$, $P < 0.01$), only %NF was included in the final model.

The scale at which habitat loss and landscape change affects *T. bullocki* distribution is unknown. Most studies looking at the effect of habitat loss at the landscape scale have used circular landscapes of a predefined size based on the species' known ecology and behaviour. Knowledge of home ranges and dispersal abilities are usually the basis for landscape scale definition, where larger landscapes are considered for species with good dispersal abilities or that migrate long distances (e.g. mammals, birds), and smaller scales are considered for low vagility species (e.g. amphibians, invertebrates). *T. bullocki* has

been found as far as ≈500 m from breeding streams, and individuals found to move up to 170 m per night (Chapter 3); however, no empirical data on maximum dispersal distances exist. To investigate the relative effect of native forest loss at different scales, three landscape sizes were defined: large, medium and small (Figure 2.2). The small scale corresponds to a square with a side length of 1,131 m (the shape of the landscape was determined by the squared shape of the neighbourhood analysis tool used in GIS) with an area of 1.28 km^2, roughly equivalent to a circle of 600 m radius. The medium scale is a square with a side length of 3,045 m, with an area of 9.3 km^2, equivalent to a circle of radius of 1,700 m. The large scale is a square with a side length of 6,003 m with an area of 36 km^2, equivalent to a 3.4 km radius circle. These scales are consistent with the range of scales at which landscape effects have been studied and found to be relevant for amphibians (Carr and Fahrig 2001, Houlahan and Findlay 2003, Semlitsch et al. 2008, 2009). The relative effect of native forest loss at different landscape scales on model performance was compared, and the most relevant scale selected as described below.

Other variables not considered for modeling (because they were highly correlated with other variables included in the model, or not available for the whole region), but that were measured to describe *T. bullocki* habitat were: upstream catchment area (UCA), which is the area of the whole catchment measured from the point of occurrence; distance to nearest native forest; and percentages of native forest and plantation in whole upstream catchment area.

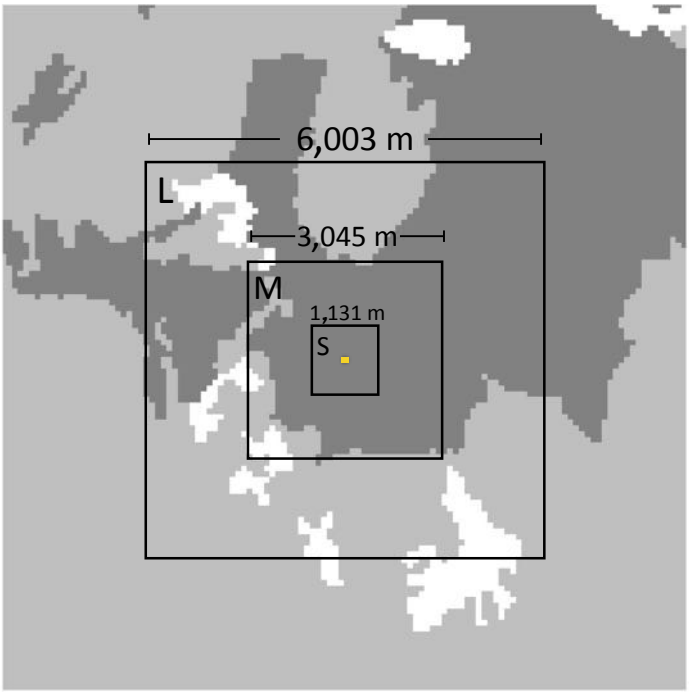


Figure 2.2. Diagram showing three different landscape scales considered in the study (L = large, M = medium, S = small). Dark grey represents native forest, light grey represents exotic plantation, and white represents non-forested areas. The percentage of native forest and exotic plantation was calculated for each scale using a moving window analysis.

Table 2.1. Summary of environmental variables selected for species distribution modeling.

Variable	Description	Unit
Bio1	Annual mean temperature	°C
Bio2	Mean diurnal range	°C
Bio12	Annual precipitation	mm
Bio17	Precipitation of driest quarter	mm
ELE	Elevation above sea level	m
TRI	Topographic Ruggedness Index	N/A
%NF	Percentage of native forest in the landscape at three different scales (%NF-L, %NF-M, %NF-S)	%

2.2.4 Maxent modeling

Presence-only data were used to model *T. bullocki* distribution using maximum entropy model in Maxent (Phillips et al. 2006, Phillips and Dudík 2008). Maxent takes presence points and environmental predictors as input, and contrasts them with a random sample of background points where presence is unknown. The logistic output of Maxent was used, which gives for every cell in the map, a value between 0 and 1 representing relative environmental suitability (0 = unsuitable habitat, 1 = optimal habitat), an estimation of probability of presence (Phillips and Dudík 2008). The selection of background points was restricted to the study area (Nahuelbuta Range), and a bias grid was created to correct for sampling bias (Phillips et al. 2009, Kramer-Schadt et al. 2013). The bias grid was constructed by creating a 1 km buffer area around all surveyed points (with or without *T. bullocki* presence), and giving these pixels a value of 1 (representing surveyed areas) while all other areas were given a value of 0.01 (representing areas not surveyed). When using a bias grid, Maxent interprets the pixel value as relative sampling effort, and uses it to weight selection of random background data used for modeling (Phillips et al. 2009). To avoid spatial autocorrelation, presence points were spatially filtered (Veloz 2009) and only points at least 1 km apart were retained for model training. Maxent software version 3.3.3k was used to run the models, with all other settings set to default values.

2.2.5 Model evaluation and selection

To evaluate model performance, a ten-fold cross-validation approach was taken. In this case, the Maxent model is built using 90% of the presence points while 10% are randomly withheld for testing. Ten replicate runs were performed and the final prediction obtained from the average of these ten runs. The ability of the model to predict the localities that

were excluded from the training dataset (predictive performance) was measured using the area under the receiver-operator curve on test data (AUC_{test}). AUC is a threshold-independent measure of predictive accuracy, that has emerged as the most widely used in the Maxent literature (Merow et al. 2013). Although AUC has been criticised as a measure for model performance (Lobo et al. 2008), its use is particularly suited and accepted for comparing single-species models within the same study region (Phillips et al. 2009, Boria et al. 2014). AUC ranges from 0 to 1 with excellent prediction for models with AUC > 0.9, fair models with AUC between 0.7 and 0.9 and poor models with AUC lower than 0.7 (Swets 1988).

Four competing hypotheses (models) were built to investigate the relative effect of native forest loss and scale on *T. bullocki* distribution. First, the model was built with the four bioclimatic and the two selected topographic variables, but without the landscape composition variable (% native forest). Then, three additional models were built, by incorporating the percentage of native forest in the landscape at each of the three scales defined. Model selection was performed by ranking all models and selecting the one with the highest AUC_{test}.

2.2.6 Model projection

In order to assess the potential impact of recent native forest loss on *T. bullocki* distribution, the Maxent model was projected into hypothetical past forest conditions. No detailed maps of original native forest cover in the Nahuelbuta Range were available for this study; however, it is known that exotic plantation forestry was established in areas that were either directly cleared from native forest, or were previously cleared for

agriculture (Cisternas et al. 1999). Thus, a reasonable hypothesis is to return current plantation areas into native forest cover to build a hypothetical past (i.e. before intensive human intervention) native forest cover surface. Then, the percentage of native forest in the landscape was calculated as explained above (landscape composition). The Maxent model for past conditions was built by replacing the landscape composition variable (percentage of native forest in the landscape) with the hypothesised past cover, while all other variables remained unchanged.

The model was also projected into future conditions under one of the predicted climate change scenarios to assess the potential impact of climate change on *T. bullocki* habitat distribution. There are four representative concentration pathways (RCP) of greenhouse gas concentration adopted by the Intergovernmental Panel on Climate Change (IPCC) for its fourth Assessment Report (i.e. RCP2.6, RCP4.5, RCP6.0, RCP8.5) (Moss et al. 2008). All four pathways are possible under present circumstances, and depend on how much greenhouse gases are emitted in the years to come. The RCP4.5, which is considered an intermediate pathway, was adopted for this study, and assumes greenhouse gas emissions will peak around 2040, then stabilize by 2100 (Thomson et al. 2011). Several future climate models exist; here the NASA GISS-E2-R model was chosen (Schmidt et al. 2006). Predicted bioclimatic data for the chosen model for year 2050 were downloaded from BIOCLIM database (http://www.worldclim.org/cmip5_30s), and resampled to match the DEM layer resolution. The Maxent projection for 2050 was built by replacing the four bioclimatic variables in the Maxent model (Bio1, Bio2, Bio12, Bio17) with their respective projection under RCP4.5 scenario, while all other variables remained unchanged. This projection

assumes there will be no further loss (or gain) of native forest, and assumes limited dispersal of the species.

2.3 Results

2.3.1 Presence points

Eighty-three surveys were conducted to search for *Telmatobufo bullocki* within the Nahuelbuta Range, and 66 presence points (individual GPS fixes) were recorded in historical and new localities (new localities were considered localities that have not been mentioned or geo-referenced in the literature before). In addition, some records were gathered from other sources (Sánchez P. et al. 2010, Rabanal and Moreno-Puig 2014, Bernardo Guzmán 2013 pers. comm.) totalling 70 recent presence records (list of coordinates is given in Appendix B). After spatial filtering to remove clumped records, 25 of the 70 presence points were retained for Maxent modeling (shown in Figure 2.3). *T. bullocki* was found in nine basins (*= new locality): 1) Rio Chivilingo, 2) Ramadillas, 3) Rio Caramávida (including Estero Las Delicias*), 4) Rio Cayucupil* (including Estero La Cueva and Estero Los Tres Viejos*), 5) Rio Butamalal, 6) Huilquehue*, 7) Estero Provoque (including Estero San Carlos*), 8) Estero Calebu*, and 9) Estero Los Lleulles. All observations occurred within the known species range, relatively close to historical records, with only a small extension of the southern limit (extended to Estero Calebu), and western limit (extended to Huilquehue in the southwest and Las Delicias within Caramávida basin). Summary statistics for variables considered in this study for all presence records (N = 70) are shown in Table 2.2.

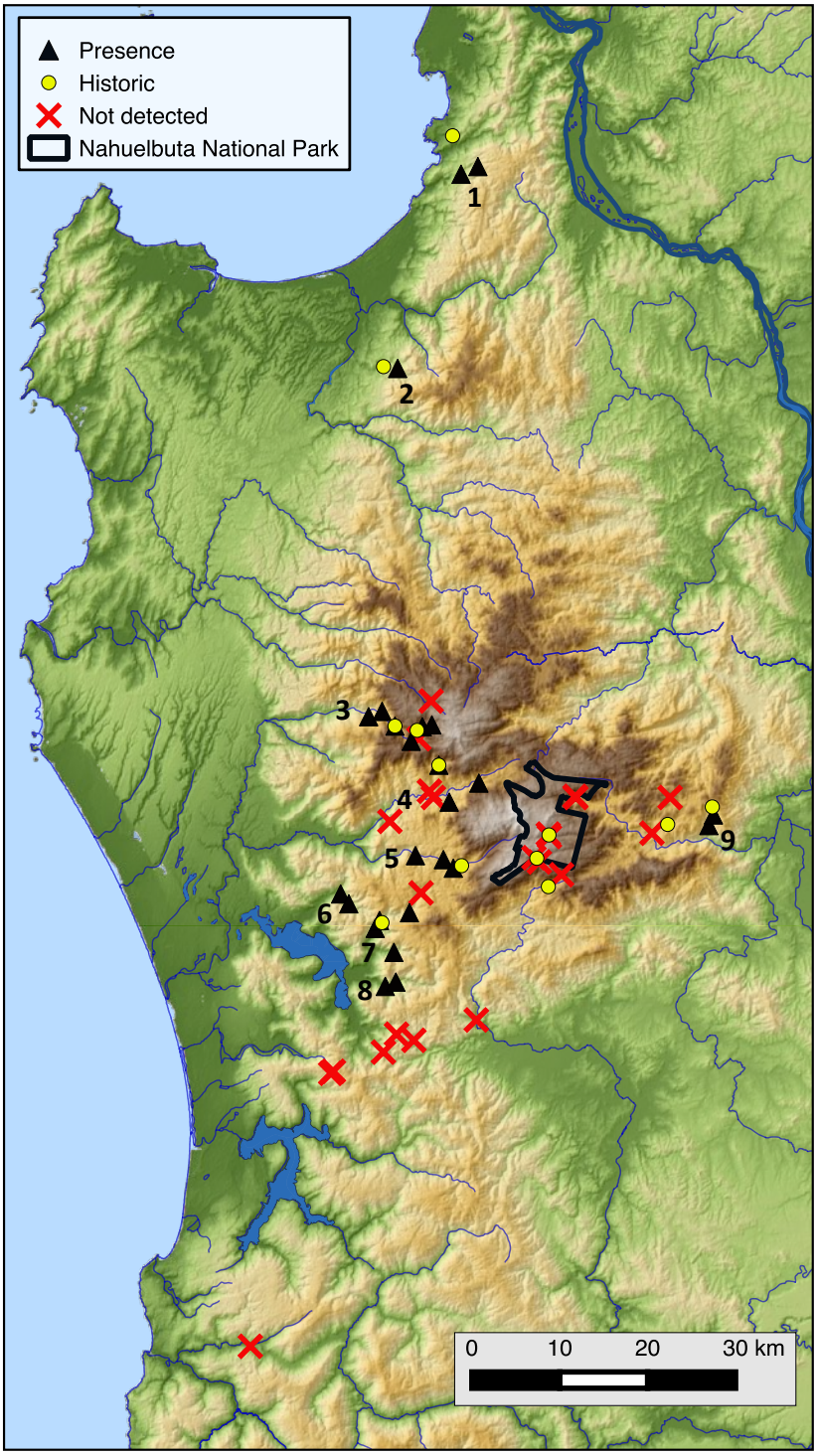


Figure 2.3. Map of Nahuelbuta showing the 25 *T. bullocki* presence points used for modeling (black triangles), historical records (yellow dots) and areas where *T. bullocki* was not detected during this study (red crosses). Main basins with *T. bullocki* presence are labeled: 1) Chivilingo, 2) Ramadillas, 3) Caramavida, 4) Cayucupil, 5) Butamalal, 6) Huilquehue, 7) Provoque, 8) Calebu, 9) Los Lleulles.

Table 2.2. Descriptive statistics for environmental variables, based on 70 presence points in the Nahuelbuta Range.

Variable	Min.	Max.	Mean	Std. Deviation
Topographical				
Upstream Catchment Area (square kilometres)	1.6	67.44	20.4	17.7
Elevation (metres above sea level)	70	1030	366.7	211.9
Slope (degrees)	2	37	21.7	10.6
Ruggedness Index	18	177	98.6	45
Landscape				
Distance to nearest native forest (metres)	0	748	123.8	145.3
Native Forest cover (%) in:				
Small Landscape	1	100	39.9	25.7
Medium Landscape	8	100	43.9	19.5
Large Landscape	10	91	43.3	16.1
Whole Upstream Catchment Area	7.3	87.8	57.7	24.5
Exotic Plantation cover (%) in:				
Small Landscape	0	100	50.4	23.6
Medium Landscape	0	91.9	50.0	19.1
Large Landscape	2.9	86.5	48.8	15.2
Whole Upstream Catchment Area	0	91.8	33.0	26.5
Bioclimatic Variables				
BIO1 - Annual Mean Temperature (°C)	7.4	12.6	10.5	1.4
BIO2 - Mean diurnal range	10.7	12.1	11.6	0.3
BIO3 - Isothermality	5.3	5.7	5.7	0.1
BIO4 - Temperature Seasonality	276.6	354.2	298.1	16.7

BIO5 - Max. Temperature of Warmest Month	19.4	25.3	22.9	1.6
BIO6 - Min. Temperature of Coldest Month	-0.1	4.1	2.4	1.1
BIO7 - Temperature Annual Range	19.2	22.5	20.5	0.7
BIO8 - Mean Temperature of Wettest Quarter	4.4	9.2	7.3	1.3
BIO9 - Mean Temperature of Driest Quarter	11.1	16.7	14.5	1.5
BIO10 - Mean Temperature of Warmest Quarter	11.1	16.7	14.5	1.5
BIO11 - Mean Temperature of Coldest Quarter	3.9	8.7	6.8	1.3
BIO12 - Annual Precipitation (mm)	1257	1682	1570.2	98.8
BIO13 - Precipitation of Wettest Month	243	311	295.5	14.6
BIO14 - Precipitation of Driest Month	20	36	30.3	3.9
BIO15 - Precipitation Seasonality	68	79	71.7	2.8
BIO16 - Precipitation of Wettest Quarter	684	853	812.9	36.9
BIO17 - Precipitation of Driest Quarter	79	129	110.9	13.4
BIO18 - Precipitation of Warmest Quarter	79	129	110.9	13.4
BIO19 - Precipitation of Coldest Quarter	641	818	777.6	39.4

2.3.2 Model evaluation and selection

The four competing models were run, ranked, and results are shown in Table 2.3. Including the landscape composition variable (%NF) improved the performance of the model in all cases. The best model corresponded to the one including the percentage of native forest cover in the large landscape (referred as landscape from here on). The predicted distribution map (continuous logistic output) for *T. bullocki* in the Nahuelbuta Range under the best model (referred as the model from here on) is depicted in Figure 2.6B (centre). AUC for training data and test data was 0.912 and 0.812 respectively, indicating a good level of predictive accuracy (Swets 1988).

Table 2.3. Mean area under the receiver operator curve (AUC) for training and test data for the four competing models (10 replicate runs).

Variables in the model	$AUC_{training}$	AUC_{test}
Bio1 + Bio2 + Bio12 + Bio17 + ELE + TRI + %NF-L	**0.912**	**0.812**
Bio1 + Bio2 + Bio12 + Bio17 + ELE + TRI + %NF-S	0.908	0.773
Bio1 + Bio2 + Bio12 + Bio17 + ELE + TRI + %NF-M	0.928	0.770
Bio1 + Bio2 + Bio12 + Bio17 + ELE + TRI	0.874	0.739

2.3.3 Relative influence of environmental variables

Among the variables included in the model, the one that was most strongly associated with *T. bullocki* occurrence was the percentage of native forest cover in the surrounding landscape, followed by elevation, annual precipitation and mean annual temperature (Table 2.4). Marginal response curves for these variables are shown in Figure 2.4. The probability of presence increases steeply with the amount of native forest in the landscape from 0-40% and remains high from 40-100%, decreases relatively constantly with elevation, is high for annual mean temperatures up to 11.5 °C then decreases rapidly for higher temperatures, and increases with the amount of annual precipitation. Mean diurnal range, precipitation of the driest quarter and topographic ruggedness index had less contribution to the model, and their response curves are not shown.

Table 2.4. Percent contribution and permutation importance of all the variables included in the best Maxent model

Variable	Percentage contribution	Permutation importance
%NF_L	46.2	48
ELE	32.3	21.2
Bio12	6.3	10.8
Bio1	5.5	11.2
Bio17	5.4	5.2
Bio2	3.7	3.4
TRI	0.6	0.1

2.3.4 Response curves

Response curves for the amount of native forest in the small- and medium-sized landscapes are shown in Figure 2.5. Although these variables (i.e. %NF_S and %NF_M) were not used in the final model, their response curves can give valuable information on the influence of native forest loss at different scales. Similar to the best model, these were the most influential variables in their respective models, confirming the importance of landscape composition in determining *T. bullocki* distribution. The response at different scales is not the same; at the small scale it appears the probability of occurrence remains high and relatively constant regardless of the amount of native forest, with high probability of occurrence at all percentages. In the medium landscape in the other hand, there is a marked threshold at just below 20% below which probability of occurrence decreases steeply.

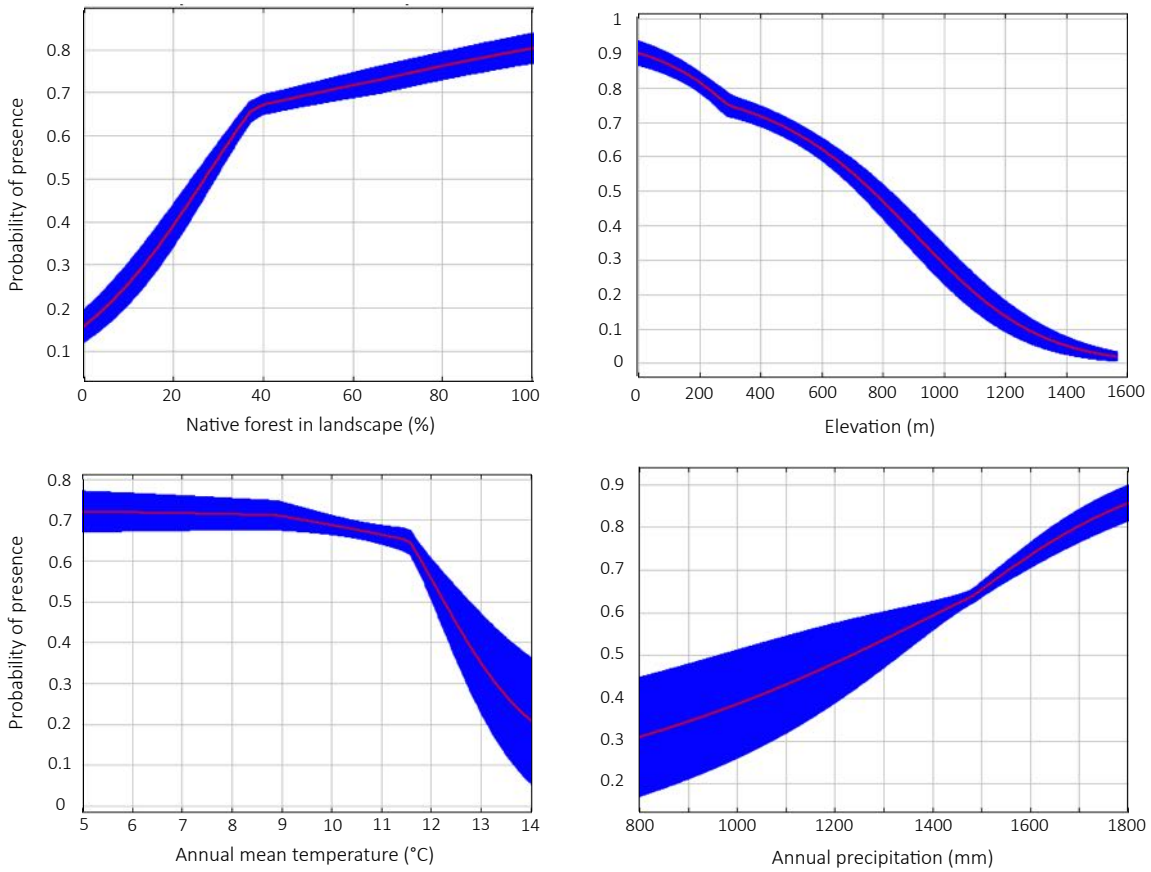


Figure 2.4. Marginal response curves of the predicted probability of *T. bullocki* presence for the four predictor variables that most contributed to the best model. The curves show the mean response of the 10 replicate Maxent runs (red) and the mean ± one standard deviation (blue).

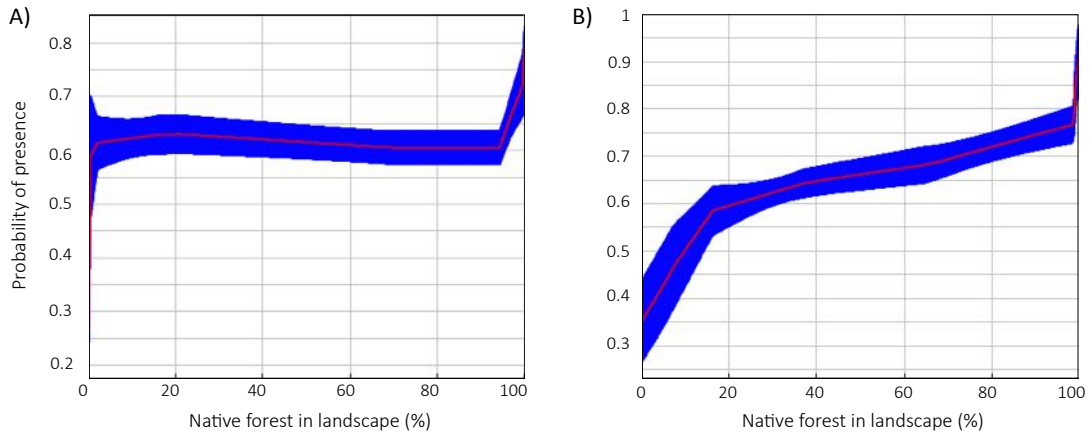


Figure 2.5. Marginal response curves for the percentage of native forest in A) small landscape, and B) medium landscape. The curves show the mean response of the 10 replicate Maxent runs (red) and the mean ± one standard deviation (blue).

2.3.5 Suitable habitat

The logistic output of the model shows *T. bullocki* is currently present in areas with probability of occurrence from 0.46, which can be considered as a minimum threshold (i.e. minimum training presence MTP threshold). When this value is applied as a minimum habitat suitability threshold to generate a binary (suitable/not suitable) output (Figure 2.7, left) the model predicts *T. bullocki* presence (or suitable habitat) in several areas that currently hold no presence records. It predicts the presence of the species in the southern part of the ranges where it has not yet been reported in the literature; however, there is a significant and potentially limiting gap separating this southern block that could act as a dispersal barrier. The total area classified as suitable under the MTP threshold is of 2,685 km^2; however, if only areas within 500 m from streams are considered (based on the maximum distance recorded, Chapter 3), the suitable habitat area is reduced by 25% to 1,980 km^2.

2.3.6 Model projections

The logistic outputs of the projected distribution into past and future environmental conditions are depicted in Figure 2.6 (left, right). Under the Maxent model, *T. bullocki* distribution follows a clear temporal trend from high suitability in the past (warmer colours) to low suitability in the future (cooler colours). Under the past scenario, 83% of the area would have been a nearly continuous surface of suitable habitat. This is an estimated reduction of 66% in habitat in recent times due to native forest loss. When projected into future environmental conditions under climate change scenario, the total suitable habitat is of only 800 km^2, which represents a further 70% reduction of the present area (Figure 2.7), and only 10% of the past distribution. Fourteen presence points (56%) fell outside suitable habitat areas.

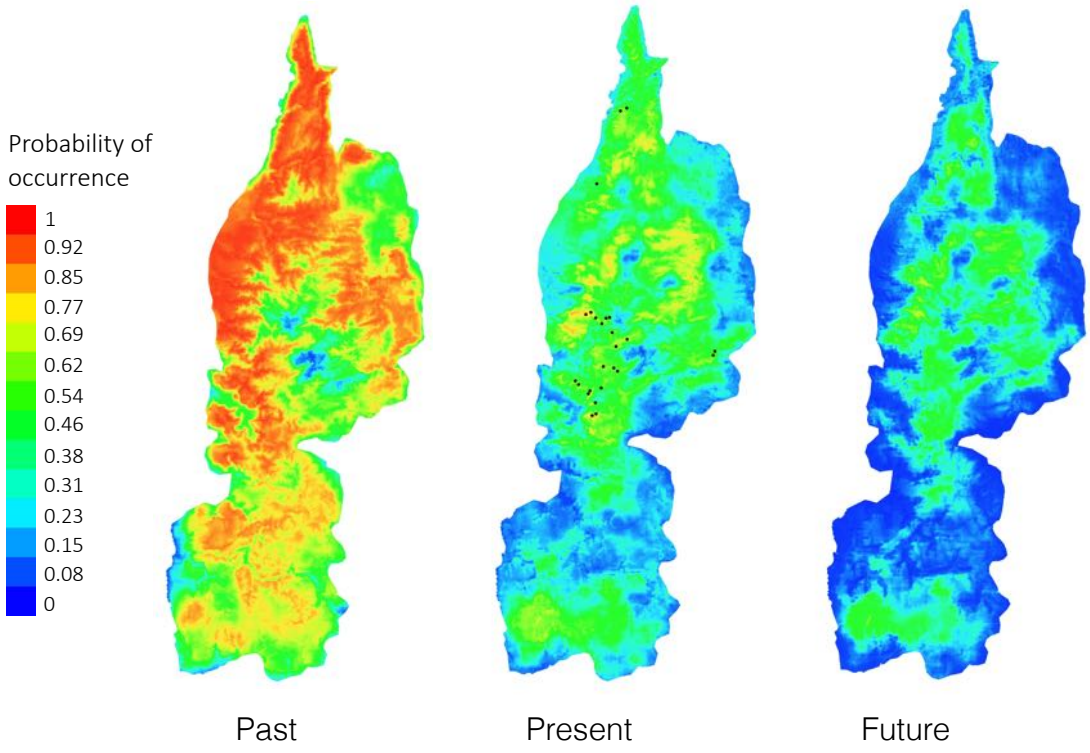


Figure 2.6. Logistic output for the predicted *Telmatobufo bullocki* distribution in the Nahuelbuta Range for past (under native forest hypothesis), present and future conditions (year 2050 under RCP4.5 scenario). Black dots are the presence points used for Maxent modeling.

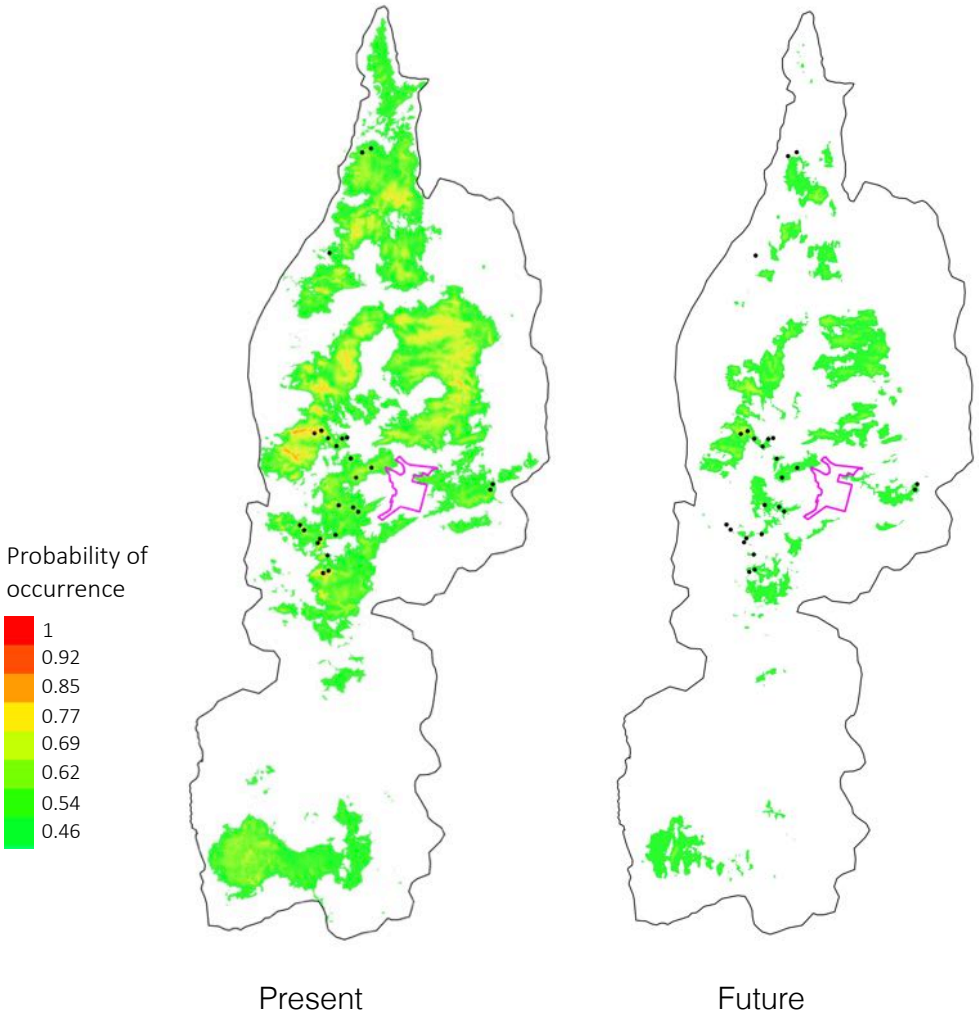


Figure 2.7. Map showing the predicted distribution of *T. bullocki* suitable habitat in the Nahuelbuta Range in present and future conditions under climate change scenario (RCP4.5, 2050). Only suitable areas (MTP probability of occurrence ≧ 0.46) are shown. Nahuelbuta National Park is shown in pink, and training presence points in black.

2.4 Discussion

The relatively high number of presence records obtained in this study, compared to the few historical observations of the species, suggests that *T. bullocki* might be more common than previously thought. Furthermore, the predictive distribution map shows many areas that could potentially hold as yet undiscovered populations. Despite this, the results show

that *T. bullocki* habitat has been reduced significantly in recent times, and suggests suitable habitat will continue to decline even further in the future.

2.4.1 Relative influence of environmental variables

The results confirm habitat loss as one of the main threats, as evidenced by the close relationship between the amount of native forest in the landscape and the current *T. bullocki* distribution. Although native forest was important at all scales, the results suggest *T. bullocki* is most affected by landscape composition changes at larger scales. At a large and medium scale, the response of *T. bullocki* to the amount of native forest in the landscape shows a threshold of 40% and 20% of native forest respectively; below this the probability of presence decreases more abruptly. This is an indication of an ecological threshold (Huggett 2005) below which *T. bullocki* habitat might become suboptimal and population persistence might be compromised. Nevertheless, *T. bullocki* was detected in one landscape with as little as 10% native forest, suggesting this population (i.e. Ramadillas) might be severely threatened due to habitat loss and is likely to be declining. At the smallest scale, the probability of presence remained high for all percentages of native forest cover, and no threshold was observed. This suggests *T. bullocki* is able to tolerate native forest loss in local areas, as long as there is enough native forest in the neighbourhood. Furthermore, *T. bullocki* was not found more than 750 m from native forest, suggesting that native forest fragments might be critical for the species survival.

However, the above threshold values should be considered carefully, as not only composition of the landscape (amount of native forest) is important for many amphibians, but also its configuration (Guerry and Hunter 2002). Considering the steam breeding habits

of *T. bullocki*, native forest in riparian areas might be more critical and valuable than native forest far from streams, as riparian forest provide immediate shelter for emerging young metamorphs and adult breeders (Chapter 3). Forest configuration, and riparian forest in particular, has been found to be important for some pond and stream breeding amphibians. For example, pond occupancy was positively correlated with native forest adjacency in two species of salamander in Maine (*Ambystoma maculatum* and *Ambystoma laterale/A. jeffersonianum*) (Guerry and Hunter 2002), while the occurrence of tailed frogs (*Ascaphus truei*), Pacific giant salamander (*Dicamptodon tenebrosus*) and torrent salamander (*Rhyacotriton* spp.), all stream-breeders, was positively associated with presence of forested habitat adjacent to the stream (Stoddard and Hayes 2005).

The second most influential variable in the model was elevation. *T. bullocki* was found at a wide range of elevations; however, its probability of occurrence declined with increasing elevation. Although Péfaur (1971) reported the species inside Nahuelbuta National Park (above 1,000 m), and despite the availability of native forest habitat, and several surveys in this historical location, *T. bullocki* was not detected inside the park (or above 1,030 m). It is possible that *T. bullocki* altitudinal tolerance is related to vegetation changes occurring at the 1,000 m of elevation, where the vegetation cover becomes a mosaic of evergreen Araucaria and Coigue, and deciduous shrub-like cover (Ñirre and Lenga). This means that most of the cover is lost during the winter, leaving only scattered patches of permanent cover. Also, at higher elevations streams are typically smaller and therefore more vulnerable to seasonal changes. It is possible that areas above 1,000 m, even when covered by 100% native forest, might constitute only marginal habitat for this species. Other potential explanations could be competitive exclusion with other amphibian species

occurring at high elevations (e.g. *Alsodes barrioi*) or more complex interactions with predators and prey that may change with elevation.

Bioclimatic variables together contributed to 20% of the model, with annual precipitation and mean annual temperature being the most influential variables. There appears to be a threshold in temperature around 11.5 °C above which the probability of *T. bullocki* being present declines more abruptly. Areas with higher annual mean temperatures are expected to have hotter summers, which is usually coupled with decreased precipitations over the summer months. These hot and dry areas might become too dry during summer, decreasing soil moisture in terrestrial habitat and stream flow of breeding habitat. The impact of bioclimatic factors on *T. bullocki* distribution is even more evident when the model is projected into future conditions under climate change (discussed below).

There have been many SDM published for amphibians worldwide, however comparing results among studies and species is extremely difficult. Several environmental variables have been tested and found to be good predictors for amphibian distribution including bioclimatic variables, land cover, percentage forest cover, soil type, elevation, slope, aspect, and topographic wetness index (Fouquet et al. 2010, Ficetola et al. 2011, Blank and Blaustein 2012, Igawa et al. 2013, Groff et al. 2014). However, important variables are highly species-specific and may differ even for closely related species (Igawa et al. 2013). In Chile, only one amphibian SDM has been published for *Rhinoderma rufum*, a critically endangered frog that has not been found since the 1980s (Bourke et al. 2012). This model included only five bioclimatic variables, identifying temperature annual range as the most influential variable, followed by annual mean temperature, precipitation of the coldest

quarter, precipitation of the warmest quarter, and annual precipition (Bourke et al. 2012). Again, these results are hard to compare as they icluded a different set of bioclimatic variables, with the only similarity being that annual mean temperature was in both cases the second most influential variable. Noticeably, the most important bioclimatic variable for *T. bullocki* distribution was annual precipitation while this was the least influential variable for *R. rufum*. Although both species are distributed within Chile temperate forest, *R. rufum* has a much wider distribution and lives and reproduces in damp seepages and intermittent and small first order streams (Bourke et al. 2012). Therefore, significant differences in ecological niche are to be expected.

Despite being a widespread conservation issue, studies incorporating the effects of land use change on SDM in Chile are scarce (Escalante et al. 2009, González et al. 2013). Most SDM incorporate only bioclimatic (i.e. climate envelope model) and topographical variables (e.g. elevation, slope and aspect), and have been developed mainly for mammals, birds, and insects (Acosta-Jamett and Simonetti 2004, Escalante et al. 2009, Tognelli et al. 2009, Moreno et al. 2011). More often, the effects of land use change have been studied at the fragment or landscape scale (Bustamante et al. 2006, Grez et al. 2006). Such studies have found that while native forest loss, fragmentation, and replacement by exotic plantations can have negative effects for some species, for others the effect might be positive (even within same group), further highlighting the species-specific effects of habitat loss and fragmentation (Estades and Temple 1999, Bustamante et al. 2006). To my knowledge, the model developed for *T. bullocki* in this chapter is the first including the effects of bioclimatic, topographical and native forest loss at a species level in Chile, the first SDM developed for a species in Nahuelbuta, and one of the two SDM developed for amphibian

species in Chile. With the rapid advance of modeling methods and softwares, and the increasing availability and accuracy of remote sensing data, it is expected more SDM will be developed for other amphibians in the near future that will allow for a better picture of the effects of land use changes on the distribution of this highly threatened group.

2.4.2 Projections

Projecting the model into past conditions under the native forest cover scenario suggests *T. bullocki* habitat quantity and quality has declined dramatically. The projection assumed all present plantation areas were once native forest, although some plantations did not directly replace native forest, but were established on agricultural land. Nevertheless, this is a close approximation to past conditions. The past projection included present bioclimatic condition, to avoid confounding the effects. However, climatic conditions have changed in recent times due to global warming, as evidenced by local weather data from the last four decades (Chapter 1). Precipitations were higher and temperatures lower, which means that *T. bullocki* distribution was less constrained by these and was likely to be found throughout the Nahuelbuta Range.

The strong dependence of amphibians on water resources and their generally low heat tolerance makes them particularly vulnerable to climatic factors, and consequently climate change (Corn 2005). Projecting the Maxent model into (one of many potential) future climatic conditions provides an insight on the potential negative impact of climate change on this species, where the already small and fragmented distribution is further reduced and becomes increasingly isolated. The future scenario used in this study is only one of the many possible climatic pathways, and all future predictions should be interpreted with

caution. Nevertheless, the predicted future distribution map can be used to identify those areas that are likely to provide refuge for this species under climate change conditions, and this information could be important in the establishment of potential protected areas.

Decreased levels and changes in patterns of precipitation are likely to reduce *T. bullocki* breeding habitat, as some previously permanent streams might become dry during summer. Precipitation also affects terrestrial habitat quality, particularly when combined with increased temperatures, which together can dramatically affect soil moisture (Corn 2005). Warmer temperatures can reduce the concentration of dissolved oxygen in aquatic habitats, further reducing stream habitat quality. Extreme events such as flooding could also affect the species negatively as heavier rain increases siltation of streams, causing sedimentation. This is expected to affect larval survival and reduce stream habitat quality by covering rocks and filling cavities that are used for egg deposition and refuge (Chapter 3).

The negative effect of climate change on the future distribution of *T. bullocki* has also been predicted for its close relative *Telmatobufo australis*, which is predicted to have a 41% reduction in distributional range (Marquet et al. 2010). Moreover, Marquet et al. (2010) identified *T. australis* as one of the species with the highest predicted loss of habitat due to climate change. Considering all *Telmatobufo* species are stream breeders, and have similar habitat adaptations, it is possible that the whole genus will be severely threatened by climate change. While the effects of climate change might be negative for *Telmatobufo* and many other amphibian species (Araújo et al. 2006, D'Amen and Bombi 2009, Blaustein et al. 2010), some models show the effects could be positive for others. For example,

climate change is expected to have a positive effect on the New Zealand endemic *Leiopelma hochstetteri*, a stream-associated frog, mainly because both temperatures and precipitation are expected to increase (Fouquet et al. 2010). Despite this, current *L. hochstetteri* distribution is highly fragmented, and therefore the potential positive effects will be hampered by the lack and isolation of habitat (Fouquet et al. 2010). Some model projections have suggested global warming could be expected to expand distributions of some European amphibians when unlimited dispersal is assumed, as warming in the cooler northern ranges of species creates new opportunities for colonization; however, when limited dispersal is assumed, the effect of climate warming is expected to be negative for most species (Araújo et al. 2006). Therefore, Araújo et al. (2006) conclude that "the ability of species to occupy future climate spaces will depend on their ability to disperse, as well on the existence of suitable pathways for dispersal". While global warming could -at least in theory- have some positive effects for some species, the decrease in precipitation projected for some areas could have devastating effects for many amphibians, and the general concensus is that amphibians in general are highly threatened by climate change (Corn 2005, Walls et al. 2013, Foden et al. 2013).

The projected *T. bullocki* distribution model into future climatic conditions assumed all other variables remained unchanged. Although this is warranted for topographic variables (at least at this short-term timescale) it is unlikely that landscape composition will remain unchanged. Furthermore, because the model is strongly influenced by landscape composition variable, it is possible that the future distribution of *T. bullocki* and this genus will be wider or narrower than predicted depending on the land use changes in the next decades. Recent reports show that although native forest loss has slowed down during the

past decades as a result of protecting laws and management regulations, nonetheless, it is still occurring (CONAF 2008). Also, the degradation of native forest (reduction in quality) is on-going and is unlikely to change in the near future. Even inside protected areas, illegal harvesting of native forest for firewood and coal occurs regularly (pers. obs.).

On a positive note, major forestry companies present in Nahuelbuta have recently obtained Forest Stewardship Council (FSC) certification. Under FSC Principles and Criteria, the substitution of native forest by plantation is only permitted under some exceptional circumstances (Criterion 6.10, FSC (2012)). To obtain certification, some companies are committed to restore native forest to its pre-1994 conditions, to compensate for native forest substitution occurred since the establishment of FSC certification. This means that potentially thousands of hectares of native forest will be restored in Nahuelbuta in the near future. Depending on where and how this restoration takes place, the future distribution of *T. bullocki* might be affected in different ways.

2.4.3 Limitations

The distribution model and its projections were built under current knowledge of species presence. As with all models, it is a simplified representation of reality, and is strongly dependent on the data used for building it (Franklin 2009). Although the variables included in the model (discussed above) are all expected to be dominant drivers of *T. bullocki* distribution, other variables not included in the model could also be important, particularly at smaller scales. Local and microhabitat conditions are likely to limit habitat suitability, particularly at breeding sites (Chapter 3). For example, local stream conditions such as the availability of cover objects could be critical for reproduction (Chapter 3). This means that

if minimum habitat conditions are not met at a local scale, *T. bullocki* could be absent from areas even if the model predicts highly suitable habitat. Also the model presented here does not incorporate any variables related to ecological interactions with competitors, predators, prey and other species (that are largely unknown), all of which can influence *T. bullocki* distribution and habitat suitability.

Due to the low number of presence records used to build the model, it is possible that the sampling is not representative of the true range of environmental conditions occupied by the species, which would lead to an underestimation of suitable habitat. It is expected that with time, new knowledge about the species, including new presence records and better resolution and accuracy of predictive variables, will allow refinement of the model. Species distributions models are by their nature dynamic, particularly in rapidly changing landscapes, and should be revised whenever new information is available. Furthermore, remote sensing methods are advancing rapidly, and future modeling will be able to include much finer resolution of land cover variables. Better accuracy and resolution could allow testing hypothesis on the response of *T. bullocki* to more finely defined land cover types, such as different types of native forest (e.g. mature, secondary) or different stand age (e.g. clear cut, mature plantation).

2.4.4 Implications for conservation and management

The results obtained in this Chapter have important implications for, and can be applied in, the management and conservation of this species. The updated distribution and clear identification of presence points can be used to target research and conservation efforts (e.g. study species ecology, identify threats, study population trends). The predictive

distribution map can be used to target survey areas of high habitat suitability which are likely to hold new populations (Tarrant and Armstrong 2013). Also, it can be used by forestry companies to identify areas at highest risk of impact by upcoming harvesting operations, and thereby take appropriate management precautions against possible habitat disturbance. Furthermore, it allows for the identification of suitable areas that (provided *T. bullocki* presence is confirmed) could be established as protected areas or corridors to ensure habitat connectivity (Chapter 5). It can also be used to identify potential suitable areas for future translocations and reintroductions (Thorn et al. 2009).

Predicting species distribution and potential range shifts under climate change scenarios has been the focus of much research recently, and the potential impacts of climate change have been assessed for many species; including some amphibians (Ficetola et al. 2007b, Fouquet et al. 2010, Marquet et al. 2010). However, translating these findings into potential management options has been less straightforward. Shoo et al. (2011) identified several management actions that are applicable to minimizing loss of amphibian biodiversity under climate change including: installation of microclimate and microhabitat refuges, enhancement and restoration of breeding sites, and manipulation of hydroperiod or water levels at breeding sites. Nevertheless, the application of management actions to avoid the impacts of climate change remains a major challenge.

The identification of several *T. bullocki* locations, including the persistence of most historical populations is encouraging: *T. bullocki* still exists in the wild. Furthermore, the predictive suitability map suggests more populations could be found: there is still suitable habitat available in the wild. Nevertheless, the on-going degradation and loss of suitable

habitat suggest high levels of threat throughout the species' range. Therefore, protecting all remaining populations and suitable habitat should be the priority for *T. bullocki* conservation. Considering Nahuelbuta National Park appears not to represent optimal *T. bullocki* habitat, the establishment (or extension) of state-protected areas where the species can be protected in the long-term should be considered. Moreover, the grim predictions for future distribution under climate change make the conservation and management of *T. bullocki* populations and its habitat more important and urgent that ever. Not only we have to *take action*, but also we have to take action *now*, as tomorrow it may be too late.

Chapter 3: Movement patterns and habitat use

3.1 Introduction

Given the recent global declines in amphibian abundance and distribution, it has become increasingly important to protect threatened species and populations; however, due to the high diversity of life strategies and complex life cycles, defining effective protection areas is challenging. It is widely understood that protection of amphibian species should consider all life stages and therefore should include all types of habitat used (i.e. aquatic and terrestrial, Wells 2010). For stream-breeding amphibians, many studies have highlighted the importance of terrestrial habitat adjacent to streams, and have called for the protection of buffer zones (deMaynadier and Hunter Jr 1995, Vesely and McComb 2002, Semlitsch and Bodie 2003). However, not all stream-breeding species have similar terrestrial needs (Semlitsch and Bodie 2003). For example, some stream-breeding amphibians only move less than ten metres away from streams, while others, move hundreds of metres away (Crawford and Semlitsch 2007). Therefore, to protect individual species, it is essential to know what types of habitats are used and when each habitat is used.

3.1.1 Amphibian migrations and movements

For most seasonal breeding species, migrations from terrestrial habitat into breeding aquatic habitat (e.g. ponds, streams), and from breeding habitat into adjacent terrestrial

home ranges during the post-breeding season are common (Wells 2010). Within terrestrial habitat, amphibians often move through the landscape to find shelter and food. Whereas some species are highly vagile, most only move short distances (Wells 2010). In addition to daily movement patterns and seasonal migrations, longer dispersal movements between breeding sites are also common in amphibians. Daily, seasonal, and dispersal movement patterns are all important for population regulation, metapopulation dynamics and the long-term persistence of species (Semlitsch 2008). It is therefore essential for the development of effective management measures that the movement patterns and habitat use of a species are understood.

3.1.2 *Telmatobufo bullocki*

Telmatobufo bullocki (Schmidt 1952) is a rare and critically endangered stream-breeding amphibian. They reproduce in fast flowing mountain streams in Chile's temperate forests, in the coastal Nahuelbuta mountain range (Formas 1988). Habitat loss and fragmentation have been identified as main threats for *T. bullocki*, and there is an urgent need to protect this species' habitat (Veloso et al. 2008, Fenolio et al. 2013). Despite being historically observed inside protected areas, there are no recent records of the species inside Nahuelbuta National Park, despite several surveys. Furthermore, most of the known populations are in areas currently covered by exotic plantations, which poses a serious threat to the species (Chapter 2). However, since little is known about this species' ecology and behaviour, management and conservation measures are difficult to assess.

T. bullocki has been found in few occasions under rocks in streams, and under logs near the stream edge (Schmidt 1952, Péfaur 1971, Formas et al. 2001), and one specimen was

found under a log 150 m from a stream (Péfaur 1971). Moreover, the stomach contents of the paratype included terrestrial invertebrates, suggesting terrestrial feeding (Schmidt 1952). Although the reproductive habits and adaptations of the species suggest a strong relationship with stream and riparian habitat, the observations also suggest *T. bullocki* use terrestrial habitat. However, the extent of the species' terrestrial habitat use remains unclear. In this chapter, *T. bullocki* habitat use was described through the use of tracking techniques and surveys in both terrestrial and aquatic habitat. The main questions behind this study were: If we want to protect *T. bullocki,* what habitats should we protect? What constitutes essential habitat? The results were used to assess potential threats and develop management recommendations that could help effectively protect this endangered species, including the estimation of terrestrial core habitat.

3.2 Methods

3.2.1 Movement patterns and terrestrial habitat use

Study area

The location for the movement and terrestrial habitat use study, El Natri, is located in a section of Provoque Stream (2 km length), near the rural locality of San Ernesto, in the Elicura Valley, Biobío Region (37°54’ S, 73°13’ W, elevation 100 m). This section of the stream is of third order (sensu Strahler[5]), with an average width and depth of 6 m and 40 cm respectively. The streambed is rocky, with boulders, cobbles, and small sand banks. The

[5] In Strahler stream ordering, the smallest tributaries are first-order, and usually don’t have other streams feeding them. When two first-order streams join, then the stream becomes a second-order stream. Similarly, when two second-order streams join, the stream becomes a third-order stream.

channel flow is moderate to fast, with many riffles and pool sequences, with a mean gradient of 3%. The valley slopes surrounding the stream are steep and covered by a mosaic of native forest and monoculture pine (*Pinus radiata*) plantation (Figure 3.1). The native forest area is part of a fragment of nearly 400 ha that has low levels of disturbance. The native vegetation is characterized by evergreen trees including: Coihue (*Nothofagus dombeyi*), Lingue (*Persea lingue*), Tiaca (*Caldcluvia paniculata*), Olivillo (*Aextoxicon punctatum*), with a dense understory dominated by Quila (*Chusquea quila*) and ferns. This type of vegetation provides a continuous and dense cover. The pine plantation area is mature (24 years), and also provides a dense cover, but lacks significant understory. It appears the area was cleared using fire prior to plantation, as burnt snags were frequently encountered. Flat riparian zones are highly disturbed; they have been cleared of vegetation, leaving a strip of open area of approximately 100 m wide, covered by grass (Figure 3.1), with some patches of shrubs (mainly invasive blackberry). Terrestrial habitat surveys were only conducted on one side of the stream (West), due to a collaboration partnership with the landowners (Forestal Arauco), and because the East side had less suitable habitat (i.e. younger plantation, no native forest).

Frog sampling

Time constrained surveys were conducted during spring and summer seasons (January-February and October-November, 2012). Terrestrial habitat adjacent to the stream was surveyed at night looking for active frogs, and during daylight by actively searching under refuges (e.g. logs, rocks). Searches were conducted by two observers for at least one hour, using a visual encounter survey (VES) technique (Crump et al. 1994). All broad types of terrestrial habitats available were included (native forest, mature pine plantation, and

open areas by the stream). Due to steep terrain, dense vegetation, and to minimize habitat disturbance, surveys followed an existing network of logging tracks (in disuse), with frequent excursions into accessible off-track areas. These tracks originate in the stream and zigzag uphill, which allowed a continuous sampling of distances from the water. Surveys extended up to 800 m from the stream edge and 500 m of elevation. Although most of the terrestrial search effort was conducted in El Natri, several one-off terrestrial searches were conducted in other *T. bullocki* populations, and the results from all surveys will be included in some of the analyses.

Figure 3.1. El Natri (front) includes areas of native forest, pine plantations, and open grass riparian areas.

Frog and environmental measurements

Frogs were captured by hand and placed in new clean plastic bags before being processed. All frogs captured were measured (snout to vent length, SVL), weighed using a digital scale, and sexed according to the presence or absence of male secondary sex characteristics (nuptial excrescences in thumbs and under the jaw). To identify potential recaptures of the same individual, all frogs were photographed in a standardized way, and the use of natural markings for individual identification was used. Measurements of microhabitat temperature and relative humidity were taken with a probe from inside the burrow or other retreat site, or at ground level when frogs were not covered. The type of habitat (i.e. pine plantation, native forest, open), and microhabitat (substrate, cover) was recorded, and the location was taken with a GPS (Garmin eTrex Vista HcX), with an accuracy of at least 10 m. The point of capture was also marked with flagging tape, with the individual ID and date/time for movement analysis. The distance to the stream was measured on site with a tape if < 20 m, and derived from a topographic map using GIS if >20 m. Data on daily rainfall for the study period were downloaded from the online national hydrological database for the closest weather station in Contulmo, only 14 km from the study site (http://snia.dga.cl/BNAConsultas/reportes).

In order to be able to give managers an estimated size needed for terrestrial habitat protection adjacent to streams, the concept of “core habitat” delevoped by Semlitsch and Jensen (2001) will be used. Such protected areas are usually called “buffer areas”; however, Semlitsch and Jensen (2001) have argued such protection areas have a function that goes beyond buffering, as these terrestrial habitats are essential for many amphibians, therefore the term "core habitat" will be used instead of "buffer zones" (Semlitsch and

Jensen 2001). Core habitats have been delineated for many amphibians; however, the criteria used for such delineation is not standard. The most common criterion is the delineation of an area that encompasses at least 90% of the terrestrial population, but some studies have used 95% or even 100%. In addition to core areas, a (true) buffer zone should be added in order to mitigate edge effects (Semlitsch and Jensen 2001). While ideally 100% of the population should be protected, this may be unrealistic in practice. Here, core habitat will be defined as an area encompassing 90 - 95% of the terrestrial population sampled during the post-breeding season.

Fluorescent powder tracking

Fluorescent pigments have been used for tracking short-term movements and studying microhabitat use in many species including small mammals (Mullican 1988), reptiles (Furman et al. 2011), and amphibians (Eggert et al. 1999, Eggert 2002, Roe and Grayson 2008). This is considered a cost-effective and safe method for amphibians, but its effectiveness can be affected by both the species' movement patterns and environmental conditions (Graeter and Rothermel 2007, Orlofske et al. 2009). This tracking technique was tested and used in combination with radio-telemetry. Dry fluorescent pigments of two different colours were used (Pink and Green, Sterling 810 Series). The ventral surface and rear legs of adult frogs were covered with pigments. This was done by carefully placing the frog in a plastic container lined with a 1 cm layer of pigments, and allowing a few seconds for the powder to stick to the frog's naturally moist skin. The frog was then released to the same place where it was found and was left alone. The place was georeferenced with a GPS and marked on site with flagging tape. The site was re-visited the following night and a LED UV torch was used to reveal the trail left by the frog as it moved through the

landscape. The trail was marked with spray paint, and measurements taken the next day during daylight hours. Although multiple applications of pigments is considered safe (Rittenhouse and Altnether 2006), each frog was only manipulated once to avoid high levels of stress.

Radiotracking

Small external radio-transmitters, each with a unique frequency, were used in this study (ATS models A2412 and A2414, Advanced Telemetry Systems USA, and Holohil model BD-2X, Holohil Systems Ltd. Ontario, Canada). These transmitters have a very thin and flexible whip antenna, which was trimmed to a length of approximately 10 cm. The transmitters were externally attached to a subset of adult frogs, using a specially designed waist belt made of cotton strand, threaded through a very fine piece of surgical silicon tubing, and tied with a knot (Figure 3.2). Together the belt and the transmitter weighted 1 to 1.5 gr, which represents less than 2-4% of an adult's body weight. The belt was designed to rot and fall off in a couple of months (6-8 weeks), in the event that the frog could not be relocated or recaptured at the end of the study. The belt was first trialled on captive frogs using a surrogate species of similar size, and then tested in the field with two wild *T. bullocki* for a week to see if they developed any wounds or rubbing on their skin.

Figure 3.2. Adult *T. bullocki* with radio-transmitter belt attached.

After transmitter attachment, frogs were relocated daily using a receiver (R-1000 Communication Specialists Inc.) and a three element folding Yagi antennae. Relocations occurred during the day when frogs were hiding, and the signal was followed until the frog was found (visual contact) or the signal source narrowed down to a 1 m^2 area. To achieve this accuracy, the receiver's aerial antenna was removed, and the gain of the receiver lowered so only very close signals were picked up. The straight-line distance between fixes was measured with a tape when possible, or based on GPS coordinates in larger distances. Magnetic bearings of the total movement direction were taken with a compass and then transformed to geographic bearings. The bearing to Provoque stream was also recorded for each movement in order to assess whether movements were related to the direction of stream.

3.2.2 Aquatic habitat

Sites

Aquatic surveys were conducted at several sites within the Nahuelbuta Range during spring and summer months. Sites included historic and potential new locations. Each site consisted of a section of stream of 20 - 200 m (depending on accessibility). Some streams were surveyed at more than one site. Sites surveyed were constrained by access, as only few public roads exist in the area.

Larval sampling

Because no standardised technique had been previously developed for *T. bullocki*, first a few techniques were trialled: 1) day surveys with kick netting 2) nocturnal surveys with kick netting 3) diurnal VES, and 4) nocturnal VES. The kick-netting technique (commonly used in aquatic macroinvertebrate sampling) involved the observer, or a group of 2 observers working upstream of a D-frame net removing rocks and kicking the substrate, so the net collects any tadpoles that would become detached and carried with the flow. This technique has been used to survey amphibians with similar stream-dwelling larvae such as the tailed frog *Ascaphus sp.* and stream salamanders (Trumbo et al. 2013). The VES technique involved the observer/s using a viewing window (custom made out of a 6" PVC pipe and clear plastic) to look under the surface. All techniques were successful in detecting *T. bullocki* presence; however, the nocturnal VES proved to be the most efficient. Kick netting was successful for diurnal and nocturnal surveys; however, it had a few drawbacks. First, because it involves getting hands and arms in the water, this was not a

good option for colder streams (even when diving gloves were used). This limited the amount of time the observer could be working. Also, removing rocks and kicking the substrate disturbs the habitat, and is energy consuming. Furthermore, larger rocks could not be moved. On the other hand the VES technique proved to be very efficient in detecting tadpoles, particularly in nocturnal surveys when the tadpoles were active. It is limited if the stream is silted (low visibility) or in sections of very fast-flowing water (white waters) as air bubbles affect visibility.

To minimise habitat disturbance, and due to the higher rate of success, the passive nocturnal VES technique was deemed the most appropriate and was therefore used in most of the surveys. At each site, two observers (wearing waders), each carrying a viewing window, a head-torch, a diving torch, and a small hand net (used in fish aquariums) slowly and thoroughly moved upstream from an initial point, in parallel, one on each side of the stream scanning the streambed for tadpoles (Figure 3.3). When a tadpole was detected, depending on the depth and accessibility it was either caught by hand or using the net and placed inside a plastic zip-lock bag half filled with stream water. The bag was left in the side of the stream and flagged, and the search continued. Stream sections of at least 100 m were targeted; however, sometimes due to deep pools or blockage from big trees the surveyed length was shorter. At the beginning and end of the survey, a GPS point (including a timestamp) was made. After finishing the survey, the bags with tadpoles were collected on the way back to the initial point where tadpoles were measured (body length and total length), and photographed. Tadpoles were returned to their point of capture after measurements.

Stream habitat characterisation

Due to time constrains, only streams where *T. bullocki* were confirmed to be present were characterised. Stream wetted width (i.e. the width of the stream at the time of survey) was measured at spaced intervals along the survey site with a tape. The depth was measured with a calibrated pole in transversal transects to include depths at the edges of the stream and in the main channel. Several transects were done in each site (at least 3) depending on accessibility. Depth measurements were taken every one step (1 m approx.) to the nearest 5 cm. Physicochemical water parameters were measured using a handheld multiparameter probe (YSI Professional Plus). Parameters measured included temperature (ºC), dissolved oxygen (mg/L), specific conductance (µS/cm) and pH. Three repeated measurements were taken from the start area in different parts of the stream (e.g. pool and riffle) to include potential variability in the parameters. Stream order (Strahler) and gradient (%) was calculated in GIS, based on the GPS coordinates for the start and end points. A general description of the stream was made including substrate (e.g. cobbles, boulders), level of turbidity (low, medium, high), and surrounding vegetation.

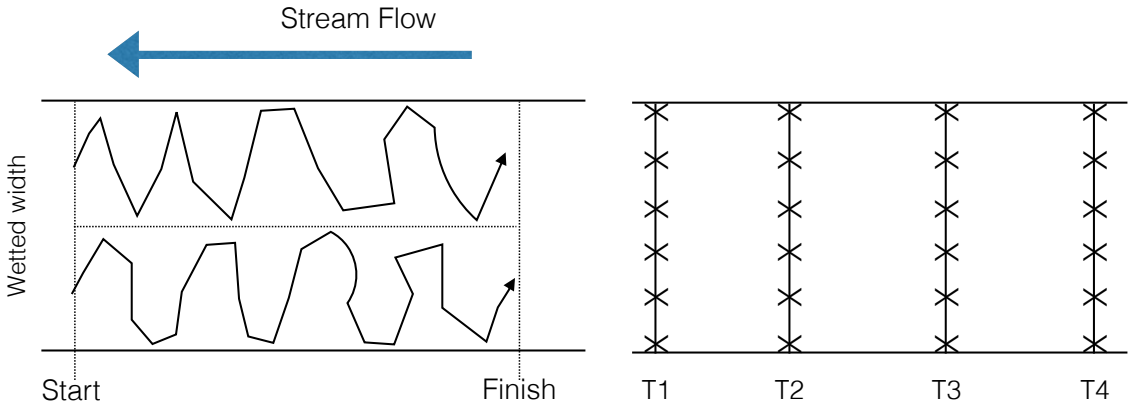


Figure 3.3. Diagram showing the VES surveying technique (left) and the stream measurements (right). The VES technique consisted of two observers moving upstream in parallel, visually scanning the streambed for tadpoles (using a viewing window and diving torch). Stream measurements included several wetted width measurements spaced through the sampling area (T1 - T4) and several depth measurements at each transect (crosses over T1 -T4) spaced approximately 1 m apart.

3.2.3 Data analysis

Many of the results were presented in a descriptive and semi-quantitative way. However, some statistical analyses were done. The software ORIANA v2 was used for directional movement analysis. Rayleigh's test for uniformity was used to determine if movement bearings were uniformly distributed (Fisher 1995). Spearman's and Pearson's correlation analyses and Kruskal-Wallis tests were done in SPSS v21. Significance levels were set at $\alpha = 0.05$. Means are presented with standard errors (SE).

3.3 Results

3.3.1 Terrestrial captures

Thirty-seven individuals were found in El Natri during 46 terrestrial surveys (306 observer hours): 6 juveniles, and 31 adults (18 females, 12 males, 1 unknown). In addition, two

juveniles were found dead, with signs of predation. Captures ranged from 0 to 8 frogs per night and from 0 to 1.33 frogs per hour. *T. bullocki* was found in 15 sampling occasions, which gives a *naive* estimate of species detection probability of 0.35. The capture rate was positively correlated to the amount of rainfall during the previous 24 hours (Pearson's r = 0.795, two-tailed $p < 0.01$, $N = 42$). Frogs were generally observed scattered in the landscape; however, a relatively high density of frogs was observed during late October, after a period of heavy rain when 7 frogs (5 males, 2 females) were found in a open grass area of approximately 20 m x 120 m adjacent to Provoque stream. These frogs were close to the stream (<20 m) and were facing away from the stream, suggesting they had recently left the water. Therefore, a density of 5.8 frogs per 100 m of stream, and a male territory size of 26 m of stream were estimated. A photographic catalogue was compiled, and individuals were identified using natural markings. All individuals had a unique dorsal gland pattern (size and disposition of glands), and unique shape and colour of the characteristic yellow inter-ocular spot. There were no re-captures (except for tracked individuals). In addition, 23 frogs were found in terrestrial habitat in other populations sampled. In total 60 frogs were found in terrestrial habitat (13 juveniles, 47 adults).

3.3.2 Terrestrial habitat use

Telmatobufo bullocki adults and juveniles made extensive use of terrestrial habitat adjacent to Provoque stream during the post-breeding season. Individuals were found from 0.5 m to 480 m from the stream (Table 3.1). The distribution of distance to the stream was different for females than males (Kruskal-Wallis Test, $P = 0.01$), with males generally closer to the stream than females. The distance to the stream was higher for adults than juveniles; however, this difference was not statistically significant. Table 3.1

summarises the occurrence distances to Provoque stream in El Natri. For a protected area to include 90% and 95% of the sampled population, terrestrial habitat up to a distance of 220 m and 240 m from the stream would need to be protected as core habitat respectively.

Post-metamorphic (juveniles and adults) frogs were found under native forest (27%) and mature plantation (41%) cover; however, most sightings occurred near the edge between both. It is possible more frogs were found under pine plantation cover as the lack of understory made detection easier. No frogs were found in core plantation areas more than 56 m away from native forest (mean = 21.9 m, SE = 3). Some individuals were found uncovered in open (non-forested) riparian areas active during the night (32%), either leaving the stream and moving towards the forest, or standing by the stream edge. Juveniles were also found in open riparian areas, during the day, hiding under logs and rocks by the stream.

Table 3.1. Descriptive statistics for occurrence distances (m) to Provoque stream for each age and sex class.

Class	N	Mean	Median	Min - Max	SE	SD
Juveniles	8	68.4	25	0.5 - 220	32.5	92
Adults*	31	124.1	130	0.4 - 480	19.9	111.1
Females	18	164.8	192.5	9 - 480	27.1	115
Males	12	53.3	19.5	0.4 - 170	17.8	61.6
Total	39	112.7	70	0.4 - 480	17.4	108.7

* Includes females, males and one unmeasured individual

When all terrestrial captures were considered (not just El Natri captures), the mean distance to stream was 94 m (SE = 12.5, *N* = 60). Similar to El Natri population, the distribution was different for males than females (Kruskal-Wallis Test, *P* = 0.01), with males closer to the stream than females. The frequency of captures at different distances to stream, according to sex and age for the full sample (*N* = 60) is shown in Figure 3.5. Frogs were found closer to the stream during October, and were farther away from November to January (Figure 3.6); however, sample size for January and February was very low. There was a significant correlation between distance to stream and frog size (Spearman's ρ = 0.424, 2-tailed, *P* < 0.01).

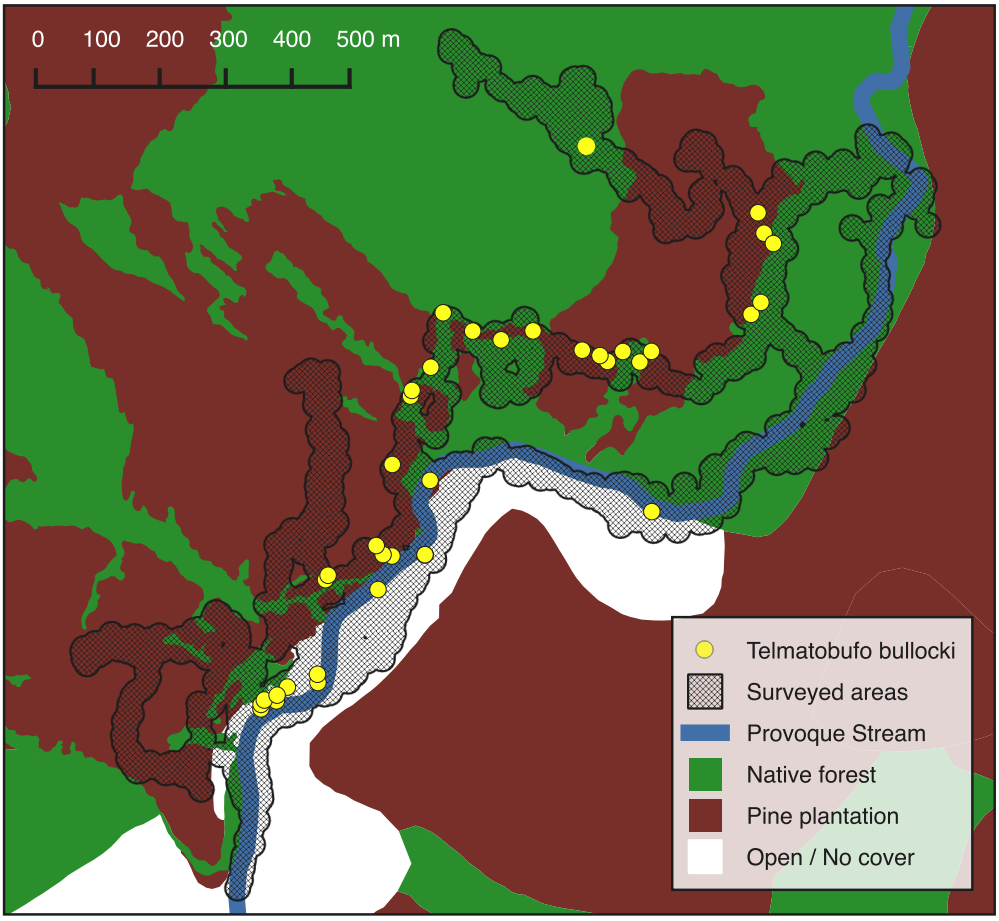


Figure 3.4. Map of the study area showing land cover type, surveyed areas, *T. bullocki* points and Provoque stream.

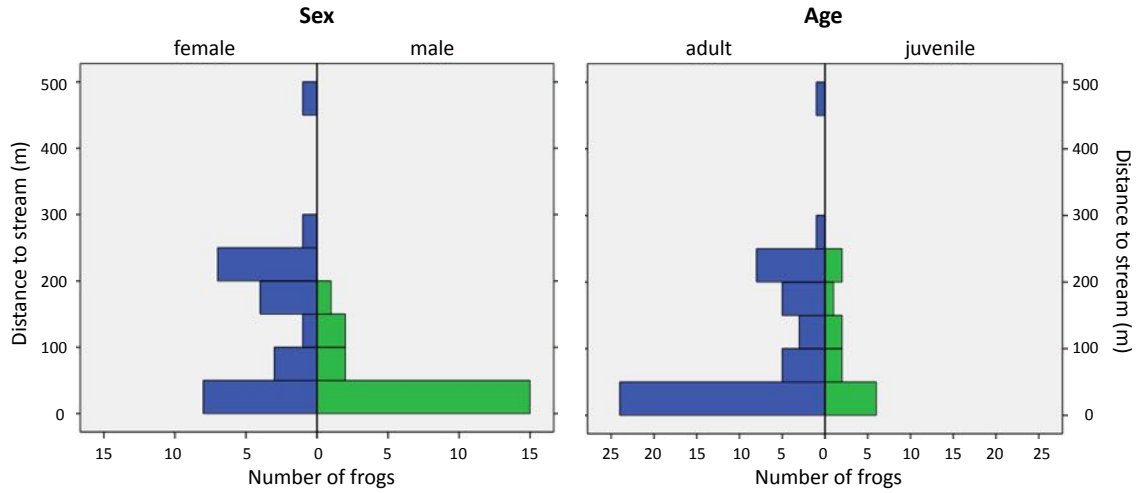


Figure 3.5. Frequency histograms of distance to stream according to sex (left) and age (right) for the full sample (*N* = 60).

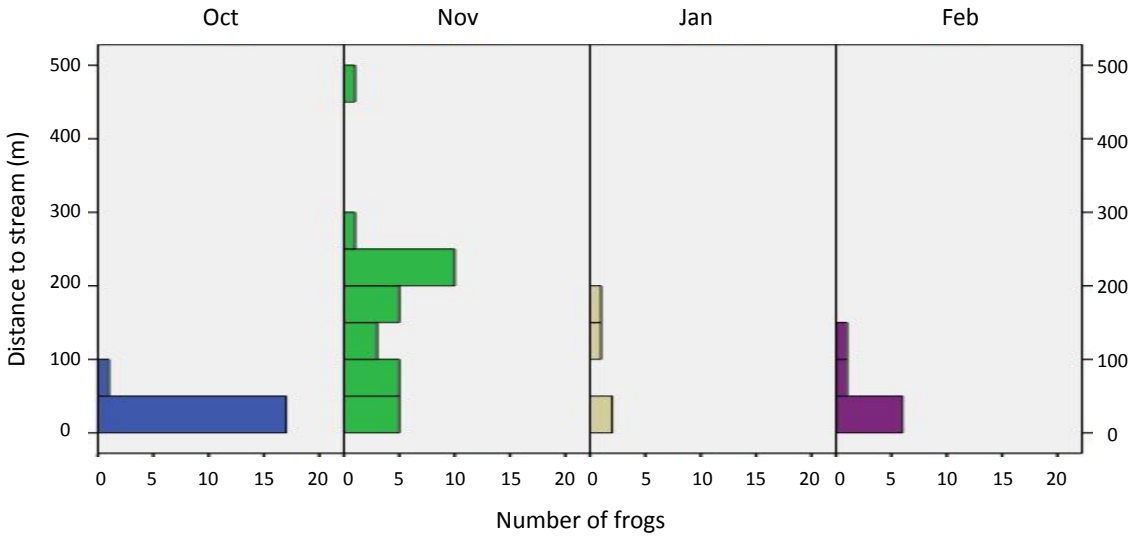


Figure 3.6. Frequency histogram for distance to stream by month of capture for the full sample (*N* = 60)

Terrestrial microhabitat

Frogs used a variety of terrestrial diurnal and nocturnal microhabitats. Diurnal refuges included logs (coarse woody debris more than 10 cm diameter), branches (woody debris

less than 10 cm diameter), rocks, vegetation, leaves, pine needles (Figure 3.7), and underground burrows (Figure 3.9). When found active during the night, frogs were either uncovered (Figure 3.8) or partially covered with only their head visible. They were frequently found at the entrance of burrows, with their heads out, likely waiting for prey (Figure 3.9, right). Some frogs were observed to burrow when released after measurements, digging with their hind legs and moving backwards in soil or leaf litter. One tracked individual was found to burrow deeper into soil and leaf litter substrate in consecutive days, reaching about 10 cm deep, but the burrow was abandoned after a period of rain. Another tracked frog was found at the end of a 36 cm long tunnel (20 cm deep underground) in a steep slope area (Figure 3.9, left). One female remained 17 days at the same burrow, under approximately 20 cm of rock and soil, without any activity, leaving the burrow after a period of heavy rain. Microhabitat temperature and relative humidity conditions averaged 11.2 °C (min = 7.7, max = 15.5, SE = 0.46) and 91.9% (min = 80.5, max = 96.8, SE = 0.96) respectively, and did not differ significantly between native forest and mature pine plantation (t-test, two-tailed $P > 0.05$).

Figure 3.7. Pine needle microhabitat: diurnal refuge (left, arrow points to hiding frog), and active frog in plantation area during the night (right).

Figure 3.8. Active adult frogs in the night.

Figure 3.9. Inactive *T. bullocki* inside burrows (left) and active frog at the entrace of burrow (right).

3.3.3 Movement patterns

Tracking

None of the two frogs tracked during the trial period developed wounds. They were both captured by the stream and remained within 5 m from their capture points, sometimes being relocated under rocks in the stream. After the trial period, transmitters were removed. Because the trial period was earlier in the season (late September), this movement data is not included in the results below. For the main study, seven adults (five females and two males) were radio-tracked for 12-25 days (average 19.3 days) during late October and early November. In addition 13 frogs were tracked from one to 12 nights using fluorescent powders only. Including radio-tracked and pigment-tracked data, a total of 37 movements were registered and included in the analysis. Fluorescent powders were effective for tracking small movements, with a mean detected path length of 14.6 m (range: 2 - 50 m, N = 15, SE = 3.0). Five trails were lost under wet weather conditions, and were excluded from analysis.

Patterns of activity and distance moved

Frogs did not move every night, and typically remained inactive for most of the tracking period. Periods of high activity were related to wet weather conditions, while frogs remained mostly inactive during dry weather. The average distance moved per night of activity was 20.4 m (SE = 5.6, N = 32). Most movements were short (<20 m), but some movements were >20 m, and the greatest distance moved was 170 m (Figure 3.10). Females moved a mean distance of 25.1 m (SE = 8.4, N = 21) and males 7.6 m (SE = 2.0, N = 9), but this difference was not statistically significant (t-test, P = 0.19). Based on the

above, two different types of movement could be identified, short and frequent daily movements (<20 m), and few but longer migration movements (> 20 m).

Two frogs, one male and one female, were radio-tracked earlier in the season (late October). They were both first found uncovered on a grass area less than 20 m from the stream after a heavy rain. They both returned to the water by the next day. The female stayed for 16 days in the same underwater refuge (Figure 3.15, left), a small cavity under boulders next to the edge, with constant but moderate flow in an area covered by riparian vegetation. She was located outside the stream only once, by the waters' edge during the night (possibly foraging), but returned to the same refuge by the next day. The male moved only small distances within the stream (<5 m), but due to strong flow, the exact hiding place could not be identified.

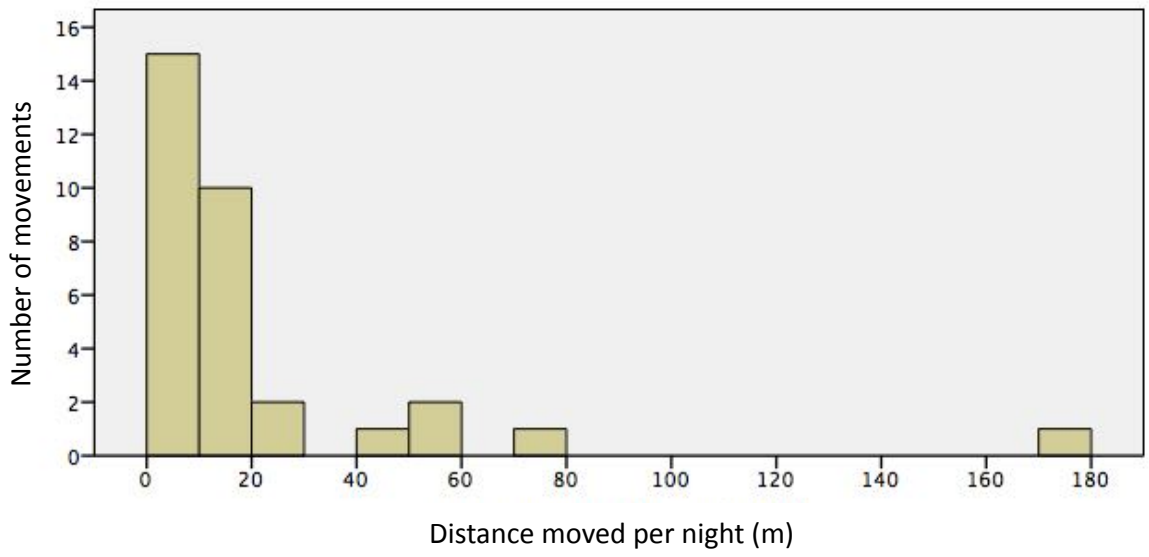


Figure 3.10. Frequency histogram of distance moved per night of activity. Includes all movements recorded from males, females and juveniles (total movements *N* = 32), using both radiotracking and fluorescent powders.

Direction of movement

Frogs did not move in random directions (Rayleigh's Uniformity Test Z = 5.59, $P < 0.01$). The average movement direction was moving away from the stream (Figure 3.11). The mean bearing of all movements was 283° (N = 31). In addition, some frogs were directly observed moving away from the stream and uphill, attempting to climb very steep slopes (nearly vertical). When unsuccessful, they bypassed the steep sections and continued uphill. This behaviour shows a strong directional movement, as they insisted in going uphill even when it was the most difficult direction to move.

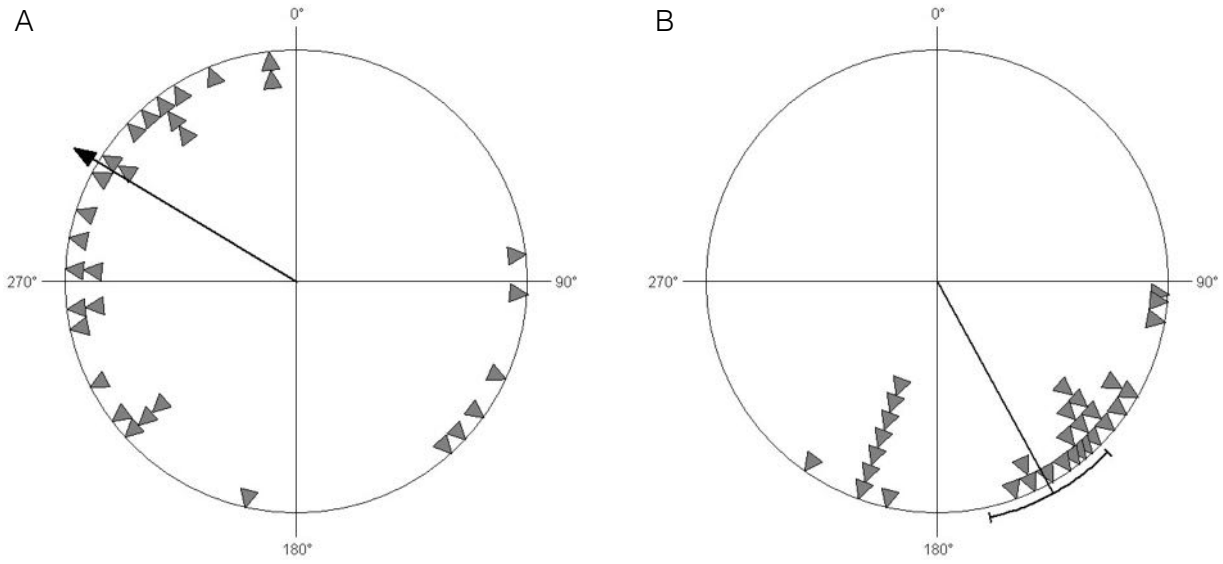


Figure 3.11. (A) Direction of movements (N = 31). The arrow is the mean weighted vector (mean direction weighted by distance moved). (B) Direction to the stream at the beginning of each movement, black line is the average with 95% CI.

3.3.4 Aquatic habitat use

Characterisation of *T. bullocki* breeding streams

Streams with *T. bullocki* presence (eggs, tadpoles, or adults) ranged from first to third order and were located from 90 to 1020 m of elevation. Physical measurements and physicochemical parameters for 15 sites are summarised in Table 3.2, and detailed in Appendix C. Streams were generally characterised by clear, cold, and fast-flowing water, with pool-riffle sequences. Riffles were characterised by coarser materials such as cobbles and large boulders, while pools were generally made up of finer gravel, sand and silt. Some streams (particularly smaller ones) had a completely closed canopy cover of dense vegetation, while others had partial or no canopy cover. The gradient of streams was generally moderate (<10 %) but a few sites were steeper (>10%) with small waterfalls and deeper pools. Details including coordinates and parameter measurements for each site can be found in Appendix C.

Table 3.2. Physical measurements and physicochemical parameters of water for streams with confirmed *T. bullocki* presence (*N* = 15).

	Min	Max	Average	SD
Average stream width (m)	2.5	10.9	5.6	2.3
Average stream depth (cm)	5.3	37.0	20.8	7.6
Stream gradient (%)	5.0	23.0	10.4	4.8
Temperature (ºC)	5.2	11.7	9.3	1.7
Specific conductance (µS/cm)	18.6	54.1	30.7	11.0
Disssolved oxygen (mg/L)	9.1	11.7	10.8	0.6
pH	6.2	7.7	7.3	0.4

Eggs

One egg mass of approximately 200 eggs was found the 18th of October 2013 in one of the historical *T. bullocki* locations (Rucapehuén, Quebrada de Caramávida) at an elevation of 800 m asl (Moreno Puig 2014). The identity of the eggs was confirmed as *T. bullocki* using mitochondrial DNA sequencing (cytochrome oxidase I subunit). The eggs were hidden under rocks (≈30 cm diameter) in the stream channel at a depth of ≈30 cm. The egg mass formed a cluster (Figure 3.12), attached together and to the substrate by a strong sticky substance. Although the cluster was in fast-flowing water, the eggs were protected from the direct current by rocks (i.e. they were not exposed). This type of reproduction corresponds to *Mode 2* (i.e. eggs and feeding tadpoles in lotic water, Duellman and Trueb 1986 p. 26). The eggs were big, with a diameter of 10 mm (including capsule of 2 mm). The stream was 6.3 m wide and average depth of 27 cm. The stream was cold, clear and with little fine sediment (temperature = 5.2 ºC, specific conductance = 18.6 µS/cm, dissolved oxygen 11.7 mg/L, and pH = 7.23), and had a full native canopy cover. An adult male *T. bullocki* was found submerged close to the egg mass (<20 cm), hiding under rocks, suggesting possible parental care.

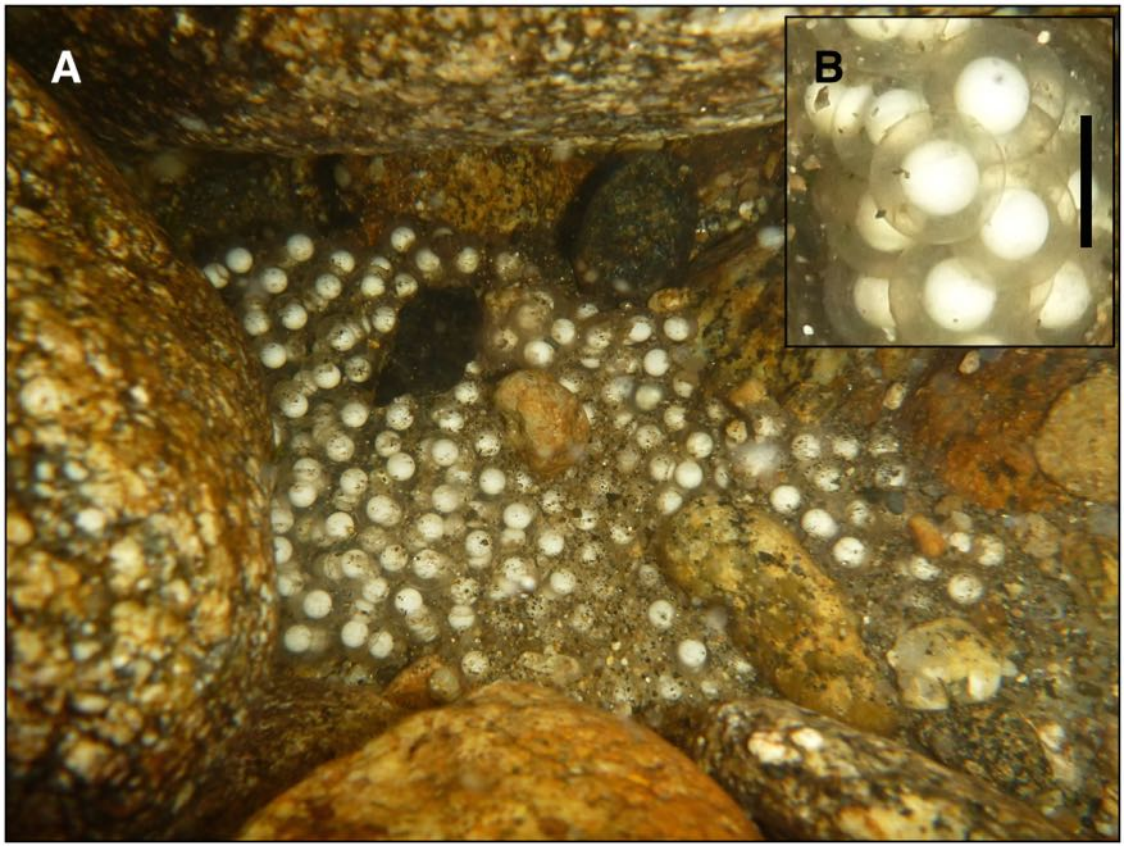


Figure 3.12. Egg cluster of approximately 200 eggs (A), and detail of eggs (B), bar size 1 cm.

Tadpoles

Ninety-three tadpoles were captured and measured during the months of October, November, and December (2013) in 18 survey sites. In addition, one tadpole was captured the 6th of January and one the 1st of October (2012). Tadpoles were found at several developmental stages (Appendix E). Exact Gosner stages of development (Gosner 1960) were difficult to assign in the field due to the presence of a large skin fold concealing hind limb development. For convenience, and to avoid prolonged manipulation of tadpoles, the size and mass of tadpoles were recorded instead. The size frequency of tadpoles is shown

according to month of sampling in Figure 3.13. Tadpoles of different sizes and at different stages of development were found simultaneously (for an example see Figure 3.14).

Tadpoles were mainly found in riffles always attached to rocks and boulders (>10 cm diameter) and never found lying on sand, finer sediment, or attached to logs. Tadpoles were present in streams from 2.5 to 10.9 m average width and 5.3 to 37 cm average depth. The relative abundance of tadpoles (where present) ranged from 0.6 to 60 tadpoles per 100 m (mean = 15.8, SE = 3.7, N = 18), and from 0.5 to 18 tadpoles per hour of survey (two observers, mean = 6.2, SE = 1.3, N = 18). Tadpoles were generally found dispersed; however, younger tadpoles were sometimes found more aggregated (several tadpoles attached to the same rock). Relative abundance for each site can be found in Appendix C.

There was a strong and significant negative correlation between number of tadpoles per 100 m and average stream width (Spearman's ρ = -0.75, two-tailed P <0.01, N = 14), and average stream depth (Spearman's ρ = -0.723, two-tailed P <0.01, N = 14), and a moderate positive correlation with water temperature (Spearman's ρ = 0.564, two-tailed P <0.05, N = 13). The number of tadpoles per hour was moderately and negatively correlated with elevation (Spearman's ρ = -0.578, two-tailed P <0.05, N = 18), and more strongly with average depth (Spearman's ρ = -0.652, two-tailed P <0.05, N = 14), and strongly positively correlated with temperature (Spearman's ρ = 0.643, two-tailed P <0.05, N = 13).

Overall, results suggest tadpoles are present in streams with a relatively wide range of characteristics (Appendix C); however, relative abundance was greater in smaller streams (2.5 to 6 m width and 5 to 25 cm depth) at moderate elavations (<500 m). Such streams

were typically characterised by comparatively higher water temperatures than larger streams and streams at higher elevations.

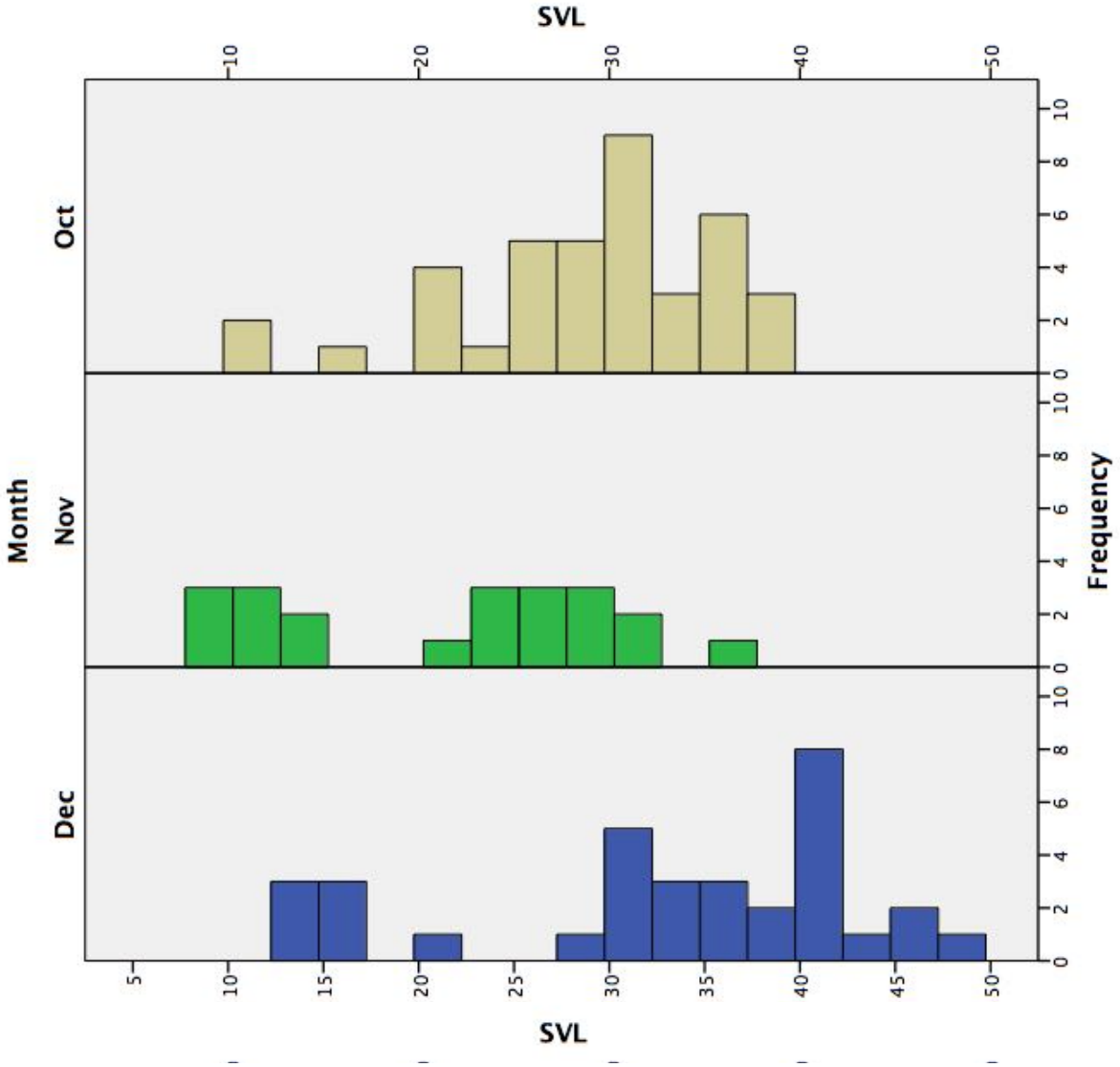


Figure 3.13. Histograms showing tadpole size (snout to vent length, mm) each month (October *N* = 39; November *N* = 21; December *N* = 33).

Figure 3.14. Two tadpoles at different stages found simultaneously (December 2013).

Adults

Adults were found in the streams, submerged underwater on six occasions from October 5th to December 17th 2013; however, five of these observations occurred during October. Frogs used cavities under big rocks and boulders (Figure 3.15) both in the main channel and in the edges of the stream. Observations included three males, two females and one undetermined.

Figure 3.15. Aquatic microhabitat used by adult *T. bullocki*: frogs were found submerged hiding under big rocks and boulders.

3.4 Discussion

The results demonstrate that *T. bullocki* has complex habitat requirements. They not only need streams as essential breeding habitat, but they also make extensive use of terrestrial habitat during the non-breeding season, moving up to 500 m away from breeding streams, and possibly more. This seasonal change in habitat use is commonly observed in aquatic-breeding amphibians, as terrestrial habitats usually provide better food and shelter resources (Wells 2010). Both females and males were found in terrestrial habitat away from streams during the non-breeding season; however, females were observed farther from streams than males. This difference has been observed in many prolonged breeding amphibians, as there is strong selection on males arriving early to breeding sites, and remaining close to the stream could represent a potential advantage (Wells 2010). Also, larger movements and home ranges of females have been attributed to the greater energetic requirements of females compared to males (larger size, egg production), or

better locomotor capacities of larger females (Wells 2010). This is possible for *T. bullocki* as females were found to be larger than males (sexual size dimorphism described in Appendix D).

A migration movement of adults from stream breeding habitat to terrestrial habitat was observed during early November, suggesting the end of the breeding season. However, adult frogs have been found in the water from August to January (this study, historic observations), tadpoles have been found from October to January (this study), presence of oocytes in January (Formas et al. 2001), and secondary sexual characters of males have been observed from July (in captive animals held at Santiago National Zoo) and August (Péfaur 1971), to March (E. Flores pers. comm.). It is clear from these observations *T. bullocki* has a prolonged breeding season (Wells 1977), possibly spanning over 6 months. In addition, differences in local characteristics such as elevation, can also induce variations on the onset and length of breeding season (Wells 2010). Considering the wide range in elevation in Nahuelbuta, such variations could be expected. Unfortunately, in this study it was not possible to collect data throughout the year, therefore the beginning of the breeding season or the presumed migration from terrestrial to aquatic breeding sites was not directly observed.

T.bullocki was detected in different types of terrestrial habitat, from undisturbed native forest, to exotic pine plantation and open riparian zones. While the greatest proportion of frogs were found under mature pine plantation cover (41%), it is important to highlight that detectability was uneven across cover types; it was much easier to spot frogs under pine plantation and open areas due to a lack of understory and vegetation, while native

forest had dense understory making it much harder to detect individuals. These results suggest that *T. bullocki* can tolerate mature exotic pine plantations, but it possibly depends on the close presence of native forest as despite an increased detectability, no frogs were found in core plantation areas. The native forest in this study is a large fragment with little disturbance, which could be acting as a reservoir, from which frogs disperse to neighbouring plantation areas. Although plantations seem to provide good cover and microhabitat conditions (e.g. frogs were found in pine needle burrows, there was no difference in microhabitat humidity or temperature between pines and native forest), they differed in many aspects, including the amount and quality of coarse woody debris and understory vegetation cover (however this was not explicitly quantified). Further studies are needed in order to determine if plantations provide good quality habitat regardless of native forest proximity, or if plantations could be acting as ecological traps (Schlaepfer et al. 2002), or potentially producing local source-sink dynamics (Pulliam 1988).

Previously, *T. bullocki* had been found mainly under logs and rocks, presumably because these hiding places had been more frequently searched during field surveys. In this study, radio-tracking allowed me to describe the use of a diversity of hiding retreats, including leaf litter, pine needle and underground burrows. The results suggest this species could be an opportunistic burrower during dry weather, rather than a strict burrower. Although typical burrowing behaviour was observed in the field (backward movement using rear legs to dig), and this was also observed in captivity (Péfaur 1971), it is unclear if frogs also use burrows made by other species (e.g. rodents). A few burrows were deep, which suggest these might have been mammal burrows. Also, frogs spent significant amounts of time in their burrows (≈3 weeks) during dry weather, without any activity, only leaving their

burrows after periods of heavy rain. This fossorial behaviour could partially explain the rareness and low detectability of the species, and indicate population sizes could be higher than previously thought.

As for all stream-breeding amphibians, stream habitat is essential for the reproduction of *T. bullocki*. For the first time, the eggs of *T. bullocki* were found, which is a significant advance in the knowledge of the reproductive ecology of the species. Aquatic habitat is also essential habitat thoughout the larval stage, which is presumably long. Long larval stages have been observed in other mountain stream amphibians such as *Ascaphus truei* and *Heleophryne purcelli*, and it has been related to the cold temperatures (leading to slow growth), and the large size needed to reach metamorphosis (Duellman and Trueb 1986). In fact, *T. bullocki* streams were characterised by cold water (mean 9.3º C), and the most developed tadpoles found reached > 40 mm SVL in size and > 20 g in weight. Therefore, it is possible *T. bullocki* tadpoles overwinter. Adults were found in the water during most of the spring and summer months. The prolonged breeding season and the presumably long larval stage suggest aquatic habitat is essential not just during a few months of the year, but possibly through the whole year.

T. bullocki breeding streams were characterised by a rocky bed, which creates the essential microhabitat for egg deposition, tadpole and adult shelter. Moreover, *T. bullocki* was absent from sections of streams that were highly silted (i.e. large rocks embedded in sediment) or that had a mainly sandy base due to recent clear-cut (data not shown). These results highlight the importance of protecting streams from siltation. Plantations and clear-cutting are known to increase sediment load in streams (Semlitsch et al. 2009), and this is

likely to have a significant negative effect on *T. bullocki* populations. Although most of the streams with *T. bullocki* presence were fast-flowing and relatively big (average width 5 m), a few of them were surprisingly small, and the smallest stream with larval presence was only 2 m wide and 5 cm deep. This suggests that although *T. bullocki* is adapted to fast-flowing water, they also have a wide tolerance range. While such small streams might represent sub-optimal habitat, these results indicate they are able to tolerate such conditions, which may be particularly important in the face of predicted decrease in precipitation due to global climate change (Chapter 2).

This study is limited by the overall small sample size and the short-term nature of the tracking study. Nevertheless, the results obtained can be considered as an important advance in the knowledge and understanding of this species' ecology and behaviour, with important implications for conservation and management. The presence of *T. bullocki* up to nearly 500 metres away from the breeding streams, and the estimated size of core habitat (220 - 240 m) suggest current regulations on buffer area protection (5-30 m depending on size of stream and slope) are not nearly enough to protect this species. Under best-case scenario (30 m buffer zone), only 35% of the sampled animals would have been within protected habitat.

This current lack of protection of *T. bullocki* terrestrial habitat is possibly one of the main threats for the species, as most of the breeding streams are currently surrounded by commercial plantations, which will be harvested periodically (every approximately 23 years). Even if good quality native habitat is present not far from streams, clear-cutting on riparian zones can have a significant impact, as it would disconnect the breeding habitat

from the terrestrial habitat (i.e. habitat split). It is therefore important in such situations, to consider an alternative management, for example by leaving un-harvested strips as corridors connecting both habitats. Fortunately (and perhaps not coincidently) some of the breeding streams have relatively large adjacent fragments of native forest that could be preserved as core habitat (e.g. El Natri). It is therefore important that these native fragments receive some kind of protection, as illegal wood extraction and forest degradation is an ongoing threat.

The weather-dependent movement patterns and capture rates, and the burrowing behaviour during dry weather, demonstrate that the species has an imperfect detection probability (detection probability less than one). This has important practical implications for monitoring and sampling, and should be considered when designing future surveys (Mackenzie 2005). Although the overall species' detectability is very low, it can be increased if surveys are done during the night and under wet weather conditions (i.e. after periods of heavy rain). High variability in detection probability can significantly bias survey results, and should be dealt with if long-term monitoring programs are established or if any comparisons (spatial or temporal) are to be made. It is also important that any risk-assessment surveys conducted within Nahuelbuta (e.g. required for hydroelectrical power plants, or other land development) are conducted when detection probability is greater. Furthermore, the results suggest at least 3 visits may be needed to detect the species in terrestrial habitat. The prolonged breeding season and presumably long larval stage, on the other hand, suggest *T. bullocki* tadpoles could be found during most of the year.

T. bullocki is currently a priority species for conservation (Baillie and Butcher 2012) facing many threats, most derived from the forestry industry. Clear-cut harvesting not only reduces the availability of core terrestrial habitat but also has an impact on the species' aquatic breeding habitat. In this study I propose a core habitat size of 220 - 240 m from breeding streams (plus 20- 50 m of buffer) should be protected (maintain full canopy cover) in order to preserve *T. bullocki* populations and protect their habitat from clear-cut operations. However, there are several other native and threatened animals that share the same habitat that would benefit from such protected areas, including other amphibians (e.g. *Eupsophus contulmoensis*, *Alsodes barrioi*, *Alsodes vanzolinii*, *Rhinoderma darwinii, Calyptocephalella gayi, Hylorina sylvatica*), birds (e.g. torrent duck *Merganetta armata*) and mammals (e.g. the iconic Darwin's fox *Pseudalopex fulvipes*, and the marsupial *Dromiciops gliroides*). Among these, the only species' that have received some conservation attention are the critically endangered amphibian *Alsodes vanzolinii*, for which some small fragments of native forest embedded in plantations have been preserved by forestry companies, and the Darwin's fox, which has been monitored and studied for the past decade. Furthermore, protecting wide riparian areas will have a significant positive effect on stream water quality, benefiting many aquatic species, some of which are also highly threatened (e.g freshwater fish) or microendemic (e.g. anomuran crab *Aegla bahamondei*). *T. bullocki* habitat could act as an effective umbrella, protecting not only this enigmatic species but also the whole unique and rich stream and riparian ecosystems in Nahuelbuta.

Chapter 4:
Genetic diversity and spatial structure of populations

4.1 Introduction

The world is currently facing unprecedented loss and degradation of biodiversity, mostly caused by human activities. Many amphibian species and populations are facing decline and extinction world-wide (Beebee and Griffiths 2005), and conservation management of populations is needed more than ever (Wake and Vredenburg 2008). Along with species and ecosystem diversity, genetic diversity is recognised as one of the fundamental levels for biodiversity conservation (McNeely et al. 1990). However, genetic information is often lacking, particularly for rare and threatened species. Recently, with the development of more affordable and automated molecular techniques, the study of population genetics has become an increasingly important tool for conservation.

Conservation genetics approaches allow us to understand current patterns of genetic diversity, and past evolutionary history, both of which are crucial knowledge needed for most management decisions. Low levels of genetic diversity can lead to increased inbreeding and reduced fitness, while high levels can increase the resilience of populations and reduce extinction risk (Reed and Frankham 2003, Rowe and Beebee 2003, Allentoft and O'Brien 2010). Low genetic diversity could be an indication of recent bottlenecks

caused by fragmentation of habitat (increased isolation of populations), but can also be related to historical processes, such as postglacial colonisations (Ficetola et al. 2007a). Therefore, understanding the level and patterns of genetic variability, along with the history of populations, helps to identify and prioritise populations at risk, and optimize management outcomes. The long-term goal in genetic management is to preserve species as dynamic entities capable of coping with environmental change (Frankham et al. 2004).

Conservation genetics takes advantage of modern molecular methods (e.g. PCR, DNA sequencing) and non-destructive sampling to aid in the conservation of wild populations. Several types of genetic markers (specific gene or DNA sequence) have been used; however, two of the most commonly used are mitochondrial DNA (mtDNA) sequences and nuclear microsatellites (Beebee 2010). Both markers are well suited to most conservation applications (i.e. intra-specific diversity, contemporary structure). Nevertheless, they have important differences and have therefore been used to answer different conservation issues (Wan et al. 2004). Mitochondrial DNA is maternally inherited (i.e. haploid genome) and undergoes no recombination. It has been particularly useful for phylogeographic studies, tracing the origin of populations back to millions of years (Avise et al. 1987, Beebee 2010). The non-coding mtDNA control region (CR) in particular, has higher rates of mutation than protein-coding regions, which makes it a useful marker to detect intra-specific population structure (Wan et al. 2004).

On the other hand, nuclear microsatellite markers are biparentally inherited (i.e. diploid) and codominant. Microsatellites are non-coding regions of genomic DNA consisting of sequences of variable number of tandem repeats (e.g. di-, tri- tetranucleotides), with

particularly high rates of mutation (Jehle and Arntzen 2002). Microsatellite loci are highly polymorphic, with alleles differing in the number of repeats (i.e. mutations add or delete one or more repeats). Alleles can therefore be separated by size (i.e. electrophoresis) and allele frequencies can be obtained for populations, allowing allele frequency based population analyses (Slatkin 1995). The development of microsatellite markers has become increasingly affordable, and is now a cost-effective alternative for population studies in novel species. Despite some limitations on their use for conservation (e.g. they do not represent variation in adaptive loci, Wang 2011), microsatellite markers have been successfully used for many amphibian population studies (Jehle and Arntzen 2002). Conservation genetics aims to develop efficient conservation strategies that depend neither on maternal nor paternal variation, but on biparental nuclear genetic variability, representing the characteristics needed to cope with environmental change (Wan et al. 2004). Consequently, mtDNA markers in conservation genetics are best used in tandem with nuclear DNA markers. Mitochondrial DNA and microsatellite markers are complementary as they reveal different aspects of the evolutionariry history of a species, and therefore using them together represents the greatest advantage (Zhang and Hewitt 2003, Wan et al. 2004).

In order to aid management, the concepts of evolutionary significant unit (ESU) and management units (MU) have been used to acknowledge two different hierarchical levels of population divergence and management (Ryder 1986, Moritz 1994). Although there is more than one definition of ESU, generally it involves populations that have been reproductively and historically isolated, or have evolved unique adaptations to their local environment (Crandall et al. 2000). Based on a genetic perspective, Moritz (1994)

proposed the use of mitochondrial DNA (mtDNA) sequences and nuclear allele frequencies to identify ESU, which are reciprocally monophyletic for mtDNA alleles (i.e. each ESU is monophyletic with respect to other ESU) and show significant divergence of allele frequencies at nuclear loci. ESUs represent historical population structure and long-term conservation needs, while MUs represent more current population structure and short-term management issues (Moritz 1994). ESUs should be managed separately, and populations or individuals from different ESUs should not be merged (Moritz 1994). MUs are considered the logical level for monitoring responses of populations to impacts and management (Moritz 1995). Despite some methodological limitations (Paetkau 1999), and criticism due to the lack of use of ecological data on adaptive differences (Crandall et al. 2000), using genetics to identify units for conservation in endangered species remains a critical application in the management of wild and captive populations, in reintroductions, and in translocations (Frankham et al. 2010).

Genetic markers can also be used to infer the effective size of populations (N_e, *number of individuals in an ideal population that loses heterozygosity at the same rate as the observed population*, Frankham et al. (2010)). N_e strongly influences microevolutionary processes, such as genetic drift, and therefore estimating contemporary N_e to detect and prioritise populations at higher risk (i.e. small populations) is of primary concern in conservation genetics (Frankham et al. 2010). Furthermore, changes in N_e can leave a signature in DNA sequences and allele frequencies that can help detect important ancient and recent demographic events, such as demographic expansions or bottleneck events. Different methods have been developed to detect such signatures, including mtDNA sequence mismatch distributions (Rogers and Harpending 1992) and Bayesian skyline plots

(Drummond et al. 2005), and allele frequency based heterozygosity excess method of Cornuet and Luikart (1996), the M-ratio test of Garza and Williamson (2001), and the coalescent Bayesian approach of Beaumont (1999).

4.1.1 *Telmatobufo bullocki*

Telmatobufo bullocki is a critically endangered frog species, and one of the rarest amphibians in Chile (IUCN 2011). It is endemic to the temperate forests of the coastal Nahuelbuta range (NR) in central-south Chile (Formas et al. 2001). Although it has been reported in two localities north of the NR (Escobar et al. 2005, Donoso et al. 2010), one of these locations has recently been described as the type locality for the newly described *T. ignotus*, while the taxonomic identity of specimens at the second locality remains unverified (Cuevas 2010). As for all *Telmatobufo* species, *T. bullocki* is adapted to fast-flowing mountain streams, where the adults breed and the tadpoles develop (Formas 1988). However, adults have also been found several hundred metres from streams, highlighting the importance of both terrestrial and aquatic habitat for species survival and conservation management (Chapter 3). Main threats for this species are habitat loss, fragmentation, and degradation, and the establishment of extensive pine plantations throughout its range (Veloso et al. 2008, Ortiz et al. 2010, Fenolio et al. 2013). Plantation forestry and clear-cuts can have detrimental effects on the connectivity of populations through habitat loss and increased mortality of dispersing individuals (Johnston and Frid 2002, Semlitsch et al. 2009). Since the late 1960s, forestry development has replaced over 50% of the native forest in NR with exotic plantations (Wolodarsky-Franke and Díaz Herrera 2011). This suggests *T. bullocki* might have experienced recent and significant population declines and isolation associated with habitat loss and fragmentation.

In this chapter, two genetic markers, mtDNA (control region and Cytochrome c oxidase subunit I) and nuclear microsatellites, were used to provide baseline genetic data for *T. bullocki* populations in the Nahuelbuta Range. The general objective was to assess conservation genetics status of the species and populations in order to prioritise and inform conservation action and management. Several aspects of the genetics of populations were assessed including; 1) current patterns and levels of genetic diversity, 2) population structure, 3) phylogeography, 4) historical demography to detect changes in population size through time, 5) contemporary effective population size. The results will be discussed from a practical management perspective, through the identification of management units (ESU and MU) and prioritisation of populations at greatest risk.

4.2 Materials and methods

4.2.1 Sample collection and pooling

DNA samples were collected during 2012 and 2013 from wild animals (tadpoles and adults) caught in several locations within the Nahuelbuta mountain range (Figure 4.1). Due to the species' rareness and limited resources, for most sampling locations the sample size was relatively low ($N < 10$). Therefore, to increase statistical power in population genetic analyses, and based on the stream breeding habits of the species, samples from within the same sub-basin were pooled together into eight populations (Figure 4.1, Table 4.1). This pooling was supported by preliminary AMOVA analysis. For analyses where geographic coordinates of populations were used, the averaged coordinates of all samples within a

population were used. Each population was identified by the name of the river (as shown in the official topographical map from *Instituto Geográfico Militar de Chile*) and a two-letter code (Table 4.1). In addition, samples were collected from captive animals held at Zoológico Nacional de Chile. These animals were collected from Butamalal during 2011, and were pooled with field samples from the same basin. DNA was collected from adults and juveniles using low-impact buccal swabs (Pidancier et al. 2003). Swabs (Copan nylon flocked dry swab Cat. No. 501CS01) were dried overnight by placing them in open vials inside an airtight container with silica beads. Tail tips were collected from tadpoles, and stored in 96% ethanol. All samples were stored at 4°C until extraction.

Table 4.1. Population name, code, geographical coordinates (UTM, zone 18S, WGS84), and sample sizes for the different markers used in this study (msat = microsatellites, COI = Cytochrome oxidase subunit I, CR = control region, COI+CR = concatenated sequence).

Basin	Code	Easting	Northing	N_{msat}	N_{COI}	N_{CR}	N_{COI+CR}
Chivilingo	CH	666500	5889152	18	8	17	8
Caramávida	CA	658842	5826864	15	16	12	11
Cayucupil	CY	664776	5819774	15	15	15	15
Butamalal	BU	663252	5811133	20	16	19	15
Huilquehue	HU	653404	5806580	11	10	11	9
Provoque	PR	657663	5803065	41	24	33	24
Calebu	CL	658810	5796889	12	9	9	8
Los Lleulles	LL	694604	5815379	10	10	9	9

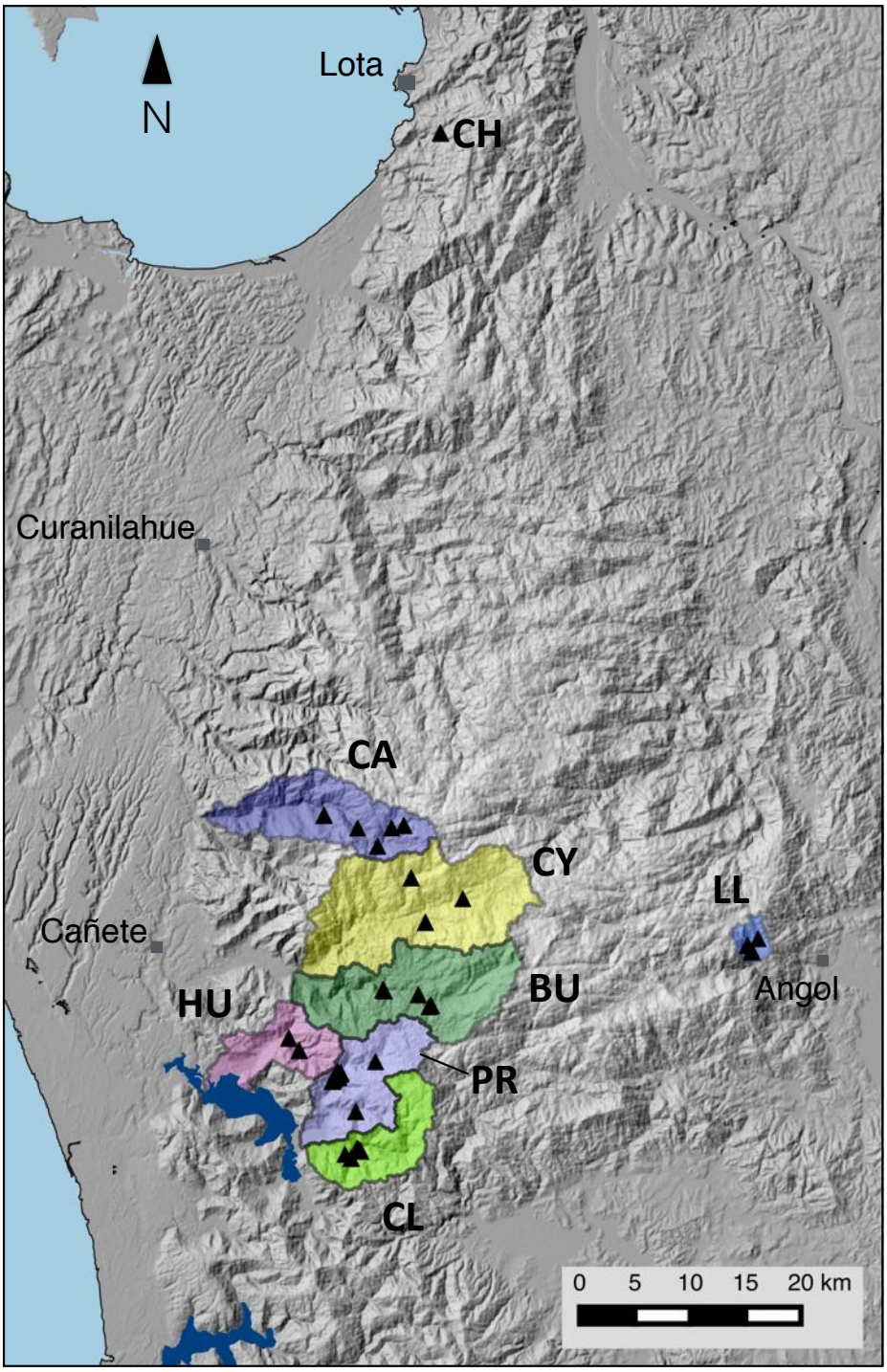


Figure 4.1. Map of genetic sampling locations (black triangles) of *T. bullocki* in the Nahuelbuta Range. Samples collected in the same sub-basin were pooled together for population analysis and assigned the following codes CH = Chivilingo, CA = Caramávida, CY = Cayucupil, BU = Butamalal, HU = Huilquehue, PR = Provoque, CL = Calebu. Main towns and cities are shown as reference.

4.2.2 DNA extraction sequencing and genotyping

DNA from swab samples was extracted using standard QIAxtractor Qiagen protocols and solutions, with a final elution volume of 70 µl. DNA from tissue was extracted using PureLink genomic DNA Kit (Life Technologies), with a final elution volume of 100 µl. Two mitochondrial DNA regions were amplified using previously published primer sequences. A ≈ 700 bp fragment of the Cytochrome C oxidase subunit I (COI) gene region was amplified using universal primers LCO1490 and HCO2198 (Folmer et al. 1994), and primers controlWrev-L and controlP-H (Goebel et al. 1999) were used to amplify a ≈ 890 bp fragment of the control region (CR) domain I. Amplification was performed in 40 ml reactions with 1 x PCR buffer, 2 mM $MgCl_2$, 0.4 mM each dNTP, 0.4 µM forward primer, 0.4 µM reverse primer, 2.0 U AmpliTaq Gold 360 DNA Polymerase, 2 µl of DNA template, 2x BSA, and deionised water. PCR conditions were 4 min at 94 ºC, 35 - 40 cycles of 30 s at 94 ºC, 30 s at Tm (44 ºC for COI; 46 ºC for CR), 45 s at 66 ºC, and a final extension 10 min at 68 ºC. PCR products were purified and sequenced using PCR primers on an ABI 3730 automated sequencer. Mitochondrial DNA sequences obtained were inspected, edited (trimmed low quality ends) and aligned using GENEIOUS.

Fifteen microsatellite loci specifically developed for *T. bullocki* were used (Moreno-Puig et al. 2014, Appendix F). All PCR conditions followed those specified in Moreno-Puig et al. (2014). PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems, Inc.) and genotyped manually using GENEIOUS microsatellite plug-in. Sampling amphibian larvae can potentially introduce bias as related individuals might be collected in one sample. To avoid this, samples from tadpoles collected at the same location (i.e. same stream reach) were screened for siblings using maximum likelihood in COLONY (Jones and

Wang 2010). Only one sample of each group of full siblings was retained for further analysis.

4.2.3 Genetic analysis

Microsatellite loci

Genotyping data was tested for errors, large allele dropout and evidence for null alleles using MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004). The frequency of null alleles was estimated using the software FREENA (Chapuis and Estoup 2007), which implements the Expectation Maximization (EM) algorithm of Dempster et al. (1977). Exact tests of Guo and Thompson (1992) for significant deviations from Hardy-Weinberg Equilibrium (HWE) were performed in GENEPOP v4.2 (Raymond and Rousset 1995) using Markov chain methods with the following specifications: dememorization = 10,000, batches = 1,000, iterations per batch = 10,000. Linkage disequilibrium (LD) between loci was also tested in GENEPOP. Significance level ($\alpha = 0.05$) was adjusted for multiple comparisons using the sequential Bonferroni correction (Holm 1979).

Genetic diversity was measured by calculating the number of alleles (N_A), number of private alleles (N_{PA}), expected (H_E) and observed (H_O) heterozygosity, for each population using GENODIVE v2.0 (Meirmans and Van Tienderen 2004). To be able to compare diversity measures among populations with different sample sizes, allelic richness (A_R) and private allelic richness (PA_R) were calculated using a rarefaction procedure to the minimum sample size (19 gene copies) as described by Kalinowski (2004) and implemented in the software HP-RARE v1.0 (Kalinowski 2005). Inbreeding coefficients (F_{IS}) and fixation indexes

(F_{ST}) were calculated for each locus and population with FSTAT v2.9.3.2 (Goudet 1995) and GENODIVE. Significance was tested using 10,000 permutations at $\alpha = 0.05$ level employing a sequential Bonferroni correction.

Population structure and differentiation

Hierarchical patterns of genetic structure were identified using different methods. Bayesian clustering of individuals was performed using multilocus data in the program STRUCTURE v2.3.4 (Pritchard et al. 2000). STRUCTURE calculates the probability $\Pr(X|K)$ of the genotype data (X) given the number of clusters (K) assuming Hardy-Weinberg and linkage equilibrium within groups, random mating within populations and free recombination between loci. The admixture model was used with and without *a priori* population definition (Hubisz et al. 2009), with populations corresponding to the 8 sub-basins shown in Figure 4.1. Ten runs of 5×10^5 iterations with a 10^5 burn-in period were conducted for each K (hypothesized number of distinct genetic clusters) from $K = 1$ to $K = 8$. The most likely number of clusters was estimated by calculating the mean $\ln \Pr(X|K)$ across the 10 runs for each K, and finding the K with the highest $\ln \Pr(X|K)$, and based on the Evanno et al. (2005) methodology by plotting the second-order rate of change in $\ln \Pr(X|K)$ for successive Ks (referred to as ΔK) with the aid of STRUCTURE HARVESTER v0.6.94 (Earl and vonHoldt 2012). The ten STRUCTURE outputs for the most likely K were combined into a single output using the cluster matching and permutation program CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007), and then visualized with the program DISTRUCT v1.1 (Rosenberg 2004). To look at the hierarchical structuring of populations, all clusters identified were further analysed independently until no further structuring was detected.

The degree of genetic differentiation among populations and groups of populations was measured by calculating different pairwise statistics based on allele frequencies. Pairwise F_{ST} (Weir and Cockerham 1984) and R_{ST} (Slatkin 1995) were calculated with ARLEQUIN v3.5.1.3 (Excoffier and Lischer 2010). In addition, pairwise Jost's D_{ST} (Jost 2008) was calculated using the R package DEMETICS v0.8-7 (Gerlach et al. 2010). The significance of genetic distances was tested by permuting individuals between populations (10,100 permutations). A sequential Bonferroni correction was used to correct for multiple tests. Frequencies of null alleles estimated with EM algorithm were used to calculate corrected F_{ST} values using the "excluding null allele" (ENA) method described by Chapuis and Estoup (2007) and implemented in FREENA software. These values were compared with uncorrected F_{ST} to assess the potential impact of null alleles on the analysis.

Bottlenecks

Three methods were implemented to detect signatures of recent reductions in effective population size. These included the heterozygosity excess method of Cornuet and Luikart (1996), the M-ratio test of Garza and Williamson (2001), and the coalescent Bayesian approach of Beaumont (1999). The heterozygosity excess method tests whether heterozygosity expected under Hardy-Weinberg equilibrium is larger than heterozygosity expected under mutation-drift equilibrium. During a bottleneck, rare alleles are lost causing an excess of heterozygosity (Cornuet and Luikart 1996). This method was implemented in BOTTLENECK v1.2.02 (Piry et al. 1999), and analyses were performed using the infinite allele model (IAM), a strict step-wise mutation model (SMM), and the two-phase model (TPM) with 95% and 90% SMM (i.e. models TPM 95%, TPM 90% respectively),

with 30% variance. The two-tailed Wilcoxon sign rank test was used to test for significance at the $P < 0.05$ level. The M-ratio test is based on the ratio of the number of microsatellite alleles to the range in allele size. During a bottleneck, the number of alleles is expected to decline faster than the range in allele size, leading to a smaller M-ratio in bottlenecked populations compared to populations at equilibrium (Garza and Williamson 2001). Tests were conducted by comparing the mean observed M-ratio across loci with the expected distribution generated from simulations under mutation-drift equilibrium. M-ratio values were calculated using M_P_VAL (Garza and Williamson 2001), and critical M values (M_c) were obtained for different mutation models (i.e. SMM, TPM) using CRITICAL_M, where M_c is set at the lower 5% tail of the simulated distribution (Garza and Williamson 2001). For TPM models the mean size of mutations was set to 3.5 and the proportion of multi step mutations as 5% and 10% (i.e. models TPM 95%, TPM 90% respectively). Several simulations were run with different values of pre-bottleneck θ (from 1 to 10, where $\theta = 4N_e\mu$, N_e = effective population size, μ = mutation rate).

Bayesian inference of past population size changes was performed using MSVAR v1.3 (Beaumont 1999). This method uses a MCMC algorithm to sample from the posterior distribution of the model parameters (i.e. current effective population size N_0, past effective population size N_1, time at which the change occurred T, and mutation rate of microsatellite loci μ). Although the inference of this method is restricted as it assumes a strict single-step mutation model (Faurby and Pertoldi 2012), it has been shown to perform better than the previous two methods (i.e. heterozygosity excess, M-ratio), particularly for species of conservation concern (Hu et al. 2010, Girod et al. 2011, Peery et al. 2012). Four independent runs were performed (2×10^9 iterations, logged every 10^5)

with different priors (Appendix G) to represent different possible demographic scenarios (e.g. stable, decreasing, or increasing N_e). A generation time of four years was used (estimation based on observed growth, development, and skeletochronology, Appendix E). Since population structure can lead to false bottleneck signals (Peter et al. 2010), the *ad-hoc* approach of sampling individuals from different groups was also used (Chikhi et al. 2010). If a bottleneck is still detected under this approach, it might be that the whole metapopulation was subject to a population size change. Convergence was checked, and summary statistics, kernel densities and 95% credible intervals (HPD) were obtained using TRACER v1.6 (Rambaut et al. 2013).

Effective population size N_e

Effective population size (N_e) was estimated based on the linkage disequilibrium (LD) method (Hill 1981), bias corrected for non-overlapping generations as described in Waples (2006), and implemented on NeESTIMATOR v.2 (Do et al. 2014), which also includes an improved method for dealing with missing data described in Peel et al. (2013). The random mating model was used with 95% CI calculated using a jacknife procedure over loci. To reduce potential bias from the presence of rare alleles, low frequency alleles were removed from the analysis following Waples and Do (2010). Effective population size was also estimated using approximate Bayesian computation as described in Tallmon et al. (2004) and implemented in the web-based software ONeSAMP v1.2 (Tallmon et al. 2008).

Mitochondrial DNA

The number of haplotypes (H_N), haplotype diversity (H_D), nucleotide diversity (π), number of segregating sites (S) and average number of nucleotide differences (k) were calculated using DNASP v5.10 (Librado and Rozas 2009), where indels were not considered. To account for uneven sample size, haplotype richness (H_R) was calculated and standardised to the smallest sample size using a rarefaction method implemented in CONTRIB v1. (Petit et al. 1998). Neutrality was tested using Tajima's D-test statistic implemented in DNASP 5.10.

Phylogeography

Concatenated mtDNA sequences obtained for all populations were used to construct phylogenetic trees. First, the best nucleotide substitution models for each partition and the concatenated sequence were selected using JMODELTEST v2.1.5 based on Akaike Information Criteria (AIC) and the BIC (Posada 2008, Darriba et al. 2012). The model selected for the COI partition was the Tamura and Nei (1993) with invariant sites (TrN+I) and for the CR was the Hasegawa Kishino Yano (1985) with invariant sites HKY+I, while for the concatenated sequence was the HKY+I. The phylogeny was then constructed using both maximum likelihood (ML) and Bayesian inference (BI) approach using MEGA v6.06 (Tamura et al. 2013) and BEAST v2.0 (Drummond and Rambaut 2007, Drummond and Bouckaert 2014) respectively. For ML phylogenetic analysis, the closely related species *Telmatobufo australis* was used as an outgroup (Formas et al. 2001). No outgroup was used for BI analysis as per author recommendations on intraspecific phylogenetic analysis (Drummond and Bouckaert 2014).

Trees were constructed using the full sample (including repeated sequences) and haplotypes only (removing repeated sequences), in all cases gaps were not considered in the analysis. ML phylogenetic trees were constructed without defining partitions, using the best substitution model for the concatenated sequence, and trees were tested using 10,000 bootstrap replications. BI trees were constructed using the appropriate models for each partition, and the coding COI partition was further split into three codon positions to allow for uneven substitution rates. The coalescent Bayesian skyline model was used for the tree prior with a strict molecular clock, with fixed mean substitution rates. Since no fossil calibration points exist for this species, and in order to be able to approximately estimate divergence times, three different substitution rates were used. The minimum and maximum clock rates usually found for vertebrate mtDNA were used (0.1-2% substitutions/My (Irisarri et al. 2012)) to give lower and upper confidence intervals for cladogenic events. In addition, a global clock rate of 0.4% substitutions/My was also used in order to compare divergence dates with those previously estimated for the genus *Telmatobufo*. Formas et al. (2001) estimated divergence times of *Telmatobufo* species using this rate of 0.4% substitutions/My citing the work of Martin and Palumbi (1993) for ectothermic vertebrates, and found congruent results with those reported by Nuñez and Formas (2000) based on an immunological approach. For each analysis four independent runs were made, each of 20 million generations, with sampling at intervals of 1,000 producing 20,000 sampled trees, of which the first 2,000 were discarded as burn-in. Convergence was checked using TRACER v1.6, and only runs where all effective sample sizes (ESS) were greater than 200 were considered successful as recommended by Drummond and Bouckaert (2014). The maximum clade credibility tree, which corresponds

to the tree with the highest product of the posterior probability of all its nodes was used to generate the final Bayesian tree with TreeAnnotator v2.1.2, annotating all nodes with a minimum posterior probability of 0.5. In addition, statistical parsimony (TCS) haplotype networks (Templeton et al. 1992, Clement et al. 2000) were constructed using the software POPART.

4.2.4 Historical demography

Mismatch distribution

Tajima's *D* (Tajima 1989) and Fu's F_S (Fu 1997) tests of selective neutrality were conducted at each hierarchical level using ARLEQUIN (Excoffier and Lischer 2010). Significance of *D* and F_S statistics was tested by comparing the observed values to the distribution of 10,000 coalescence simulations under the hypothesis of selective neutrality and population equilibrium. Significant and negative *D* and F_S values are indications of possible past population expansions. Parameters of pure demographic expansion (past population size θ_0, present population size θ_1, and time since expansion τ) were estimated using mismatch distribution analysis in ARLEQUIN, which implements the generalized non-linear least-square approach of Schneider and Excoffier (1999). A population that has undergone a recent sudden demographic expansion is expected to have a unimodal and smooth mismatch distribution, while a population with constant historical size will have a more ragged, multimodal distribution (Rogers and Harpending 1992). Harpending's raggedness index (*r*) and the sum of squared deviations statistics (SSD) were also calculated to assess model fit. Only the control region fragment was used for the mismatch distribution, as more sequences were available.

Bayesian Skyline Plots

The population dynamics of each lineage and for the full sample were constructed using the coalescent reconstruction of Bayesian Skyline Plots (BSP) using BEAST v2.0. Bayesian skyline plots uses standard MCMC sampling procedures to estimate a posterior distribution of effective population size through time from a sample of gene sequences, and allows to fit a wide range of demographic scenarios (Drummond et al. 2005). Each analysis consisted of 20 million iterations, discarding 10% as burn-in, and sampled every 2,000th iteration under a strict molecular clock. The mutation rate was fixed to a minimum of 0.01% and a maximum of 2% to obtain estimated times as explained above for the phylogenetic tree. For Clade 1 (CH), only the CR sequence was used, as more samples were available, while for Clade 2 the concatenated sequence was used. Plots for each analysis were visualized in TRACER v1.6.

4.3 Results

4.3.1 Microsatellite loci

A total of 144 individuals were genotyped at 15 microsatellite loci. Data was not obtained from some individuals due mainly to low quality DNA, which was more common for swab samples than tail tip samples. Two individuals were removed from the analyses after being identified as close siblings, yielding a final sample size of $N = 142$. There was no evidence for large allele dropout or scoring errors at any of the 15 loci. Excess of homozygotes was detected for locus *Tbu19* in LL, *Tbu33* in BU, *Tbu39* in CL, and *Tbu48* at 4 populations (CA,

BU, PR, CL), suggesting possible null alleles. After correcting for multiple comparisons, significant departures from Hardy-Weinberg equilibrium were found for locus *Tbu19* in LL only, *Tbu23* in BU, and *Tbu48* deviated in PR (Appendix G). Due to the high amount of missing data and possible null alleles, locus *Tbu48* was removed from further population analyses. Loci that showed null allele evidence or deviation from HWE at only one population were retained for subsequent analyses.

Population structure

The most likely number of genetically distinct populations according to STRUCTURE was $K = 4$, while the Evanno methodology supported $K = 2$, with a secondary peak at $K = 4$ (Appendix G). The hierarchical analysis of the data suggests that at the highest level ($K = 2$) the northern Chivilingo (CH) population separates from all the other sampled populations. At the next level ($K = 4$), when the southern cluster (CA, CY, BU, HU, PR, CL, LL) is further analysed, the results suggest 3 distinct groups. Individuals from HU, PR and CL were all clustered together (Group C) with very little admixture with other populations. Individuals from CA, CY and BU were clustered together (Group B); however, BU individuals showed some level of admixture with Group C. Individuals from LL were all clustered together. Despite the suggestion that BU might represent a different group, no further structure was detected within each group.

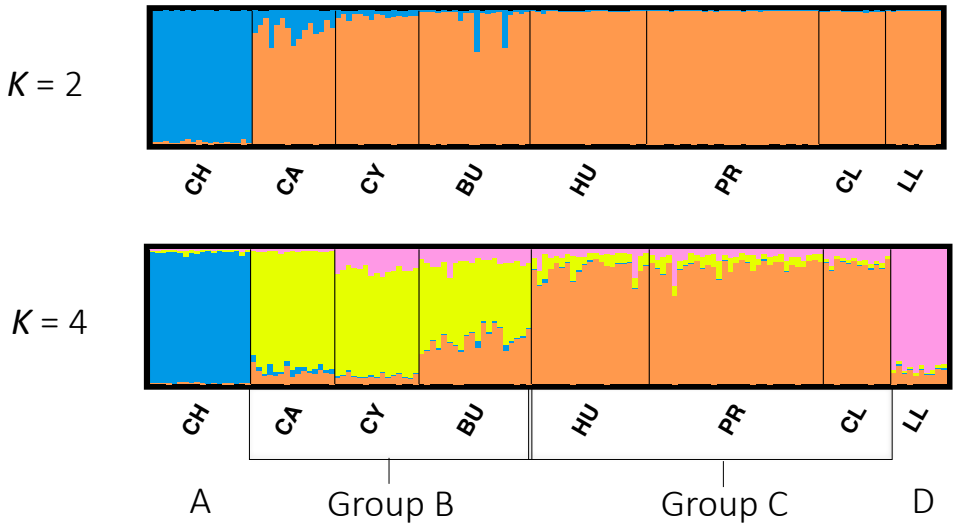


Figure 4.2. Hierarchical clustering of individuals and populations given in STRUCTURE when *K* = 2 and *K* = 4. Each vertical bar represents an individual's probability of population origin, with each colour representing a different population. The original sampling population is shown with the two-letter code assigned.

Genetic diversity

Genetic diversity indices (for mitochondrial and microsatellite loci) and differentiation statistics for each population are summarised in the following table (Table 4.2). To help visualise patterns and relative levels of diversity, main indices were classified into three equal-interval classes based on the minimum and maximum values observed. Each class was colour-coded with red representing relatively low, yellow medium, and green high levels of diversity.

Table 4.2. Genetic diversity indices for each basin and group. Left: Molecular diversity for the 1265 bp concatenated (COI+CR) mtDNA sequence: number of samples *N*, number of segregating sites *S*, number of haplotypes H_N, haplotype diversity H_D, nucleotide diversity π, average number of nucleotide differences *k*, and haplotype richness after rarefaction to the smallest sample size H_R. Right: Genetic diversity at 14 microsatellite loci: Number of individuals *N*, average number of alleles N_A, number of private alleles N_{PA}, allelic richness A_R and private allelic richness PA_R, average observed H_o and expected H_E heterozygosity, average inbreeding coefficient F_{IS}. Red = low, yellow = medium, and green = high relative diversity. ns = non significant.

			Mitochondrial DNA							**Microsatellite loci**							
	Basin	Code	*N*	*S*	H_N	$H_D \pm SD$	$\pi \pm SD$	*k*	H_R	*N*	N_A	N_{PA}	A_R	PA_R	H_O	H_E	F_{IS}
A	Chivilingo	CH	8	9	6	0.89 ± 0.11	0.0022 ± 0.00053	2.79	5.00	18	5.00	7	4.27	0.52	0.590	0.575	-0.025 ns
B	Caramávida	CA	11	24	10	0.98 ± 0.05	0.0062 ± 0.00113	7.78	6.49	15	5.93	3	5.25	0.25	0.686	0.680	-0.006 ns
	Cayucupil	CY	15	26	10	0.91 ± 0.06	0.0043 ± 0.00087	5.45	5.16	15	5.79	2	5.01	0.17	0.681	0.660	-0.031 ns
	Butamalal	BU	15	13	10	0.92 ± 0.05	0.0032 ± 0.00034	3.98	5.31	20	5.93	3	4.97	0.19	0.603	0.675	0.107 ns
	TOTAL B		41	36	26	0.94 ± 0.03	0.0048 ± 0.00056	6.04	5.71	50	7.64	13	5.21	0.87	0.651	0.683	0.047 ns
C	Huilquehue	HU	10	7	5	0.80 ± 0.10	0.0022 ± 0.00052	2.73	3.40	11	4.93	1	4.64	0.10	0.558	0.575	0.029 ns
	Provoque	PR	24	10	7	0.81 ± 0.05	0.0021 ± 0.00027	2.70	3.40	41	7.14	6	4.74	0.19	0.575	0.601	0.052 ns
	Calebu	CL	8	11	5	0.79 ± 0.15	0.0023 ± 0.00082	2.93	4.00	12	5.00	1	4.71	0.15	0.624	0.608	-0.007 ns
	TOTAL C		42	17	13	0.83 ± 0.03	0.0025 ± 0.00020	3.17	3.82	64	7.86	11	4.70	0.55	0.582	0.600	0.039 ns
D	Los Lleulles	LL	9	5	5	0.72 ± 0.16	0.0011 ± 0.00029	1.39	3.56	10	4.00	0	3.95	0.13	0.550	0.555	0.011 ns

Pairwise differentiation

Pairwise distances between populations show moderate to high levels of differentiation (Table 4.3). The results agree with STRUCTURE grouping, with F_{ST} distances within groups generally smaller and less significant (shaded cells, Table 4.3) than among groups. The only inter-group F_{ST} distance that was not significant was for HU-BU. The greatest distance was for CH-LL. Pairwise distances between the four groups were all significant, and were small for B-C and B-D, and highest for A-D (Table 4.4). Similar results were found for Jost's D_{ST} distance for basin and group pairwise distances (tables provided in Appendix G).

Table 4.3. Pairwise F_{ST} between populations above diagonal (Weir and Cockerham 1984), R_{ST} (Slatkin 1995) below diagonal. Shading is used to highlight populations grouped together according to STRUCTURE results.

	CH	CA	CY	BU	HU	PR	CL	LL
CH	-	0.110*	0.156*	0.140*	0.183*	0.181*	0.183*	0.259*
CA	0.104§	-	0.022§	0.025§	0.058*	0.046*	0.045*	0.110*
CY	-0.019	0.069§	-	0.024§	0.064*	0.068*	0.075*	0.070*
BU	0.028	0.084§	-0.017	-	0.017	0.041*	0.037*	0.066*
HU	0.065	-0.016	-0.008	0.018	-	0.008	0.017	0.093*
PR	0.030	0.047	-0.036	-0.011	0.000	-	-0.006	0.126*
CL	0.045	-0.023	-0.044	0.000	-0.040	-0.007	-	0.109*
LL	0.076	0.109	0.005	-0.018	0.047	0.019	0.032	-

* significant after Bonferroni correction , § significant at $\alpha = 0.05$ level

Table 4.4. Pairwise F_{ST} between groups above diagonal R_{ST} below diagonal.

	A	B	C	D
A	-	0.123*	0.181*	0.259*
B	0.034	-	0.040*	0.069*
C	0.041[§]	-0.013	-	0.116*
D	0.076	0.018	0.025	-

* significant after Bonferroni correction

Bottlenecks and N_e

The heterozygosity excess test showed no significant excess of heterozygosity under SMM or TPM in any of the groups (Appendix G). Significant excess of heterozygosity was found in Groups B and D under the IAM; however, this result is possibly due to an inappropriate mutation model. Significant deficiency of heterozygosity was found in Groups B and C. The M-ratio test results (Figure 4.3) show how critical values of M-ratio are sensitive to the chosen mutation model and pre-bottleneck θ. For population CH (Figure 4.3A), a significant bottleneck was detected under the strict SMM regardless of the value of the pre-bottleneck θ, while for the TPM 95% model, a bottleneck was detected only for low values of initial θ. For Groups B and C (Figure 4.3B and C), significant bottleneck evidence was found under SMM model only for ancestral $\theta < 2$ and $\theta < 5$ respectively. For LL population (Figure 4.3D) significant bottleneck evidence was found under SMM, for pre-bottleneck $\theta < 5$, and under TPM 95% for $\theta < 2$. Under the TPM 90% model, no population or group revealed significant evidence of bottleneck events.

MSVAR results, on the other hand, revealed significant population declines for all groups. The combined results from all the runs for each group and for the full sample are given in

Table 4.5. All runs from all groups, and the full sample, converged to similar values for the three parameters of interest (Table 4.5, Figure 4.4), showing no significant differences among groups. The posterior distributions for log(N_0) and log(N_1) were non overlapping (Figure 4.4), which suggests a significant reduction in effective population size. The estimated time since the decline is T = 448 years (95% HPD = 61 - 3376 years).

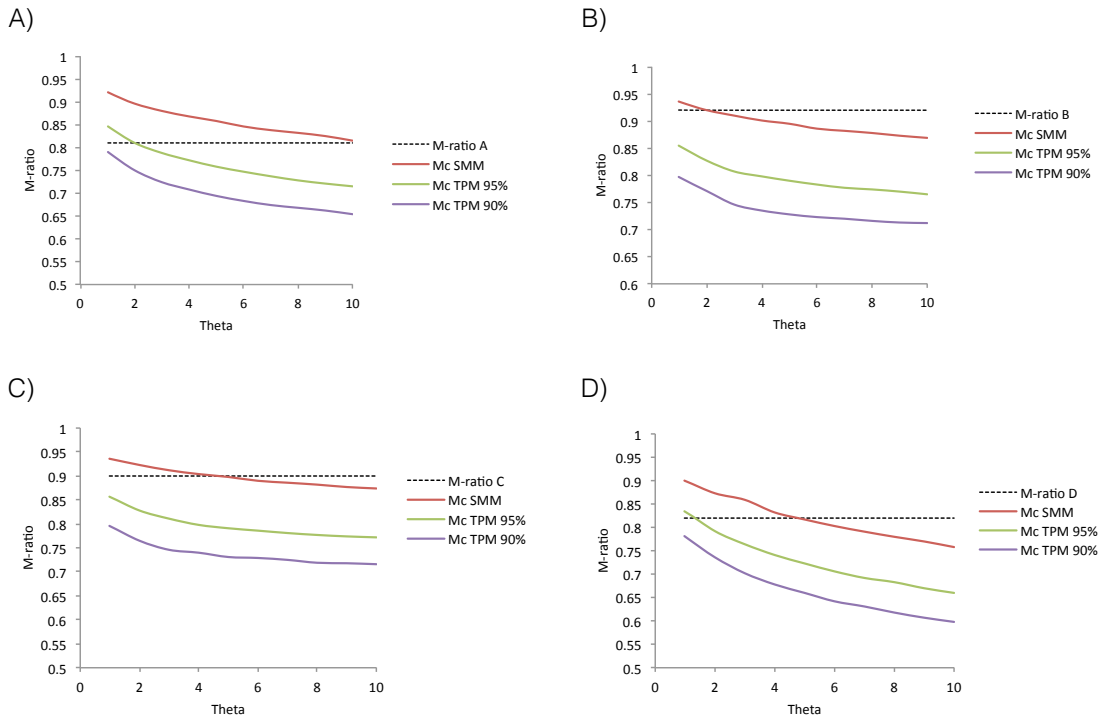


Figure 4.3. M-ratio simulations for the four populations or groups of populations (A = CH; B = CA, CY, BU; C = HU, PR, CL; D = LL). In each case, the calculated M-ratio is shown (dashed line) and the simulated critical values (M_c) for each model (i.e. SMM, TPM 95%, TPM 90%) are shown as a function of pre-bottleneck theta. Evidence for bottleneck is significant for models that are above the dashed line.

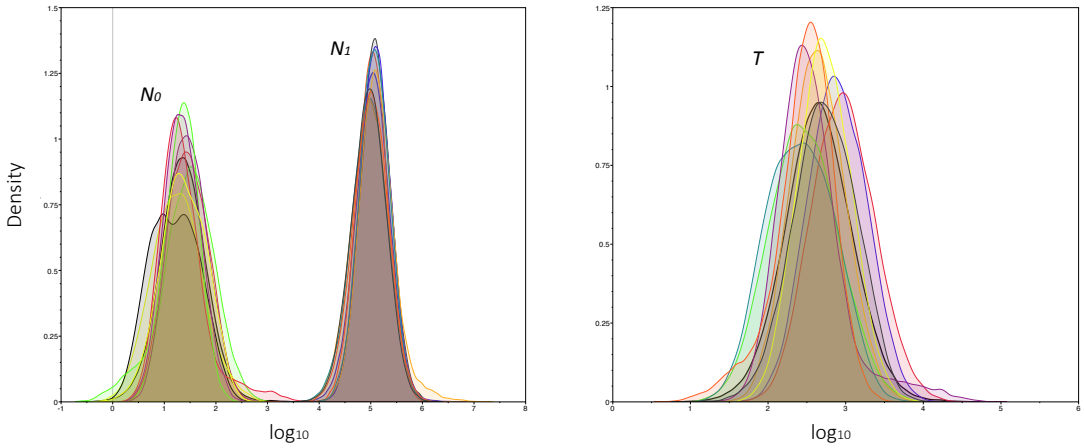


Figure 4.4. Marginal probability distributions for log(N_0) and log(N_1) in the left, and log(T) in the right for all runs including all groups. Posterior distributions were generated in MSVAR and kernel densities calculated in TRACER.

Table 4.5. Estimated parameters given by the combination of all runs in MSVAR for each group. for current effective population size N_0, ancient effective population size N_1, and time since population change T on a log scale.

Group	**log(N_0)**	**log(N_1)**	**log(T)**
A			
Mean	1.36	4.98	2.73
Variance	0.159	0.115	0.142
95% HPD	0.62 - 2.16	4.31 - 5.63	2.01 - 3.47
B			
Mean	1.25	5.07	2.46
Variance	0.149	0.086	0.127
95% HPD	0.48 - 2.00	4.50 - 5.66	1.75 - 3.15
C			
Mean	1.23	5.09	2.44
Variance	0.233	0.087	0.192

95% HPD	0.33 - 2.18	4.52 - 5.67	1.61 - 3.29
D			
Mean	1.49	4.97	2.93
Variance	0.170	0.122	0.152
95% HPD	0.69 - 2.28	4.27 - 5.64	2.17 - 3.68
OVERALL			
Mean	1.36	5.03	2.65
Variance	0.197	0.109	0.195
95% HPD	0.499 - 2.24	4.37 - 5.66	1.79 - 3.53

Contemporary effective population size

Effective population size was estimated at the group level, following STRUCTURE clustering results. The LD method gave estimates for contemporary N_e with wide confidence intervals (Table 4.6). In all cases, the upper 95% CI was infinite, which can be expected for larger populations (Waples and Do 2010). As expected, estimated N_e were positively biased when rare alleles were included. Due to the low sample size, results for LL were inconclusive (i.e. $N_e = \infty$), and further sampling would be needed to obtain an estimate. Still, a low lower limit of $N_e = 43$ was obtained. The lowest estimated N_e corresponds to CH population, with a lower CI of 34. However, this population has a low sample size ($N < 20$), thus results should be interpreted with care. Groups B and C have relatively large estimates of N_e, with similar lower CIs of 594 and 465 respectively. Approximate Bayesian computation gave estimates for groups A, B, and C (Table 4.6).

Table 4.6. Estimated effective population size (N_e) and 95% confidence intervals using the linkage disequilibrium (LD) method excluding rare alleles (Waples and Do 2010), and approximate Bayesian computation (ABC) of Tallmon et al. (2008). The cut-off value (P_{crit}) to exclude rare alleles was calculated for each sample size (*S*).

			LD Excluding rare alleles			ABC	
Group	Populations	*S*	P_{crit}	*Ne*	95% CI	*Ne*	95% CI
A	CH	18	0.03	156	34 - ∞	52	33 - 148
B	CA - CY - BU	50	0.02	594	195 - ∞	90	64 - 159
C	PR - HU - CL	64	0.01	465	202 - ∞	1724	888-5219
D	LL	10	0.05	∞	43 - ∞	-	-

4.3.2 Mitochondrial DNA

Diversity

After trimming low-quality ends, a 718 bp fragment of the control region (CR) was obtained for 125 *T. bullocki* individuals, while a 547 bp fragment of the COI was obtained for 108 individuals. A concatenated (COI+CR) sequence of 1,264 bp was obtained for a total of 100 individuals, including samples from eight sub-basins. The noncoding CR sequence was overall more variable than the COI (Table 4.7), and included 7 indels and 35 parsimony informative sites, while the COI had 26 parsimony informative sites, and no indels. Tajima's test of neutrality indicated that mutations in both segments were selectively neutral (data not shown). The analysis of the 100 *T. bullocki* concatenated mitochondrial sequences revealed a total of 50 haplotypes (excluding 7 indel sites), with 76 segregating sites (50 parsimony informative, 26 singletons). Overall haplotype diversity

(H_D) of 0.96 ± 0.01 SD and nucleotide diversity of (π) 0.0071 ± 0.00076 SD, average number of nucleotide differences (k) of 8.94. Uncorrected divergence (p-distances) between *T. bullocki* haplotypes ranged from 0.08% to 2.72%, with a mean divergence of 0.89%. Mean divergence with the close relative *T. australis* was 12.96%. Genetic diversity for each basin revealed relatively high levels of both haplotype and nucleotide diversities (Table 4.2). The highest diversity indices were found in Caramávida (CA), while the lowest overall diversity was found in Los Lleulles (LL). Huilquehue (HU), Provoque (PR) and Calebu (CL) also had lower levels of haplotype richness compared to the rest.

Table 4.7. Diversity indices for the two mtDNA markers used. Sample size *N*, segregating sites *S*, number of haplotype H_N, haplotype diversity H_D, nucleotide diversity π, average number of nucleotide differences *k*.

Partition	Size (bp)	*N*	*S*	H_N	H_D ± SD	π ± SD	*k*
COI	547	108	34	30	0.92 ± 0.01	0.0071 ± 0.00074	3.90
CR	718	125	48	43	0.95 ± 0.01	0.0085 ± 0.00077	6.06
COI+CR	1265	100	76	50	0.96 ± 0.01	0.0071 ± 0.00076	8.94

Phylogeography

Phylogenetic analysis of the concatenated mtDNA sequence revealed two well-supported clades corresponding to the northern Chivilingo (CH) population (Clade 1) and all the other sampled populations (Clade 2), as shown in Figure 4.5. Both maximum likelihood (Appendix G) and Bayesian inference significantly supported both branches with 99% and 90% bootstrap support and 100% and 100% posterior probabilities for Clades 1 and 2 respectively. These two clades are reciprocally monophyletic. Within the southern clade,

Los Lleulles (LL) population appeared consistently as a monophyletic group with moderate support (56% bootstrap support, 92% posterior probability), suggesting this population represents a distinct lineage with a shared common ancestor with individuals from Group B. Individuals from HU, PR, and CL (Group C) were monophyletic but with lower bootstrap support (<50%) and 60% posterior probability. Individuals from CA, CY, and BU (Group B) were paraphyletic. Four individuals from CA and CY basins formed a well-supported clade (86% bootstrap support, 100% posterior probability) that forms the sister clade of all other individuals from Clade 2. The minimum and maximum substitution rates used gave an estimated divergence time for Clades 1 and 2 of 8.8 and 0.70 Mya respectively (composite 95% HPD between 0.45 and 13.8 Mya. The time to most recent common ancestor (TMRCA) for CH individuals was 0.916 Mya and 85.9 kya (26.5 kya to 1.962 Mya 95% HPD), and for Clade 2 individuals was 3.592 Mya and 0.3211 Mya (0.1727 - 5.6201 Mya 95% HPD). The TMRCA for Group C was 1.32 and 0.12 Mya (59.1 kya to 2.18 Mya composite 95% HPD), while for LL it was 572 kya to 52.7 kya (19.8 kya to 1.12 Mya composite 95% HPD).

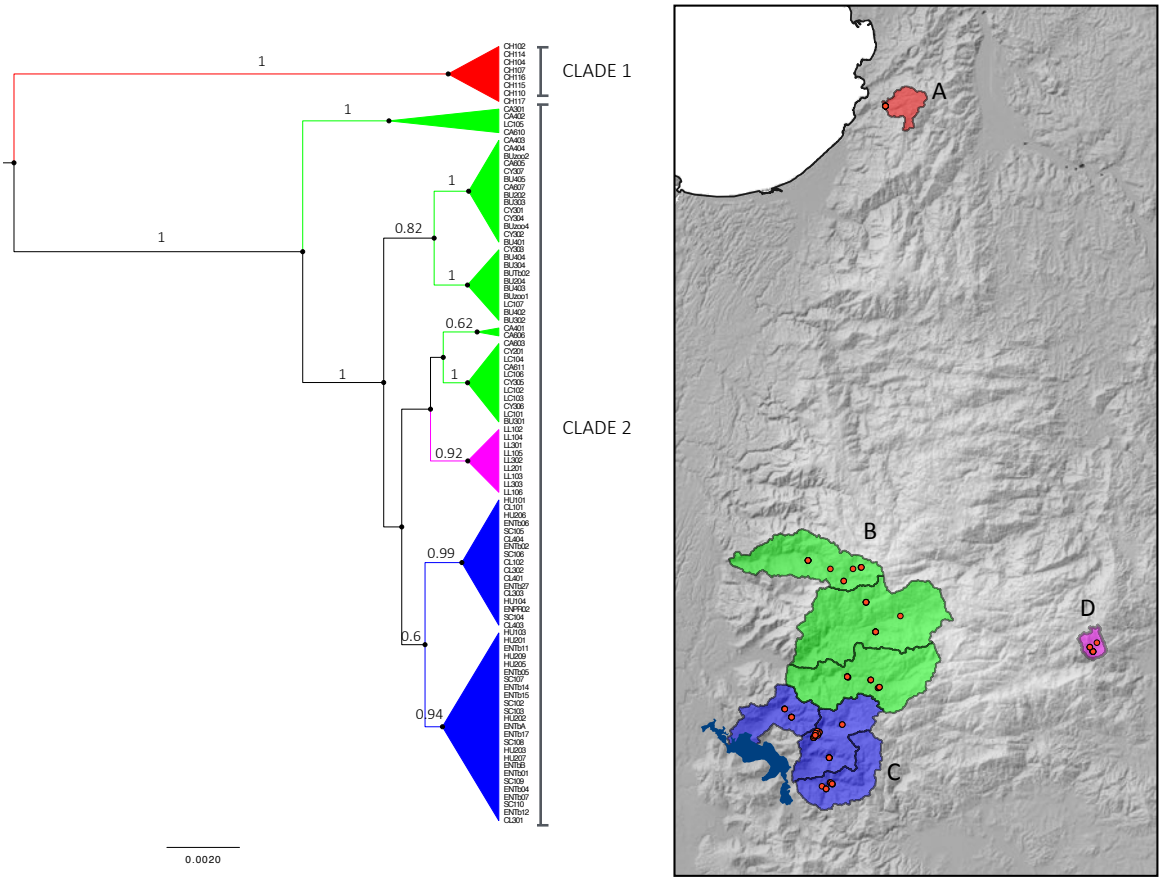


Figure 4.5. Gene tree constructed from 10,000 posterior trees output by BEAST (left). Branch labels correspond to posterior probabilities (only probabilities > 0.5 are shown), and scale is substitutions per site. The colours correspond to the geographic group of origin as shown in the map (right).

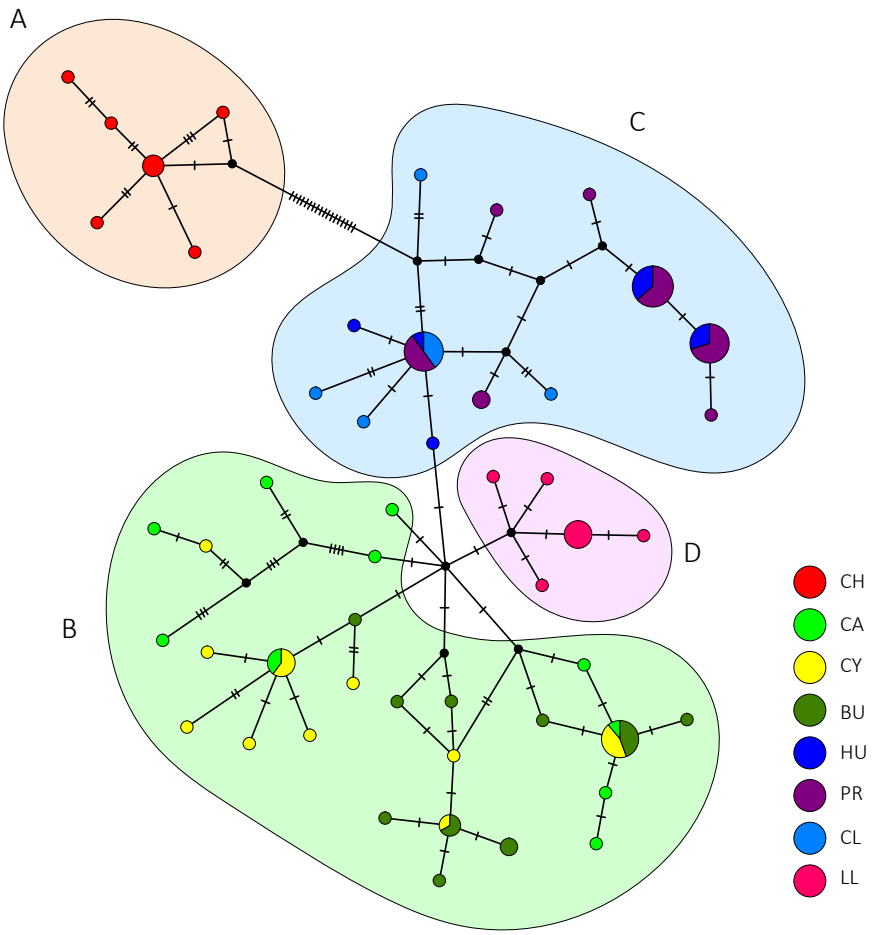


Figure 4.6. TCS network of concatenated mtDNA haplotypes. Population of origin is represented by colour. The size of each circle is relative to the number of individuals sharing the same haplotype. Black dots represent inferred missing haplotypes (nodes). Hatch marks represent the number of substitutions occurring on that branch. Shading represents clustering of individuals according to STRUCTURE results.

Mismatch distributions

Mismatch distribution of the CR sequence for the two major phylogenetic clades and for the full sample are shown in Figure 4.7. All frequency distributions fitted the model of demographic expansion (Figure 4.7) and had non-significant raggedness index values indicating a smooth unimodal distribution. Results for spatial expansion were similar

(Appendix G). Fu's F_s was significant and negative ($p < 0.02$) for the full sample and for Clade 2, indicating an excess of low-frequency haplotypes consistent with demographic expansion (Appendix G). Fu's F_s for Clade 1 was nearly significant ($0.02 < p < 0.05$). Mismatch distribution of the full sample appears bimodal; however, the raggedness index is low and not significant ($r = 0.006$, Appendix G). Multi-modal distributions that fit sudden expansion models can be an indication of population structuring (Castoe et al. 2007), which is consistent with the structuring found in the other analyses. According to these results, *T. bullocki* populations have undergone both demographic and spatial expansion throughout the species' range.

Skyline plots, N_e

Bayesian coalescent skyline plots (BSP) of the mtDNA data for the full sample and the two main clades are shown in Figure 4.8. The BSP for the full sample and Clade 2 were nearly identical, and showed a statistically significant change in population size. In agreement with the mismatch distribution analysis, these plots show a recent demographic explosion. In contrast, BSP for Clade 1 did not show any statistically significant change in population size; however, confidence intervals were wide due to the small sample size. Due to a lack of calibration points, the exact timing of the demographic explosion could not be defined. However, as a rough estimate, the time of the explosion was estimated using a minimum and maximum rates of substitution of 0.1-2% per My respectively. This gave an estimated time for the demographic explosion during the Pleistocene era between ~500,000 and ~20,000 years ago.

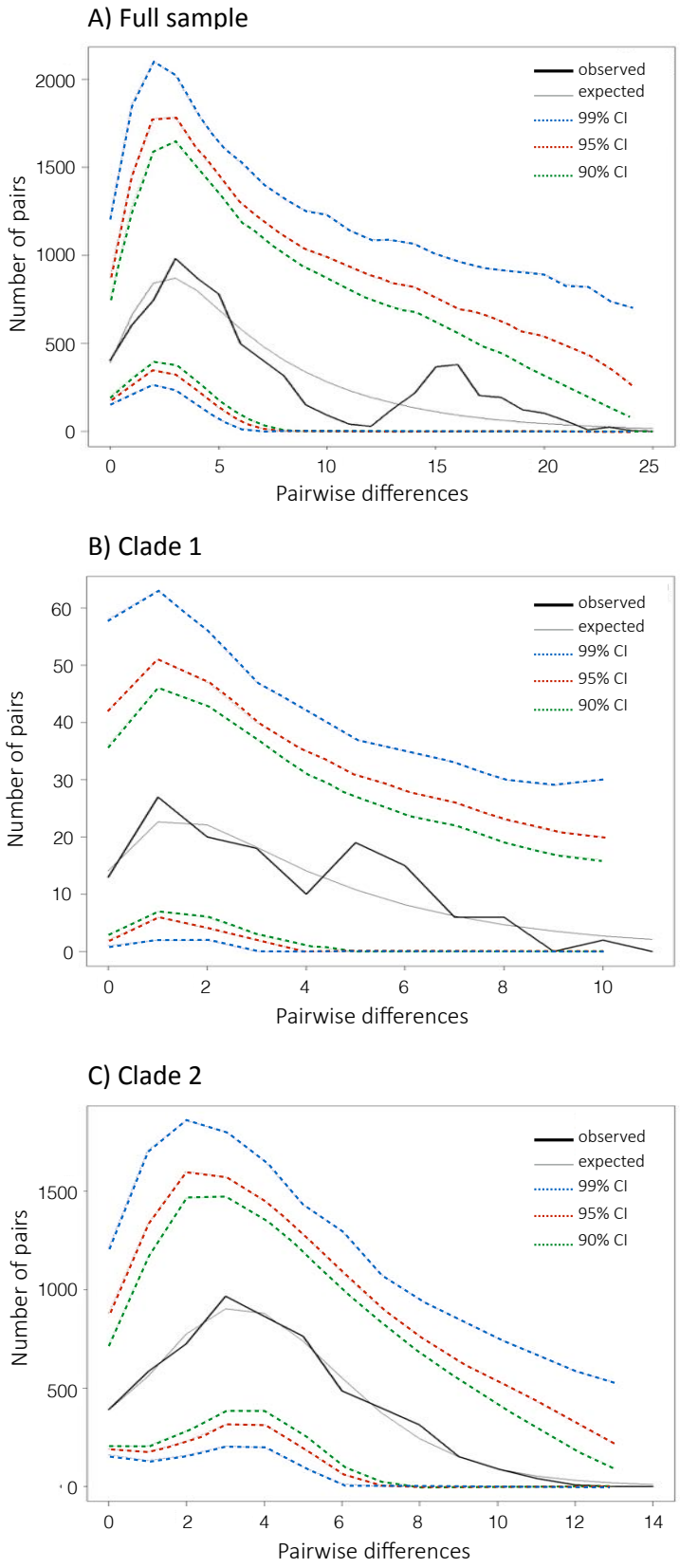


Figure 4.7. Observed mismatch distribution is shown (solid black line), along with expectations under the demographic expansion model (black dashed), and its confidence intervals (colour dashed).

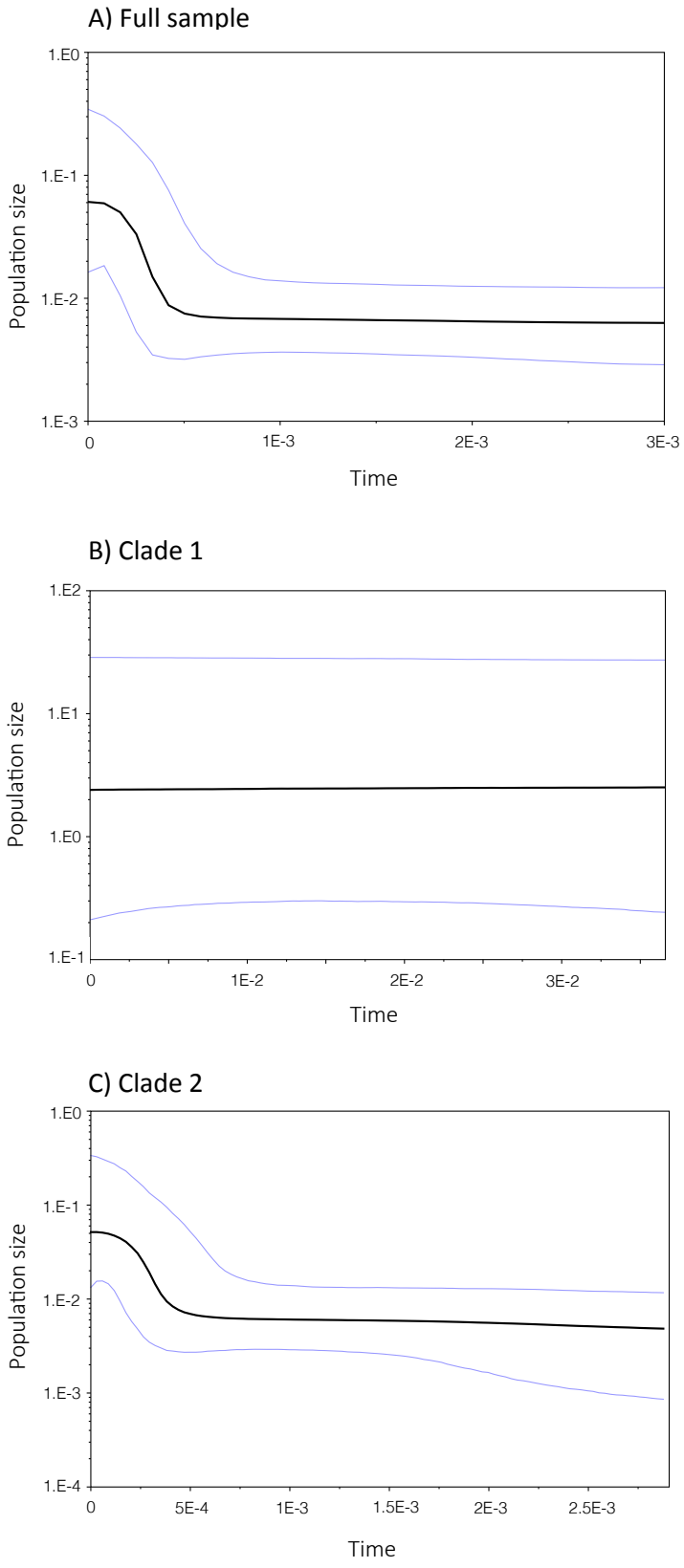


Figure 4.8 Bayesian Skyline Plots (BSP) show the median of the population size (solid black), expressed as effective population size per generation time (log transformed), with 95% HPD (blue lines). *X*-axis is time expressed in units of substitutions/site.

4.4 Discussion

4.4.1 Genetic diversity

Both mitochondrial and microsatellite markers revealed relatively high levels of genetic diversity in *T. bullocki* populations, particularly when compared to what could be expected from a rare and critically endangered species (Frankham 2005). Although nucleotide diversity values for *T. bullocki* populations were relatively low (average $\pi = 0.003$, overall $\pi = 0.007$, range $0.001 \leq \pi \leq 0.006$), haplotype diversity was high ($0.72 \leq H_D \leq 0.98$). Nucleotide diversity was similar to other threatened amphibians such as *Litoria aurea* (mean $\pi = 0.003$ Burns et al. (2007)), *Pchyhynobius shangchengensis* ($\pi = 0.002$, Pan et al. (2014)), and considerably lower than for the widespread Chilean temperate forest species *Eupsophus calcaratus* (average $\pi = 0.069$, Núñez et al. (2011)). However, comparisons between different species, markers, and index measures are extremely difficult.

Heterozygosity at microsatellite loci varied between $0.555 \leq H_E \leq 0.683$ (overall $H_E = 0.664$). These values are larger than those found in some threatened amphibians (*Rana pretiosa*, vulnerable, $H_E = 0.31$ (Blouin et al. 2010), *Randon sibiricus*, endangered $0.077 \leq H_E \leq 0.086$ (Chen et al. 2012)). However, similar and even higher levels of heterozygosity have also been found in other critically endangered amphibians (*Pseudophryne corroboree*, $H_E = 0.607$, *P. pengilleyi* $H_E = 0.885$, Morgan et al. (2008), *Ambystoma leorae*, $H_E = 0.613$ Sunny et al. (2014)).

This relatively high level of genetic diversity does not reflect the current endangered status of *T. bullocki*. High levels of genetic diversity could be an indication of large historic effective population sizes and/or high mobility and dispersal (i.e. gene flow). Although *T. bullocki* has generally been observed at very low numbers, recent studies have shown that the species detection probability is low and highly dependent on weather conditions and survey technique (Chapter 3). Although direct studies on *T. bullocki* dispersal are still lacking, frogs have been found up to 500 m from breeding streams, and movements of over a hundred metres per night have been recorded (Chapter 3), which suggests dispersal between close streams might be frequent. Because significant habitat disturbance has occurred only recently in most cases (within the last 40 years), it is possible that populations have declined too recently for the genetic signature to be detected.

Perhaps more valuable for conservation management than overall genetic diversity is the comparison of diversity levels between populations, as this can help identify populations of concern. Genetic diversity was not homogeneous at the population level (Table 4.2). The lowest diversity was found in the disjunct population of Los Lleulles (LL), which had the lowest mtDNA nucleotide and haplotype diversity, low haplotype richness, and had the lowest observed heterozygosity and allelic richness at microsatellite loci. However, this population also had the smallest sample size, which could be biasing the results. Nevertheless, lower genetic diversity at peripheral populations could be expected, as these populations are more isolated from sources of migrants, and thus more prone to genetic bottlenecks (Rowe and Beebee 2003, Hoffman and Blouin 2004). Reduced diversity could also be an indication of recent reductions in population size (bottlenecks will be discussed below). Genetic diversity at CH, which is also isolated and peripheral, was slightly higher

than LL, and similar to populations in Group C (PR, HU, CL), which are also peripheral (but less isolated). In contrast, and in agreement with the central-peripheral hypothesis, populations CA, CY and BU had the highest genetic diversity for both markers.

The pattern of distribution of genetic diversity is also concordant with current levels of habitat disturbance. The greatest diversity was found in Caramávida (CA), which has the highest amount of native and protected forest, while the lowest diversity (LL) was found for an area that has very little native forest and is dominated by exotic pine plantations. Disentangling the relative significance of historical effects (peripheral population) from more recent events (habitat loss) is difficult, however, as these variables are highly correlated (areas at the edge of the species' range are also the areas most affected by habitat loss).

Both markers used to measure diversity are considered neutral; however, evolutionary potential (which is the real concern in conservation biology) is best reflected by adaptive variation. Although neutral loci variation has been widely used as a surrogate measure, it is unclear whether neutral variation alone can reflect adaptive potential. Recent developments, particularly using the major histocompatibility complex (MHC) could allow us to look at variation in adaptive loci in the near future.

4.4.2 Phylogeography and population structure

Previous work within the *Telmatobufo* genus suggested that *T. bullocki* is a lineage that diverged from its close relative *T. australis* ~20 Mya (Nuñez and Formas 2000, Formas et

al. 2001). Phylogenetic analysis of mtDNA sequences confirmed this ancient origin with an estimated TMRCA for the sampled populations between 0.79 and 8.8 Mya (0.45-13.8 Mya composite 95 % HPD), when a maximum rate of 2% and minimum of 0.1% substitutions per My are considered. The phylogenetic tree shows there are two reciprocally monophyletic clades corresponding to the northern CH population and the southern cluster (the remaining populations) that have been reproductively isolated for at least half a million years. Furthermore, there is also a significant divergence in allele frequencies at microsatellite loci, suggesting the two clades should be treated as two separate evolutionary units (Moritz 1994). Although the geographic distance separating the two ESUs is relatively small (63 km), local environmental conditions could be significantly different (particularly under climate change conditions), and it is possible that local adaptations have developed. It is possible that CH individuals are best adapted to warmer and drier conditions than the more southern populations, as there is a marked latitudinal gradient of precipitation and temperature in Nahuelbuta (Smith-Ramírez 2004). Also, the CH population is at a lower elevation and close to the sea, which is also associated with warmer conditions. In fact, water temperature in CH stream was 2 degrees ˚C higher than the mean temperature of all other streams in the southern clade (Chapter 3). Due to the relatively high level of evolutionary divergence, and the potential for local adaptations, CH should be treated as a separate ESU from other known populations.

As well as the clear and prolonged genetic isolation between CH and the rest of the populations in the southern clade, significant population structuring was also found within the southern cluster of populations. Microsatellite markers revealed that the southern cluster is structured in three groups, as shown in the map in Figure 4.5. As expected due to

its geographical isolation, LL in the eastern slopes of Nahuelbuta diverged from all other populations at both genetic markers. Phylogenetic reconstruction placed LL individuals as a distinct internal lineage; however, its origin was not well resolved. TMRCA for LL individuals was 0.0198 - 1.119 Mya suggesting isolation of this population from the western lineages occurred before human intervention.

All populations from streams draining towards the Lanalhue lake in the south (HU, PR and CL) were clustered together based on allele frequencies (Group C), and no haplotypes were shared with other populations outside the cluster. Furthermore, the phylogenetic tree (Figure 4.5) shows these populations form a distinct and relatively old lineage (TMRCA from 59,100 years to 2,177 My). This suggests frequent historical female migration and functional connectivity between neighbouring basins HU, PR and CL. This connectivity means dispersal between breeding streams must occur overland, and can reach distances of at least 2-4 km. High dispersal and movement between breeding sites have been related to an increased vulnerability to habitat fragmentation in frogs (Funk et al. 2005), possibly due to increased mortality of juvenile dispersers. In contrast, no haplotypes from Group C were shared with the neighbouring cluster (CA, CY, BU), suggesting no female migration between them. However, microsatellite markers show some degree of admixture between BU and Group C, and genetic distance (F_{ST}) was not significant for BU - HU. This could be an indication of some degree of connectivity between the groups, which could be attributable to male migration. Distance between BU and Group C is approximately 10 km, which may represent the upper dispersal limit of this species. Dispersal and migration distances of 10 km and greater have been found for some amphibians (Funk et al. 2005), and this could be possible for *T. bullocki,* particularly if habitat is continuous. CA, CY and BU populations

were clustered together (Group B) based on allele frequencies, however, they were paraphyletic for mtDNA sequences. This also supports the idea that *T. bullocki* can migrate and disperse between neighbouring breeding streams.

The phylogenetic relationships and structuring discussed above are based on the sampled populations, which include all currently known populations except for one recently located population near Ramadillas. However, the great majority of Nahuelbuta has not been surveyed for *T. bullocki*, and it is possible there are several unsampled populations, and that the apparently disjunct distribution could be in fact more continuous. Phylogenetic relationships and structuring of populations should be re-evaluated and updated as new data become available.

4.4.3 Historical demography and bottlenecks

By using two different types of markers, it was possible to infer the demographic history of populations at different time scales. Several climatic changes affected the Southern Hemisphere during the Pleistocene era, with glacial advances (and retreats) shaping the physical and biological environment (Villagrán 2001). Pleistocene glaciations in South America include the most recent Patagonian glaciation (180 kya), the last glacial maximum (LGM, ~25-23 kya), and the coldest Pleistocene glaciation (~0.7 Mya) (Núñez et al. 2011). Mitochondrial DNA markers revealed a strong signature of a sudden population expansion in the southern populations (both demographic and spatial), which is likely to have started during the early/middle Pleistocene, between 20 and 500 kya. This is consistent with an event of glacial retreat (possibly post LGM), as previously unsuitable habitat becomes

available and colonised. Although Nahuelbuta was not directly covered by the LGM ice sheet, forests were restricted only to lower altitudes (Villagrán and Armesto 2005), and higher elevations would have been unsuitable to sustain frog populations. *Telmatobufo bullocki* populations in the southern cluster would have survived glacial periods in lower altitudes. In contrast, the demographic history of the northern CH population, as inferred by mtDNA markers, showed less evidence for recent population changes, suggesting lower latitude and altitude might have created a glacial refugium. However, this population had a smaller sample size, and more samples would be needed to confirm this hypothesis (Peery et al. 2012).

Microsatellite markers, on the other hand, allow me to infer demographic changes that occurred more recently. Although the heterozygosity excess method failed to detect any bottlenecks (or expansions), and the M-ratio test revealed weak evidence for bottlenecks in CH and LL only, the Bayesian inference method implemented in MSVAR strongly suggests that a demographic bottleneck occurred throughout *T. bullocki* populations ~400 years ago. Considering the estimated timeframe (95% HPD ~57- 3000 years), this bottleneck could be related to recent human-induced habitat loss and fragmentation. However, recent simulation studies suggest that MSVAR could be unreliable under violations of the single-step mutation assumption, which tends to underestimate current population size and overestimate ancient population size (Faurby and Pertoldi 2012). The authors suggest that although the absolute values of the parameters might be unreliable, the relative differences between N_0 and N_1, and N_e between populations of the same species will generally be more reliable. Therefore, the results obtained can be interpreted as evidence for a recent decrease in effective population size in *T. bullocki* populations.

However, the magnitude of the decrease, or the absolute numbers, remain unclear. The relatively weak evidence for reductions in population size could be due to the very recent timing of the expected bottleneck (~40 years), as methods available usually perform better for longer time periods.

4.4.4 Effective population sizes

Lower confidence limits for estimated effective population sizes with the linkage disequilibrium method were low for the peripheral and disjunct populations of Los Lleulles (LL; lower 95% CI N_e = 43) and Chivilingo (CH; lower 95% CI N_e = 34). With approximate Bayesian computation, results were similar for CH. The result supports the idea that LL and CH are at higher risk of extinction than the other populations, and that one important management objective should be to increase population sizes at these sites. With the LD method Groups B and C had lower limits of nearly two hundred and estimated N_e were relatively high (~500), while ABC method gave larger N_e for Group C (N_e = 1724) and smaller for Group B (N_e = 90). This is consequent with the biggest geographic area encompassing these populations, and the fact that these are groups of populations (larger metapopulations). This also agrees with the higher diversity found in these groups. The fact that higher confidence limits could not be defined also is an indication of larger population sizes.

4.4.5 Implications for conservation and management

In-situ management

One of the main objectives of this study was to be able to give practical guidance for population management, based on genetic evidence. Among the first steps toward this, is the delineation of evolutionarily significant and management units (Frankham et al. 2010). As discussed above, two ESUs and four MUs were identified for *T. bullocki*, all of which should receive conservation attention. Ideally, all populations should be protected, particularly in small ranging species such as *T. bullocki*; however, resources are generally limited, and some prioritisation might be needed. Usually, population prioritisation based on genetic markers favours the most unique and diverse populations, to maximise both representation and persistence of diversity (Petit et al. 1998, Moritz 2002). However, prioritisation should also consider current levels of protection, immediate threats, and potential conservation opportunities.

Considering the greatest genetic diversity was found in populations that are less immediately threatened by habitat loss (Group B: Caramávida, Cayucupil, Butamalal), and therefore are considered to have a lower risk of extinction, priority could be given to the more genetically unique but less diverse populations, which are at the highest imminent risk. The results show that the disjunct, peripheral, and genetically unique populations of Los Lleulles and Chivilingo are both smaller and less genetically diverse, and therefore are under more immediate threat. The small estimated population sizes, greater isolation from other populations, along with the increased disturbance at these sites, makes these populations highly vulnerable to extinction (Rowe and Beebee 2003). Since levels of genetic diversity are still relatively high, and no significant inbreeding was detected,

management should aim primarily at increasing the effective sizes of these populations to ensure current levels of diversity are preserved. Considering the main immediate threat to both populations is the loss and degradation of the natural habitat due to forestry plantations, it is important that these are managed in a way that minimises the impact on *T. bullocki* habitat. Some level of protection is currently given to CH population, as some of the area has been declared of high conservation value and therefore remaining native forest is being protected. Nevertheless, considering this population represents an independent ESU, current protection and native buffer areas might be less than adequate (Chapter 3), and further protection measures are needed.

Ways to increase population sizes through management (and therefore effective sizes) are to directly increase the habitat available for the species. Since *T. bullocki* is adapted to native forest habitat, ecological restoration of native forest might be needed at these locations. Mortality in frogs is typically higher at younger stages (Wells 2010), thus increased recruitment of young metamorphs could also help increase population size. This could be achieved through the protection of critical breeding habitat (Chapter 3), for example through the establishment of wide native riparian zones rich in cover objects (e.g. logs, rocks). Particular care should be taken when harvesting plantations near these populations, as this could reduce the population size even further and cause a severe genetic bottleneck or local extinction. The lack of neighbouring populations means dispersal and/or recolonisation is unlikely, and populations might not be able to recover.

For the other populations, which are bigger, more diverse, and more connected, management should be focused on maintaining the high levels of diversity and current

population sizes through habitat protection and maintenance of connectivity between breeding sites. Since Group B and C were identified as separate management units, management should aim to maintain functional connectivity among populations within groups. This way, connectivity between Huilquehue, Provoque, and Calebu should be enhanced through the maintenance of existing native forest and establishment of protected corridors. By maintaining local connectivity the risk of extinction is reduced, as populations can be "rescued" or recolonized by neighbouring populations if local bottlenecks or extinctions occur (whether from natural or human-induced causes). In a similar way, habitat protection and connectivity between Caramávida, Cayucupil and Butamalal should be the primary focus of management at these locations. Considering the relatively large extent of native forest protected in Caramávida, management should aim at identifying the corridors that connect this area to other native fragments and populations. This group of populations contains the greatest numbers and diversity for this species and could be considered a stronghold population. Long term monitoring of populations, including their genetic diversity, should be considered for the four management units.

Ex-situ conservation

Although the results shown here do not suggest captive breeding should be the main priority for *T. bullocki* conservation, some captive management recommendations can also be drawn from the results. Genetic profiling of captive individuals held at the National Zoo correctly identified the population of origin as Butamalal. Based on the results, captive breeding should consider the existence of two ESU and four MU. If the objective of captive breeding is to maintain the diversity and evolutionary potential within a species, effort

should be made to keep viable populations representing at least the four genetic groups identified in this study. Individuals from CH should be kept as a separate captive population, while individuals from the other populations could be mixed. However, founders should come from all populations. If new populations are discovered, the genetic resources should be assessed through genotyping a sample of individuals. Depending on the results, new populations could be treated as part of the existing MU or they could represent differentiated populations that should be treated independently.

4.4.6 Future directions

It is important to highlight that all of these recommendations come from a genetic conservation point of view, which is by no means the only criterion for population conservation. Several other issues should be considered when defining and prioritising populations of concern. Ideally, multiple criteria should be used. For example, to define management units, the proposed concepts of ecological and genetic exchangeability could be used if more ecological data became available (Templeton 1989, Crandall et al. 2000). This approach incorporates genetic distinctiveness (e.g. low levels of gene flow) and local ecological adaptations (e.g same life history traits, morphology) to define units that can be exchangeable.

The genetic data obtained in this study for mtDNA and microsatellite markers opens the possibility for a more detailed reconstruction of the evolutionary history of the genus. Particularly, the level of genetic divergence between *T. bullocki* and the recently described *T. ignotus* has not been assessed, and phylogenetic relationships of *T. ignotus* within the genus remain unknown. Now that we have baseline genetic data for *T. bullocki* populations

in the Nahuelbuta Range, a natural next step would be to assess the genetic divergence of *T. ignotus* (Los Queules population) and the Quirihue population compared to variability within *T. bullocki* populations. Microsatellite markers developed for this study have amplified DNA from *T. venustus*, and could presumably amplify DNA from the other *Telmatobufo* species.

Chapter 5:
Landscape genetics and dispersal pathways

5.1 Introduction

5.1.1 Habitat fragmentation and extinction

Human-induced habitat loss and fragmentation are regarded as major causes of recent biodiversity declines and extinctions (Cushman 2006, Sodhi and Ehrlich 2010). Increased isolation caused by fragmentation may disrupt critical population processes such as the migration and dispersal of individuals (Fahrig 2003), and consequently gene flow among populations. Low rates of gene flow can increase the extinction risk of populations through the negative effects of genetic drift, accumulation of deleterious mutations, and inbreeding depression (Andersen et al. 2004, Frankham 2005). Connectivity between populations is also essential for the long-term persistence of metapopulation dynamics (Hanski 1998), as dispersing animals can re-colonize habitats that have experienced local extinctions. Consequently, maintaining connectivity among populations has become one of the main objectives in the conservation of many species, including amphibians in fragmented habitats (Fahrig and Merriam 1994, Joly et al. 2003). However, assessing and maintaining connectivity of populations in heterogeneous landscapes remains challenging, particularly when little is known about the habitat needs, movements, and dispersal behaviour of organisms.

5.1.2 Landscape genetics

One increasingly successful approach to assess how different environmental factors affect connectivity of populations is the use of landscape genetics (Manel et al. 2003). Landscape genetics approaches integrate elements of population genetics, landscape ecology, and spatial statistics, and are commonly used to understand how different landscape features affect microevolutionary processes of gene flow, drift and selection (Manel et al. 2003, Holderegger and Wagner 2006). In particular, landscape genetics has become an important conservation tool used to infer how human-induced landscape changes affect gene flow, and identify potentially important dispersal routes or significant barriers (Cushman et al. 2009, van Strien et al. 2013).

Spatial genetic patterns of neutral variation arise naturally when dispersal of individuals is limited, due to the increased probability of mating with nearby conspecifics compared to more distant individuals. Consequently, individuals or populations that are more geographically distant will tend to be more genetically differentiated (at neutral loci), a pattern known as isolation by distance (IBD) (Wright 1943). In addition to IBD, population structure can also arise due to the presence of distinct features acting as geographic barriers (isolation by barrier, IBB) such as large rivers, mountains, and highways (Holderegger and Di Giulio 2010). In complex and heterogeneous landscapes, small-scale gene flow and genetic structuring of populations will depend on the composition and spatial structure of the different elements, and their relative influence on movements and dispersal (Storfer et al. 2006). Under the isolation by resistance hypothesis (IBR), landscapes are perceived as continuous surfaces of resistance gradients representing the ability of an individual to move through the landscape. By using a modeling approach, IBR

hypotheses can be tested against IBD and IBB in order to detect landscape effects, and identify particular features that facilitate or hinder dispersal (Spear et al. 2010).

Within the isolation-by-resistance framework, least-cost paths (LCP) analyses can be used to represent the relative effect of landscape features to gene flow (Spear et al. 2010). Using GIS tools, landscape resistance surfaces representing IBR hypotheses can be created, and the path that minimises the cost of moving from source to destination (i.e. the least cost path) can be found. Using statistical tests and a modeling approach, the correlation between the LCP distance (landscape distance) and genetic distance can be assessed and compared between different resistance hypotheses and alternative IBD and IBB hypotheses. In particular, Mantel and partial Mantel tests correlating genetic and landscape distance matrices have been commonly used (Manel and Holderegger 2013). The use of Mantel and partial Mantel tests has been critisized due to the inflated type I error rate or low power, and its usefulness in population genetics has been debated (Raufaste and Rousset 2001, Castellano and Balletto 2002, Guillot and Rousset 2013). Despite critizism, some authors have demonstrated that Mantel and partial Mantel tests remain valuable when hypotheses are strictly based on distances (Legendre et al. 2002, Legendre and Fortin 2010), and when the relative support among a full combination of alternative hypotheses is used to assess model support (Cushman et al. 2013). Furthermore, when used in conjunction with a causal modeling approach, they can correctly identify drivers of gene flow and reject incorrect and correlated hypotheses (Cushman and Landguth 2010).

5.1.3 *Telmatobufo bullocki* and fragmentation

Telmatobufo bullocki is a rare and endangered frog restricted to the Nahuelbuta Range in central south Chile. One of the main threats to this species is the recent and intensive loss and fragmentation of native forest that has been replaced by commercial monoculture plantations of exotic pine (*Pinus radiata*) and eucalyptus (mainly *Eucalyptus globulus*), which now covers nearly 70% of the species' range (Wolodarsky-Franke and Díaz Herrera 2011). Remaining native forests are mostly secondary and these forests are unevenly distributed across one big native core and thousands of smaller and scattered native fragments.

There are important characteristics of monoculture forestry plantations that could pose a substantial threat to native species, including amphibians. Exotic tree planatations can change both water quantity and quality in catchments (Huber et al. 2010) and hence indirectly result in changes in macroinvertebrate fauna (Mancilla et al. 2009). *E. globulus* plantations have been found to alter soil hydrological properties and sediment transport, and they release toxic allelochemicals that may influence the composition and structure of the understory (Oyarzún et al. 2011). Additionally, leaf litter leachates can result in deoxygenated and more acidic water, with increased phenolic contents and conductivity, which can in turn affect the viability and ecology of macroinvertebrate fauna (Canhoto and Laranjeira 2007). But perhaps the greatest threat to amphibians is the periodical harvesting of plantations through clear-cutting (Popescu and Hunter 2010). Full canopy removal has been found to have a strong negative effect on forest amphibian richness and abundance, as this process exposes the soil to direct sunlight and winds, leading to higher temperatures and drier conditions (Petranka et al. 1993, Semlitsch et al. 2009). Due to

physiological constraints, most forest amphibians will not be able to tolerate clear-cut conditions, making such areas probable barriers for dispersal and gene flow.

Nevertheless, plantation ecosystems in Chile are not a biological desert, and several native species, particularly birds, can use mature plantations to some degree (Grez et al. 2006). In Chapter 3, I described how *T. bullocki* was found living in mature pine plantation habitat adjacent to native forest, suggesting endangered native amphibians could be using mature plantations to some degree.

In Chapter 4, I described patterns of genetic diversity and population differentiation in *T. bullocki*. I showed how some neighbouring populations are connected through gene flow forming clusters; however, I did not test specific hypotheses about whether IBD alone or IBLR patterns were shaping the observed distributions. In this chapter, I will: 1) use a causal modeling approach and LCP analysis to assess the relative effects of natural and human-induced landscape features on functional connectivity in *T. bullocki,* and 2) identify potential dispersal routes. I hypothesize that gene flow and dispersal routes will be more closely associated to landscape features that are important for *T. bullocki* based on current knowledge of their ecology and behaviour. In particular, the effects of streams, slope, elevation, and land-use change from native forest to exotic plantation will be assessed.

5.2 Methods

5.2.1 Study area

The area selected for this study is located within the Nahuelbuta Range in central-south Chile (Figure 5.1), and includes a cluster of most of the known *T. bullocki* localities. The study area is covered by a complex mosaic of native forest (40.31%), exotic plantation (41.01%), agriculture (8.23%), scrub (8.71%), urban (0.11%), wetlands (0.42%), and lakes (1.17%; Figure 5.3). Native forest is mostly secondary (80%) as it has been logged for timber and wood extraction since human settlement, leaving mostly small and isolated fragments of adult native forest (7.4%) and mixed adult/secondary (12.7%). Most of the remaining adult native forest is at higher elevations inside the Nahuelbuta National Park, and in the privately owned High Conservation Value Area *Quebrada de Caramávida*. The area has a small human population, and most towns, cities, and agricultural lands are at low elevations.

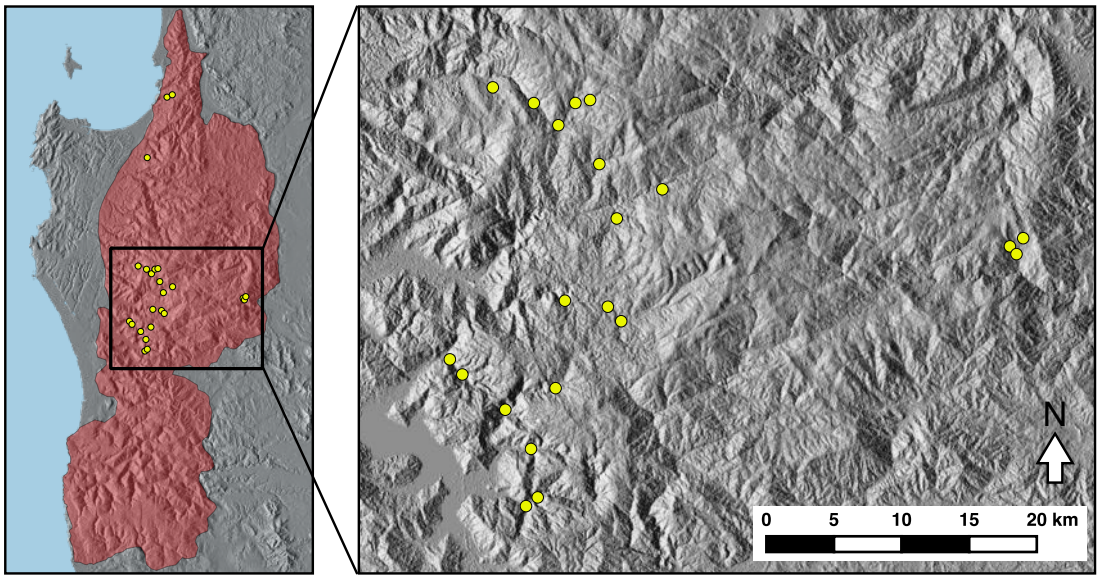


Figure 5.1. Study area in the Nahuelbuta Range, Chile (red shaded area in the left). Yellow dots in the left represent all recent *T. bullocki* sightings. Yellow dots in the right represent sampling locations included in this study.

5.2.2 DNA sampling and genotyping

Telmatobufo bullocki DNA samples were collected during 2012 and 2013 from wild animals within the study area (Figure 5.1). DNA samples from adults and juveniles were collected using low-impact buccal swabs (Copan nylon flocked dry swab Cat. No. 501CS01), which involves only a short manipulation of the animal, thus minimising stress and avoiding the negative impacts of other commonly used methods such as toe-clipping (Pidancier et al. 2003). Swabs were completely dried overnight by placing the open vials in an airtight container filled with silica beads. DNA samples were collected from tadpoles by clipping a small amount of tail tissue (1-2 mm), which was stored in 96% ethanol. DNA from swab samples was extracted using standard QIAxtractor Qiagen protocols and solutions, with a final elution volume of 70 µl. DNA from tissue was extracted using PureLink genomic DNA Kit (Life Technologies), with a final elution volume of 100 µl. Fifteen microsatellite loci

specifically developed for *T. bullocki* were used (Moreno-Puig et al. 2014). All PCR conditions followed those specified in the article. PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems, Inc.) and genotyped manually using GENEIOUS microsatellite plug-in. Individuals with more than two alleles missing (due to poor amplification or scoring difficulties) were removed from the analysis. Genotyping data was tested for errors and evidence of null alleles using MICROCHECKER v2.2.3 (Van Oosterhout et al. 2004).

5.2.3 Genetic analysis

Because of the rareness and low abundance of *T. bullocki*, the number of individuals sampled at each location was generally low (i.e. <10). To improve the spatial sampling, an individual-based approach was deemed more appropriate than a population-based approach (Prunier et al. 2013). Although individual-based landscape genetic analyses have been mostly used for species with continuous distributions that can't be easily assigned to discrete populations (e.g. American black bears (Cushman et al. 2006)), a recent study has shown that, provided pseudo-replication issues are taken into account, an individual-based approach represents an efficient way to maximise representativeness of landscape heterogeneity (Prunier et al. 2013). Genetic differentiation between individuals was measured using Rousset's a_r (Rousset 2000), which is analogous to the inter-population pairwise distance $F_{ST}/(1-F_{ST})$ commonly used in the assessment of spatial genetic variation. The square matrix of pair-wise a_r values between all individuals was calculated using the software SPAGeDI (Hardy and Vekemans 2002).

5.2.4 Landscape analysis

The relationship between landscape factors and gene flow was examined by testing multiple alternative hypotheses under a causal modeling approach (Cushman et al. 2006, Wasserman et al. 2010). Potential factors driving functional connectivity were identified as geographic distance (i.e. IBD hypothesis), and isolation by landscape resistance (IBLR), which represents the hypothesis that landscape characteristics affect movement, dispersal and gene flow. The relative influence of natural and human-induced landscape variables was examined by testing the correlations of several landscape-resistance hypotheses, with the observed genetic distances. Euclidean distances were measured as straight-line distances connecting two individuals, and landscape distances were calculated using least-cost path (LCP) analysis in ArcGIS (Spear et al. 2010, Etherington 2011). To account for the three-dimensional movement of individuals crossing mountainous areas, all LCP distances and Euclidean distances were corrected for topography by calculating the surface length (overland distance) using 3D Analyst tools in ArcGIS.

Under a causal modeling approach, the different IBR hypotheses were tested against the null hypothesis of IBD. The causal modeling approach defines certain statistical expectations that have to be met in order for a model to be supported independently from alternative models (Cushman et al. 2006). In this study, for a landscape model to be considered an independent driver of genetic structure it would have to 1) have a significant and positive correlation with genetic distance, 2) have a significant and positive correlation with genetic distance after controlling for Euclidean distance, and 3) have a non-significant relationship between the Euclidean distance and geographic distance when controlling for the landscape model. Mantel and partial Mantel tests were used to test the

correlation between the individual genetic distance matrix and the different LCP distance matrices (following Wasserman et al. 2010, Cushman et al. 2013). Model support was based on the Mantel *r* and partial Mantel *r* statistic and its significance ($\alpha = 0.05$). To avoid pseudoreplication of clumped individuals, significance was tested using 10000 restricted permutations following Prunier et al. (2013), where permuted objects are the aggregates rather than the individuals. Calculations were done in MATLAB using the script provided in Prunier et al. (2013).

Resistance surfaces

Resistance surfaces representing different landscape resistance hypotheses were developed based on factors that are likely to influence *T. bullocki* movements and gene flow based on the species' known ecology and behaviour (Chapters 1, 2, 3). Natural landscape features hypothesised to be correlated to gene flow were slope, elevation, and presence of streams. The effect of human-induced landscape change was assessed through the creation of several resistance surfaces based on land cover type. In particular, the effect of land-use change from native forest to exotic plantation was explored by assigning different costs for plantations compared to native forest. In order to optimise the relationship between landscape variables and genetic distance, multiple candidate resistance models were created for each variable using different resistance values and functional responses to gene flow (e.g. linear and power functions) as described below, following Spear et al. (2010). The best transformation was selected based on the highest correlation with genetic distance (highest Mantel *r*).

Slope

In mountainous areas such as Nahuelbuta, rugged terrain might be hindering movements of small species such as amphibians, which have comparatively limited dispersal. Therefore, slope was selected as a potentially important variable related to movements, dispersal, and gene flow. Slope was derived (as % rise) from the ASTER GDEM digital elevation model (30 m resolution) (Tachikawa et al. 2011), and rescaled with a linear function using ArcGIS 10.2. Three different levels of slope resistance were tested. The lowest resistance, corresponding to flat areas was set to 1, while the maximum resistance was set to 2, 5 and 10 for the low ($Slope_L$), medium ($Slope_M$), and high ($Slope_H$) slope resistance models, respectively. These models represent hypotheses that high slope areas are more difficult to cross, but they differ on the relative impact of slope on gene flow. In addition, slope was transformed using power functions of 0.2, 0.4, 0.6, 0.8, 2nd, and 3rd power, and rescaled to 1-10 (Wasserman et al. 2010). These models were named $Slope_x$, where x corresponds to the exponent in the power function (e.g. $Slope_{0.2}$, $Slope_{0.4}$). In total, 9 resistance surfaces were created for the slope variable, representing different functional relationships between genetic distances and LCP distance.

Elevation

Elevation results in several gradients in the Nahuelbuta range that might be important for *T. bullocki*, including shifts in climate as well as vegetation structure and composition (Wolodarsky-Franke and Díaz Herrera 2011). Recent distribution records show *T. bullocki* can be found from 70 m to 1030 m a.s.l., and despite several surveys, the species has not been found at higher elevations, suggesting very low and very high elevations are less

suitable (CHAPTER 2). Resistance surfaces were created based on the ASTER GDEM elevation data. First, a categorical surface was created where elevations from 50 to 1050 m were given a low resistance value (1 = suitable), and all other elevations (<50 and >1050) were given high resistance (10 = unsuitable); this model was denoted as Ele_{1_10}. The species distribution model created in CHAPTER 2, found a nearly linear function between habitat suitability and elevation, with suitability decreasing as elevation increases. Therefore, three continuous resistance surfaces were also created using a linear transformation from 1 to 2, 5 and 10 (Ele_L, Ele_M, Ele_H, respectively), between 50 and 1050 m a.s.l. while all other elevations (<50 and >1050) were given a high resistance value (10). Similarly to the slope models, these surfaces represent the hypotheses that resistance increases linearly with elevation, but they differ on the impact of elevation on dispersal. In addition, elevation was transformed with power functions in the same way as described above for slope, and models were denominated Ele_x, where x is the exponent of the power transformation. In all cases, elevations <50 and >1050 were given the maximum resistance value (10). In total, 10 rescaled and transformed resistance surfaces were tested for correlation between genetic distance and LCP distance (1 categorical, 3 linear, 6 power).

Streams

Telmatobufo bullocki is associated with fast-flowing streams, where adults breed and tadpoles spend months and possibly a year until metamorphosis (Chapter 3). Therefore, streams and riparian habitat can be expected to be acting as important connectivity corridors. However, *T. bullocki* also uses terrestrial habitat during the post-breeding season, where post-metamorphic frogs have been found over 500 m from streams

(Chapter 3). To test the relative resistance of terrestrial vs. stream movements, first a categorical resistance surface was created where streams were considered corridors (given low resistance value of 1), while terrestrial habitat was given high resistance (10), this model was denoted $Stream_{1\_10}$ similar to the other categorical models described above. In addition, a proximity surface was created to represent distance to streams as a continuous variable, where the resistance value corresponds to the distance (in pixels) from stream. A continuous surface might be a more realistic representation, as it better represents the spatial association of *T. bullocki* to both stream and riparian habitat. The proximity raster was rescaled so streams had a resistance value of 1, while resistance increased linearly as distance to stream increased, reaching maximum resistances of 2, 5, and 10 ($Stream_L$, $Stream_M$, $Stream_H$ respectively). The proximity raster was also transformed and rescaled (from 1 to 10) using power functions (0.2, 0.4, 0.6, 2, 3). In conjunction, these hypotheses test whether the resistance to movement increases rapidly with distance to stream and then stabilises, or increases slowly and then suddenly increases when reaching a threshold.

Land cover

Land cover data were obtained from the Chile national native forest survey (CONAF 2008). The original vector layer was transformed to a categorical raster layer (30 m resolution), and reclassified into 8 classes (e.g. all native forest types including secondary and adult were merged into one single native class). Resistance values from 1 to 10 were assigned to different land cover types according to the different hypotheses summarised in Table 5.1. Urban, agriculture, and scrub cover, were *a priori* considered less suitable for *T. bullocki* and therefore were assigned high resistance values. The first hypothesis considered all

forest types (native and exotic) to have low resistance (1), while non-forest cover types were assigned high resistance values (10). This resistance surface (LC_1) represents the null hypothesis that there is no difference between the resistance of native forest and exotic plantation. Surface LC_2 represents the hypothesis of high resistance of clear-cuts and young plantations and moderate resistance of mature plantations compared to native forest. LC_3 considered young and mature plantations to have higher but moderate resistance compared to native forest. LC_4 considered only native cover as having low resistance while all other land cover types had equally high resistance. LC_5 considered mature plantations and native forest as having equally low resistance, while young plantations and clear-cuts have high resistance. Finally, two alternative resistance surfaces (LC_6 and LC_7) were created representing the hypothesis of lower resistance of plantations compared to native forest.

Table 5.1. Land cover classes and resistance values assigned in each resistance hypothesis. A small value of 1 represents lower landscape resistance to movement (i.e. corridor), while the highest value 10 represents high landscape resistance to movement (i.e. barrier).

Land cover class	LC_1	LC_2	LC_3	LC_4	LC_5	LC_6	LC_7
Urban	10	10	10	10	10	10	10
Agriculture	10	10	10	10	10	10	10
Scrub	10	2	10	10	10	10	10
Plantation (old)	1	2	2	10	1	1	1
Plantation (young)	1	10	2	10	10	1	1
Native	1	1	1	1	1	2	10
Wetlands	10	2	10	10	10	10	10
Water	10	10	10	10	10	10	10

Multivariate models

Once the most supported scale transformation and resistance parameterisation for each variable was identified, 15 additive models were built combining the four variables. In addition to the factorial combination of the four variables, the habitat suitability index model (HSI) developed in Chapter 2 was also tested. This model was built using maximum entropy modeling, and includes climatic, topographic, and land cover variables (Chapter 2). Some studies have used habitat suitability as surrogate for landscape resistance (Wang et al. 2008, Igawa et al. 2013), assuming dispersal occurs primarily through suitable habitat. Here, the HSI model was included to test the hypothesis that habitat suitability could be used as a proxy for landscape resistance.

The modeling approach followed the two-stage causal modeling of Wasserman et al. (2010). In the first step, the 16 resistance models described above were tested against the null hypothesis (IBD). For a model to be supported in the first step of causal modeling, it had to remain significantly correlated to genetic distance when partialling out Euclidean distance, while the correlation of Euclidean distance would be non-significant after partialling out the effect of the candidate resistance model. Only models that met those statistical expectations were included in the second step of causal modeling. The second step tested 1) whether the top model of the first step (based on partial Mantel *r* value) had significantly more support than the other supported models, 2) if any of the alternative models could explain genetic distances independently of the top model. For the top model to be identified as the only hypothesis supported, the correlation of the top model with genetic distance, partialling out all the alternative models, would have to remain

significant, while the correlation of the alternative model with genetic distance would have to be non-significant after partialling out the top model.

Dispersal network

Potential dispersal routes were identified based on the top resistance model. LCPs represent single routes (links) connecting two locations (nodes); however, dispersal in natural systems and heterogeneous landscapes is unlikely to occur through a single route. Instead, multiple links between two locations could exist. In order to better visualize the full connectivity of the landscape, the least-cost corridor tool for ArcGIS included in the SDMtoolbox was used (Brown 2014). This tool uses a density analysis to create a raster surface with categories including all paths that are slightly more costly than the LCP.

5.3 Results

5.3.1 Genotyping

A total of 113 individual genotypes were sampled at 15 loci (121 individuals were sampled: 8 individuals had too few data and were removed). Clumped samples less than 500 m away were considered to be from the same site, and the average coordinate was used. The final dataset comprised 21 locations with 1 to 25 samples each, with an average of 5.4 individuals per site. The minimum and maximum distances between sites was 775 m and 44.2 km, respectively, with an average distance of 20.1 km. Genetic distance was significantly correlated with Euclidean distance (Mantel $r = 0.2419$, $P = 0.0001$).

5.3.2 Landscape genetic analysis

Univariate analysis

There was a positive and significant correlation between genetic distance and all LCP measurements for all univariate resistance models (Table 5.2). Ranking of models according to Mantel *r* for each variable is shown in Table 5.2. The best model representing the stream variable was $Stream_{0.2}$, the proximity to stream transformed with a power function (from 1 to 10) with exponent 0.2 (Mantel r = 0.2588, P = 0). The same transformation (Appendix H) was selected for slope where the best model was $Slope_{0.2}$ (Mantel r = 0.2427, P = 0). The best model for land cover was LC_4, which assigned low resistance to native forest, and high resistance to plantations and all other cover types (Mantel r = 0.2443, P = 0). For elevation, the best model was the categorical Ele_{1_10} with low resistance between 50 and 1050 m a.s.l. and high resistance otherwise (Mantel r = 0.2396, P = 0). All correlations except for Ele_{1_10} were more correlated to genetic distances than the Euclidean model. These four top resistance surfaces were subsequently used to build the multivariate models.

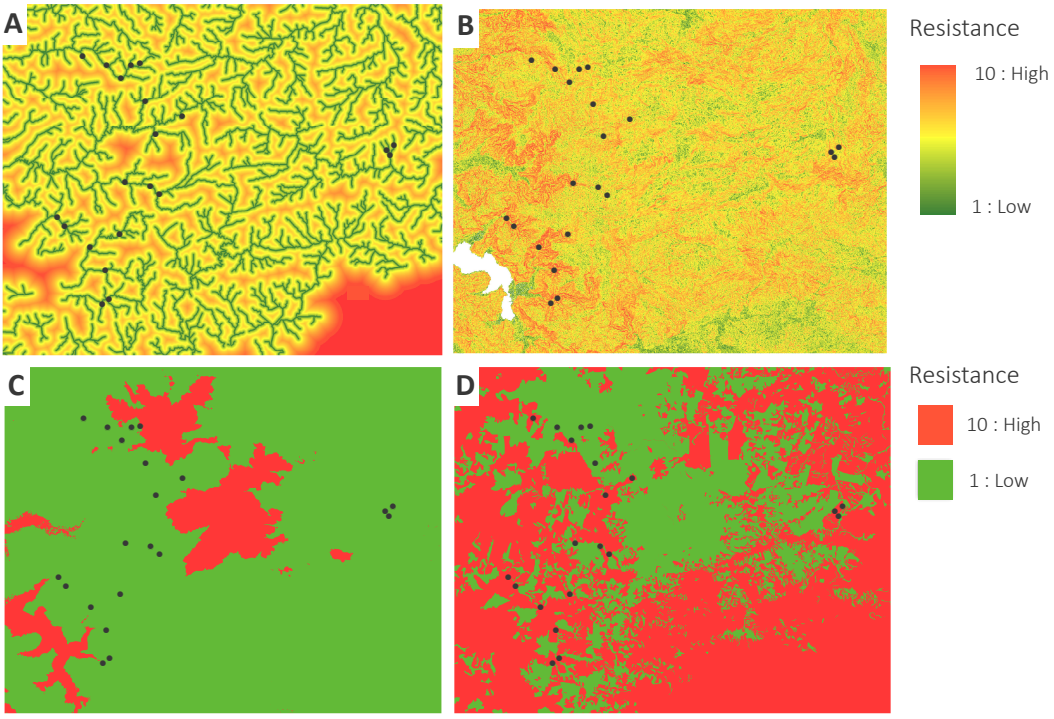


Figure 5.2. Resistance surfaces for each variable used for modeling. A) Streams: Proximity to streams as a power function with exponent 0.2. B) Slope: percentage rise in slope transformed with a power function with exponent 0.2. C) Elevation: categorical with low resistance between 50 and 1050 m a.s.l and high resistance for all other elevations. D) Land cover: categorical with native forest with low resistance and all other types with high resistance. Black dots represent the 21 sampling locations.

Table 5.2. Mantel *r* and *P*-values (10000 restricted permutations) for the correlation between pairwise genetic distance and LCP distance for 113 individuals across each resistance surface for the four variables considered. Models are ranked with the highest correlation on top (grey shaded rows). The top model for each variable was used in subsequent causal modeling approaches.

STREAM			ELEVATION		
Model	Mantel *r*	*P*-value	Model	Mantel *r*	*P*-value
$Stream_{02}$	0.2588	0	Ele_{1_10}	0.2396	0
$Stream_{04}$	0.2574	0	Ele_{L}	0.2359	0
$Stream_{1\_10}$	0.2555	0.0001	Ele_{02}	0.2319	0.0001
$Stream_{06}$	0.2499	0	Ele_{3}	0.2240	0.0004
$Stream_{H}$	0.2466	0	Ele_{06}	0.2228	0
$Stream_{08}$	0.2457	0.0001	Ele_{M}	0.2184	0
$Stream_{M}$	0.2452	0	Ele_{2}	0.2123	0.0010
$Stream_{2}$	0.2442	0	Ele_{H}	0.2107	0.0007
$Stream_{3}$	0.2421	0.0002	Ele_{08}	0.2079	0.0005
$Stream_{L}$	0.2420	0	Ele_{04}	0.2017	0.0005
SLOPE			LAND COVER		
Model	Mantel *r*	*P*-value	Model	Mantel *r*	*P*-value
$Slope_{02}$	0.2427	0	LC_{4}	0.2443	0
$Slope_{2}$	0.2424	0	LC_{2}	0.2425	0.0001
$Slope_{L}$	0.2420	0	LC_{3}	0.2402	0
$Slope_{3}$	0.2420	0	LC_{5}	0.2395	0
$Slope_{M}$	0.2414	0	LC_{1}	0.2379	0
$Slope_{04}$	0.2403	0	LC_{7}	0.2343	0
$Slope_{H}$	0.2392	0	LC_{6}	0.2232	0.0001
$Slope_{06}$	0.2383	0.0001			
$Slope_{08}$	0.2371	0			

Multivariate analysis

In the first step of causal modeling, all models were significantly correlated with genetic distance; however, only five remained significant after partialling out the effects of Euclidean distance (Table 5.3). Euclidean distance was not independently able to explain genetic distance after partialling out the effects of the five supported models. Therefore, these five models (highlighted in Table 5.3) were selected for the second step of causal modeling.

Proximity to streams (St) was the top supported model based on partial Mantel *r* and *P*-value ($r = 0.1297$ $P = 0$) in the first step of causal modeling. In the second step, the ability of the top model (St) to independently explain genetic distances was assessed by removing the effects of the other four supported models (Table 5.4). Proximity to streams remained significantly correlated to genetic distance after partialling out the effects of three of the alternative models (E + St, St + Sl +LC, Sl), and was not significantly correlated when partialling out the effects of the St + LC model. None of the four alternative models were significant after partialling out the effect of St. These results show that proximity to streams is the landscape feature that best explains current patterns of genetic differentiation in *T. bullocki*. However, the second best model, which included proximity to streams and land cover, could not be rejected.

Dispersal network

The single least-cost paths between all sampling locations over the top resistance model are shown in Figure 5.3 (with land cover as base map), and the least-cost corridors are

shown in Figure 5.4. The dispersal network (Figure 5.4) includes paths that are up to 10% more costly than the LCP.

Table 5.3. Results of Mantel and partial Mantel tests for the null model (Euclidean) and the 16 landscape resistance models tested, ranked according to their Mantel *r* value. Variables included in the models are proximity to streams (St), slope (Sl), elevation (E), and land cover (LC). Mantel tests were performed between the pairwise genetic distance matrix (a_r) between 113 individuals and the Euclidean and least cost path distance (LCP) matrices. Models that meet the causal modeling statistical expectations are highlighted.

Model	Mantel test		Partial Mantel test			
			LCP|Euclidean		Euclidean|LCP	
	Mantel r	*p*-value	Mantel *r*	*p*-value	Mantel *r*	*p*-value
St	0.2588	0	0.1297	0	-0.0889	0.9969
St + LC	0.2575	0	0.0921	0.0039	-0.0142	0.6216
E + St	0.2475	0	0.0578	0.0278	-0.0208	0.7674
St + Sl + LC	0.2451	0.0001	0.0548	0.0411	-0.0365	0.8727
H.S.I.	0.2449	0	0.0409	0.0651	0.009	0.4431
LC	0.2443	0	0.0461	0.1389	0.0297	0.3172
E + Sl + LC	0.2437	0	0.0404	0.0891	-0.0259	0.8006
Sl	0.2427	0	0.0405	0.0460	-0.0345	0.9317
Sl + LC	0.2425	0	0.0195	0.2861	0.0050	0.4764
E + St + Sl	0.2423	0.0001	0.0149	0.2727	0.0026	0.4535
Euclidean	0.2419	0.0001				
E + St + Sl + LC	0.2419	0	0.0079	0.3736	0.0066	0.4124
E + Sl	0.2408	0	-0.0080	0.6163	0.0246	0.2017
E + LC	0.2403	0	0.0078	0.3794	0.0288	0.2240
E	0.2394	0	-0.0155	0.6760	0.0388	0.1185
E + St + LC	0.2390	0	-0.0029	0.5020	0.0382	0.1480
St + Sl	0.2375	0.0003	-0.0558	0.9574	0.0729	0.0113

Table 5.4. Results of the second step of causal modeling. The top model identified in Table 5.3 (St) is tested against the other models also supported.

Model	Partial Mantel test			
	St|model		model|St	
	Mantel *r*	*p*-value	Mantel *r*	*p*-value
St + LC	0.0440	0.1881	0.0349	0.1572
E + St	0.0895	0.0004	-0.0438	0.9298
St + Sl + LC	0.1075	0.0002	-0.0651	0.9812
Sl	0.1234	0.0001	-0.0821	0.9935

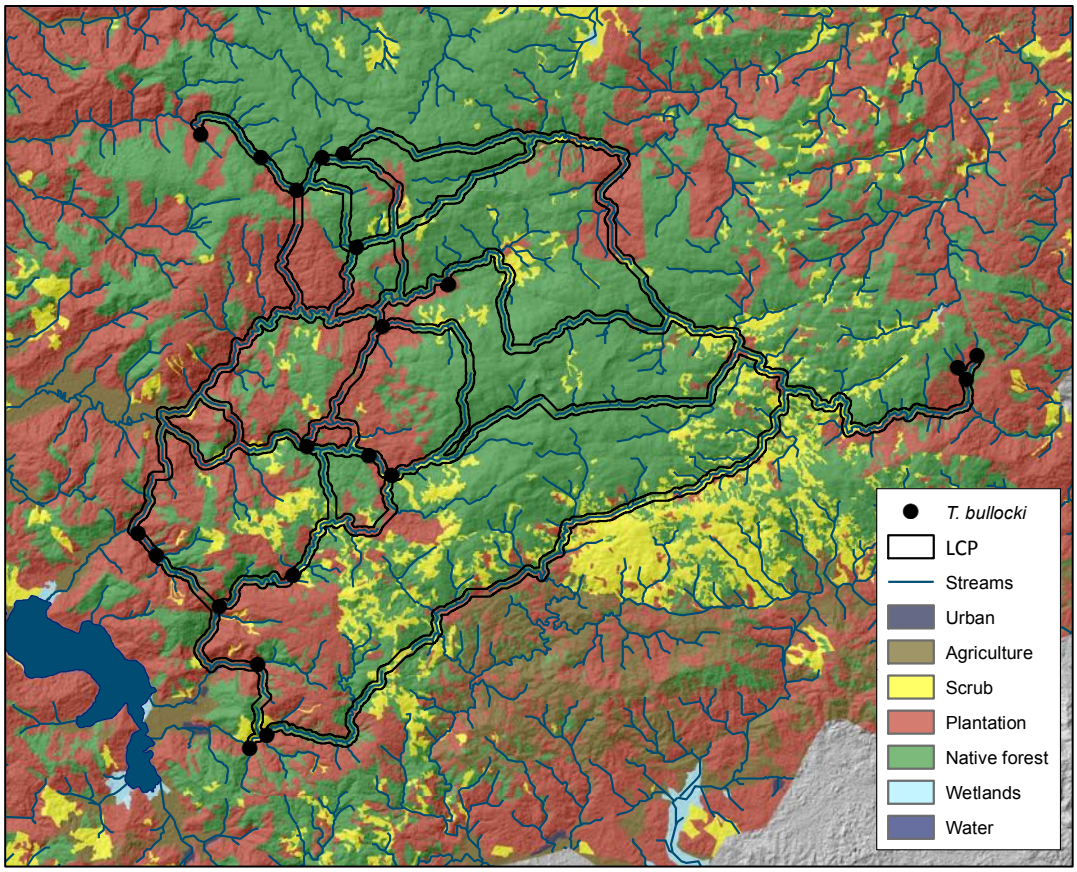


Figure 5.3. Map showing the least cost paths (LCP) connecting *T. bullocki* sampling locations and the land-use classification based on CONAF (2008). LCP shown represent potential historical dispersal routes under the best-supported model of landscape resistance. The paths follow stream and riparian habitat.

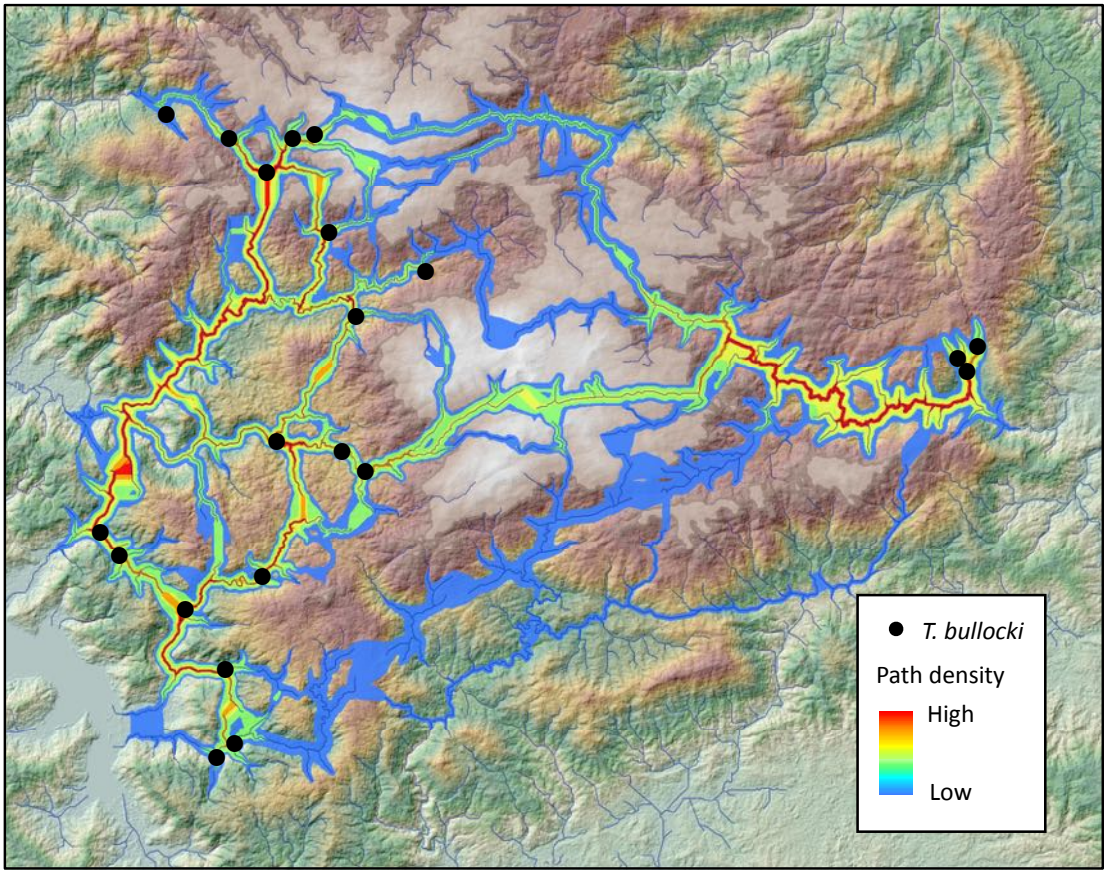


Figure 5.4. Map showing the dispersal network (least-cost corridors). Corridors include routes that are up to 10% more costly than the LCP. The colour represents the relative amount of paths that go through that corridor (path density). Warmer colours represent higher gene flow and connectivity.

5.4 Discussion

5.4.1 Effect of landscape features on *T. bullocki* functional connectivity

Streams

My results provide strong evidence that dispersal in *T. bullocki* is associated with streams and riparian habitat (top four models included St = proximity to stream, and St was the top ranked model). The ranking of scaled transformations for the stream variable suggests that terrestrial habitat resistance increases rapidly with distance to stream, but riparian habitat also contributes to genetic connectivity (top model power function 0.2 ranked better than the categorical model), highlighting the close association of *T. bullocki* to streams and riparian habitat.

In dendritic stream networks, dispersal can follow two pathways: 1) within network (intra-basin) or 2) between networks (inter-basin). The tadpoles of *T. bullocki* have a long growth stage (Chapter 3), and are highly adapted to fast-flowing conditions (Formas 1988). It is possible that tadpoles dispersing through streams are responsible for most of the intra-basin dispersal. Nevertheless, adults are also well adapted to stream conditions, and can spend most of the breeding season in the streams (Chapter 3). Movement of adults within streams in search of suitable breeding habitat and mates could also contribute to strong intra-basin dispersal. Riparian zones are critical habitat for most aquatic breeding amphibians (Semlitsch 2000, Semlitsch and Jensen 2001, Semlitsch and Bodie 2003). Riparian areas are essential habitat for young metamorphs during the transition from aquatic to terrestrial habitat use, and young *T. bullocki* have been found under rocks mainly within a few metres from the water (Chapter 3).

Despite the strong association with streams and riparian habitat discussed above, *T. bullocki* is able to disperse between basins (Chapter 4). Such inter-basin dispersal movements are key to population connectivity, and imply moving overland through non-riparian habitat and crossing ridges. The results suggest inter-basin dispersal movements minimise the total distance travelled over non-riparian habitat, which results in inter-basin connectivity through headwaters and mountain passes. However, a recent study on a similar stream-breeding amphibian (*Ascaphus montanus*) comparing effects of anthropogenic disturbance suggests patterns of dispersal associated to riparian habitat in managed forests might be a result of the impact of timber harvest (Spear and Storfer 2010). Dispersing individuals would be more likely to stay close to streams to avoid the lack of cover in recently harvested areas, and therefore dispersal routes in managed forests would tend to follow riparian areas. Whether *T. bullocki* naturally uses riparian habitat as dispersal corridors, or if this is a result of the high levels of disturbance of non-riparian areas (or more likely a combination of both), is not yet possible to determine.

Slope and elevation

In contrast to the influence of streams, slope and elevation were poor predictors of gene flow, suggesting these variables are not as strongly related to dispersal as proximity to streams. Although high slope has been related to dispersal in some amphibians (Richards-Zawacki 2009), and could not be *a priori* ruled out as an important factor, this result is unsurprising. *T. bullocki* is a species that has evolved in a landscape of steep slopes for very long time; in fact, high slope areas could be essential for the species. High slopes create complex microhabitats, and are characteristic of ravine and riparian areas of mountain

streams. In Chapter 3, adult frogs were observed burrowing and moving uphill through nearly vertical slopes (at a small scale), suggesting they can manage to cross small high-slope areas. It is important to note, however, that the scale at which slope becomes important might be smaller than the resolution of the data used in this study, potentially affecting the results, as the effect of slope becomes homogenized through the landscape.

Elevation was not significantly correlated with dispersal, and I believe this result is likely to be due to data, sampling, and statistical constraints. Although *T. bullocki* has been found at a wide range of elevations (from 70 m to 1030 m a.s.l., Chapter 2), it hasn't been found in the highest areas of Nahuelbuta (above 1030 m) despite the wide availability of native forest habitat (e.g. Nahuelbuta National Park), and repeated surveys over the study period. Moreover, the habitat suitability model built in Chapter 2 suggests a negative correlation between habitat suitability and elevation. Considering the changes in climate, vegetation, and habitat at higher altitudes, it was expected dispersal would be diminished across high elevations; however, such effect was not found (most models including elevation were poorly ranked, and the elevation model had less support than the Euclidean model). This result is possibly due to the low number of samples and lack of independent replication, as unfortunately only one population is known to persist in the eastern slopes of Nahuelbuta. Despite this, the ranking of alternative models for the elevation variable confirmed the elevation effect is better described as a categorical effect rather than a continuous effect (the categorical model had greater support than linear and power functions).

The habitat suitability model of Chapter 2 was not supported under the causal modeling approach; however, it was more strongly correlated to genetic distances than the

Euclidean model. Habitat models have been found to be good predictors of gene flow in some species (Wang et al. 2008, Igawa et al. 2013), but not all (Wasserman et al. 2010). Non-significant correlations of gene flow and habitat suitability have been explained as a result of the different processes involved. Habitat use is more related to the behaviour of individuals within home ranges, while gene flow is driven by mating and dispersal events (Wasserman et al. 2010). Therefore, dispersal is not necessarily restricted to highly suitable habitat, and could eventually occur through less suitable habitat for example, provided "stepping stones" are available through the path.

Effect of exotic plantations on functional connectivity

While the effect of streams on connectivity is clear, the effect of exotic plantations was not as clearly detected (LC model was not significant after controlling for Euclidean distance). However, the second-best model, which contained proximity to streams and land cover as variables (St + LC) could not be ruled out by the causal modeling approach. It is important to highlight although the land cover model was not supported statistically (after controlling for the effect of geographic distance), this does not mean land cover has no effect on gene flow. It is possible that the correlation of gene flow to natural features (i.e. proximity to streams) was more easily detected than the human-induced land use change, as it has been acting over a longer period of time. In contrast, land-use change has occurred mainly during the last few decades. In simulation studies, the lag time between the appearance of barriers to dispersal and the detectability of genetic signatures in individual-based approaches using Mantel tests was 1 to 15 generations, depending on dispersal distances (Landguth et al. 2010). The generation time of *T. bullocki* has not been empirically studied, however considering the long larval stage, it can be estimated to be at least ~4-5 years.

Combined with a relatively low dispersal (<10 km), the effect of new barriers could take up to 75 years to be detected. In contrast, the removal of historical barriers could take up to 500 generations (Landguth et al. 2010). Considering plantations might not be acting as absolute barriers, actual lag times could be even higher.

In addition to the time lag discussed above, the resolution and accuracy of the land cover data used was limited. The National Native Forest Survey (*Catastro Nacional de Bosque Nativo*) used in this study has limited resolution and accuracy. It was first developed in 1998 and partially updated in 2007-2008, so rapidly changing landscapes such as plantation areas are unlikely to be 100% accurately represented. Comparing land cover data from the survey with recent satellite images (ArcGIS base map), reveals small and discrete features (e.g. narrow riparian strips) are poorly represented and classification of land-cover types is not 100% accurate. Undoubtedly, more reliable results could be expected if more accurate data become available. Furthermore, it is possible that the effects of land cover were poorly detected due to sampling limitations (e.g. low numbers, poor replication) resulting in low statistical power. Despite these limitations, the comparison of the different land cover resistance hypotheses suggest exotic plantations have higher resistance to movement than native forest. The best supported model for land cover assigned low resistance to native forest and high resistance to exotic plantations and was better ranked than Euclidean model, while the models representing the opposite situation (exotic forest facilitating dispersal) were ranked poorly.

Managed pine and eucalyptus plantations have a short (23 year) cycle, designed to maximise production. Clear-cuts are followed by a short period of succession from open

canopy, to complete canopy closure in pine and partial cover in eucalyptus plantations. Depending on the particular management (and managed species), mature plantations may contain some amount of understory and/or coarse woody debris, providing temporary suitable habitat. *T. bullocki* use of mature pine plantations was documented in Chapter 3, where adults were found in plantation areas adjacent to native forest. The pine needle mat that accumulates under pine plantations appears to provide some degree of cover to *T. bullocki* (Chapter 3). This suggests resistance of pine plantation matrix fluctuates between very high (when mortality of overland dispersers will be high and connectivity between breeding sites might be lost), and moderate (when connectivity is restored to some degree). The degree of functional connectivity resulting from this temporal pattern will depend, among many factors, on the length of the connectivity period and the quality of the habitat (availability of refuge and stepping stones). *T. bullocki* has not been found in managed eucalyptus plantations, which differ in many aspects from pines (Huber et al., 2010), but formal surveys are lacking. Despite this, it could be expected that eucalyptus plantations do not provide suitable terrestrial habitat for *T. bullocki*, even when mature. It is possible eucalyptus plantations differ from pines in their ability to connect *T. bullocki* habitat, if so, eucalyptus could be a greater threat to *T. bullocki* survival. This should be formally assessed, particularly in the face of recent more rapid expansion of eucalyptus compared to pine plantations.

5.4.2 Dispersal network and implications for management

The results strongly highlight the importance of streams and riparian areas for the long-term persistence of populations, and suggest that protecting these areas might be one of the most important steps in the protection of the species, particularly in plantation areas.

Plantations can affect connectivity by disrupting both intra- and inter-basin dispersal. The effect of clear-cuts can be particularly strong in disrupting inter-basin movements, if there are no alternate routes of dispersal. For example if plantations have been established on ridges (as it is often the case), basins might become temporarily disconnected after clear-cutting. Plantations can also affect intra-basin connectivity. For example, in the similarly stream-adapted species *Ascaphus truei*, movements of stream-dwelling tadpoles were affected by harvesting (Wahbe and Bunnell 2001). Tadpoles moved 7.4 times as far in unmanaged forest compared to clear-cuts, possibly due to the increased abundance of imbedded debris and logjams. After clear cutting, large amounts of sediments are carried to streams, particularly in steep-sloped areas. This can degrade *T. bullocki* stream habitat as fine sediment fills the cavities used as cover by *T. bullocki* during their aquatic phase (Chapter 3), and which are used for oviposition (Moreno Puig 2014). Thus clear cuts could disconnect previously continuous habitat and contribute to the increased isolation of populations.

Combining the connectivity network and the habitat suitability map could also help identify which of the connectivity corridors are currently more threatened by native habitat loss and degradation (Appendix H), and where management and protection effort could be most efficiently allocated. A low degree of connectivity between eastern and western populations was found in Chapter 4, and this could be due to paths connecting eastern and western populations going through high elevation areas, and therefore possibly constraining movement (Figure 5.4). Paths going through the highest elevations may not be valid dispersal routes, and paths bypassing higher elevations might be more realistic. Regardless, the results highlight the current isolation of the eastern population of Los

Lleulles from the known western locations. My work purports that unless other "stepping stone" populations actually exist in the paths connecting eastern and western populations, conservation management should first focus on connectivity among the critical western populations rather than restoring east-west pathways.

The objective of this study was to identify landscape features that facilitate or impede gene flow, so that appropriate measures could be developed to secure the long-term connectivity of populations. Stream and riparian habitat was identified as the most important feature among those tested, and based on this knowledge potential dispersal routes were identified. The next step should be to involve other stakeholders (e.g. forestry companies, landowners, government agencies) in the design of a protected network to enhance the connectivity of populations. In conjunction with core habitat protection (Semlitsch and Jensen 2001), a connectivity network will help maintain metapopulation dynamics and avoid the isolation of populations. The design of connectivity corridors should carefully consider the current and future threats, as well as costs, and opportunity among others. Only by maintaining and enhancing protection of habitat and connectivity can we expect populations to be self-sustainable and persist in the long term. Finally, the intersection of LCPs, dispersal network, and habitat suitability map can help lead future field surveys in search of new populations, as the combination of models predicts that the probability of finding *T. bullocki* will be higher in suitable habitat that is also part of a historical dispersal route.

Chapter 6: General Discussion

Prior to this study, *T. bullocki* was rarely seen and poorly studied. There was little information on the true conservation status of this species (other than its rareness), as all assessments had been based on a few historic sightings (Veloso et al. 2008, ZSL 2011). In other words, *T. bullocki* was a mystery. In the previous chapters I have presented several results that contibute to the knowledge of the species, making a significant contribution to its conservation and management. Below, the main findings of this thesis and their significance are discussed. Management guidelines and recommendations are given, and future research directions suggested.

6.1 Main findings

6.1.1 *T. bullocki* distribution and populations

One of the main objectives of this thesis was to give an updated account of current *T. bullocki* distribution and abundance. This was achieved by surveying historic populations to assess their persistence and surveying new locations in search of new populations. The updated distribution (described in Chapter 2) includes the confirmed presence of *T. bullocki* in nine main basins (and several sub-basins): Chivilingo, Ramadillas, Caramávida (including Estero Las Delicias), Cayucupil (including Estero La Cueva and Estero Los Tres Viejos), Butamalal, Huilquehue, Provoque (including Estero San Carlos), Calebu and Los

Lleulles. In spite of the considerable amount of effort of this study, limited time and resources did not allow for a full coverage, and large areas of Nahuelbuta remain un-surveyed. Nevertheless, the habitat suitability model developed in Chapter 2 suggests more populations could be found, and this information should be used to guide future survey efforts. These results represent an important improvement in the knowledge of this species' distribution and the location of extant populations.

One of the unexpected results was the non-detection of *T. bullocki* in the two historic sites inside Nahuelbuta National Park (NNP): Vanerías (Estero Coimallín = Agua de los Gringos) and Estero Cabrerías[6] reported by Péfaur (1971). Both areas were surveyed on more than one occasion using different techniques, and had high-quality habitat (i.e. clear rocky bed streams in native forest). Nevertheless, the distribution model of Chapter 2 explains the absence of *T. bullocki* from NNP as likely due to high elevation, and it is possible that the historical observations were actually made at lower altitudes. However, other factors could be involved (e.g. past fire disturbance, presence of predators, stochastic fluctuations). Presence of *T. bullocki* in these streams should not be ruled out, and visiting them at lower altitudes might increase the chance of finding the species. Whether *T. bullocki* is inside the only National Park in its range or not, is important information needed for risk assessment, and should be clarified. Furthermore, if it is not there, then the reasons for its absence should be determined too.

[6] Of these, only Vanerías is a confirmed collection point, as the exact location of Cabrerías is unclear (two locations with the same name).

Likewise, *T. bullocki* was not found in Cei's (1962) map location of Vegas Blancas or in Péfaur's (1971) Vegas de Rucapillán; however, there was little detail given about the exact location of these historical populations (other than a dot in the map), and the streams visited might not correspond to the historical locations. Vegas de Rucapillán is a large open meadow and not the typical *T. bullocki* stream, therefore it is possible the name was used in a generic way. Nevertheless, these two areas are on the eastern slopes of Nahuelbuta and are highly disturbed. At both areas visited, native forest had been lost and degraded, leaving mostly open canopy, which combined with the naturally lower amounts of precipitation (rain shadow effect) resulted in noticeably drier conditions. Furthermore, harvesting of native forest for wood and coal production is on-going, and was frequently observed during fieldwork. Many families in the area have been doing this for generations, and rely on this income for their subsistence. It is possible that *T. bullocki* distribution has declined in these areas, and that populations have been lost. This highlights the importance of protecting the Los Lleulles population as the only extant population in the eastern slopes. Surveying and protecting remaining native habitat in the eastern slopes should likewise be prioritised.

Another unexpected result was finding *T. bullocki* still present in disjunct populations in Chivilingo, Ramadillas, and Los Lleulles, where native habitat replacement by plantations has been more severe. This shows a relatively high resilience to this environmental change, which is encouraging. This apparent resilience was first observed by Péfaur (1971), as he described the habitat in Los Lleulles as being under "*not too intense but constant anthropogenic intervention* ". Unfortunately, the lack of baseline data on abundance does not allow for an assessment of population declines, and presence of *T. bullocki* on highly

disturbed areas does not necessarily mean populations are stable. It has been shown there can be important time lags or extinction-debts in fragmented landscapes (Tilman et al. 1994, Bennett and Saunders 2010), and it may take several decades of disturbances for a declining population to go extinct. Therefore, it is impossible to assume these disjunct populations will persist under current conditions. Moreover, *T. bullocki* was not found in the disturbed area mentioned by Péfaur (1971), suggesting that continued disturbance may have a long-term effect[7]. To determine if these populations are in decline, long-term monitoring should be established.

6.1.2 Habitat use

Through the use of radio-telemetry and field observations we now have a better understanding of the complex habitat requirements of *T. bullocki*. Clean, cold, rocky, and fast-flowing oxygenated streams are critical habitat for reproduction, while large areas of terrestrial habitat (e.g. up to 500 m from breeding streams) are used during the non-breeding season. Within aquatic habitat, cavities under rocks and boulders are essential microhabitat necessary for egg deposition and shelter. In terrestrial habitat, *T. bullocki* relies on canopy cover and the availability of cool and moist microhabitats (e.g. under logs, rocks, or underground burrows) to avoid desiccation during dry weather. At a larger scale, *T. bullocki* habitat suitability was strongly and positively related to the amount of native forest in the landscape, while stream and riparian habitat were identified as main dispersal pathways needed for functional population connectivity (i.e. gene flow).

[7] The population in Los Lleulles persists; however, *T. bullocki* was not found in the area described by Péfaur (1971), which is presumably close to the public road. The stream here was very small and silted.

6.1.3 Movements, behaviour, and detectability

A seasonal migration from stream habitat into adjacent terrestrial habitat was observed during early November, when a few longer (maximum 170 m) and strongly directional movements were tracked, and frogs were observed leaving the streams and moving away into the forest after periods of heavy rain. Frogs were observed moving uphill and climbing through very steep terrain. Unfortunately, the short-term nature of the tracking study and the lack of surveys through the whole year (particularly during winter) did not enable me to observe the presumed migration to breeding sites at the beginning of the breeding season. Patterns of activity and behaviour during other seasons remain unknown, as *T. bullocki* has not been observed during winter season. It is possible that *T. bullocki* overwinters in burrows and this could explain the lack of sightings.

T. bullocki patterns of activity were typical of many amphibians: they were active during the night, particularly under wet weather conditions, and remained inactive in burrows during the day, and for prolonged periods of time (i.e. 3 weeks) during dry weather. Even when active, frogs were generally sedentary and typically moved only short distances (10-20 m). Frogs active (i.e. uncovered) during the night were generally static, suggesting sit-and-wait predator behaviour rather than active foraging. Consequently, the probability of detecting the species was low and strongly related to weather patterns, and time of survey. Detecting active individuals at night proved more efficient than the more common diurnal searches (i.e. looking under logs and rocks during the day). Furthermore, detectability greatly increased after periods of heavy rain, when maximum abundances of 6-8 frogs per night were observed. The fossorial behaviour during dry weather and

nocturnal habits partially explains the small number of individuals found historically, and suggests that *T. bullocki* might be more abundant than previously thought. Nevertheless, despite the improvements made in our ability to detect the species, adult *T. bullocki* were difficult to find.

Imperfect detection probability remains an important sampling issue for *T. bullocki*, and future surveys or long-term monitoring should take this into account if any reliable inferences on population changes or comparisons are to be made. Other than maximising detection probability by surveying during optimal environmental conditions (i.e. at night after periods of rain), there are some statistically robust methods that can deal with imperfect detection probability that could be implemented. For example, Royle (2004) *N*-mixture model allows to estimate both abundance and detection parameters simultaneosly based on replicated counts at multiple sites; however, this can be resource intensive and may require significant funding to get implemented. Similarly robust, but slightly less intensive, is the site-occupancy model of MacKenzie et al. (2002), where the parameter of interest is the probability of occurence (or the proportion of area occupied by a species) rather than abundance. For this model, only presence-absence data is needed, but it still relies on replicated surveys at multiple sites. Both models and several related models can be easily implemented (given adequate data) using the freely available software PRESENCE, which makes this an attractive option for the long-term monitoring of amphibians.

6.1.4 Population genetic structure and genetic diversity

For the first time, *T. bullocki* intra-specific genetic diversity, population structure, and phylogeography were assessed. Two ESUs were identified, with the northern Chivilingo population being reciprocally monophyletic to all other extant populations sampled. *T. bullocki* had relatively high levels of genetic variability at both mtDNA and microsatellite loci (compared to what could be expected for a critically endangered species); however, this was not homogeneously distributed. Lowest levels of genetic diversity (e.g. nucleotide diversity, allelic richness) were observed in the disjunct Los Lleulles population, while the highest diversity was found in Caramávida, Cayucupil and Butamalal (populations with low levels of disturbance and high connectivity).

Disjunct populations of Chivilingo and Los Lleulles were identified as distinct management units. The remaining cluster of populations formed two groups of basins (1. Caramávida/Cayucupil/Butamalal; 2. Huilquehue/Provoque/Calebu), with relatively low levels of gene flow between them, but high levels of intra-group gene-flow. The results suggests inter-basin dispersal and gene flow occurs among neighbouring basins, in a stepping-stone manner. Estimates for effective population sizes suggest that disjunct populations are smaller and therefore at greatest risk of inbreeding depression and stochastic events (however, estimates were not precise).

6.1.5 Main threats

One of the hypotheses of this thesis was that native forest loss and its replacement by exotic plantations was one of the main threats to *T. bullocki*, and this was confirmed by the results. Habitat suitability for *T. bullocki* was positively related to the amount of native

forest in the landscape. Furthermore, the projection of the model into hypothetical past conditions of native forest cover resulted in an estimated decline of 66% of the amount of suitable habitat in recent times. Despite the presence of *T. bullocki* under mature pine plantations, and the ability of the species to use microhabitats within this exotic environment (e.g. pine needle burrows) *T. bullocki* was not found in core pine plantation areas away from native forest, suggesting only a limited tolerance to this exotic environment.

Periodical harvesting of plantations through clear-cutting of large areas is a major disturbance that has known negative consequences for forest amphibians (Petranka et al. 1993, Semlitsch et al. 2009, Popescu and Hunter 2010), and represents one of the major threats to *T. bullocki*. Current management regulations allow clear-cutting as near as 5-30 m from streams (*Decreto 82, Ley 20.283, Reglamento de Suelos Aguas y Humedales*), which could directly affect *T. bullocki* if plantations have been colonised, causing direct mortality of individuals. The large size of clear-cuts combined with the steep mountainous terrain of Nahuelbuta, and little riparian protection, can dramatically increase soil erosion and sediment load in the streams. Increased siltation of streams affects critical aquatic habitat for *T. bullocki*, as silt covers the cavities that are essential refuges for adults and egg-laying during the breeding season, and essential larval habitat during most of the year (possibly throughout the year). Furthermore, this threat could be even greater under climate change as more intense periods of rain are expected. Moreover, extensive clear-cuts, poor riparian protection, and degraded stream quality affect the functional connectivity of populations (i.e. gene flow) as streams and riparian areas are main dispersal pathways (Chapter 5). Lastly, the threats associated with exotic plantations are

widespread: over 50% of Nahuelbuta is covered by plantations, and the majority of *T. bullocki* populations found in this study (> 60%) are in areas dominated by plantations and are threatened by future harvesting operations.

Forestry is currently an important part of Chile's economy. It has been growing for the last several decades, and with global demand increasing, there is no indication that this growth will change in the near future. However, this economic growth has been achieved through highly unsustainable practices, with significant ecological impacts (Frêne Conget and Núñez Ávila 2008). More recently, major forestry companies (i.e. Arauco, CMPC) have moved towards more sustainable practices under FSC certification, and this has opened an opportunity for conservation. Nevertheless, it has become increasingly clear that important changes are needed to the laws and regulations at a national level. Furthermore, the potential use of native species in the forestry industry should be encouraged, which could considerably reduce forestry impact. Several examples exist of more sustainable forestry practices (e.g. Swedish model), but moving towards long-term sustainability has not been a priority in the political agenda. Therefore, oversight to ensure that FSC certification principles are met might represent the best chance to protect *T. bullocki* within plantation areas in the short-term.

In addition to habitat loss and plantations, climate change was identified as a potentially important threat. Maxent model projections of distribution into potential future climates suggest that higher temperatures and lower precipitation will reduce the amount of suitable habitat available for *T. bullocki*. Less rain and higher temperatures can severely affect soil moisture and decrease the number of suitable amphibian microhabitats

(Blaustein et al. 2010). Less rain will reduce flow in small first-order streams, some of which could become too dry to sustain tadpoles during the summer. Under drier conditions, full canopy cover becomes even more essential for maintaining microclimate and microhabitat conditions. Therefore, a preventive measure under climate change threat would be to maintain and restore native cover in degraded riparian areas where canopy has been removed. Canopy cover can protect streams from increased solar radiation and helps keep water cool and oxygenated. Retention or supplementation of natural and artificial shelters (e.g. logs, cover boards) to reduce desiccation and thermal stress could also be implemented temporarily while native forests regenerate. The model suggests that several populations are at risk, but some areas containing populations will remain highly suitable (Caramávida, Cayucupil, Butamalal). Furthermore, this projection can be used to target surveys and protection of habitat in areas less threatened by climate change.

6.2 Other threats

Although this thesis focused predominantly on the most pervasive threats (i.e. habitat loss and exotic plantations), there are several other threats that could affect conservation of *T. bullocki*. Particular attention has been recently drawn to the potential impact of run-of-the-river hydroelectric power stations, as they become more widespread in Nahuelbuta. These power stations are generally small (5-20 MW), and are considered "green" as they are based on a renewable resource. However, they can still have an impact on the environment. The main threat stems from the diversion of water from a section of the stream (usually 1-2 km), significantly reducing stream flow in this area. Therefore, aquatic

and semi-aquatic species are more directly affected. For example, if the remaining flow (also denominated "ecological or environmental flow[8]") in the intervention area is only 10% of the original flow, there would be a direct reduction of 90% of aquatic habitat. Nevertheless, tadpoles of *T. bullocki* have been found in relatively high abundance in small streams (Chapter 3), suggesting a reduction in flow could be tolerated. However, flow reduction could affect the equilibrium of the stream ecosystem, causing indirect, cascading, or unpredictable effects (e.g. changes in water temperature can alter dissolved oxygen levels). In addition, the construction of power plants involves building roads and transmission lines with the use of heavy machinery, and this could affect terrestrial habitat of *T. bullocki*. Therefore, the potential impact of run-of-the-river stations cannot be disregarded, and should be formally assessed[9].

Introduced trout species (mainly Brown trout *Salmo truta fario* and Rainbow trout *Oncorhynchus mykiss*) are widespread in southern Chile (Soto et al. 2006), and have been identified as potential threats to *T. bullocki* (Soto-Azat et al. 2012, Fenolio et al. 2013). Trout could be directly preying on amphibian eggs and tadpoles, competing for resources, or having indirect effects (Kats and Ferrer 2003). However, trout have been found throughout Nahuelbuta, and found coexisting with *T. bullocki* tadpoles in most of the streams visited during this thesis. While this does not mean that trout have no impact on *T. bullocki*, it does suggest that populations can persist regardless of the presence of trout. Moreover, the first trout establishment in Chile occurred in 1880 in Chivilingo River

[8] The ecological flow is commonly defined as the minimal flow required in a stream or river to maintain its ecological functions.

[9] Two projects have been approved in stream reaches likely to hold *T. bullocki* populations: Hiroeléctrica Cayucupil and Hidroeléctrica Picoiquén.

(Sernapesca 2009), where a *T. bullocki* population still persists after more than 130 years. While trout certainly represent potential threats to *T. bullocki*, their invasive nature makes them difficult to eradicate, and therefore this is not a threat that could be easily managed. Nevertheless, successful eradication of trout from streams is not impossible, and has been accomplished with benefits for native species elsewhere (Lintermans 2000). Further studies on the impact of trout on *T. bullocki* should be conducted, and the restoration of streams through trout eradication should be considered as a potential measure to improve *T. bullocki* populations.

Another potential threat to *T. bullocki* is the introduction of the chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which has caused severe population declines and extinctions of some amphibian species (Lips et al. 2006). During this thesis, chytrid swab samples were collected from all *T. bullocki* adults encountered and for several other amphibian species encountered (data not shown), and some of the results were published in Fenolio et al. (2013), where we reported the detection of Bd in eight out of 45 *T. bullocki* samples tested, including positives from three different populations. Furthermore, Bd was detected in two other widespread species of amphibians in Nahuelbuta (i.e *Eupsophus sp.* and *Pleurodema thaul*) suggesting that Bd might be widespread in the area (unpublished results). Despite the potential threat, the impact of Bd is not clear, as the response to Bd can be highly species-specific; some species are able to resist Bd through the secretion of antimicrobial skin peptides (Woodhams et al. 2007). Future research is needed in order to assess the threat of Bd to *T. bullocki*.

6.3 What can be done to protect *T. bullocki* populations and habitat?

6.3.1 Management

Despite being potentially more widespread than anticipated, *T. bullocki* distribution remains restricted to a few populations in nine basins within Nahuelbuta: it remains an endemic species with a small and restricted distribution. Considering this, the low abundances observed, the complex habitat requirements, and great levels of threat, conservation of *T. bullocki* should aim to maintain and enhance all known populations. In order to do this, the first priority should be to secure habitat protection. At each known population, core habitat should be protected, including aquatic (stream) habitat and ≈250 m of adjacent terrestrial habitat (core habitat + buffer) on each side of the stream. In this area native forest should be maintained and/or restored. In addition, a connectivity network of native corridors should be established connecting adjacent populations to maintain meta-population dynamics. Although the minimum amount of habitat needed for a viable population is yet unknown, larger areas will protect more individuals (ultimately the amount of habitat protected will depend on landowner's commitment and conservation priorities).

Extant populations can be grouped into five management units (MU): the four MU identified in Chapter 4 based on genetic connectivity, plus one disjunct population (Ramadillas, not sampled). Based on current levels of connectivity, the five MUs can be classified into two types, requiring different management approaches: 1) Disjunct "island" populations (i.e. Chivilingo, Ramadillas, Los Lleulles) and 2) Meta-populations (i.e. Caramavida/Cayucupil/Butamalal and Huilquehue/Provoque/Calebu). Small and disjunct

populations of Chivilingo, Ramadillas and Los Lleulles are at greater risk due to small size and increased isolation. Habitat protection and restoration should be prioritised at these locations, while surveys should aim to locate neighbouring populations. Special measures should be taken when harvesting plantations at these locations. For example, prior to clear-cutting, plantations should be surveyed for the presence of *T. bullocki*, particularly in riparian areas or areas adjacent to native forest. If present, individuals could be rescued and re-located to suitable habitat nearby. Populations that are part of larger meta-populations are at lower risk if habitat and connectivity are maintained. Therefore maintaining core habitat and connectivity between adjacent populations should be the main objective for these populations. Connectivity should be maintained through the maintenance and restoration of riparian and stream habitat, for example as described in Olson et al. (2007) and Olson and Burnett (2009), where they suggest a combination of riparian buffer zones, patch reserves (e.g. at headwaters and stream junctions), and partial harvest and/or leave islands to provide connectivity functions between watersheds. A proposed connectivity network is given in Appendix I, along with specific management and conservation opportunities for each management unit.

6.3.2 Other recommendations

1) Re-assess *T. bullocki* threat classification at a national level (RCE) on the basis of updated distribution and new findings. For this, the identity of the Quirihue population (Escobar et al. 2005) should be established in order to define the correct extent of occurrence of *T. bullocki* and *T. ignotus*. It is likely that current threat classification (Vulnerable and Rare) is underestimating the levels of threat, and *T. bullocki* could potentially be moved at least to

Endangered status if updated information on threats is considered (e.g. potential threat of climate change, estimated decline in habitat suitability).

2) Develop a species recovery plan. Although some recommendations and guidelines are given in this thesis, a full conservation and recovery plan was beyond the scope of this thesis. A plan should be developed by the relevant authorities (Ministerio de Medio Ambiente) and should include specific short and long-term goals. Main stakeholders (e.g. forestry companies) should be involved. Existing threatened species recovery plans developed for other endangered amphibians could be used as a guide (e.g. http://www.doc.govt.nz/Documents/science-and-technical/tsrp63entire.pdf)

3) Continue surveying streams to detect *T. bullocki* populations. For this, the habitat suitability map could be used to target areas.

4) Establish robust long-term monitoring of priority populations and continue developing standardised field techniques (Blaustein et al. 1994)

6.3.3 Future research

This thesis encompassed a wide range of disciplines and methods converging on one common objective. It included aspects and concepts of spatial ecology, spatial statistics, niche modelling, population genetics, biogeography, phylogeography, ecology, ethology, phenology, and landscape genetics, all applied to conservation. However, it only scratched the surface of our understanding on any single topic. Below I suggest some future research mainly from a conservation perspective; however, most aspects of the biology of *T.*

bullocki remain unknown and will not be discussed here as they have already been mentioned above.

Ecological studies

The impact of plantation forestry has been a central focus of this thesis, where potential threats were identified based on the acquired knowledge of the species' use of habitat and the effects of clear cuts and forestry on amphibians described in the literature. However, this impact or its mechanisms were not directly/empirically studied. The long-term impact of plantation forestry should be studied in more detail in purpose-designed ecological studies (i.e. replicated, random sampling). The use of plantations by endangered amphibians should be further studied in order to determine the extent of its use, and the quality of the habitat provided. In this thesis *T. bullocki* was not found in core mature pine plantation, but no other plantations were surveyed intensively. Frogs were found in mature plantation areas close to native forest, but it is still not known at what stage pine plantations become suitable (i.e. colonised after clear-cutting) or the quality of habitat they provide. Furthermore, the use of eucalyptus plantations has not been assessed. The persistence of *T. bullocki* in plantation areas with little native forest (e.g. Ramadillas) suggest that either plantations provide good-quality habitat, or that populations in these areas could be declining due to habitat loss. While native forest should be targeted for conservation, can exotic plantations provide habitat when there is no native forest left? In other words, can riparian plantation areas serve as core habitat for *T. bullocki* and be established for protection purposes? Are populations in areas with small native riparian areas doomed?

Extant populations are good candidates for a natural-experiment approach, as they range from undisturbed native forest to highly disturbed plantation areas. While replication and randomisation remain issues when only a few populations are known, some opportunities were identified to study the impact of forestry on *T. bullocki* more closely. For example, some streams with *T. bullocki* presence (e.g. La Cueva) go through both native and recently harvested exotic plantation. Preliminary observations suggest that the stream habitat quality was affected by recent harvesting in plantation areas (e.g. silted base), no tadpoles were found, whereas tadpoles were relatively abundant under native cover 3 km upstream. Comparing habitat and abundance within the same stream allows statistical control of some factors, and can help identify differences derived from forestry practices.

While there is an opportunity for natural experiments, controlled field experiments could allow for true experimental replication. For example, different treatments (experimental harvesting) could be applied in replicated forest arrays to test the effectiveness of retaining coarse woody debris (CWD) to mitigate the negative effects of clear-cutting on amphibians following the experiments in Semlitsch et al. (2009). These experiments could be replicated with some modifications, and could help improve our understanding of the effects of harvesting and identify effective measures for protection. A combined experimental approach and radio-telemetry could also be used to test the effectiveness of different riparian buffer zone sizes as in Veysey et al. (2009) and Freidenfelds et al. (2011). In a similar way, experimental harvesting could be studied for *T. bullocki* by comparing the response to different treatments (e.g. leaving coarse woody debris, leaving "stepping stones" of vegetation or strips connecting nearest habitat, different buffer widths). Upcoming harvesting in *T. bullocki* habitat could serve as an opportunity to implement

some experimental harvesting options and to directly monitor the impacts by measuring response variables (e.g. abundance) before and after harvesting. Furthermore, because *T. bullocki* shares habitat with many other amphibian species, such experiments could allow for the study of multiple threatened amphibians (i.e. *Rhinoderma darwinii*, *Calyptocephalella gayi*, *Eupsophus contulmoensis*, *Eupsophus nahuelbutensis*, *Alsodes barrioi*, *Alsodes vanzolinii*) and responses could be measured at the community level.

Detecting population changes relies on being able to reliably measure population vital rates such as abundance, survival, and reproduction. Some robust methods such as mark-recapture could be used to estimate *T. bullocki* population sizes. Frogs found in this thesis had a unique dorsal gland pattern, and colour and shape of the inter-ocular yellow spot. This allowed for the use of natural markings for individual identification, an appropriate low-impact alternative for endangered animals. In addition, marking of tadpoles with visible implant elastomers (VIE) could be useful in establishing growth and survival parameters (Bainbridge et al. 2015). Although these are relatively low-cost techniques, re-capturing animals does require intensive and repeated surveys. Nevertheless, some *T. bullocki* populations are more easily accessed than others and could be good candidates for long-term and experimental population studies.

Management

Effective management measures should be developed and applied to protect *T. bullocki* populations and habitat, particularly at most disturbed and threatened locations. The potential use and effectiveness of relocation and translocations should be evaluated. If frogs are commonly found in plantation areas, rescuing prior to clear-cutting could

mitigate the effects of harvesting. Frogs can be relocated to close suitable habitat or translocated into other areas provided suitable habitat is available. However, relocations and translocations of amphibians are not always successful (Germano and Bishop 2009), and should not be considered effective measures *a priori* (Dodd Jr. and Seigel 1991). Furthermore, the fossorial behaviour of *T. bullocki* means only a small fraction of the population might be detected and relocated. Nevertheless it is an option that should be considered and researched.

Captive breeding has been used as a conservation tool for some threatened amphibians with both positive and negative outcomes (Griffiths and Pavajeau 2008). However, this should not be considered a priority for *T. bullocki,* particularly if funds could be otherwise used for direct habitat protection. While captive populations could serve for research and education purposes, there are still opportunities for *in-situ* conservation that would exceed the benefits of potentially successful captive breeding. Therefore captive breeding should be considered only if populations become under imminent threat and no *in-situ* conservation alternative remains, in other words captive breeding should not be ruled out, but should only be considered as a last resort.

Restoration of *T. bullocki* habitat should be a long-term goal in disturbed populations, and research on habitat restoration should be encouraged. Native vegetation restoration can be achieved through passive regeneration or active reforestation. However, not only terrestrial habitat should be restored; stream habitat should be restored too. As part of that restoration, the potential use and effectiveness of trout removal to reinstate *T. bullocki* breeding habitat should be assessed. Given that trout are likely to affect egg and

larval stages mainly, removal of trout could have a significant impact on the population through increased hatching success and larval survival, which could increase juvenile recruitment. In addition, restoration of streams would also benefit imperilled native freshwater fish and invertebrates.

6.4 Conclusion

The focus of this thesis was the conservation of the rare and endangered amphibian *Telmatobufo bullocki* in fragmented forests of Nahuelbuta, Chile. The four data chapters included represent a significant improvement in current knowledge of *T. bullocki*, with important implications for its management and long-term conservation. Nevertheless, it is only the beginning of our understanding on this species' ecology and behaviour. While it will always be better to have complete knowledge about a species for the effectiveness of management, for some rare and declining species such as *T. bullocki* this is difficult to achieve. Therefore, habitat protection and management should be implemented urgently regardless of incomplete knowledge.

Overall, the results obtained for *T. bullocki* distribution are encouraging. Currently, there are as many populations known as there were before (and it is likely more populations will be discovered). Furthermore, many more *T. bullocki* individuals were found compared to the scarce historical observations. However, the number of populations and relative abundance are still low and *T. bullocki* remains a rare and micro-endemic species, facing multiple threats. Although this thesis was based on a single endangered species, the conservation issues (i.e. native forest loss and fragmentation) are shared by many other

threatened species of plants and animals in Nahuelbuta and other fragmented forests in Chile. Native forest loss, degradation, and fragmentation are on-going processes affecting biodiversity at multiple levels. While for some endangered species a species-specific approach to conservation might be needed, conservation should ideally be focused on maintaining the entire native and functioning ecosystems. Nevertheless, conservation of species and ecosystems is ultimately a social issue involving not just scientists but also authorities, legislators, politicians, communities, corporations, etc. I have, from the conservation biology perspective, provided guidelines and recommendations that could significantly help in the conservation of *T. bullocki* and its native riparian ecosystem. But this knowledge won't make a difference *per-se*; it will only be valuable to conservation if applied. I hope that authorities (Ministerio de Medio Ambiente) and main stakeholders (forestry companies) will be open to enforce and implement some of the suggested management, and will endeavour to commit to the long-term conservation of *T. bullocki* and other endangered native species.

References

Acosta-Jamett, G., and J. A. Simonetti. 2004. Habitat use by *Oncifelis guigna* and *Pseudalopex culpaeus* in a fragmented forest landscape in central Chile. Biodiversity and Conservation 13:1135–1151.

Allentoft, M. E., and J. O'Brien. 2010. Global amphibian declines, loss of genetic diversity and fitness: a review. Diversity 2:47–71.

Andersen, L. W., K. Fog, and C. Damgaard. 2004. Habitat fragmentation causes bottlenecks and inbreeding in the European tree frog (*Hyla arborea*). Proceedings of the Royal Society of London 271:1293–1302.

Araújo, M. B., W. Thuiller, and R. G. Pearson. 2006. Climate warming and the decline of amphibians and reptiles in Europe. Journal of Biogeography 33:1712–1728.

Avise, J. C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb, and N. C. Saunders. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics:489–522.

Baillie, J. E. M., and E. R. Butcher. 2012. Priceless or Worthless? The world's most threatened species. Zoological Society of London, United Kingdom.

Bainbridge, L., M. Stockwell, J. Valdez, K. Klop-Toker, S. Clulow, J. Clulow, and M. Mahony. 2015. Tagging tadpoles: retention rates and impacts of visible implant elastomer (VIE) tags from the larval to adult amphibian stages. The Herpetological Journal 25:133–140.

Beaumont, M. A. 1999. Detecting population expansion and decline using microsatellites. Genetics 153:2013–2029.

Beebee, T. J. C. 2010. Genetics in field ecology and conservation. Pages 407–427 *in* C. K. Dodd, editor. Amphibian Ecology and Conservation: A Handbook of Techniques. Oxford University Press Inc., New York.

Beebee, T. J. C., and R. A. Griffiths. 2005. The amphibian decline crisis: A watershed for conservation biology? Biological Conservation 125:271–285.

Bennett, A. F., and D. A. Saunders. 2010. Habitat fragmentation and landscape change. Pages 88–104 *in* N. S. Sodhi and P. R. Ehrlich, editors. Conservation Biology for All. Oxford University Press.

Blank, L., and L. Blaustein. 2012. Using ecological niche modeling to predict the

distributions of two endangered amphibian species in aquatic breeding sites. Hydrobiologia 693:157–167.

Blaustein, A. R., C. Searle, B. A. Bancroft, and J. Lawler. 2012. Amphibian population declines and climate change. Pages 29–53 *in* E. A. Beever and J. L. Belant, editors. Ecological Consequences of Climate Change: Mechanisms, Conservation, and Management. Taylor & Francis Group.

Blaustein, A. R., D. B. Wake, and W. P. Sousa. 1994. Amphibian declines: Judging stability, persistence, and susceptibility of populations to local and global extinctions. Conservation Biology 8:60–71.

Blaustein, A. R., S. C. Walls, B. A. Bancroft, J. J. Lawler, C. L. Searle, and S. S. Gervasi. 2010. Direct and indirect effects of climate change on amphibian populations. Diversity 2:281–313.

Boria, R. A., L. E. Olson, S. M. Goodman, and R. P. Anderson. 2014. Spatial filtering to reduce sampling bias can improve the performance of ecological niche models. Ecological Modelling 275:73–77.

Bourke, J., K. Busse, and W. Bohme. 2012. Searching for a lost frog (*Rhinoderma rufum*): identification of the most promising areas for future surveys and possible reasons of its enigmatic decline. North-Western Journal of Zoology 8:99–106.

Brown, J. L. 2014. SDMtoolbox: a python-based GIS toolkit for landscape genetic, biogeographic and species distribution model analyses. Methods in Ecology and Evolution 5:694–700.

Bullock, D. S. 1954. Una especie nueva de Rana de Nahuelbuta. Boletín Jardín Zoológico de Concepción 1:19–21.

Bustamante, R. O., A. A. Grez, and J. A. Simonetti. 2006. Efectos de la fragmentacíon del bosque maulino sobre la abundancia y diversidad de especies nativas. Pages 83–98 Biodiversidad en ambientes fragmentados de Chile: patrones y procesos a diferentes escalas.

Canhoto, C., and C. Laranjeira. 2007. Leachates of *Eucalyptus globulus* in intermittent streams affect water parameters and invertebrates. International Review of Hydrobiology 92:173–182.

Carr, L. W., and L. Fahrig. 2001. Effect of road traffic on two amphibian species of differing vagility. Conservation Biology 15:1071–1078.

Castellano, S., and E. Balletto. 2002. Is the partial Mantel test inadequate? Evolution 56:1871.

Castoe, T. A., C. L. Spencer, and C. L. Parkinson. 2007. Phylogeographic structure and

historical demography of the western diamondback rattlesnake (*Crotalus atrox*): A perspective on North American desert biogeography. Molecular Phylogenetics and Evolution 42:193–212.

Cei, J. M. 1962. Batracios de Chile. Ediciones de la Universidad de Chile, Santiago, Chile.

Chapuis, M.-P., and A. Estoup. 2007. Microsatellite null alleles and estimation of population differentiation. Molecular Biology and Evolution 24:621–631.

Chikhi, L., V. C. Sousa, P. Luisi, B. Goossens, and M. A. Beaumont. 2010. The confounding effects of population structure, genetic diversity and the sampling scheme on the detection and quantification of population size changes. Genetics 186:983–995.

Cisternas, M., P. Martínez, C. Oyarzún, and P. Debels. 1999. Caracterización del proceso de reemplazo de vegetación nativa por plantaciones forestales en una cuenca lacustre de la Cordillera de Nahuelbuta, VIII Región, Chile. Revista Chilena de Historia Natural 72:661–676.

Clement, M., D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9:1657–1659.

Collins, J. P., and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9:89–98.

CONAF. 2008. Catastro y Evaluación de los Recursos Vegetacionales Nativos de Chile. Corporación Nacional Forestal, Santiago, Chile.

CONAMA. 2008. Plan de acción nacional de cambio climático. Gobierno de Chile.

Corn, P. S. 2005. Climate change and amphibians. Animal Biodiversity and Conservation 29:59–67.

Corn, P. S., and R. B. Bury. 1989. Logging in Western Oregon: Responses of headwater habitats and stream amphibians. Forest Ecology and Management 29:39–57.

Cornuet, J. M., and G. Luikart. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014.

Crandall, K. A., O. R. P. Bininda-Emonds, G. M. Mace, and R. K. Wayne. 2000. Considering evolutionary processes in conservation biology. Trends in Ecology & Evolution 15:290–295.

Crawford, J. A., and R. D. Semlitsch. 2007. Estimation of core terrestrial habitat for stream-breeding salamanders and delineation of riparian buffers for protection of biodiversity. Conservation Biology 21:152–158.

Crump, M. L., N. J. Scott, and M. S. Foster. 1994. Visual Encounter Surveys. *in* W. R. Heyer, M. A. Donnelly, R. W. McDiarmid, L.-A. C. Hayek, and M. S. Foster, editors. Measuring and Monitoring Biological Diversity: Standard Methods for Amphibians. The Smithsonian Institution.

Cuevas, C. C. 2010. A new species of *Telmatobufo* (Schmidt 1852) (Anura, Calyptocephalellidae) from a remnant of the Maulino forest, central Chile. Gayana 74:102–112.

Cushman, S. A. 2006. Effects of habitat loss and fragmentation on amphibians: A review and prospectus. Biological Conservation 128:231–240.

Cushman, S. A., and E. L. Landguth. 2010. Spurious correlations and inference in landscape genetics. Molecular Ecology 19:3592–3602.

Cushman, S. A., K. McKelvey, J. Hayden, and M. Schwartz. 2006. Gene flow in complex landscapes: testing multiple hypotheses with causal modeling. The American Naturalist 168:486–499.

Cushman, S. A., K. S. McKelvey, and M. K. Schwartz. 2009. Use of empirically derived source-destination models to map regional conservation corridors. Conservation Biology 23:368–376.

Cushman, S., T. Wasserman, E. Landguth, and A. Shirk. 2013. Re-evaluating causal modeling with Mantel tests in landscape genetics. Diversity 5:51–72.

D'Amen, M., and P. Bombi. 2009. Global warming and biodiversity: Evidence of climate-linked amphibian declines in Italy. Biological Conservation 142:3060–3067.

Darriba, D., G. L. Taboada, R. Doallo, and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Meth 9:772.

deMaynadier, P. G., and M. L. Hunter Jr. 1995. The relationship between forest management and amphibian ecology: a review of the North American literature. Environmental Reviews 3:230–261.

Dempster, A. P., N. M. Laird, and D. B. Rubin. 1977. Maximum likelihood from incomplete data via the EM algorithm. Journal of the Royal Statistical Society. Series B (Methodological):1–38.

Díaz-Páez, H., J. J. Núñez, and J. C. Ortiz. 2008. Estado de conservación de anfibios y reptiles. Pages 233–267 *in* M. A. Vidal Maldonado and A. Labra Lillo, editors. Herpetología de Chile. Science Verlag Chile, Santiago de Chile.

Díaz-Páez, H., and J. C. Ortiz. 2003. Evaluación del estado de conservación de los anfibios en Chile. Revista Chilena de Historia Natural 76:509–525.

Do, C., R. S. Waples, D. Peel, G. M. Macbeth, B. J. Tillett, and J. R. Ovenden. 2014. NeEstimator v2: re-implementation of software for the estimation of contemporary effective population size (*N*e) from genetic data. Molecular Ecology Resources 14:209–214.

Dodd Jr., C. K., and R. A. Seigel. 1991. Relocation, repatriation, and translocation of amphibians and reptiles: are they conservation strategies that work? Herpetologica 47:336–350.

Donoso, D. S., C. Correa Q., P. Henríquez, N. F. Lagos, and M. A. Méndez. 2010. Amphibia, Anura, Calyptocephalellidae, *Telmatobufo bullocki* Schmidt, 1952: Distribution extension, habitat use and geographic distribution map. Check List 6:298–300.

Donoso-Barros, R. 1972. Contribución al conocimiento del género *Aruncus* Phillippi. Boletín de la Sociedad de Biología de Concepción 44:109–116.

Drummond, A. J., and R. R. Bouckaert. 2014. Bayesian evolutionary analysis with BEAST 2. Cambridge University Press.

Drummond, A. J., and A. Rambaut. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC evolutionary biology 7:214.

Drummond, A. J., A. Rambaut, B. Shapiro, and O. G. Pybus. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. Molecular Biology and Evolution 22:1185–1192.

Duellman, W. E., and L. Trueb. 1986. Biology of Amphibians. McGraw-Hill, Inc., Baltimore, Maryland.

Van Dyke, F. 2003. Conservation biology: foundations, concepts, applications. McGraw-Hill, New York.

Earl, D., and B. vonHoldt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4:359–361.

Echeverria, C., D. Coomes, J. Salas, J. M. Rey-Benayas, A. Lara, and A. Newton. 2006. Rapid deforestation and fragmentation of Chilean Temperate Forests. Biological Conservation 130:481–494.

Eggert, C. 2002. Use of fluorescent pigments and implantable transmitters to track a fossorial toad (*Pelobates fuscus*). Herpetological Journal 12:69–74.

Eggert, C., P.-H. Peyret, and R. Guyétant. 1999. Two complementary methods for studying amphibian terrestrial movements. Pages 95–97 *in* C. Miaud and R. Guyétant, editors. Current Studies in Herpetology. Societas Europaea

Herpetologica, Le Bourget du Lac (SEH).

Elith, J., and J. R. Leathwick. 2009. Species distribution models: Ecological explanation and prediction across space and time. Annual Review of Ecology, Evolution, and Systematics 40:677–697.

Elith, J., S. J. Phillips, T. Hastie, M. Dudík, Y. E. Chee, and C. J. Yates. 2011. A statistical explanation of MaxEnt for ecologists. Diversity and Distributions 17:43–57.

Escalante, T., M. Linaje, P. IlloldiI-Rangel, M. Rivas, P. Estrada, F. Neira, and J. J. Morrone. 2009. Ecological niche models and patterns of richness and endemism of the southern Andean genus *Eurymetopum* (Coleoptera, Cleridae). Revista Brasileira de Entomologia 53:379–385.

Escobar, M. A., C. F. Estades, M. Falcy, and M. A. Vukasovic. 2005. Geographic distribution. *Telmatobufo bullocki* (Bullock's Frog). Herpetological Review 36:77.

Estades, C. F., and M. A. Escobar. 2005. Plantation ecosystems in the Coastal Range. Pages 600–616 *in* C. Smith-Ramírez, J. J. Armesto, and C. Valdovinos, editors. Historia, biodiversidad y ecología de los bosques costeros de Chile. Science Verlag Chile, Santiago de Chile.

Estades, C. F., and S. A. Temple. 1999. Deciduous-forest bird communities in a fragmented landscape dominated by exotic pine plantations. Ecological Applications 9:573–585.

Etherington, T. R. 2011. Python based GIS tools for landscape genetics: visualising genetic relatedness and measuring landscape connectivity. Methods in Ecology and Evolution 2:52–55.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14:2611–2620.

Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567.

Fahrig, L. 2003. Effects of habitat fragmentation on biodiversity. Annual Review of Ecology, Evolution, and Systematics 34:487–515.

Fahrig, L., and G. Merriam. 1994. Conservation of fragmented populations. Conservation Biology 8:50–59.

Faurby, S., and C. Pertoldi. 2012. The consequences of the unlikely but critical assumption of stepwise mutation in the population genetic software, MSVAR. Evolutionary Ecology Research 14:859–879.

Fenolio, D. B., V. Moreno-Puig, M. G. Levy, J. J. Nuñez, W. W. Lamar, M. O. Fabry, M. S. Tirado, M. L. Crump, and A. Charrier. 2013. Status and conservation of a Gondwana legacy: Bullock's false toad, *Telmatobufo bullocki* (Amphibia: Anura: Calyptocephalellidae). Herpetological Review 44:583–590.

Ferrière, G. 1963. Aspectos ecologicos del Parque Nacional de Nahuelbuta: Tesis de prueba para optar al grado de Licenciado en Ciencias Pecuarias y Medico Veterinarias.

Ficetola, G. F., T. W. J. Garner, and F. De Bernardi. 2007a. Genetic diversity, but not hatching success, is jointly affected by postglacial colonization and isolation in the threatened frog, *Rana latastei*. Molecular Ecology 16:1787–1797.

Ficetola, G. F., L. Marziali, B. Rossaro, F. De Bernardi, and E. Padoa-Schioppa. 2011. Landscape-stream interactions and habitat conservation for amphibians. Ecological Applications 21:1272–1282.

Ficetola, G. F., W. Thuiller, and C. Miaud. 2007b. Prediction and validation of the potential global distribution of a problematic alien invasive species — the American bullfrog. Diversity and Distributions 13:476–485.

Fisher, N. I. 1995. Statistical Analysis of Circular Data. Cambridge University Press.

Foden, W. B., S. H. M. Butchart, S. N. Stuart, J.-C. Vié, H. R. Akçakaya, A. Angulo, L. M. DeVantier, A. Gutsche, E. Turak, L. Cao, S. D. Donner, V. Katariya, R. Bernard, R. A. Holland, A. F. Hughes, S. E. O'Hanlon, S. T. Garnett, C. H. Sekercioğlu, and G. M. Mace. 2013. Identifying the world's most climate change vulnerable species: a systematic trait-based assessment of all birds, amphibians and corals. PloS one 8:e65427.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3:294–299.

Formas, J. R. 1988. The tadpole of *Telmatobufo bullocki*. Herpetologica 44:458–460.

Formas, J. R., and N. D. Espinoza. 1975. Karyological relationships of frogs of the genus *Telmatobufo* (Anura: Leptodactylidae). Herpetologica 31:429–432.

Formas, J. R., J. J. Núñez, and L. M. Brieva. 2001. Osteology, taxonomy and phylogenetic relationships of the frog genus *Telmatobufo* (Leptodactylidae). Revista Chilena de Historia Natural 74:365–387.

Formas, J. R., and A. Veloso. 1982. Taxonomy of *Bufo venustus* Philippi, 1899 (Anura: Leptodactylidae) from central Chile. Proceedings of the Biological Society of Washington 95:688–693.

Fouquet, A., G. F. Ficetola, A. Haigh, and N. Gemmell. 2010. Using ecological niche modelling to infer past, present and future environmental suitability for *Leiopelma hochstetteri*, an endangered New Zealand native frog. Biological Conservation 143:1375–1384.

Frankham, R. 2005. Genetics and extinction. Biological Conservation 126:131–140.

Frankham, R., J. D. Ballou, and D. A. Briscoe. 2004. A primer of conservation genetics. Cambridge University Press.

Frankham, R., J. D. Ballou, and D. A. Briscoe. 2010. Introduction to Conservation Genetics. Cambridge University Press, Cambridge.

Franklin, J. 2009. Mapping Species Distributions: Spatial Inference and Prediction. Ecology, Biodiversity and Conservation. Cambridge University Press, Cambridge.

Freidenfelds, N. A., J. L. Purrenhage, and K. J. Babbitt. 2011. The effects of clearcuts and forest buffer size on post-breeding emigration of adult wood frogs (*Lithobates sylvaticus*). Forest Ecology and Management 261:2115–2122.

Frêne Conget, C., and M. Núñez Ávila. 2008. Hacia un Nuevo modelo forestal en Chile. Revista Bosque Nativo 47:25–35.

Frost, D. R. 2014. Amphibian species of the world: an online reference. Version 6. New York, USA. http://research.amnh.org/herpetology/amphibia/index.html.

FSC. 2012. Principios y Criterios del FSC para el Manejo Forestal Responsable (BORRADOR FINAL).

Fu, Y.-X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925.

Funk, W. C., A. E. Greene, P. S. Corn, and F. W. Allendorf. 2005. High dispersal in a frog species suggests that it is vulnerable to habitat fragmentation. Biology Letters 1:13–16.

Furman, B. L. S., B. R. Scheffers, and C. A. Paszkowski. 2011. The use of fluorescent powdered pigments as a tracking technique for snakes. Herpetological Conservation and Biology 6:473–478.

Garza, J. C., and E. G. Williamson. 2001. Detection of reduction in population size using data from microsatellite loci. Molecular Ecology 10:305–318.

Gerlach, G., A. Jueterbock, P. Kraemer, J. Deppermann, and P. Harmand. 2010. Calculations of population differentiation based on GST and D: forget GST but not all of statistics! Molecular Ecology 19:3845–3852.

Germano, J. M., and P. J. Bishop. 2009. Suitability of amphibians and reptiles for

translocation. Conservation Biology 23:7–15.

Girod, C., R. Vitalis, R. Leblois, and H. Fréville. 2011. Inferring population decline and expansion from microsatellite data: a simulation-based evaluation of the Msvar method. Genetics 188:165–179.

Girvetz, E. H., C. Zganjar, G. T. Raber, E. P. Maurer, P. Kareiva, and J. J. Lawler. 2009. Applied climate-change analysis: The climate wizard tool. PLoS ONE 4:e8320.

Goebel, A. M., J. M. Donnelly, and M. E. Atz. 1999. PCR primers and amplification methods for 12S ribosomal DNA, the control region, cytochrome oxidase I, and cytochrome b in Bufonids and other frogs, and an overview of PCR primers which have amplified DNA in amphibians successfully. Molecular Phylogenetics and Evolution 11:163–199.

González, B. A., H. Samaniego, J. C. Marín, and C. F. Estades. 2013. Unveiling current Guanaco distribution in chile based upon niche structure of phylogeographic lineages: Andean puna to subpolar forests. PloS one 8:e78894.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Goudet, J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. Journal of Heredity 86:485–486.

Graeter, G. J., and B. B. Rothermel. 2007. The effectiveness of fluorescent powdered pigments as a tracking technique for amphibians. Herpetological Review 38:162–166.

Grez, A. A., J. A. Simonetti, and R. O. Bustamante. 2006. Biodiversidad en ambientes fragmentados de Chile: patrones y procesos a diferentes escalas. Editorial Universitaria, Santiago de Chile.

Griffiths, R. A., and L. Pavajeau. 2008. Captive breeding, reintroduction, and the conservation of amphibians. Conservation Biology 22:852–861.

Groff, L. A., S. B. Marks, and M. P. Hayes. 2014. Using ecological niche models to direct rare amphibian surveys: a case study using the oregon spotted frog (*Rana pretiosa*). Herpetological Conservation and Biology 9:354–368.

Guerry, A. D., and M. L. Hunter. 2002. Amphibian distributions in a landscape of forests and agriculture: an examination of landscape composition and conf iguration. Conservation Biology 16:745–754.

Guillot, G., and F. Rousset. 2013. Dismantling the Mantel tests. Methods in Ecology and Evolution 4:336–344.

Guisan, A., and N. E. Zimmermann. 2000. Predictive habitat distribution models in ecology. Ecological Modelling 135:147–186.

Guo, S. W., and E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361–372.

Hanski, I. 1998. Metapopulation dynamics. Nature 396:41–49.

Hardy, O. J., and X. Vekemans. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. Molecular Ecology Notes 2:618–620.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22:160–174.

Hernandez, P. A., C. H. Graham, L. L. Master, and D. L. Albert. 2006. The effect of sample size and species characteristics on performance of different species distribution modeling methods. Ecography 29:773–785.

Hijmans, R. J., S. E. Cameron, J. L. Parra, P. G. Jones, and A. Jarvis. 2005. Very high resolution interpolated climate surfaces for global land areas. International Journal of Climatology 25:1965–1978.

Hill, W. G. 1981. Estimation of effective population size from data on linkage disequilibrium. Genetical Research 38:209–216.

Hoffman, E. A., and M. S. Blouin. 2004. Historical data refute recent range contraction as cause of low genetic diversity in isolated frog populations. Molecular Ecology 13:271–276.

Holderegger, R., and M. Di Giulio. 2010. The genetic effects of roads: A review of empirical evidence. Basic and Applied Ecology 11:522–531.

Holderegger, R., and H. H. Wagner. 2006. A brief guide to landscape genetics. Landscape Ecology 21:793–796.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6:65–70.

Houlahan, J. E., and C. S. Findlay. 2003. The effects of adjacent land use on wetland amphibian species richness and community composition. Canadian Journal of Fisheries and Aquatic Sciences 60:1078–1094.

Hu, Y., D. Qi, H. Wang, and F. Wei. 2010. Genetic evidence of recent population contraction in the southernmost population of giant pandas. Genetica 138:1297–1306.

Huber, A., A. Iroumé, C. Mohr, and C. Frrêne. 2010. Efecto de plantaciones de *Pinus*

radiata y *Eucalyptus globulus* sobre el recurso agua en la Cordillera de la Costa de la región del Biobío. BOSQUE 31:219–230.

Hubisz, M. J., D. Falush, M. Stephens, and J. K. Pritchard. 2009. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources 9:1322–1332.

Huggett, A. J. 2005. The concept and utility of "ecological thresholds" in biodiversity conservation. Biological Conservation 124:301–310.

Ibarra-Vidal, H. 1989. Impacto de las actividades humanas sobre la herpetofauna en Chile. Comunicaciones del Museo Regional de Concepción 3:33–39.

Igawa, T., S. Oumi, S. Katsuren, and M. Sumida. 2013. Population structure and landscape genetics of two endangered frog species of genus *Odorrana*: different scenarios on two islands. Heredity 110:46–56.

Irisarri, I., D. San Mauro, F. Abascal, A. Ohler, M. Vences, and R. Zardoya. 2012. The origin of modern frogs (Neobatrachia) was accompanied by acceleration in mitochondrial and nuclear substitution rates. BMC Genomics 13:626.

Isaac, N. J. B., D. W. Redding, H. M. Meredith, and K. Safi. 2012. Phylogenetically-informed priorities for amphibian conservation. PLoS ONE 7:e43912.

IUCN. 2011. IUCN Red List of Threatened Species. Version 2011.1. http://www.iucnredlist.org.

Jakobsson, M., and N. A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806.

Jehle, R., and J. W. Arntzen. 2002. Review: microsatellite markers in amphibian conservation genetics. Herpetological Journal 12:1–9.

Johnston, B., and L. Frid. 2002. Clearcut logging restricts the movements of terrestrial Pacific giant salamanders (*Dicamptodon tenebrosus* Good). Canadian Journal of Zoology 80:2170–2177.

Joly, P., C. Morand, and A. Cohas. 2003. Habitat fragmentation and amphibian conservation: building a tool for assessing landscape matrix connectivity. Comptes Rendus Biologies 326:132–139.

Jones, O. R., and J. Wang. 2010. COLONY: a program for parentage and sibship inference from multilocus genotype data. Molecular Ecology Resources 10:551–555.

Jost, L. 2008. GST and its relatives do not measure differentiation. Molecular Ecology

17:4015–4026.

Kalinowski, S. T. 2004. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. Conservation Genetics 5:539–543.

Kalinowski, S. T. 2005. HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. Molecular Ecology Notes 5:187–189.

Kats, L. B., and R. P. Ferrer. 2003. Alien predators and amphibian declines: review of two decades of science and the transition to conservation. Diversity and Distributions 9:99–110.

Kramer-Schadt, S., J. Niedballa, J. D. Pilgrim, B. Schröder, J. Lindenborn, V. Reinfelder, M. Stillfried, I. Heckmann, A. K. Scharf, D. M. Augeri, S. M. Cheyne, A. J. Hearn, J. Ross, D. W. Macdonald, J. Mathai, J. Eaton, A. J. Marshall, G. Semiadi, R. Rustam, H. Bernard, R. Alfred, H. Samejima, J. W. Duckworth, C. Breitenmoser-Wuersten, J. L. Belant, H. Hofer, and A. Wilting. 2013. The importance of correcting for sampling bias in MaxEnt species distribution models. Diversity and Distributions 19:1366–1379.

Landguth, E. L., S. A. Cushman, M. K. Schwartz, K. S. McKelvey, M. Murphy, and G. Luikart. 2010. Quantifying the lag time to detect barriers in landscape genetics. Molecular Ecology 19:4179–4191.

Legendre, P., M. R. T. Dale, M.-J. Fortin, J. Gurevitch, M. Hohn, and D. Myers. 2002. The consequences of spatial structure for the design and analysis of ecological field surveys. Ecography 25:601–615.

Legendre, P., and M. Fortin. 2010. Comparison of the Mantel test and alternative approaches for detecting complex multivariate relationships in the spatial analysis of genetic data. Molecular Ecology Resources 10:831–844.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Lintermans, M. 2000. Recolonization by the mountain galaxias *Galaxias olidus* of a montane stream after the eradication of rainbow trout *Oncorhynchus mykiss*. Marine and Freshwater Research 51:799–804.

Lips, K. R., F. Brem, R. Brenes, J. Reeve D., R. A. Alford, J. Voyles, C. Carey, L. Livo, A. P. Pessier, and J. P. Collins. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. PNAS 103:3165–3170.

Little, C., A. Lara, J. McPhee, and R. Urrutia. 2009. Revealing the impact of forest exotic plantations on water yield in large scale watersheds in South-Central Chile. Journal of Hydrology 374:162–170.

Lobo, J. M., A. Jiménez-Valverde, and R. Real. 2008. AUC: a misleading measure of the performance of predictive distribution models. Global Ecology and Biogeography 17:145–151.

Mackenzie, D. I. 2005. Was it there? Dealing with imperfect detection for species presence/absence data. Australia & New Zealand Journal of Statistics 47:65–74.

MacKenzie, D. I., J. D. Nichols, G. B. Lachman, S. Droege, J. Andrew Royle, and C. A. Langtimm. 2002. Estimating site occupancy rates when detection probabilities are less than one. Ecology 83:2248–2255.

Mancilla, G., C. Valdovinos, M. Azócar, P. Jorquera, and R. Figueroa. 2009. Replacement effect of riparian native vegetation on benthic macroinvertebrates community in temperate climate streams, Central Chile. Hidrobiológica 19:193–203.

Manel, S., and R. Holderegger. 2013. Ten years of landscape genetics. Trends in Ecology & Evolution 28:614–621.

Manel, S., M. K. Schwartz, G. Luikart, and P. Taberlet. 2003. Landscape genetics: combining landscape ecology and population genetics. Trends in Ecology and Evolution 18:189–197.

Mardones, M. 2005. La Cordillera de la Costa: caracterización físico-ambiental y regiones morfoestructurales. *in* C. Smith-Ramírez, J. J. Armesto, and C. Valdovinos, editors. Historia, biodiversidad y ecología de los bosques costeros de Chile. Editorial Universitaria, Santiago Chile.

Marquet, D. P., D. S. Abades, D. J. Armesto, S. I. Barria, D. M. T. K. Arroyo, D. L. Cavieres, D. R. Gajardo, L. C. Garín, D. F. Labra, D. F. Meza, D. P. Pliscoff, D. C. Prado, D. P. R. de Arellano, and D. S. Vicuña. 2010. Estudio de la vulnerabilidad de la biodiversidad terrestre en la eco-región Mediterránes, a nivel de ecosistemas y especies, y medidas de adaptación frente a escenarios de cambio climático: Licitación N°1588-133-LE09. Ministerio de Medio Ambiente, Gobierno de Chile.

Martin, A. P., and S. R. Palumbi. 1993. Body size, metabolic rate, generation time, and the molecular clock. Proceedings of the National Academy of Sciences 90:4087–4091.

McMenamin, S. K., E. A. Hadly, and C. K. Wright. 2008. Climatic change and wetland desiccation cause amphibian decline in Yellowstone National Park. Proceedings of the National Academy of Sciences 105:16988–16993.

McNeely, J. A., K. R. Miller, W. V Reid, R. A. Mittermeier, and T. B. Werner. 1990. Conserving the world's biological diversity. IUCN Gland.

Meirmans, P. G., and P. H. Van Tienderen. 2004. GENOTYPE and GENODIVE: Two

programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes 4:792–794.

Méndez, M. A., and C. Correa Q. 2008. Anfibios. Page 640 Biodiversidad de Chile, Patrimonio y Desafíos.

Merow, C., M. J. Smith, and J. A. Silander. 2013. A practical guide to MaxEnt for modeling species' distributions: what it does, and why inputs and settings matter. Ecography 36:1058–1069.

Molina, A., M. J. Reigosa, and A. Carballeira. 1991. Release of allelochemical agents from litter, throughfall, and topsoil in plantations of Eucalyptus globulus; Labill in Spain. Journal of Chemical Ecology 17:147–160.

Moreno Puig, V. 2014. La ovipostura de *Telmatobufo bullocki* Schmidt, 1952 (Amphibia, Anura, Calyptocepalella). Boletín Chileno de Herpetología 1:17.

Moreno, R., R. Zamora, J. R. Molina, A. Vasquez, and M. Á. Herrera. 2011. Predictive modeling of microhabitats for endemic birds in South Chilean temperate forests using Maximum entropy (Maxent). Ecological Informatics 6:364–370.

Moreno-Puig, V., Y. Yildirim, and D. Brunton. 2014. Development of microsatellite markers for the critically endangered frog *Telmatobufo bullocki* and cross-species amplification in two related species. Conservation Genetics Resources 6:883–884.

Moritz, C. 1994. Defining evolutionarily significant units for conservation. Trends in Ecology & Evolution 9:373–375.

Moritz, C. 1995. Uses of molecular phylogenies for conservation. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 349:113–118.

Moritz, C. 2002. Strategies to protect biological diversity and the evolutionary processes that sustain it. Systematic biology 51:238–54.

Moss, R. H., M. Babiker, S. Brinkman, E. Calvo, T. Carter, J. A. Edmonds, I. Elgizouli, S. Emori, E. Lin, and K. Hibbard. 2008. Towards new scenarios for analysis of emissions, climate change, impacts, and response strategies. IPCC, Geneva (Switzerland).

Mullican, T. R. 1988. Radio telemetry and fluorescent pigments: A comparison of techniques. The Journal of Wildlife Management 52:627–631.

Myers, N., R. A. Mittermeier, C. G. Mittermeier, G. A. B. da Fonseca, and J. Kent. 2000. Biodiversity hotspots for conservation priorities. Nature 403:853–858.

Nuñez, J. J., and J. R. Formas. 2000. Evolutionary history of the Chilean frog genus

Telmatobufo (Leptodactylidae): an immunological approach. Amphibia-Reptilia 21:351–356.

Núñez, J. J., N. K. Wood, F. E. Rabanal, F. M. Fontanella, and J. W. Sites Jr. 2011. Amphibian phylogeography in the Antipodes: Refugia and postglacial colonization explain mitochondrial haplotype distribution in the Patagonian frog *Eupsophus calcaratus* (Cycloramphidae). Molecular Phylogenetics and Evolution 58:343–352.

Olson, D. H., P. D. Anderson, C. A. Frissell, H. H. Welsh Jr, and D. F. Bradford. 2007. Biodiversity management approaches for stream-riparian areas: Perspectives for Pacific Northwest headwater forests, microclimates, and amphibians. Forest Ecology and Management 246:81–107.

Olson, D. H., and K. M. Burnett. 2009. Design and management of linkage areas across headwater drainages to conserve biodiversity in forest ecosystems. Forest Ecology and Management 258, Suppl:S117–S126.

Van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4:535–538.

Orlofske, S. A., K. L. Grayson, and W. A. Hopkins. 2009. The effects of fluorescent tracking powder on oxygen consumption in salamanders using either cutaneous or bimodal respiration. Copeia 2009:623–627.

Ortiz, J. C., and H. Díaz-Páez. 2006. Estado de conocimiento de los anfibios de Chile. Gayana 70:114–121.

Ortiz, J. C., H. Heatwole, and H. Heatwole. 2010. Status of conservation and decline of the amphibians of Chile. Pages 20–29 *in* H. Heatwole, editor. Amphibian Biology.

Ortiz, J. C., and H. Ibarra-Vidal. 2005. Anfibios y reptiles de la cordillera de Nahuelbuta. Pages 427–440 *in* C. Smith-Ramírez, J. J. Armesto, and C. Valdovinos, editors. Historia, biodiversidad y ecología de los bosques costeros de Chile. Editorial Universitaria, Santiago de Chile.

Oyarzún, C. E., C. Frêne, G. Lacrampe, A. Huber, and P. Hervé. 2011. Soil hydrological properties and sediment transport in two headwater catchments with different vegetative cover at the Coastal Mountain Range in southern Chile. BOSQUE 32:10–19.

Paetkau, D. 1999. Using genetics to identify intraspecific conservation units: a critique of current methods. Conservation Biology 13:1507–1509.

Parris, K. M., and D. B. Lindenmayer. 2004. Evidence that creation of a *Pinus radiata* plantation in south-eastern Australia has reduced habitat for frogs. Acta

Oecologica 25:93–101.

Pearson, R. G., C. J. Raxworthy, M. Nakamura, and A. Townsend Peterson. 2007. Predicting species distributions from small numbers of occurrence records: a test case using cryptic geckos in Madagascar. Journal of Biogeography 34:102–117.

Peel, D., R. S. Waples, G. M. Macbeth, C. Do, and J. R. Ovenden. 2013. Accounting for missing data in the estimation of contemporary genetic effective population size (Ne). Molecular Ecology Resources 13:243–253.

Peery, M. Z., R. Kirby, B. N. Reid, R. Stoelting, E. Doucet-Bëer, S. Robinson, C. Vásquez-Carrillo, J. N. Pauli, and P. J. Palsboll. 2012. Reliability of genetic bottleneck tests for detecting recent population declines. Molecular Ecology 21:3403–3418.

Péfaur, J. 1971. Nota sobre *Telmatobufo bullocki* Schmidt (Anura, Leptodactylidae). Boletín del Museo Nacional de Historia Natural Chile 32:215–225.

Peter, B. M., D. Wegmann, and L. Excoffier. 2010. Distinguishing between population bottleneck and population subdivision by a Bayesian model choice procedure. Molecular Ecology 19:4648–4660.

Petit, R. J., A. El Mousadik, and O. Pons. 1998. Identifying populations for conservation on the basis of genetic markers. Conservation Biology 12:844–855.

Petranka, J. W., M. E. Eldridge, and K. E. Haley. 1993. Effects of timber harvesting on southern Appalachian salamanders. Conservation Biology 7:363–370.

Phillips, S. J., R. P. Anderson, and R. E. Schapire. 2006. Maximum entropy modeling of species geographic distributions. Ecological Modelling 190:231–259.

Phillips, S. J., and M. Dudík. 2008. Modeling of species distributions with Maxent: new extensions and a comprehensive evaluation. Ecography 31:161–175.

Phillips, S. J., M. Dudík, J. Elith, C. H. Graham, A. Lehmann, J. Leathwick, and S. Ferrier. 2009. Sample selection bias and presence-only distribution models: implications for background and pseudo-absence data. Ecological Applications 19:181–197.

Pidancier, N., C. Miquel, and C. Miaud. 2003. Buccal swabs as a non-destructive tissue sampling method for DNA analysis in amphibians. Herpetological Journal 13:175–178.

Piry, S., G. Luikart, and J.-M. Cornuet. 1999. BOTTLENECK: a program for detecting recent effective population size reductions from allele data frequencies. The Journal of Heredity 90:502–503.

Ponce-Reyes, R., E. Nicholson, P. W. J. Baxter, R. A. Fuller, and H. Possingham. 2013. Extinction risk in cloud forest fragments under climate change and habitat loss.

Diversity and Distributions 19:518–529.

Popescu, V. D., and M. L. Hunter. 2010. Clear-cutting affects habitat connectivity for a forest amphibian by decreasing permeability to juvenile movements. Ecological Applications 21:1283–1295.

Posada, D. 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25:1253–1256.

Pounds, J. A., M. R. Bustamante, L. A. Coloma, J. A. Consuegra, M. P. L. Fogden, P. N. Foster, E. La Marca, K. L. Masters, A. Merino-Viteri, R. Puschendorf, S. R. Ron, G. A. Sánchez-Azofeifa, C. J. Still, and B. E. Young. 2006. Widespread amphibian extinctions from epidemic disease driven by global warming. Ecology 439:161–167.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Prunier, J. G., B. Kaufmann, S. Fenet, D. Picard, F. Pompanon, P. Joly, and J. P. Lena. 2013. Optimizing the trade-off between spatial and genetic sampling efforts in patchy populations: towards a better assessment of functional connectivity using an individual-based sampling scheme. Molecular Ecology 22:5516–5530.

Pulliam, H. R. 1988. Sources, sinks, and population regulation. The American Naturalist 132:652–661.

Pyron, R. A., and J. J. Wiens. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Molecular Phylogenetics and Evolution 61:543–583.

Rabanal, F. E., and D. Alarcón. 2010. Amphibia, Anura, Cycloramphidae, Alsodes vanzolinii (Donoso-Barros, 1974): Rediscovery in nature, latitudinal and altitudinal extension in Nahuelbuta Range, southern Chile. Check List 6:362–363.

Rabanal, F. E., and V. Moreno-Puig. 2014. New distribution records of the critically endangered frog *Telmatobufo bullocki* Schmidt, 1952 (Anura: Calyptocephalellidae) in southern Chile. Check List 10:428–431.

Rambaut, A., M. A. Suchard, W. Xie, and A. J. Drummond. 2013. Tracer v1.6.0 Available from http://tree.bio.ed.ac.uk/software/tracer/, accessed at July 2014.

Raufaste, N., and F. Rousset. 2001. Are partial Mantel tests adequate? Evolution 55:1703–1705.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–249.

Reed, D. H., and R. Frankham. 2003. Correlation between fitness and genetic diversity.

Conservation Biology 17:230–237.

Richards-Zawacki, C. L. 2009. Effects of slope and riparian habitat connectivity on gene flow in an endangered Panamanian frog, *Atelopus varius*. Diversity and Distributions 15:796–806.

Riley, S. J., S. D. DeGloria, and R. Elliot. 1999. A terrain ruggedness index that quantifies topographic heterogeneity. Intermountain Journal of Sciences 5:23–27.

Rittenhouse, T. A. G., and T. T. Altnether. 2006. Fluorescent powder pigments as a harmless tracking method for Ambystomatids and Ranids. Herpetological Review 37:188–191.

Roe, A. W., and K. L. Grayson. 2008. Terrestrial movements and habitat use of juvenile and emigrating adult eastern red-spotted newts, *Notophthalmus viridescens*. Journal of Herpetology 42:22–30.

Rogers, A. R., and H. Harpending. 1992. Population growth makes waves in the distribution of pairwise genetic differences. Molecular Biology and Evolution 9:552–569.

Rosenberg, N. A. 2004. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4:137–138.

Rosenblitt B., J., and R. Nazer A. (n.d.). Entre el mar y Nahuelbuta: Historia del asentamiento humano en Arauco.

Rousset. 2000. Genetic differentiation between individuals. Journal of Evolutionary Biology 13:58–62.

Rowe, G., and T. J. C. Beebee. 2003. Population on the verge of a mutational meltdown? Fitness costs of genetic load for an amphibian in the wild. Evolution 57:177–181.

Royle, J. A. 2004. N-mixture models for estimating population size from spatially replicated counts. Biometrics 60:108–15.

Ryder, O. A. 1986. Species conservation and systematics: the dilemma of subspecies. Trends in Ecology & Evolution 1:9–10.

Sánchez P., P., B. Guiñez L., E. Hauenstein B., and M. Guerrero A. 2010. Informe Final: Recopilación de información científica y técnica y elaboración de una propuesta de plan de conservación para Telmatobufo bullocki Schmidt 1952, en torno al área de influencia directa del Parque Nacional Nahuelbuta, Región de la Araucanía. Temuco, Chile.

Schlaepfer, M. A., M. C. Runge, and P. W. Sherman. 2002. Ecological and evolutionary traps. Trends in Ecology & Evolution 17:474–480.

Schmidt, G. A., R. Ruedy, J. E. Hansen, I. Aleinov, N. Bell, M. Bauer, S. Bauer, B. Cairns, V. Canuto, and Y. Cheng. 2006. Present-day atmospheric simulations using GISS ModelE: Comparison to in situ, satellite, and reanalysis data. Journal of Climate 19:153–192.

Schmidt, K. P. 1952. A new Leptodactylid frog from Chile. Fieldiana Zoology 34:11–15.

Schneider, S., and L. Excoffier. 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: Application to human mitochondrial DNA. Genetics 152:1079–1089.

Semlitsch, R. D. 2000. Principles for management of aquatic-breeding amphibians. The Journal of Wildlife Management 64:615–631.

Semlitsch, R. D. 2008. Differentiating migration and dispersal processes for pond-breeding amphibians. The Journal of Wildlife Management 72:260–267.

Semlitsch, R. D., and J. R. Bodie. 2003. Biological criteria for buffer zones around wetlands and riparian habitats for amphibians and reptiles. Conservation Biology 17:1219–1228.

Semlitsch, R. D., C. A. Conner, D. J. Hocking, T. A. G. Rittenhouse, and E. B. Harper. 2008. Effects of timber harvesting on pond-breeding amphibian persistence: testing the evacuation hypothesis. Ecological Applications 18:283–289.

Semlitsch, R. D., and J. B. Jensen. 2001. Core habitat, not buffer zone. National Wetlands Newsletter 24:5–11.

Semlitsch, R. D., B. D. Todd, S. M. Blomquist, A. J. K. Calhoun, J. W. Gibbons, J. P. Gibbs, G. J. Graeter, E. B. Harper, D. J. Hocking, M. L. Hunter Jr, D. A. Patrick, T. A. G. Rittenhouse, and B. B. Rothermel. 2009. Effects of timber harvest on amphibian populations: Understanding mechanisms from forest experiments. BioScience 59:853–862.

Sernapesca. 2009. History of the introduction of Species. http://pescarecreativa.sernapesca.cl/index.php?option=com_content&view=article&id=84&Itemid=146&lang=en.

Shoo, L. P., D. H. Olson, S. K. McMenamin, K. A. Murray, M. Van Sluys, M. A. Donnelly, D. Stratford, J. Terhivuo, A. Merino-Viteri, S. M. Herbert, P. J. Bishop, P. S. Corn, L. Dovey, R. A. Griffiths, K. Lowe, M. Mahony, H. McCallum, J. D. Shuker, C. Simpkins, L. F. Skerratt, S. E. Williams, and J.-M. Hero. 2011. Engineering a future for amphibians under climate change. Journal of Applied Ecology 48:487–492.

Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462.

Smith-Ramírez, C. 2004. The Chilean coastal range: a vanishing center of biodiversity and endemism in South American temperate rainforests. Biodiversity and Conservation 13:373–393.

Sodhi, N. S., and P. R. Ehrlich. 2010. Conservation biology for all. Oxford University Press, New York.

Soto, D., I. Arismendi, J. González, J. Sanzana, and F. Jara. 2006. Southern Chile, trout and salmon country: invasion patterns and threats for native species. Revista Chilena de Historia Natural 79:97–117.

Soto-Azat, C., C. Cuevas, E. Flores, and A. Valenzuela-Sánchez. 2012. Conservación de *Telmatobufo bullocki* (Sapo de Bullock) y su hábitat en los bosques degradados de Nahuelbuta. *in* C. Soto-Azat and A. Valenzuela-Sánchez, editors. Conservación de Anfibios de Chile: Memorias del taller de conservación de anfibios para organismos públicos. Universidad Nacional Andrés Bello, Chile.

Soulé, M. E. 1985. What is Conservation Biology?: A new synthetic discipline addresses the dynamics and problems of perturbed species, communities, and ecosystems. BioScience 35:727–734.

Spear, S. F., N. Balkenhol, M.-J. Fortin, B. H. McRae, and K. I. M. Scribner. 2010. Use of resistance surfaces for landscape genetic studies: considerations for parameterization and analysis. Molecular Ecology 19:3576–3591.

Spear, S. F., and A. Storfer. 2010. Anthropogenic and natural disturbance lead to differing patterns of gene flow in the Rocky Mountain tailed frog, *Ascaphus montanus*. Biological Conservation 143:778–786.

Stoddard, M. A., and J. P. Hayes. 2005. The influence of forest management on headwater stream amphibians at multiple spatial scales. Ecological Applications 15:811–823.

Storfer, A., M. A. Murphy, J. S. Evans, C. S. Goldberg, S. Robinson, S. F. Spear, R. Dezzani, E. Delmelle, L. Vierling, and L. P. Waits. 2006. Putting the “landscape” in landscape genetics. Heredity 98:128–142.

van Strien, M. J., D. Keller, R. Holderegger, J. Ghazoul, F. Kienast, and J. Bolliger. 2013. Landscape genetics as a tool for conservation planning: predicting the effects of landscape change on gene flow. Ecological Applications 24:327–339.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783–1786.

Swets, J. A. 1988. Measuring the accuracy of diagnostic systems. Science 240 :1285–

1293.

Tachikawa, T., M. Hato, and A. Iwasaki. 2011. The characteristics of ASTER GDEM version 2. IGARSS. Vancouver.

Tajima, F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tallmon, D. A., A. Koyuk, G. Luikart, and M. A. Beaumont. 2008. COMPUTER PROGRAMS: onesamp: a program to estimate effective population size using approximate Bayesian computation. Molecular Ecology Resources 8:299–301.

Tallmon, D. A., G. Luikart, and M. A. Beaumont. 2004. Comparative evaluation of a new effective population size estimator based on approximate Bayesian computation. Genetics 167:977–988.

Tamura, K., and M. Nei. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512–526.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30:2725–2729.

Tarrant, J., and A. J. Armstrong. 2013. Using predictive modelling to guide the conservation of a critically endangered coastal wetland amphibian. Journal for Nature Conservation 21:369–381.

Templeton, A. R. 1989. The meaning of species and speciation: a genetic perspective. Pages 159–183 *in* M. Ereshefsky, editor. The units of evolution: Essays on the nature of species. MIT press.

Templeton, A. R., K. A. Crandall, and C. F. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619–633.

Thomson, A., K. Calvin, S. Smith, G. P. Kyle, A. Volke, P. Patel, S. Delgado-Arias, B. Bond-Lamberty, M. Wise, L. Clarke, and J. Edmonds. 2011. RCP4.5: a pathway for stabilization of radiative forcing by 2100. Climatic Change 109:77–94.

Thorn, J. S., V. Nijman, D. Smith, and K. A. I. Nekaris. 2009. Ecological niche modelling as a technique for assessing threats and setting conservation priorities for Asian slow lorises (Primates: Nycticebus). Diversity and Distributions 15:289–298.

Tilman, D., R. M. May, C. L. Lehman, and M. A. Nowak. 1994. Habitat destruction and the extinction debt. Nature 371:65–66.

Tognelli, M. F., S. A. Roig-Junent, A. E. Marvaldi, G. E. Flores, and J. M. Lobo. 2009. An evaluation of methods for modelling distribution of Patagonian insects. Revista Chilena de Historia Natural 82:347–360.

Trumbo, D. R., S. F. Spear, J. Baumsteiger, and A. Storfer. 2013. Rangewide landscape genetics of an endemic Pacific northwestern salamander. Molecular Ecology 22:1250–1266.

Veloso, A., H. Núñez, and R. Formas. 2008. *Telmatobufo bullocki*. In: IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. www.iucnredlist.org. http://www.iucnredlist.org/details/21623/0.

Veloz, S. D. 2009. Spatially autocorrelated sampling falsely inflates measures of accuracy for presence-only niche models. Journal of Biogeography 36:2290–2299.

Venegas S., W. 1975. Los cromosomas de *Aruncus venustus* (Philippi) 1899 (=*Telmatobufo bullocki* Schmidt, 1952). Boletín Sociedad de Biología de Concepción 49:71–77.

Vesely, D. G., and W. C. McComb. 2002. Salamander abundance and amphibian species richness in riparian buffer strips in the Oregon Coast Range. Forest Science 48:291–297.

Veysey, J. S., K. J. Babbitt, and A. Cooper. 2009. An experimental assessment of buffer width: Implications for salamander migratory behavior. Biological Conservation 142:2227–2239.

Vidal Maldonado, M. A., and A. Labra Lillo. 2008. Herpetología de Chile. Primera Ed. Science Verlag Chile, Santiago de Chile.

Villagrán, C. 2001. Un modelo de la historia de la vegetación de la Cordillera de La Costa de Chile central-sur: la hipótesis glacial de Darwin. Revista chilena de historia natural 74:793–803.

Villagrán, C., and J. J. Armesto. 2005. Historical phytogeography of the Chilean Coastal Range. Pages 99–116 *in* C. Smith-Ramírez, J. J. Armesto, and C. Valdovinos, editors. Historia, biodiversidad y ecología de los bosques costeros de Chile. Editorial Universitaria, Santiago de Chile.

Wahbe, T. R., and F. L. Bunnell. 2001. Preliminary observations on movements of tailed frog tadpoles (*Ascaphus truei*) in streams through harvested and natural forests.

Wake, D. B., and V. T. Vredenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. PNAS 105:11466–11473.

Walls, S., W. Barichivich, and M. Brown. 2013. Drought, deluge and declines: The impact of precipitation extremes on amphibians in a changing climate. Biology 2:399–418.

Wan, Q.-H., H. Wu, T. Fujihara, and S.-G. Fang. 2004. Which genetic marker for which conservation genetics issue? Electrophoresis 25:2165–76.

Wang, I. J. 2011. Choosing appropriate genetic markers and analytical methods for testing landscape genetic hypotheses. Molecular Ecology 20:2480–2482.

Wang, Y.-H., K.-C. Yang, C. Bridgman, and L.-K. Lin. 2008. Habitat suitability modelling to correlate gene flow with landscape connectivity. Landscape Ecology 23:989–1000.

Waples, R. S. 2006. A bias correction for estimates of effective population size based on linkage disequilibrium at unlinked gene loci. Conservation Genetics 7:167–184.

Waples, R. S., and C. Do. 2010. Linkage disequilibrium estimates of contemporary Ne using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. Evolutionary Applications 3:244–262.

Wasserman, T. N., S. A. Cushman, M. K. Schwartz, and D. O. Wallin. 2010. Spatial scaling and multi-model inference in landscape genetics: *Martes americana* in northern Idaho. Landscape Ecology 25:1601–1612.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358–1370.

Wells, K. D. 1977. The social behaviour of anuran amphibians. Animal Behaviour 25:666–693.

Wells, K. D. 2010. The Ecology and Behavior of Amphibians. University of Chicago Press, Chicago.

Wolodarsky-Franke, A., and S. Díaz Herrera. 2011. Cordillera de Nahuelbuta. Reserva Mundial de Biodiversidad . WWF, Valdivia, Chile.

Woodhams, D. C., K. Ardipradja, R. A. Alford, G. Marantelli, L. K. Reinert, and L. A. Rollins-Smith. 2007. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation 10:409–417.

Wright, S. 1943. Isolation by distance. Genetics 28:114–38.

Zhang, D.-X., and G. M. Hewitt. 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Molecular Ecology 12:563–584.

ZSL. 2011. EDGE: Top 100 Evolutionarily Distinct & Globally Endangered amphibians. http://www.edgeofexistence.org/amphibians/top_100.php.

Appendix A. Historical records for *Telmatobufo bullocki* from 1931 to 2006.

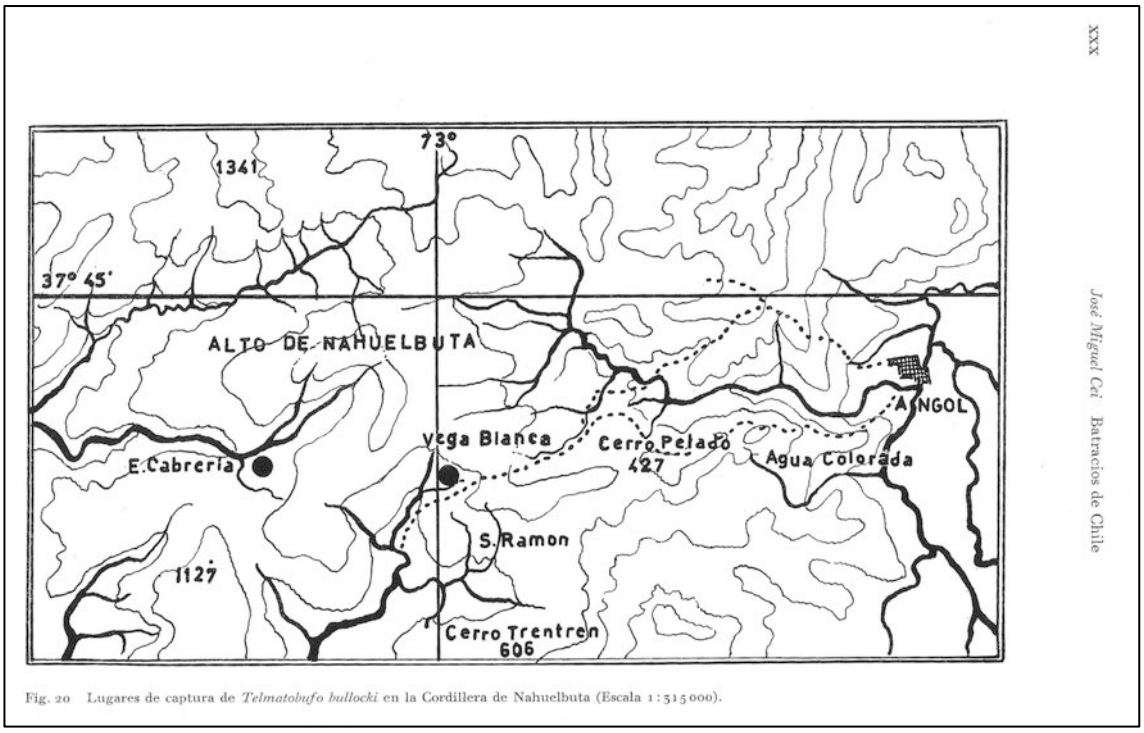
List of records, location names and sources.

Date	Location Name	Collector, source and notes
12 October 1931	Nahuelbuta	Dillman S. Bullock, Schmidt (1952). Type specimen, Chicago Natural History Museum (CNHM/FMNH #23842), USA. Adult male 63 mm SVL found under a log in the hills behind Angol (possibly referred as Vegas Blancas in Cei 1962).
27 November 1936	Nahuelbuta	Dillman S. Bullock, Schmidt (1952) Paratype, Chicago Natural History Museum (FMNH #31618), USA. Male, 64 mm SVL, found under log in the hills behind Angol. Stomach contents described.
2 April 1942	Nahuelbuta	Dillman S. Bullock, Bullock (1954) Museo el Vergel, Angol. Found under log.
31 December 1957	Cordillera de Nahuelbuta, Los Alpes	W. J. Eyerdam, http://collections.calacademy.org/herp/ Collected, California Academy of Science Herpetology Collection (CAS-HERP Catalog #143364) Misidentified as *T. australis*
February 1960	Estero Cabrerías, Alto de Nahuelbuta	R. Donoso-Barros? Cei (1962 p. 53) Colección Donoso-Barros, U. De Concepcion. Female.
September 1963	Vegas de Rucapillán	Péfaur (1971) Mentioned in the publication but no details given, possibly a generic name given to the area visited by Dr. Bullock.
7 August 1968	Vanerías Nahuelbuta National Park	Hermosino Cárcamo, Péfaur (1971) Collected, Facultad de Ciencias Universidad de Chile. Adult male found under rotting *Araucaria* log, 150 m from Coimallín Stream inside the National Park. Based on the map provided in Ferriere (1963), Coimallin Stream corrsponds to *Agua de los Gringos* Stream.
10 August 1968	Estero Los Lleulles	J. Péfaur (1971) Collected, Facultad de Ciencias Universidad de Chile. Adult male found in the stream. This and the previous record were studied in captivity for a couple of months.
1968	Estero Los Lleulles	

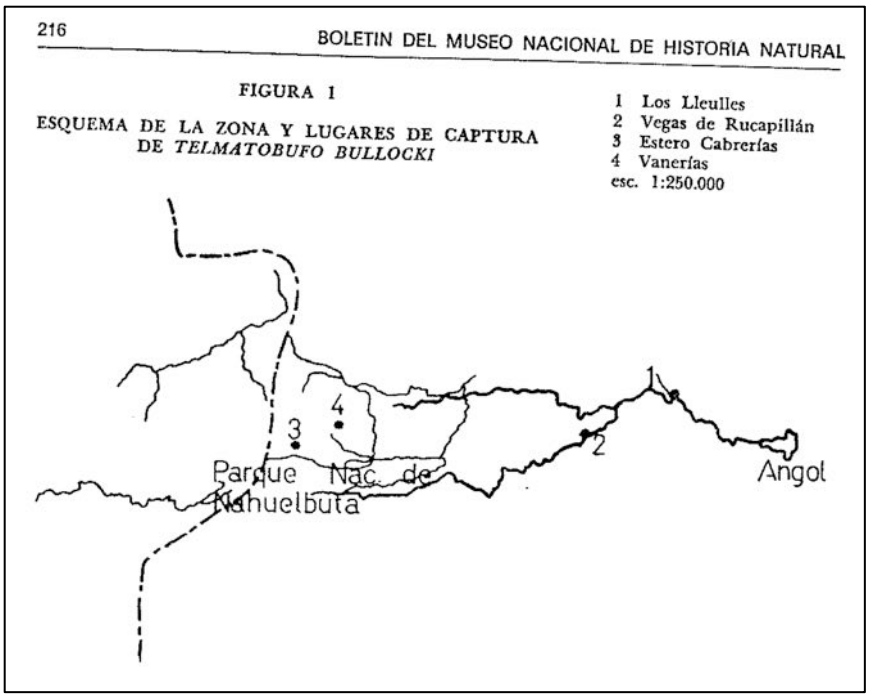
		http://collections.biodiversity.ku.edu/KUHerps/ Misidentified as *T. venustus* in This is possibly one of Péfaur's specimens (same date and location?
20 April 1969	Fundo San Ernesto Elicura	I. Sanfeliu http://mczbase.mcz.harvard.edu Collected, Harvard Collection Herpetology MCZbase (A-78829)
1970s	Ramadillas	R. Donoso-Barros (1972) The author mentions *T. bullocki* had been recently found in Ramadillas, Provincia de Arauco, in montaneous *Nothofagus* forest, later in the text he added Contulmo, Ramadilla.
1970s (?)	Lota	Venegas (1975) This author mentions for the first time the presence of *T. bullocki* near Lota, Collected, Museo de Zoología Universidad de Concepción (MUZUC #11644)
1970s (?)	Ramadillas	Formas and Veloso (1982) (MUZUC #12266)
1970s (?)	Elicura	Formas and Veloso (1982) (MUZUC #12276)
5 January 1975	Arroyo Los Lleulles	W. E. Duellman http://collections.biodiversity.ku.edu/KUHerps/ 2 frogs collected Kansas University Herpetology Collection (Catalog KU #161438, #161439) Misidentified as *T. venustus* Frogs were found under rocks in Los Lleulles Stream 11 km W of Angol
14 February 1977	Malleco	G. Moreno http://fm1.fieldmuseum.org/collections/search.cgi?dest=herps&action=form One adult collected, Field Museum of Natural History (Catalog FMNH #209291), Chicago USA
24 January 1984	Rio La Cueva, Antihuala	Dr. Roberto Schlatter, Formas (1988) Tadpoles collected and described in Formas (1988) In Formas (1988), La Cueva is wrongly referenced as being South of Canete. It is actually East of Antihuala, and close to Rucapehuen but drains to Cayucupil River instead of Caramavida.
November (year?)	Ramadillas	

		R. Formas, J.J. Nuñez, Formas and Veloso (1982) One juvenile collected, Instituto Zoologia Universidad Austral (IZUA #1563, lost)
November (year?)	Rucapehuen	R. Formas, J.J. Nuñez, Nuñez and Formas (2000) One adult male collected (37°40' S; 73°25 W), Arauco province, (IZUA #3157, lost).
(?)	Rio Caramavida	Formas & Veloso (1982) (IZUA #1858, #1859)
Noviembre (year?)	Rio Caramavida	R. Formas, J.J. Nuñez Formas et al. (2001) Two adults collected next to Rio Caramavida (IZUA #1820, #1821, lost)
June 2006	Los Lleulles	Jaime Carcamo, Sánchez (2010)

Maps



The first map provided for *T. bullocki* collection points in *Batracios de Chile* (Cei 1962). It included two points.



Map containing four *T. bullocki* collection points published in Péfaur (1971).

Map of Nahuelbuta National Park obtained from Ferrière (1963), showing the location Vanerías (in red) and Estero Coimallín (in blue) inside the park (circles added). In 1968 one *T. bullocki* adult was found in Vanerías 150 m from Estero Coimallín (Péfaur 1971).

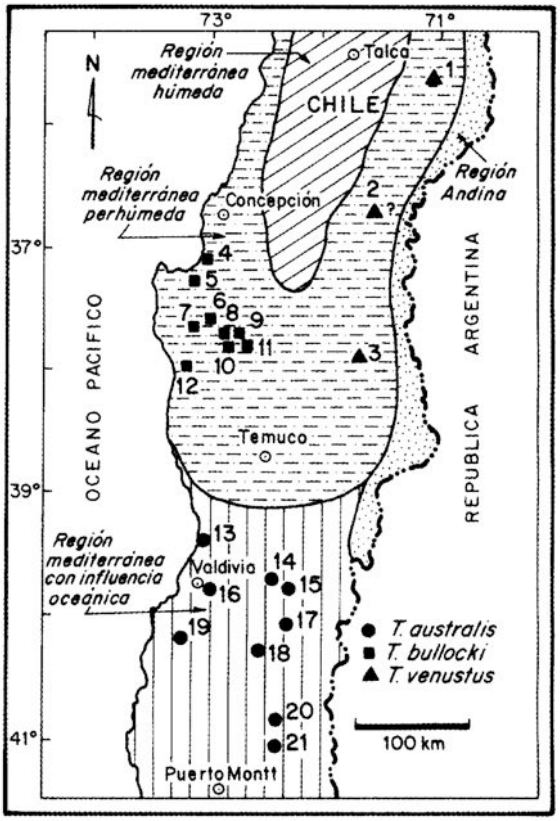


Fig. 4: Distribución geográfica de las especies de *Telmatobufo*. (1) Altos de Vilches, (2) *Cordillera de Chillán (lugar indeterminado), (3) **Ralco, (4) Lota, (5) Ramadillas, (6) Ruca-Pehuén, (7) Río Caramávida, (8) ***Estero Cabrerías, (9) ***Vanerías, (10) ***Vegas de Rucapillán, (11) Los Lleulles, (12) Elicura, (13) Mehuín, (14) Panguipulli, (15) Riñihue, (16) Llancahue, (17) Coñaripe, (18) Río Quimán, (19) Chiverías, (20) Piedras Negras, (21) Cerro Püschel. Según: *Philippi (1899), **Díaz et al. (1983), ***Péfaur (1971).

Geographical distribution of the *Telmatobufo* species. (1) Altos de Vilches, (2) *Cordillera de Chillán (undeterminated place), (3) **Ralco, (4) Lota, (5) Ramadillas, (6) Ruca-Pehuén, (7) Caramavida river, (8) ***Estero Cabrerías, (9) ***Vanerías, (10) ***Vegas de Rucapillán, (11) Los Lleulles, (12) Elicura, (13) Mehuín, (14) Panguipulli, (15) Riñihue, (16) Llancahue, (17) Coñaripe, (18) Quimán river, (19) Chiverías, (20) Piedras Negras,(21) Cerro Püschel. According to: *Philippi (1899), **Díaz et al. (1983), ***Péfaur (1971).

Map from Formas et al. (2001), with nine *T. bullocki* points.

Appendix B. List of presence records

Geographic coordinates (UTM 18S, WGS80) for all recent *T. bullocki* sightings (*N* = 70, since 2006). Records obtained from other sources are marked as follows: * = Rabanal and Moreno-Puig (2014); ** = Edgardo Flores, pers. comm.; *** = Sánchez (2010); **** Bernardo Guzman 2013, pers. comm. Some locations represent more than one individual.

ID	Basin	Easting	Northing
BUTb-1	Rio Butamalal	665479	5810127
BU-2-01	Rio Butamalal	661317	5811650
BU-2-02	Rio Butamalal	661331	5811673
BU-2-03	Rio Butamalal	661331	5811670
BU-2-04	Rio Butamalal	661319	5811664
BU-3-01	Rio Butamalal	664493	5811203
BU-4-01	Rio Butamalal	665622	5810228
BU-Tb-1	Rio Butamalal	661432	5811558
BU-Tb-2	Rio Butamalal	661312	5811652
BU-2011_1*	Rio Butamalal	661777	5811586
BU-2011_2*	Rio Butamalal	661797	5811453
CL-1-01	Estero Calebu	659305	5797068
CL-2-01	Estero Calebu	657878	5796782
CL-3-01	Estero Calebu (small tributary)	658438	5796423
CL-4-01	Estero Calebu	659132	5797164
CL-4-03	Estero Calebu	659044	5797187
CL-4-04	Estero Calebu	658971	5797317
CL-4-05	Estero Calebu	659078	5797276
CL-4-06	Estero Calebu	659254	5797116
CA-2-01	Caramavida	660832	5824658
CA-3-01	Caramavida	662093	5826306
CA-4-01	Caramavida	663179	5826526
CA-5-01	Caramavida	659019	5826298
CA-EF_1**	Caramavida	657513	5828058
CA-2012	Caramavida	658981	5826386
CH-1-01	Chivilingo	666500	5889152
CH-FR*	Chivilingo	668417	5890025
CA-6	Estero Las Delicias (Caramávida)	655985	5827462
CY-3-01	Estero Los Tres Viejos (Cayucupil)	665154	5817742
SC-1-01	Estero San Carlos	658857	5800655
HU-1-01	Huilquehue	652799	5807300
HU-2-01	Huilquehue	653729	5806181

LC-1-01	La Cueva	663862	5821776
LC-2012	La Cueva	663972	5821941
LL-1-01	Los Lleulles	694601	5815101
LL-1-02	Los Lleulles	694691	5815076
LL-2-01	Los Lleulles	695214	5816283
LL-3-01	Los Lleulles	694257	5815676
LL-2006***	Los Lleulles	694320	5815375
EN-04-1-01	Provoque	656908	5803567
EN-PR-01	Provoque	660628	5805166
EN-Tb-01	Provoque	657337	5803697
EN-Tb-02	Provoque	656727	5803376
EN-Tb-03	Provoque	656727	5803383
EN-Tb-04	Provoque	656732	5803390
EN-Tb-05	Provoque	656768	5803411
EN-Tb-06	Provoque	656816	5803419
EN-Tb-07	Provoque	656815	5803432
EN-Tb-08	Provoque	656752	5803398
EN-Tb-09	Provoque	656987	5803740
EN-Tb-10	Provoque	656751	5803389
EN-Tb-11	Provoque	656929	5803620
EN-Tb-12	Provoque	656915	5803622
EN-Tb-13	Provoque	656985	5803918
EN-Tb-14	Provoque	657145	5803978
EN-Tb-15	Provoque	657263	5803932
EN-Tb-17	Provoque	657488	5804010
EN-Tb-18	Provoque	657496	5804170
EN-Tb-19	Provoque	657050	5803976
EN-Tb-20	Provoque	656829	5803588
EN-Tb-21	Provoque	657223	5803949
EN-Tb-22	Provoque	657251	5803941
EN-Tb-24	Provoque	657506	5804138
EN-Tb-25	Provoque	657521	5804122
EN-Tb-26	Provoque	657224	5804270
EN-Tb-27	Provoque	657287	5803948
EN-Tb-32	Provoque	657003	5804004
EN-TbA	Provoque	656967	5803706
Ramadillas****	Ramadillas	659284	5867062
CY-2-01	Rio Cayucupil	668526	5819904

.

Appendix C. Stream surveys and tadpole relative abundance in 18 sites in Nahuelbuta.

Table C.1. List of 18 streams surveyed in this thesis and their location in UTM 18S (WGS80), stream characterisation, water physicochemical parameters (SC = specific conductance, DO = dissolved oxygen).

Site ID	Date	Location	Easting	Northing	Elevation (m)	Width (m)	Depth (cm)	Temp (ºC)	SC (uS/cm)	DO (mg/L)	pH	gradient %
1	29/11/13	Butamalal (lower)	664493	5811203	622							
2	1/12/13	Butamalal (upper)	665622	5810228	648	9.0	37.0	10.65	28.10	10.63	7.54	9.8
3	8/11/13	Caramavida	662093	5826306	865	4.9	20.0	7.8	20.00	11.06	7.31	12.6
4	8/11/13	Caramavida	663179	5826526	1020	4.3	24.4	7.1	18.76	11.01	7.28	12.4
5	16/12/13	Las Delicias (Caramavida)	655976	5827468	452	4.8	19.2	11.7	48.00	10.57	7.63	16
6	10/10/13	Chivilingo (tributary)	666500	5889152	181	4.9	11.2	11.1	24.35	9.13	6.21	8.8
7	9/10/13	Calebu	659305	5797068	124	10.9	20.4	8.7	25.97	11.67	7.23	12
8	3/11/13	Calebu (tributary)	658438	5796423	102							
9	3/12/13	Cayucupil	668526	5819904	686	8.5	30.2	9.2	27.10	10.58	7.51	5.5
10	14/12/13	Los 3 Viejos (Cayucupil)	665154	5817742	553	4.1	18.4	10.4	37.00	10.68	7.45	7.3
11	17/10/13	La Cueva (Cayucupil)	663862	5821776	700	5.1	24.1	8.2	24.13	10.74	7.40	7
12	5/12/13	Huilquehue	652799	5807300	90	4.1	13.8	10.3	28.37	11.14	7.15	5
13	9/12/13	Huilquehue	653729	5806181	165							
14	25/10/13	Los Lleulles	694691	5815076	381	3.1	16.7	9.3	47.08	10.91	7.71	14.5
15	27/10/13	Los Lleulles	695214	5816283	595							
16	27/10/13	Los Lleulles (tributary)	694257	5815676	520	2.5	5.3	9.8	54.10	10.57	7.71	23
17	5/10/13	Provoque	660628	5805166	431	7.0	26.1					5
18	2/11/13	San Carlos (Provoque)	658857	5800655	153	4.0	18.4	11.3	28.10	11.00	7.37	11.5

Table C.2. Tadpole survey results for sites in Table C.1. Not all tadpoles were captured and measured.

Site ID	Date	Survey effort		Relative abundance		
		Time (hours)	Distance (m)	Tadpoles	Tadpoles/100 m	Tadpoles/hr
1	29/11/13	1.25	70	4	5.7	3.2
2	1/12/13	2	100	5	5.0	2.5
3	8/11/13	1	30	1	3.3	1.0
4	8/11/13	1.63	50	6	12.0	3.7
5	16/12/13	1.25	50	15	30.0	12.0
6	10/10/13	1.4	80	18	22.5	12.9
7	9/10/13	0.5	50	2	4.0	4.0
8	3/11/13	0.66	80	6	7.5	9.1
9	3/12/13	2	160	1	0.6	0.5
10	14/12/13	1.75	100	7	7.0	4.0
11	17/10/13	2	50	7	14.0	3.5
12	5/12/13	2	50	15	30.0	7.5
13	9/12/13	1	60	15	25.0	15.0
14	25/10/13	5	20	5	25.0	1.0
15	27/10/13	1.25	80	1	1.3	0.8
16	27/10/13	0.25	10	3	30.0	12.0
17	5/10/13	1	50	1	2.0	1.0
18	2/11/13	1	30	18	60.0	18.0

Appendix D. Sexual size dimorphism in *T. bullocki*

Table D.1. Mean values and standard deviations (SD) for snout to vent length (SVL) and weight. Sex identification was based on the presence or absence of nuptial excrescences (all surveys were done during breeding season).

	N	SVL (mm)	SD	Weight (g)	SD
Female	26	79.46	3.39	55.21	9.50
Male	20	71.80	3.27	48.25	4.63
TOTAL	46	76.13	5.06	52.19	8.45

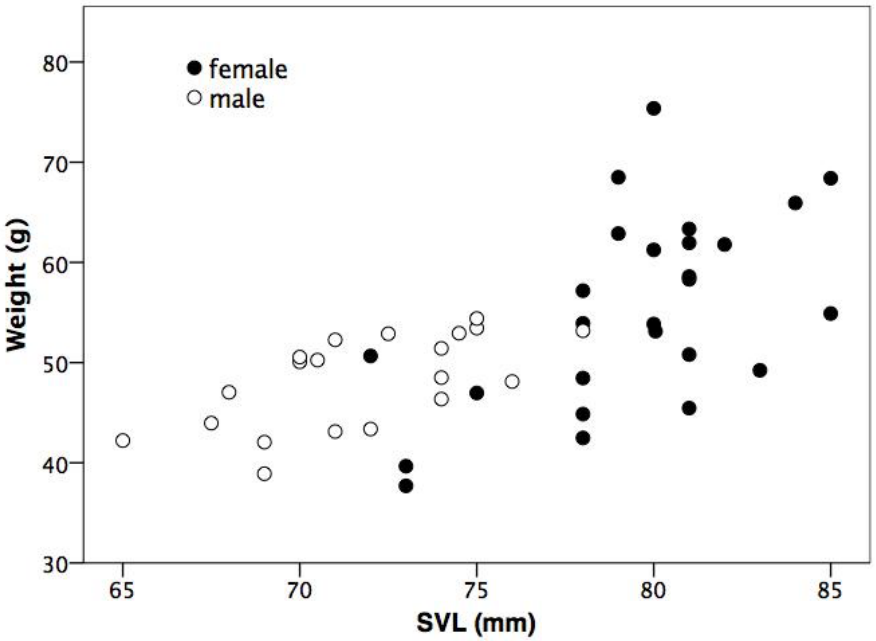


Figure D.1. Scatterplot showing the relationship between snout-to-vent (SVL) length (mm) and weight (g) for female and male adult frogs (*N* = 46). Females are generally both larger and heavier than males.

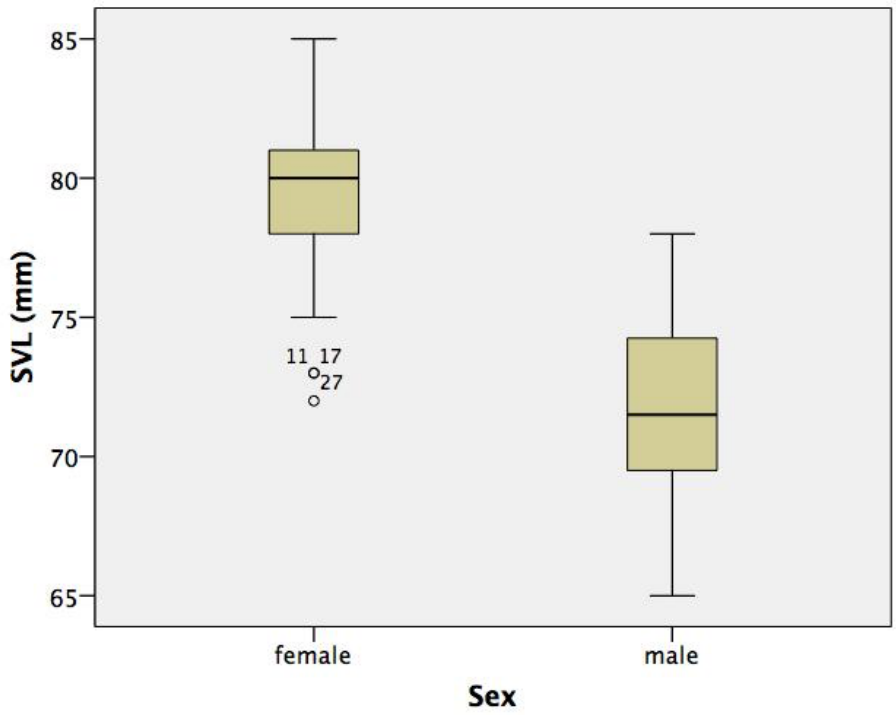


Figure D.2. Boxplot for data on Table D.1 showing the median (horizontal line), maximum and minimum (whiskers), and suspected outliers (dots outside box for female data) for observed females (*N* = 26) and males (*N* = 20). Female outliers could correspond to males misidentified as females due to lack of secondary sex characteristics.

Appendix E. Observations on *T. bullocki* growth and development.

Tadpoles

Figure E.1 shows some pictures of different developmental stages in *T. bullocki* larvae. Exact Gosner stages of development were difficult to assign due to the large skin fold concealing hind limb development, so the stages are approximate. Figure E.1 shows ventral view (left) and dorsal view (right) of tadpoles. From top to bottom: At stage 28, tadpole total length (TL) is 45 mm and body length (BL) is 19 mm, hind limb bud visible through semi-transparent skin fold as small white dots (roughly round). At stage 30-35, TL = 85 mm, BL = 37 mm, weight = 11.55 g, hind limb visible through skin fold as white elongated spots (length > 2x diameter). At stage 37-39 TL = 82 BL = 39 mm weight 8.5 g, hind limbs clearly visible through skin fold and foot developed, skin fold not fully covering toes, dorsal parotoid glands start to develop. Stage 40-41, TL = 96 mm, BL = 48 mm, weight = 15.7 g, hind legs well developed and not covered by skin fold, dorsal parotoid gland clearly developed and yellow inter-ocular spot visible. Stage 41, TL = 101 mm, BL = 42 mm, weight = 19.95 g, body stocky and strong legs, larval mouthparts breaking down.

Metamorphs

Young metamorphs were found mainly in the stream and only few metres away. Figure E.2 shows metamorphs at different developmental stages. From top to bottom and left to right: at stage 42, fore limbs have developed, but larval mouthparts still remain, dorsal glands and yellow spot evident. At stage 43, mouth parts have changed, and skin glands

developed. At stages 44-45-46, the tail shortens progressively, while larval mouthparts are completely lost.

Adults

Figure E.3 shows a cross section of a phalangeal bone stained with Delafields Haematoxylin and mounted in glycerine of an adult *T. bullocki* female (80 mm SVL) found dead in a stream. Two LAG lines can be seen, indicating an approximate minimum age of 3 years. Slides and picture prepared by Dr. Matthew R. F. Perrot (BVSc.) from the Pathobiology Group at the Institute of Veterinary Animal and Biomedical Science, Massey University, Palmerston North.

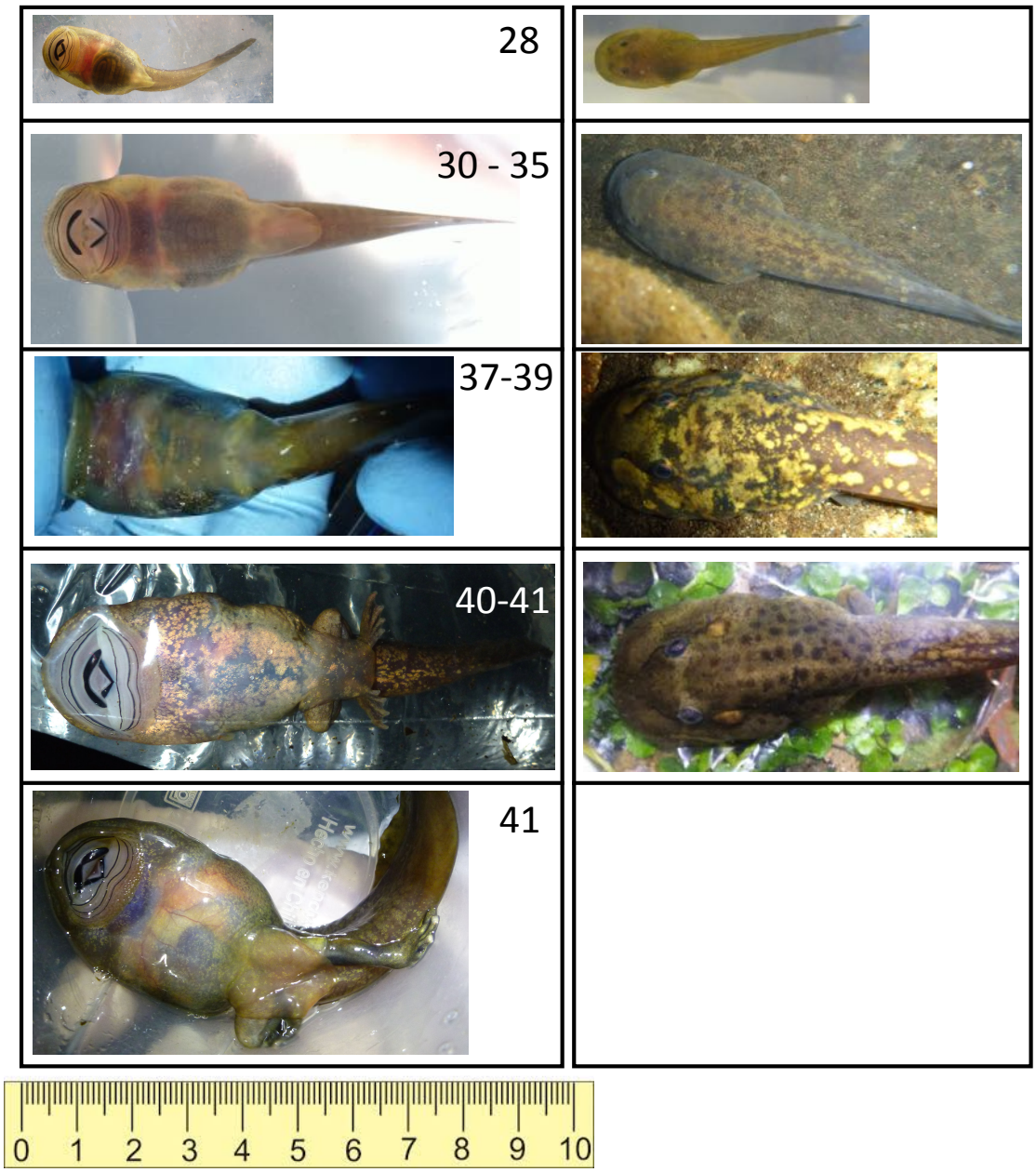


Figure E.1. Tadpole growth, number corresponds to estimated Gosner stage. Left column shows ventral view, right column shows dorsal view for same stage.

Figure E.2. Metamorphs (42, photo Felipe Rabanal, 43 photo Bernardo Guzman)

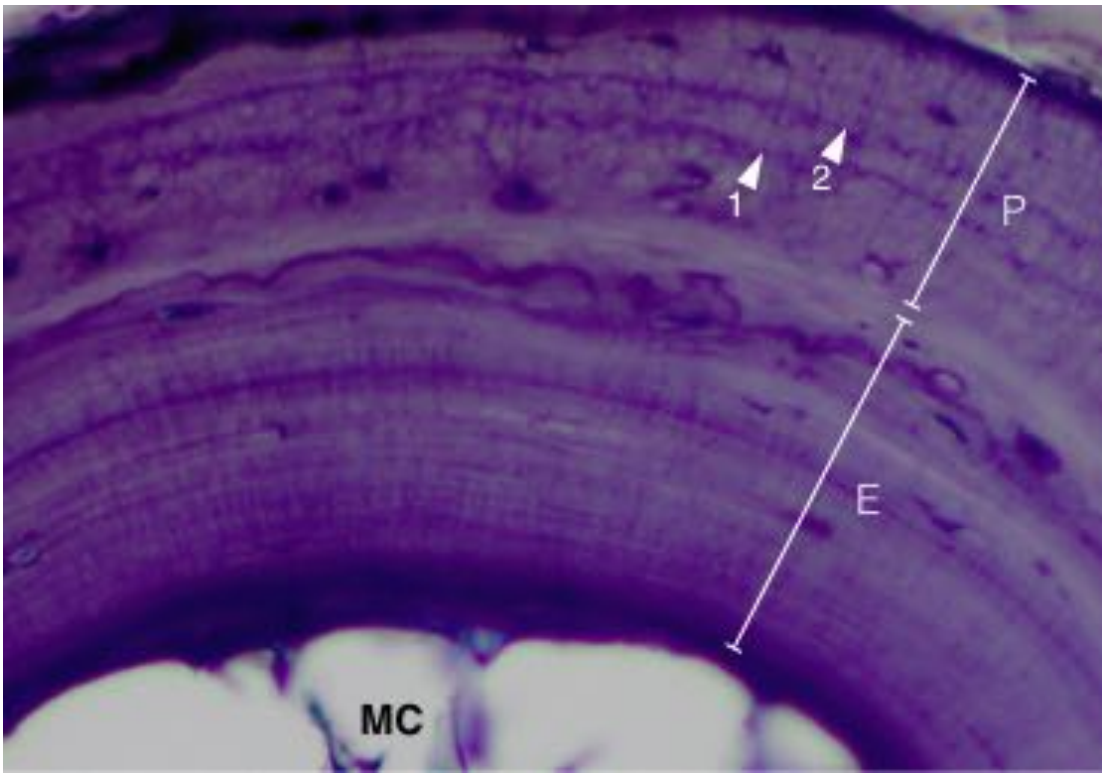


Figure E.3. Cross section of phalangeal bone of adult female *T. bullocki* showing two lines of arrested growth. MC = marrow cavity, E = endosteal, P = perosteal, numbered arrows = lines of arrested growth (LAG). Slide and picture prepared by Dr. Matthew R. F. Perrott, Massey University.

Appendix F. Development of microsatellite markers for the critically endangered frog *Telmatobufo bullocki* and cross-species amplification in two related species.

Published in Conservation Genetics Resources (2014) 6, 883-884. The final publication is available at link.spinger.com (http://link.springer.com./article/10.1007/s12686-014-0231-7)

Appendix G. Additional tables and graphs for Chapter 4.

G. 1 Bayesian MSVAR run parameters

For all runs, the starting values for ancestral (N_1) and current population (N_0) sizes that were assumed to be similar and large (10^4). These values are updated during the MCMC, using hyperpriors defined by columns 6-9.

	Starting values (mean and variance)				Hyperpriors (α σ β τ)			
	log(N_0)	log(N_1)	log(μ)	log(T)	log(N_0)	log(N_1)	log(μ)	log(T)
Run 1	4 1	4 1	-3.5 1	5 1	4 3 0 0.5	3 3 0 0.5	-3.5 0.5 0 2	4 3 0 0.5
Run 2	4 1	4 1	-3.5 1	5 1	4 3 0 0.5	5 3 0 0.5	-3.5 0.5 0 2	4 3 0 0.5
Run 3	4 1	4 1	-3.5 1	5 1	4 3 0 0.5	4 3 0 0.5	-3.5 0.5 0 2	4 3 0 0.5
Run 4	4 2	4 2	-3.5 1	5 3	5 3 0 0.5	3 2 0 0.5	-3.5 0.25 0 0. 5	5 2 0 0.5

G. 2 Hardy-Weinberg Equilibrium test

P-values for probability of deviation from Hardy Weinberg equilibrium (HWE) as calculated in GENEPOP (probability test) for 15 microsatellite loci in *T. bullocki* (*N* = 142) at 8 populations (basins). *** monomorphic. * Significant departures from HWE after sequential Bonferroni correction.

Locus	CH	CA	CY	BU	HU	PR	CL	LL
Tbu03	0.0958	0.6652	0.8511	0.4980	0.3567	0.2704	0.3978	0.8675
Tbu06	1.0000	0.8578	0.9467	0.3242	0.3064	0.2342	1.0000	0.5200
Tbu19	0.6521	0.0954	0.7064	1.0000	1.0000	0.3211	1.0000	0.0000*
Tbu20	0.4913	0.8021	0.1554	0.0933	0.8432	0.2620	0.3266	1.000
Tbu23	0.1545	0.4334	0.1203	0.0001*	1.0000	0.3288	0.7041	0.0337
Tbu24	0.1194	0.4455	0.9162	0.0627	0.1084	0.9550	0.0418	0.3412
Tbu28	0.1443	0.4465	0.0041	0.3804	1.0000	0.2013	0.6029	0.8397
Tbu33	0.7695	0.8972	0.1431	0.0257	1.0000	0.1697	1.000	0.3066
Tbu34	0.0314	0.7975	0.0563	0.0854	1.0000	0.0084	0.2586	***
Tbu35	0.6831	0.1686	0.4248	0.2430	0.3905	0.8109	0.6650	0.1811
Tbu39	0.5610	1.000	0.4025	0.2293	1.000	0.0810	0.0284	1.0000
Tbu42	***	1.000	1.000	1.0000	0.5429	0.4537	0.2135	1.0000
Tbu44	0.8908	0.0060	0.2371	0.1503	0.4935	0.9731	0.7079	0.6952
Tbu47	1.0000	1.0000	1.000	0.5770	1.0000	0.9723	0.7973	0.5636
Tbu48	0.8050	0.0014	0.7474	0.0056	0.3781	0.0002*	0.0006	0.3558

G. 3 Evanno method to detect number of genetic clusters (*K*)

Plots of the mean of estimated natural log probability of K [P(*K*)] and delta *K* (Δ*K*) to determine number of populations (*K*). Plotted using the Evanno method (Evanno et al. 2005) in program Structure Harvester Web 0.6.92 (Earl and von Holdt 2012).

Full dataset (8 basins)

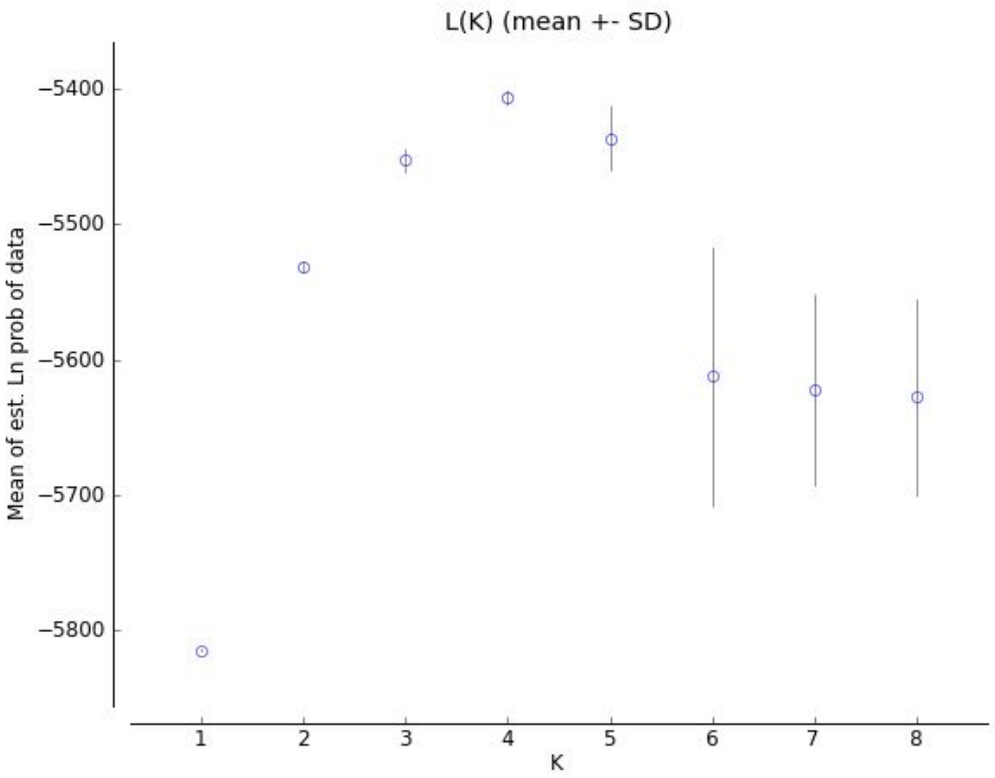


Figure G.3.1. Mean of estimated natural log probability of K [P(*K*)] is highest and plateaus at *K* = 4.

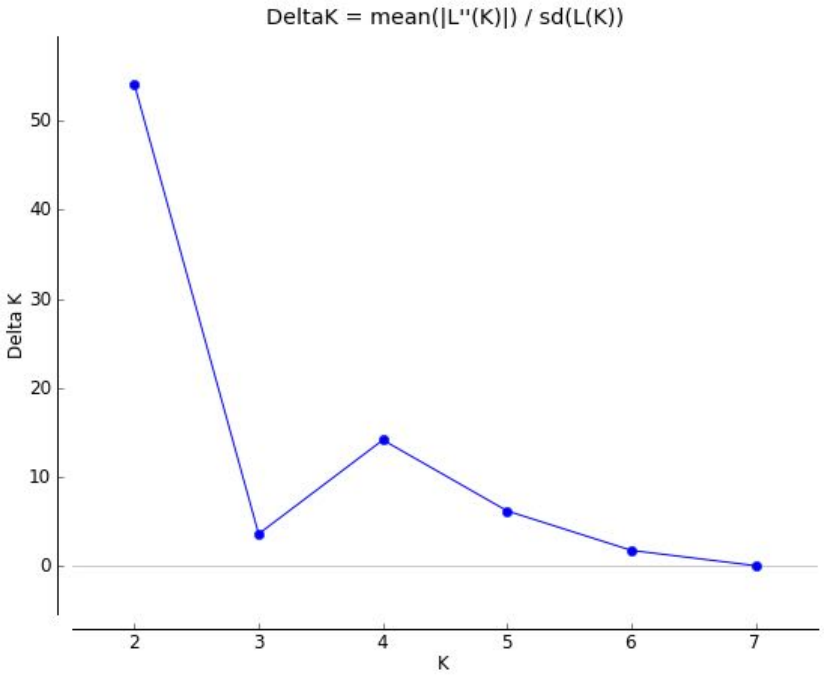


Figure G.3.2. Highest Δ*K* is for *K* = 2, with a secondary peak at *K* = 4

Chivilingo excluded (7 basins).

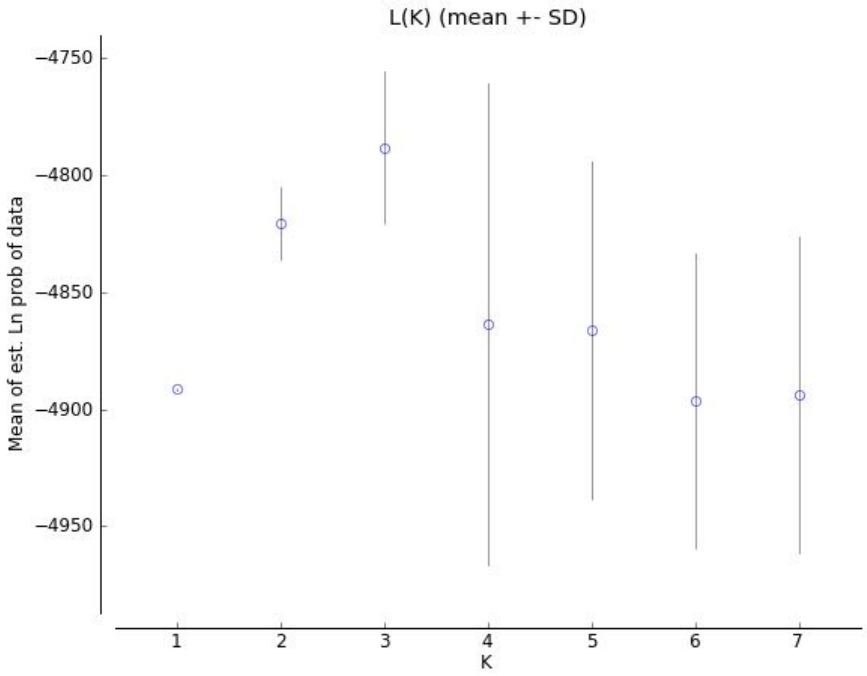


Figure G.3.3. Mean of estimated natural log probability of K [P(K)] plateaus at *K* = 3, suggesting 3 clusters.

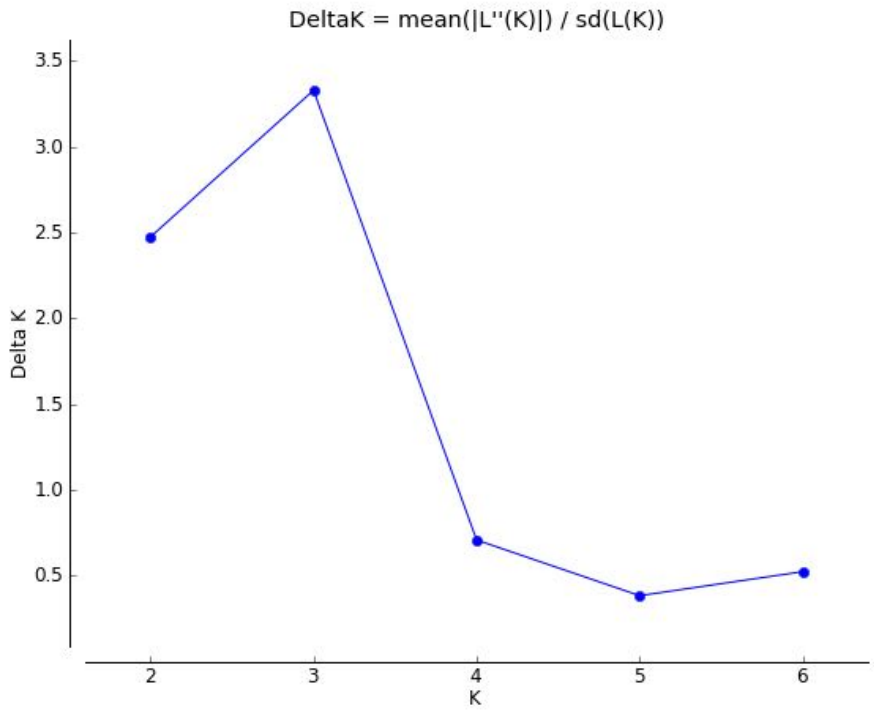


Figure G.3.4. Highest Δ*K* is for *K* = 3, supporting 3 clusters.

G. 4 Genetic diversity and *G*-statistics for 14 microsatellite loci

Genetic diversity and *G*-statistics (Nei 1987) for all loci used in the study. Number of alleles N_A, observed heterozygosity H_O, expected heterozygosity H_E, inbreeding coefficient G_{IS}, fixation index G_{ST}, Nei corrected fixation index G'_{ST}(Nei), Hedrick standardised fixation index G'_{ST}(Hed), corrected standardised fixation index G''_{ST}

Locus	N_A	H_O	H_E	G_{IS}	G_{ST}	G'_{ST}(Nei)	G'_{ST}(Hed)	G''_{ST}
Tbu03	7	0.746	0.760	-0.035	0.052	0.059*	0.204	0.209
Tbu06	6	0.527	0.660	-0.062	0.249	0.275*	0.529	0.545
Tbu19	31	0.893	0.955	0.059	0.005	0.006	0.120	0.121
Tbu20	14	0.737	0.742	0.007	-0.001	-0.001	-0.003	-0.003
Tbu23	7	0.683	0.734	-0.042	0.107	0.121*	0.340	0.350
Tbu24	7	0.583	0.642	0.055	0.039	0.044*	0.110	0.115
Tbu28	8	0.648	0.673	-0.008	0.045	0.051*	0.139	0.144
Tbu33	7	0.619	0.643	-0.010	0.047	0.053*	0.131	0.137
Tbu34	7	0.320	0.390	0.155	0.031	0.035*	0.052	0.056
Tbu35	8	0.700	0.749	0.006	0.060	0.068*	0.223	0.230
Tbu39	6	0.421	0.514	0.086	0.103	0.116*	0.203	0.215
Tbu42	3	0.433	0.516	-0.070	0.216	0.239*	0.383	0.402
Tbu44	16	0.689	0.825	0.083	0.089	0.100*	0.396	0.404
Tbu47	6	0.517	0.527	-0.047	0.065	0.073*	0.137	0.145
Overall	9.5	0.608	0.667	0.013	0.075	0.085*	0.213	0.221

G. 5 Pairwise Jost D_{st} distances between basins and groups

Pairwise Jost D_{st} below diagonal, *p* value above diagonal. Shading is used to highlight basins grouped together according to STRUCTURE results.

		CH	CA	CY	BU	HU	PR	CL	LL
A	CH	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001
B	CA	0.234*	-	0.019	0.002	0.001	0.001	0.001	0.001
	CY	0.273*	0.040	-	0.008	0.001	0.001	0.001	0.001
	BU	0.258*	0.069	0.051	-	0.098	0.001	0.001	0.001
C	HU	0.267*	0.111*	0.111*	0.024	-	0.508	0.118	0.001
	PR	0.310*	0.101*	0.120*	0.090*	-0.002	-	0.525	0.001
	CL	0.284*	0.100*	0.136*	0.073*	0.022	-0.003	-	0.001
D	LL	0.425*	0.201*	0.135*	0.151*	0.156*	0.191*	0.143*	-

* significant after Bonferroni correction, § significant at $\alpha = 0.05$ level

Pairwise Jost D_{st} between groups.

	A	B	C	D
A	-			
B	0.241*	-		
C	0.300*	0.083*	-	
D	0.425*	0.147*	0.176*	-

* significant after Bonferroni correction

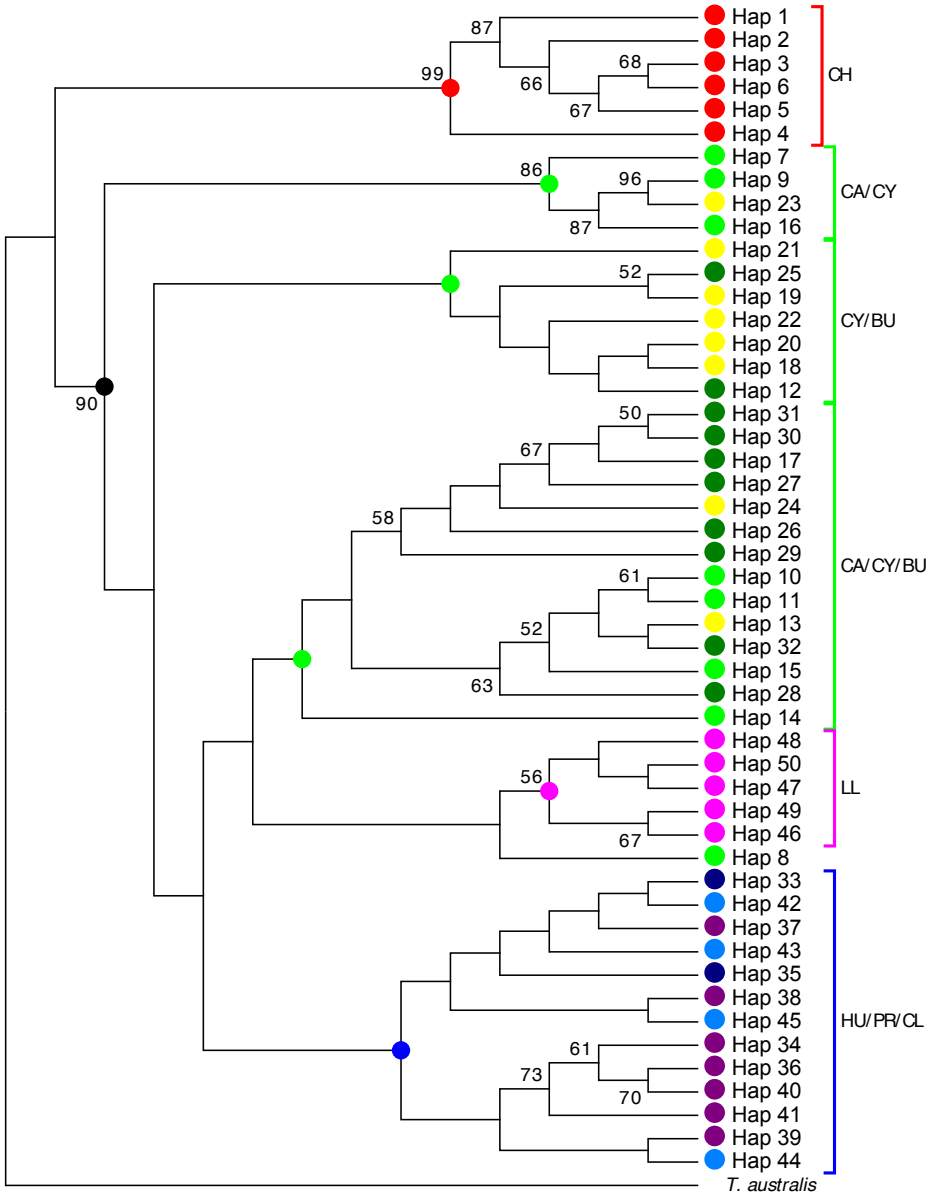
G. 6 Heterozygosity excess test results for the detection of genetic bottlenecks

P-values for the two-tailed Wilcoxon sign for heterozygosity excess (e) or deficiency (d) at the group level. Significant tests are in bold.

	SMM	TPM 95%	TPM 90%	IAM
A	0.95	0.76	0.54	0.09
B	**0.05 d**	0.09	0.54	**0.00 e**
C	**0.00 d**	**0.00 d**	**0.03 d**	0.39
D	0.84	0.54	0.37	**0.00 e**

G. 7 Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. The tree with the highest log likelihood (-3073.6827) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches when >50%. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 8.9314% sites). The analysis involved 51 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1251 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.



G. 8 Mismatch distribution: Test of spatial expansion

Observed mismatch distribution of the control region is shown (solid black line), along with expectations under the spatial expansion model (grey), and its confidence intervals (colour dashed) for the two main clades. Clade 1 corresponds to CH population (Figure G.8.1), and Clade 2 (Figure G.8.2) includes all other sampled populations (CA, CY, BU, HU, PR, CL, LL).

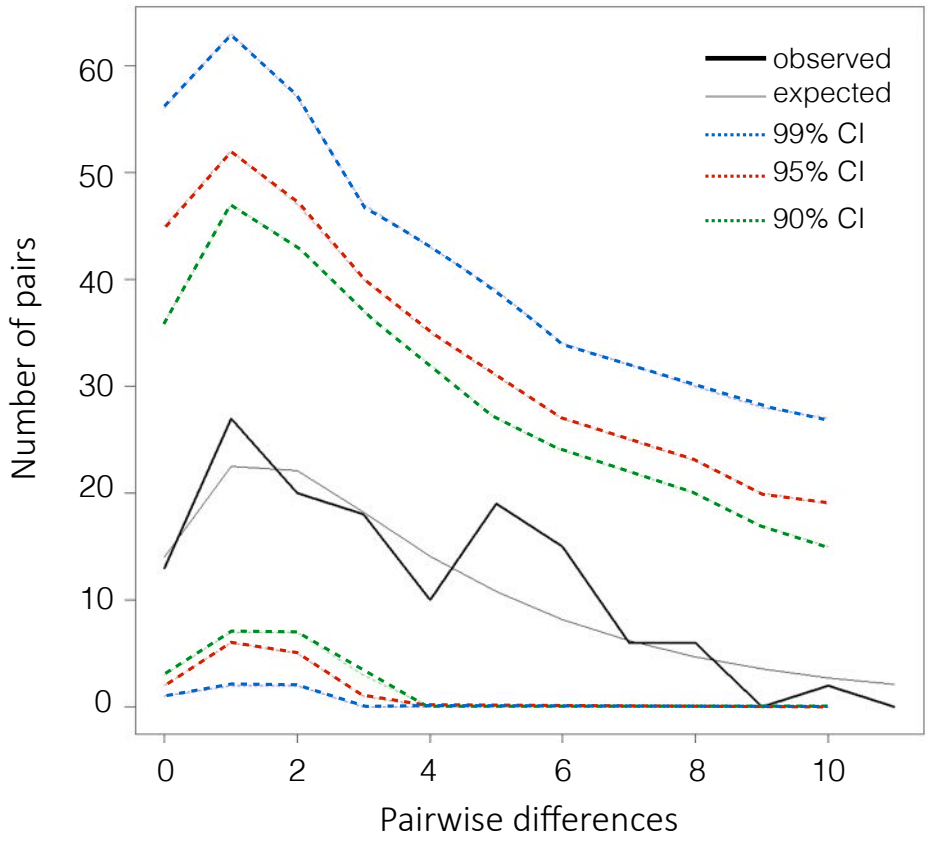


Figure G.8.1. Observed mismatch distribution of the control region for Clade 1 (Chivilingo).

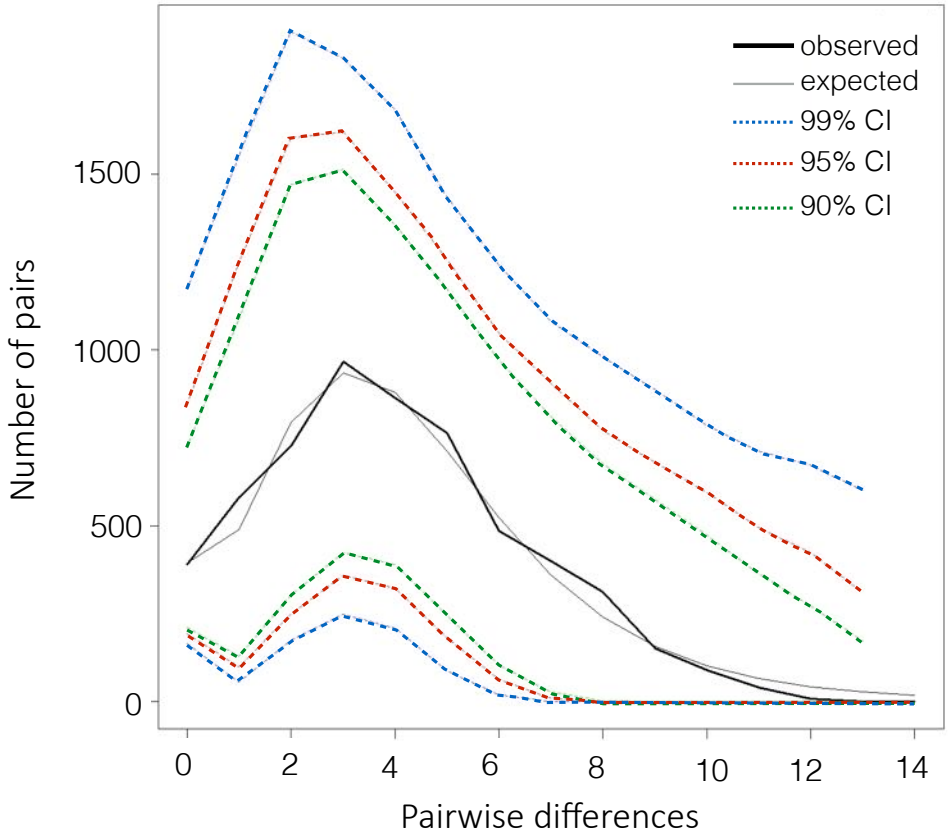


Figure G.8.2. Observed mismatch distribution of the control region for Clade 2 .

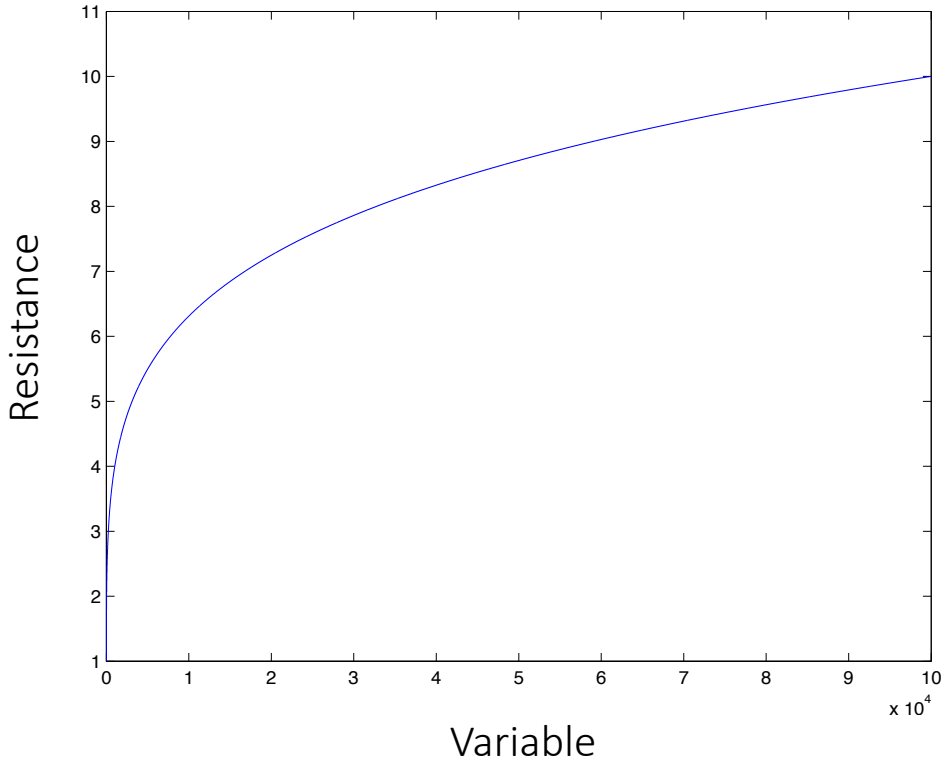
G. 9 Parameters of demographic expansion and neutrality tests calculated using the CR sequence

Parameters of demographic expansion and neutrality tests calculated using the CR sequence. ϑ_0 population size before the expansion, ϑ_1 after expansion (ϑ = 2Neμ), $\boldsymbol{\tau}$ generations since the expansion (τ = 2tμ). Harpending's raggedness index *r*

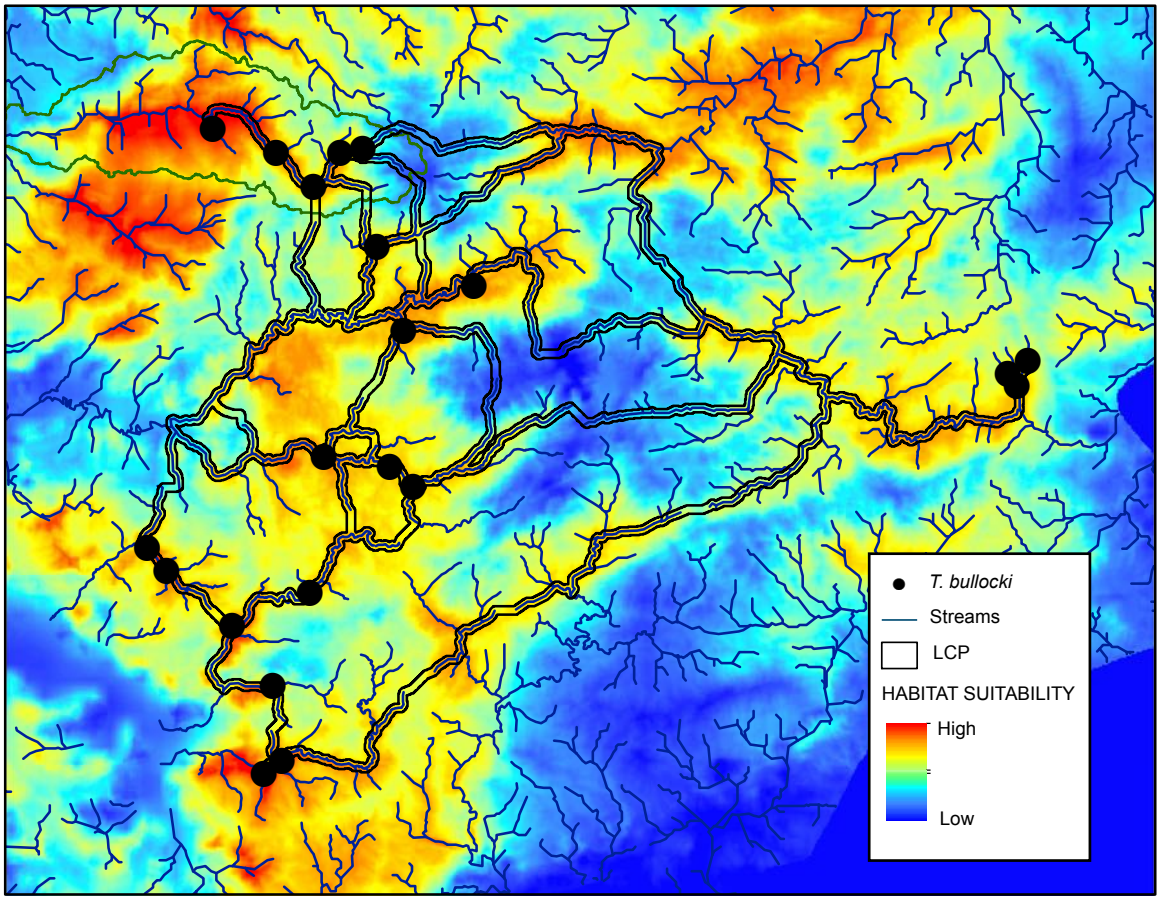
	N	τ (95% CI)	θ_0 (95% CI)	θ_1 (95% CI)	SSD	*r*	Tajima's *D*	Fu's F_S
Full	125	1.77 (0.15 - 13.04)	4.95 (0 - 13.1)	35.12 (10.22 - 99999)	0.007 ns	0.006 ns	-0.98 ns	-16.41 §
Clade 1	17	0.84 (0 - 7.58)	3.16 (0 - 8.29)	99999 (7.4 - 99999)	0.009 ns	0.029 ns	-1.61*	-3.00*
Clade 2	108	3.2 (1.28 - 8.99)	1.41 (0 - 3.50)	17.08 (7.83 - 99999)	0.001 ns	0.008 ns	-1.20 ns	-16.68 §

ns = non significant, * = $p < 0.05$, § = $p < 0.02$

Appendix H. Additional figures for Chapter 5.



Power function (exponent 0.2) used to transform proximity to stream and slope variables.



Map showing the least cost paths (LCP) connecting *T. bullocki* sampling locations and the habitat suitability map of Chapter 2. LCP shown represent potential historical dispersal routes under the best-supported model of landscape resistance. The paths follow stream and riparian habitat.

Appendix I. Management guidelines for *T. bullocki* populations.

T. bullocki populations can be grouped into five management units (MU) as shown in Figure I.1. This includes three disjunct populations (1. Chivilingo, 2. Ramadillas, and 3. Los Lleulles) and two larger metapopulations (4. Caramávida/Cayucupil/Butamalal, and 5. Huilquehue/Provoque/Calebu).

1. CHIVILINGO

This population is at the northern edge of the distribution and has significantly diverged genetically from all other populations; therefore it is considered a separate evolutionary significant unit. Some level of protection exists as some of the area has been declared a High Conservation Value Area (HCVA) under FSC Principles and Criteria due to the presence of endangered vegetation (Forestal Arauco). However, riparian protection is poor in some sections of the stream where only 10-30 m of vegetation was retained (Figure I.2). An effort should be made to increase native riparian areas and restore existing native forest through the strengthening and expansion of the HCVA. Benefits from increased riparian protection could also positively affect the quality and quantity of water in the catchment, which supplies nearby towns. This could become particularly important under climate change. This population is close to urban areas and is under high levels of disturbance. Neighbouring basins should be surveyed to assess the presence of other nearby *T. bullocki* populations.

2. RAMADILLAS

This location has the lowest amount of remaining native habitat, and is potentially at highest risk. Plantations have been established with minimal riparian protection (≈10 m, Figure I.3). In addition, most of the area was harvested recently and planted with Eucalyptus, which has yet unknown impact on *T. bullocki*. Unfortunately this population could not be sampled; therefore there is little information about current population status other than *T. bullocki* presence (e.g. the levels of genetic diversity or effective population size were not assessed). This population is within Forestal Arauco land ownership, and a small protected HCVA was established in 2011 for the protection of the endangered plant *Gaultheria renjifoana* (Chaura de Laraquete). Adding *T. bullocki* as a high conservation value should reinforce this existing conservation effort. Moreover, the also critically endangered amphibian *Alsodes valzolinii* is present only 2 km from this point, highlighting the importance of the area as a hot-spot for conservation. A bigger HCVA should be established protecting all remaining native forest and including the three endangered species. Restoration of native habitat should be the long-term goal. This population should be surveyed and long-term abundance monitoring established as soon as possible.

3. LOS LLEULLES

This is one of *T. bullocki* historical locations, and the only known population from eastern slopes of Nahuelbuta. It is surrounded by pine plantation owned by Forestal Arauco and CMPC. Some native forest remains in a fragment adjacent to Los Lleulles stream (owned by CMPC) where protection should be strengthened (Figure I.4). Restoration of riparian areas with native vegetation should be prioritised. Surveys in nearby streams and Picoiquén River should be conducted to identify potential neighbouring populations. The run-of-the-river project Rio Picoiquén (approved) will be established soon, therefore baseline data

should be urgently collected for the area of intervention. Connectivity between stream habitat and remaining native forest (some privately owned) should be improved.

4) Caramávida/Cayucupil/ Butamalal

This cluster of populations represents a stronghold for *T. bullocki*. It contains high levels of genetic diversity, presumably large population sizes, and high amount of continuous remaining native forest protected under the HCVA Quebrada de Caramávida (over 20,000 ha). Management of these populations should focus in maintaining and enhancing inter-basin dispersal through the establishment of a connectivity network connecting large fragments of native forest (Figure I.5). A large clear-cut area has potentially affected Cayucupil population, and the impact should be assessed. Furthermore, a run-of-the-river power plant has already been approved in Cayucupil (Hidroelectrica Cayucupil). The area of intervention should be surveyed to obtain baseline data. Another run-of-the river plant has been proposed in Butamalal River, which could potentially affect high-quality *T. bullocki* habitat. Protection and mitigation measures should be evaluated and implemented.

5) Huilquehue/Provoque/Calebu

This set of populations is at the edge of the distribution as it is bounded by Lanalhue Lake in the South. As with the previous MU, management should focus at maintaining high levels of inter-basin connectivity. Some protection is given to *T. bullocki* by two HCVA, one from Forestal Arauco (El Natri, proposed) and one from CMPC (Calebu, established), which contain large continuous areas of mature native forest. Management should aim to keep these areas connected through the establishment of native forest corridors

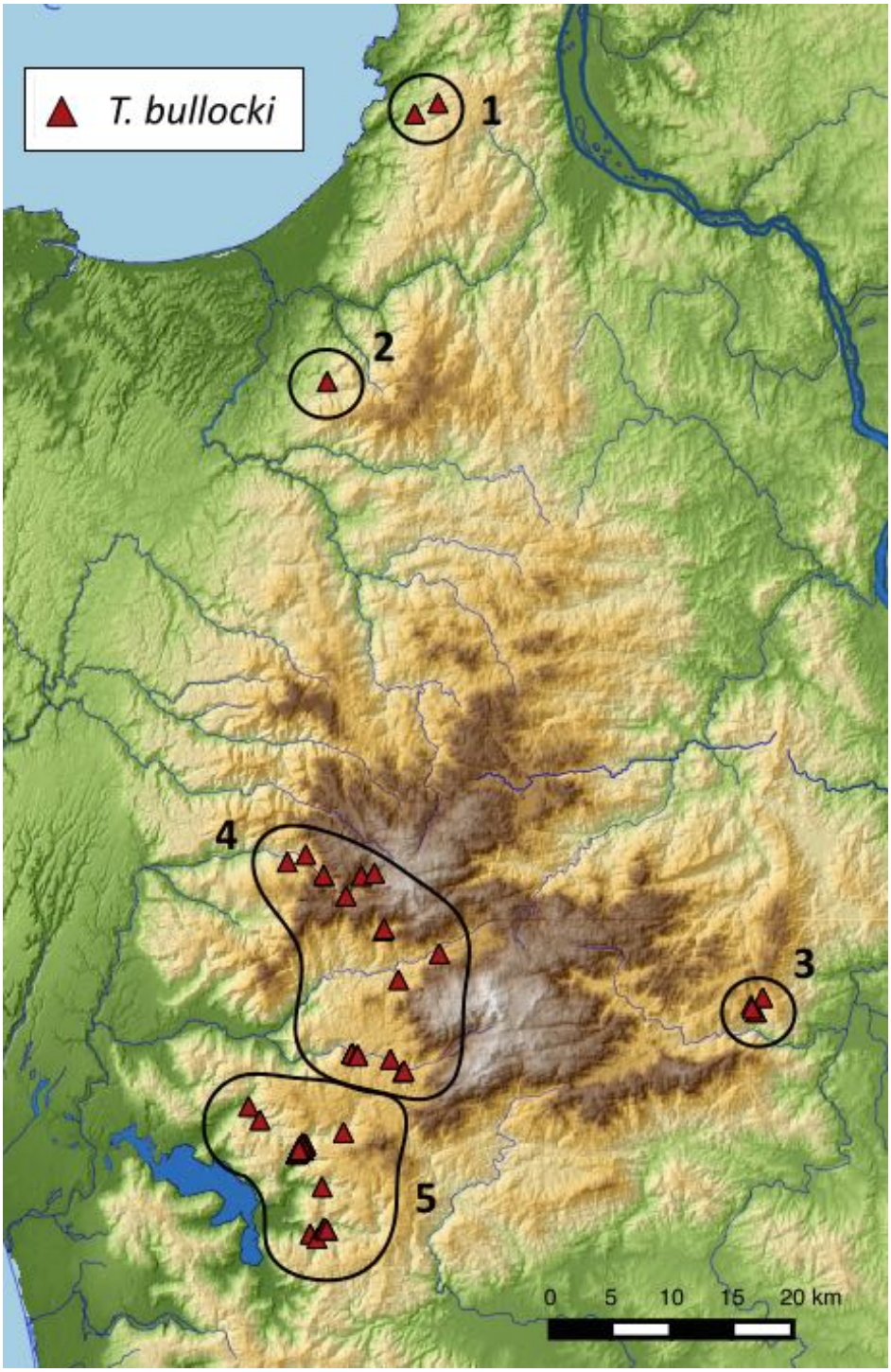


Figure I.1. Map with the proposed management units 1) Chivilingo, 2) Ramadillas, 3) Los Lleulles, 4) Caramávida/Cayucupil/Butamalal, and 5) Huilquehue/Provoque/Calebu.

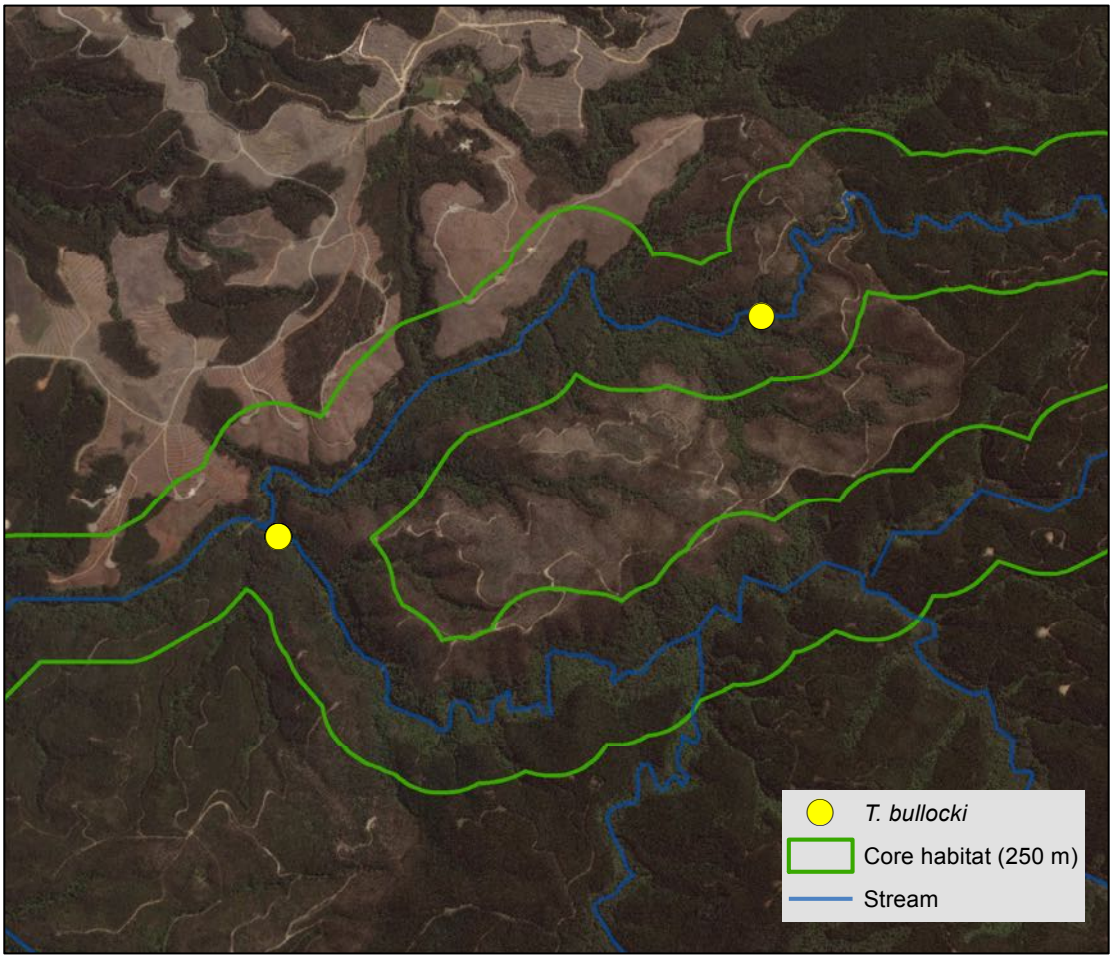


Figure I.2. *T. bullocki* presence points in Chivilingo catchment, figure shows a proposed protected are of 250 m (each side of the breeding stream).

Figure I.3. The single collection point in Ramadillas (yellow dot) is embedded in plantation matrix. The image shows the extent of clear-cutting and the proposed protection area of 250 m (image date 2002, Google Earth Digital Globe, 2015).

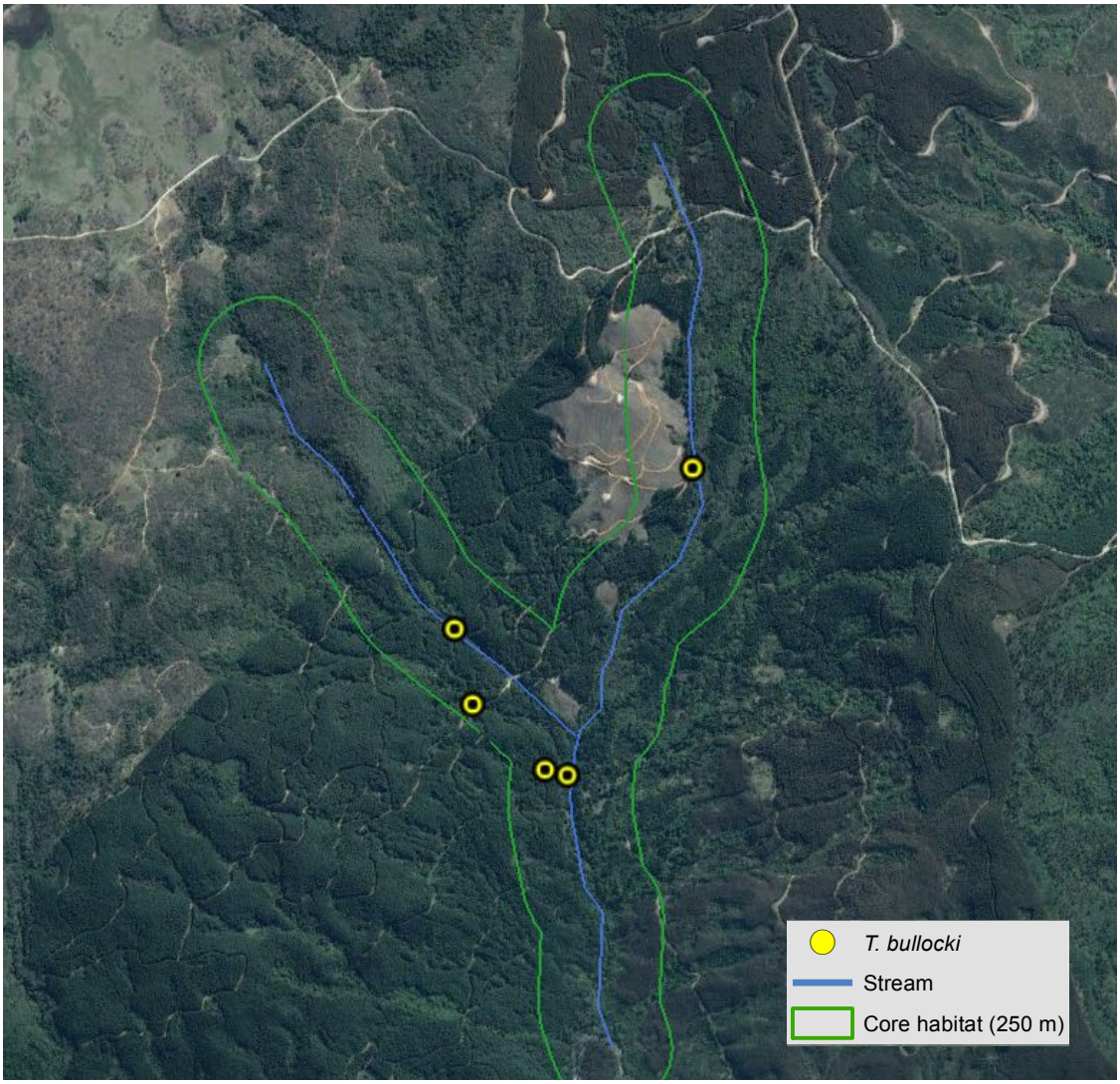


Figure I.4. *T. bullocki* sightings in Los Lleulles, an area covered mainly by pine plantation (dark green).

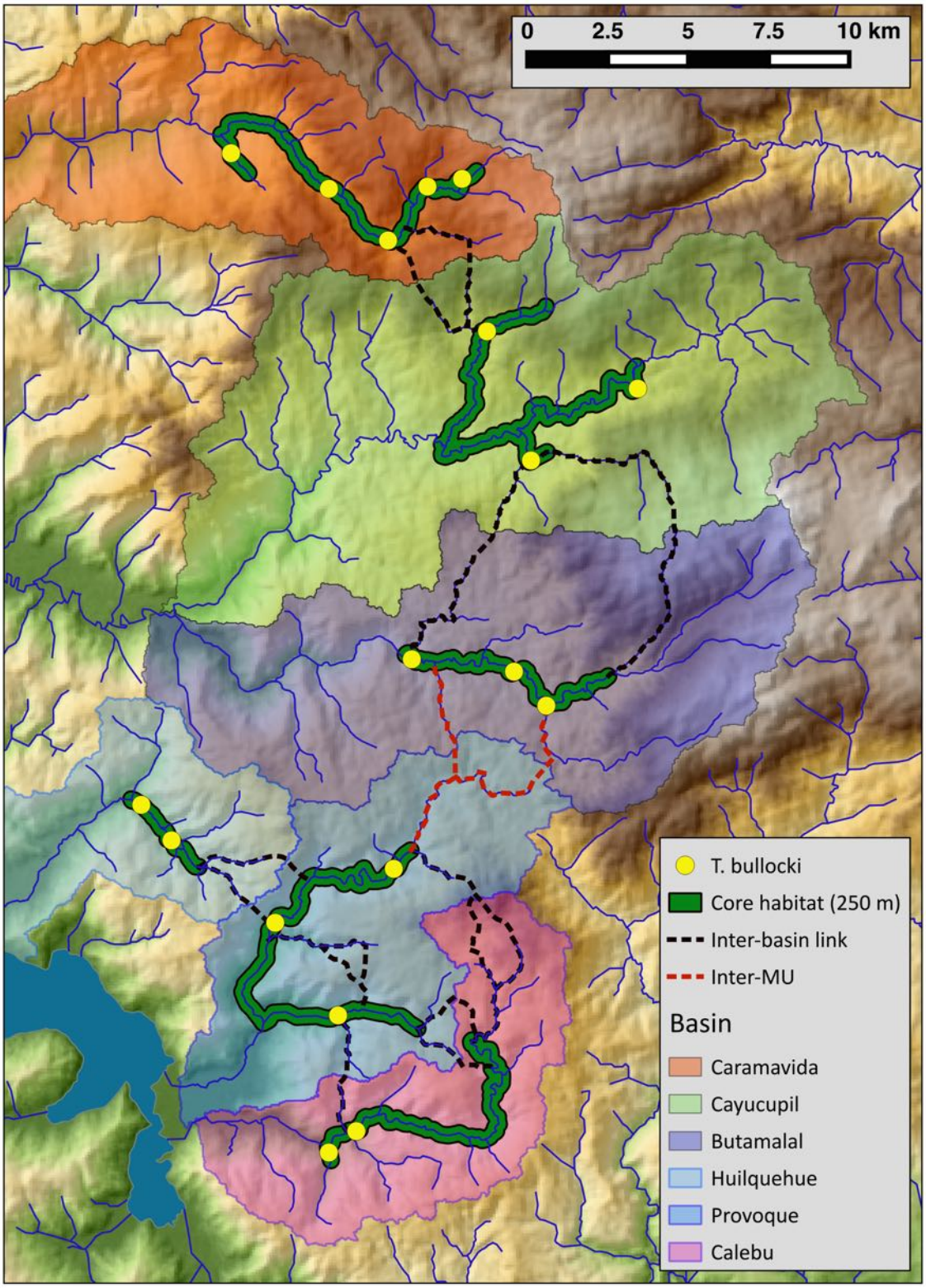


Figure I.5. Proposed core habitat protection and metapopulation connectivity network for *T. bullocki* management. Two of the identified management units (MU) are shown: Caramávida/Cayucupil/Butamalal and Huilquehue/Provoque/Calebu

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Virginia Moreno-Puig

Name/Title of Principal Supervisor: Prof. Dianne H. Brunton

Name of Published Research Output and full reference:

Moreno-Puig,V., Y.Yildirim,and D. Brunton. 2014. Development of microsatellite markers for the critically endangered frog Telmatobufo bullocki and cross species amplification in two related species. Conservation Genetics Resources 6:883–884.

In which Chapter is the Published Work: Appendix F

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%

  and / or
- Describe the contribution that the candidate has made to the Published Work:

  Virginia, designed implemented and wrote this publication.

Virginia Moreno — 30/03/2015

Candidate's Signature — Date

Dianne H Brunton — 30/3/2015

Principal Supervisor's signature — Date