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Faculdade de Ciências

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Population Analysis of *Hyla arborea* and *Hyla meridionalis* (Amphibia, Anura) in Portugal: A Molecular Genetic and Bioacoustic Approach

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À memória do meu tio João, com saudade.
A tese já chegou demasiado tarde
e ele partiu demasiado cedo.

À Barracuda, com olhos postos no futuro.

Nota Prévia

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Catarina Nascimento Moreira

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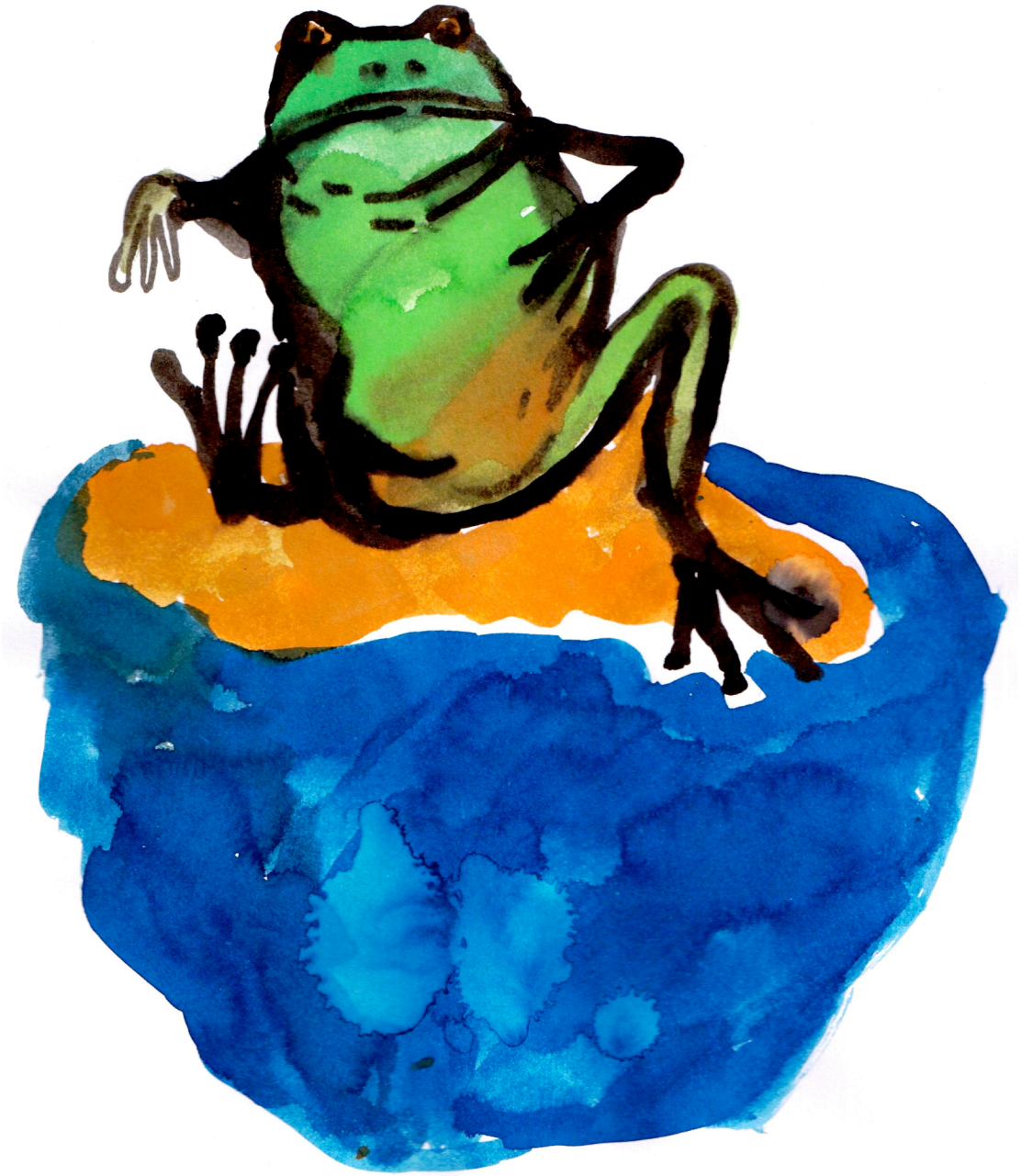

If you refuse to let me go, I will plague the whole country with frogs.

Exodus 8:1

Table of Contents

List of Figures ..... **iii**
List of Tables ..... **iv**
List of Appendices ..... **v**
Acknowledgements ..... **vii**
Sumário ..... **xi**
Summary ..... **xvii**
Chapter I. Introduction **1**
1. Biogeographical History of the Western Mediterranean and Iberian Peninsula and Amphibian Distribution (Origins and Colonisation) 4
1.1. The Western Mediterranean Region in the Context of Global Tectonic Movements 4
1.2. Temporary Land Corridors between Iberia and Africa 5
1.3. The Uplift of the Pyrenees 7
1.4. Rivers as Zoogeographical Barriers 8
1.5. Effects of Climate Change Cycles 9
2. Phylogeography and Evolutionary Genetics Advances History 10
2.1. Patterns of European Phylogeography: Mediterranean Refugia 10
2.2. Iberian Refugia: 'Refugia within Refugia' 11
2.3. Molecular Techniques and the Use of Different Molecular Markers 12
3. Bioacoustics of Anurans 16
3.1. Chorus and Mating Strategies 18
3.2. Sexual Selection and Variation of the Advertisement Calls 20
3.3. Mating Calls Characteristics 20
3.4. Ecological Factors 21
3.5. Male Size Effect on Calls: Vocal Apparatus Characteristics 22
3.6. Temperature Effect on Calls: Environmental and Male Body Temperatures 22
4. Species Interaction 23
4.1. Character Displacement and Speciation 23
4.2. Species Hybridisation: The Ambivalent Topic of Species Contact 24
5. Combining Approaches: Geographic Variation in Genetic Structure and Advertisement Calls 26
6. Natural and Evolutionary History of Hylidae Rafinesque, 1815 29
6.1. Taxonomy 29
6.2. Distribution 32
6.3. Colonisations 33
6.4. The Holarctic Hylids 35
6.5. The Iberian Hylids: *Hyla meridionalis* and *Hyla arborea* 38
7. Objectives of the Thesis 55
Chapter II. Materials and Methods **59**
1. Fieldwork Procedures: Specimen Collection and Sampling Strategy 61
1.1. Sampling Sites 61
1.2. Male Advertisement Call Recordings 73
1.3. Tissue Sample Collection 75
1.4. Ethical Note 76
2. Laboratory Procedures 76
2.1. Molecular Techniques 76
2.2. Species Hybridisation in the Laboratory: Interspecific Crosses 82
3. Data Analysis 84
3.1. Molecular Analysis of Mitochondrial DNA sequences 84
3.2. Sound Analyses of Advertisement Calls 87
Chapter III: Results **93**
1. Molecular Genetic Analyses 95
1.1. *Hyla arborea* 95

1.2. *Hyla meridionalis*.....102
2. Sound Analyses of Advertisement Calls.....112
2.1. *Hyla arborea*.....113
2.2. *Hyla meridionalis*.....130
3. Species Interaction: Comparison of populations in Allopatry versus Sympatry of *Hyla meridionalis* and *Hyla arborea*.....145
3.1. Hybrid Fingerprinting using Restriction Fragment Length Polymorphisms (RFLPs).....145
3.2. Advertisement Calls: Allopatry versus Sympatry.....147
Chapter IV: Discussion.....151
1. The COI Mitochondrial Gene as a Molecular Marker.....154
1.1. *Hyla arborea*.....155
1.2. *Hyla meridionalis*.....158
2. Microsatellites.....162
3. The Advertisement Calls as a Bioacoustic Marker.....164
3.1. *Hyla arborea*.....164
3.2. *Hyla meridionalis*.....167
4. Species Interaction: Hybrids and Reproductive Character Displacement in Calls.....168
Chapter V: Conclusions and Future Prospects.....173
Chapter VI. References.....179
Appendix.....227

List of Figures

Figure 1.1. Reconstruction of the Western Mediterranean, from the early Cretaceous (~145.5 Mya) to the present....6
Figure 1.2. Mechanism of sound production in Anurans. 17
Figure 1.3. Phylogenetic tree of anurans. 31
Figure 1.4. World distribution of Hylids. 33
Figure 1.5. *Hyla meridionalis* geographic distribution. 39
Figure 1.6. *H. arborea* geographic distribution. 40
Figure 1.7. Iberian distribution of *Hyla meridionalis* and *H. arborea*. 41
Figure 1.8. Distribution of *Hyla meridionalis* and *Hyla arborea* in Portugal. 42
Figure 1.9. Males from *Hyla meridionalis* and *H. arborea*. 43
Figure 1.10. *Hyla meridionalis* and *H. arborea* hybrid male. 44
Figure 1.11. Advertisement calls of *Hyla arborea*, *H. meridionalis* and their F1 hybrid. 45
Figure 1.12. *Hyla arborea* species group phylogeny. 47
Figure 1.13. *Hyla meridionalis* phylogenetic tree. 49
Figure 1.14. *Hyla meridionalis* advertisement call. 51
Figure 1.15. *Hyla arborea* advertisement call. 53
Figure 2.1. Sampled sites of *Hyla meridionalis* for the molecular genetic analysis. 63
Figure 2.2. Sampled sites of *Hyla arborea* for the molecular genetic analysis. 64
Figure 2.3. Sampled sites of *Hyla arborea* and *H. meridionalis* for the bioacoustic analysis. 65
Figure 2.4. Measuring treefrogs' body size. 75
Figure 2.5. *Hyla arborea* gravid female. 83
Figure 2.6. Parameters measured in the advertisement calls of *Hyla arborea*. 89
Figure 2.7. Parameters measured in the advertisement calls of *Hyla meridionalis*. 89
Figure 3.1. Sample sites used in COI analysis within Iberian Peninsula. 96
Figure 3.2. ML phylogenetic tree for COI for *Hyla arborea*. 97
Figure 3.3. Mitochondrial COI genealogy of Iberian *H. arborea*. 98
Figure 3.4. COI haplotype distribution map of *Hyla arborea* in the Iberian Peninsula. 99
Figure 3.5. Mismatch distributions of *Hyla arborea* COI samples. 101
Figure 3.6. Genetic distance vs. Geographic distance in *Hyla arborea*. 102
Figure 3.7. Bayesian phylogenetic tree for COI for *Hyla meridionalis*. 104
Figure 3.8. Mitochondrial COI genealogy of *Hyla meridionalis*. 105
Figure 3.9. COI haplotype distribution map of *Hyla meridionalis* in the Iberian Peninsula and Northern Morocco. 106
Figure 3.10. Mismatch distributions of *Hyla meridionalis* COI samples. 110
Figure 3.11. Genetic distance vs. Geographic distance in *Hyla meridionalis*. 111
Figure 3.12. Distribution of acoustic sample sites of *Hyla meridionalis* and *H. arborea* in Portugal. 112
Figure 3.13. Body size of *Hyla arborea* calling males in sampled populations in Portugal. 117
Figure 3.14. Temperature distribution in *Hyla arborea* sampled populations in Portugal. 119
Figure 3.15. Relationship between call parameters and male temperature or male mass in *Hyla arborea* sampled populations. 122
Figure 3.16. Call parameters distribution in *Hyla arborea* sampled populations in Portugal. 126
Figure 3.17. Canonical coefficients of the first two canonical functions extracted in *Hyla arborea*. .. 128
Figure 3.18. PCA scatterplot of the first two principal components. 130
Figure 3.19 Body size distribution of calling males of *Hyla meridionalis* sampled populations in Portugal. 133
Figure 3.20. Temperature distribution in *Hyla meridionalis* sampled populations in Portugal. 135
Figure 3.21. Relationships between call parameters and male temperature or male mass in *Hyla meridionalis*. 138
Figure 3.22. Call parameters distribution in *Hyla meridionalis* sampled populations in Portugal. 141
Figure 3.23. Canonical coefficients of the first two canonical functions extracted for *Hyla meridionalis* advertisement call parameters. 143
Figures 3.24. PCA scatterplot of the first two principal components with Varimax rotation for *Hyla meridionalis*. 144
Figure 3.25. Restriction fragments obtained for RAG1 and Tyr1. 146

List of Tables

Table 1.1. Organismal classification of Holarctic Hyline treefrogs. 35
Table 1.2. Classification of Hyline treefrogs based on albumin data. 36
Table 2.1. *Hyla meridionalis* COI samples information. 66
Table 2.2. *Hyla arborea* COI samples information. 70
Table 2.3. Bioacoustic Sampled Locations in Portugal. 72
Table 2.4. Primers used in this study. 79
Table 2.5. Characterization of 36 microsatellite loci for the European tree frog *Hyla arborea*. 80
Table 2.6. Restriction enzyme reactions. 81
Table 3.1. Genetic diversity estimates and neutrality tests for *Hyla arborea*. 99
Table 3.2. Genetic and geographic distances among *Hyla arborea* populations. 101
Table 3.3. Genetic diversity estimates and neutrality tests for *Hyla meridionalis*. 107
Table 3.4. Pairwise *P*-uncorrected distances for COI in *Hyla meridionalis*. 107
Table 3.5. Genetic and geographic distances among *H. meridionalis* populations. 110
Table 3.6. Within-call group coefficient of variation of *Hyla arborea*. 113
Table 3.7. Population within-call group coefficient of variation of *Hyla arborea*. 114
Table 3.8. Within-individual coefficient of variation of the *Hyla arborea*. 115
Table 3.9. Population within-individual coefficient of variation of the *Hyla arborea*. 116
Table 3.10. Temperature correlation matrix for *Hyla arborea* sampled populations. 118
Table 3.11. DFA on call parameters among populations of the *Hyla arborea*. 127
Table 3.12. Principal Component Analysis of the advertisement call properties of *Hyla arborea*. 129
Table 3.13. Within-individual coefficient of variation of the *Hyla meridionalis* call acoustic properties. 131
Table 3.14. Population within-individual coefficient of variation of the *H. meridionalis*. 132
Table 3.15. Temperature correlation matrix for *Hyla arborea* sampled populations. 135
Table 3.16. Correlation matrix of call properties and male temperature and mass. 136
Table 3.17. DFA on call parameters among sampled populations of *Hyla meridionalis*. 142
Table 3.18. Principal Component Analysis of the advertisement call parameters of *Hyla meridionalis*. 144
Table 3.19. Samples typed with RFLP of the two species of *Hyla*. 147
Table 3.20. Influence of Allopatry vs Sympatry in Advertisement Call Parameters. 148

List of Appendices

Appendix 1. Variability in the lateral bands in *H. arborea* and *H. meridionalis* in sampled populations of Portugal. 229
Appendix 2. Temperatures and Body Measurements of Audio-Recorded Populations of *Hyla arborea*. 232
Appendix 3. Advertisement Call Parameters of *Hyla arborea* males recorded in Portugal. 233
Appendix 3 (cont.). Advertisement Call Parameters of *Hyla arborea* males recorded in Portugal. 236
Appendix 4 .Temperatures and Body Measurements of Audio-Recorded Populations of *Hyla meridionalis*..... 239
Appendix 5. Advertisement Call Parameters of *Hyla meridionalis* males recorded in Portugal..... 240
Appendix 6. Allopatry versus Sympatry Call Parameters distribution in *Hyla arborea* sampled populations in Portugal..... 246
Appendix 7. Allopatry versus Sympatry call parameters distribution in *Hyla meridionalis* sampled populations in Portugal..... 250

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Sumário

As relas do género *Hyla* da Europa e do Norte de África pentencentes à família Hylidae, da ordem dos Anuros, repartem-se por duas espécies na Península Ibérica, a rela-europeia, *Hyla arborea* e a rela-meridional, *H. meridionalis*. Durante muito tempo estas duas espécies, bem como outras do mesmo género que habitam o continente europeu, eram consideradas meras subspécies de *H. arborea*. Estudos morfológicos, genéticos e bioacústicos foram contudo determinando a atribuição do seu carácter de espécie a muitas destas formas. Actualmente são 16 reconhecidas espécies repartidas por dois grandes grupos, *H. arborea* e *H. japonica*, correspondentes aos extremos da sua área de distribuição global, uma a ocidente da Eurásia e outro no seu extremo Oriental.

Considerada uma espécie distinta da sua congénere por critérios morfológicos, imunológicos e bioacústicos (estes últimos salienta-se serviriam de base ao seu reconhecimento original. A *H. meridionalis*, é peculiarmente o único Hilídeo que ocorre em África. As chamadas de acasalamento dos machos, comparadas com as da *H. arborea*, são mais longas (aproximadamente cinco vezes para a mesma temperatura ambiental) e mais graves (as suas frequências são relativamente mais baixas). Estudos genéticos recentes baseados na análise de ADN mitocondrial e nuclear, sugerirem que a *H. meridionalis* se caracteriza por uma baixa diversidade genética patente nas amostras provenientes dos dois clados bem diferenciados: 1) um ocidental que inlcui Marrocos, o Sudoeste da Europa (incluindo a Península Ibérica) e as ilhas Canárias e 2) um oriental que inclui a Tunísia. As amostras pertencentes à Tunísia formariam um clado altamente divergente das restantes, o que sugere ter havido uma separação bastante antiga destas duas linhagens. A presente distribuição da espécie teria resultado de dois grandes eventos de dispersão a partir do Norte de África provavelmente recentes (e a introdução, mediada por humanos ou em 'jangadas' naturais arrastadas por correntes, nas Ilhas Canárias e na Madeira). Um primeiro evento de dispersão, mais antigo, teria ocorrido com a colonização do Sul da Península Ibérica por indíviduos vindos originalmente do Sul de Marrocos, que teriam migrado primeiro para o Norte de Marrocos e daí para a Península Ibérica. Num segundo evento, mais recente, como atrás referimos, alguns elementos provenientes do Norte de Marrocos teriam sido introduzidos na costa mediterrânica de França e daí migrado para Norte, Este e Sul, ocupando o Sul de França e o Nordeste de Espanha. As diferenças encontradas entre estes indivíduos e os do primeiro evento de expansão (pertencem a dois grupos distintos), podem ser resultado de uma recolonização do Norte de Marrocos, após a primeira dispersão para o continente europeu, por uma população com algum grau de diferenciação genética em relação aos primeiros.

Quanto à *H. arborea*, os seus estudos são geralmente mais abrangentes do ponto de vista geográfico, uma vez que a distribuição da espécie se estende desde a Europa Central até à Pensínsula Ibérica. Recentemente, a descrição da variabilidade genética ao nível dos ADN mitocondrial e nuclear, de muitas das suas populações europeias, demonstrou a existência de grupos altamente diferenciados e com uma forte associação com a sua distribuição geográfica. Alguns desses grupos correspondem a espécies actualmente aceites como espécies distintas de que falámos, como são os casos da *H. sarda* (endémica da Sardenha e da Córsega), *H. orientalis* (presente na Europa do Leste, nomeadamente, Ucrânia, Roménia e Turquia), *H. savignyi* (Médio Oriente, incluindo Chipre, Turquia, Síria, Irão e Iraque), e da *H. intermedia* (sul de Itália, na Península Apenina e na Sicília). Um dos resultados concordantes nos vários estudos realizados foi a acentuada diferenciação genética entre *H. arborea* da Europa Central e a da Península Ibérica, tendo sido, por isso, proposta a ressureição, da antiga sugestão, do carácter de espécie distinta para a forma da Península Ibérica, *Hyla molleri* Bedriaga 1890.

Na Península Ibérica estas duas espécies ocorrem em simpatria numa vasta área das suas distribuições geográficas, e são conhecidas várias situações de sintopia em que indíviduos das duas espécies partilham o mesmo charco (ou outra zona húmida). Através do canto de acasalamento, foram detectados híbridos (machos, dados serem estes os únicos a emitir chamadas) em Portugal e Espanha. Todos os híbridos encontrados apresentavam características intermédias às das espécies parentais relativamente às chamadas de acasalamento, aos padrões de variação de aloenzimas (híbrido encontrado em Portugal). A nível morfológico, a banda lateral em alguns elementos estendia-se até metade dos flancos ou apresentava um 'escudo' rudimentar na região inguinal (típico da *H. arborea*). Todos os indivíduos híbridos capturados, revelaram ser estéreis, com testículos sem células espermatogénicas.

Com base nestes conhecimentos prévios, estabelecemos como objectivo central desta tese o aprofundar do estudo sobre a história evolutiva de *H. arborea e H. meridionalis*, na tentativa de avaliar, confirmando ou infirmando as hipóteses anteriormente formuladas com recurso a uma dupla abordagem genética e bioacústica, acerca dos vários aspectos que poderiam informar o melhor conhecimento do que podemos considerar as suas filogeografias e interrelação no nosso território. Assim, mais especificamente propusemo-nos 1) determinar os padrões biogeográficos da diversidade genética das populações das duas espécies na Pensínsula Ibérica (sobretudo em Portugal), utilizando diferentes marcadores moleculares, fazendo uma re-análise dos padrões filogeográficos, anteriormente propostos, ao nível da Europa e do Norte de África e uma análise a uma escala mais fina dos padrões ibéricos e mais em particular dos de Portugal; 2) paralelamente procurámos investigar quais os padrões geográficos da diversidade bioacústica em Portugal ao nível dos cantos de acasalamento dos

machos de ambas as espécies, pelas potencialidades que este tipo de abordagem oferece em termos de contribuições de índole ecológica e evolutiva; e 3) tentar obter informação acerca das relações interespecíficas através da análise integrada de dados genéticos e bioacústicos, explorando aspectos ligados a eventuais processos de hibridação/introgressão das duas espécies e correlativamente da ocorrência de fenómenos de 'deslocação de caracteres'.

Nestas espécies para conseguirmos concretizar os objectivos propostos apostámos numa abordagem dupla e complementar, baseada na análise de dados genético-moleculares e bioacústicos. Para fazer face à parca amostragem, em estudos destas espécies, efectuados em Portugal, o esforço de amostragem (para recolha de tecido e para gravações áudio dos cantos de acasalamento) foi significativamente aumentado, tentando-se cobrir uma área bastante mais abrangente da área de distribuição das espécies. A amostragem de campo decorreu entre 2005-2010. Recolheram-se amostras de tecido de indivíduos adultos e de girinos e foram gravados os cantos de acasalamento dos machos de ambas as espécies. Para completar a cobertura geográfica no que refere a recolha de amostras para ulterior estudos genéticos, foram aproveitadas amostras cedidas por outros investigadores, bem como se usaram as sequências já publicadas em estudos anteriores. O gene escolhido do ADN mitocondrial foi o Citocromo Oxidase I (COI) dado ter já sido utilizado noutros estudos para estas mesmas espécies. No que concerne ao estudo das interacções das espécies, através da técnica de RFLP (do inglês, 'Restriction Fragment Length Polymorphism') testaram-se os indivíduos provenientes de locais sintópicos na busca de possíveis híbridos F1 e, paralelamente, compararam-se os pârametros dos cantos de acasalamento de machos de locais sintópicos com os de locais alopátricos (que serviram de referência).

No que diz respeito à *H. arborea*, da análise das sequências de COI obtidas, confirmou-se a grande divergência entre o grupo europeu e ibérico, com um distância genética (*p-uncorrected*) de 0.125. Da análise destes dados resultou a obetnção de uma árvore filogenética robusta, em que estes dois clados são suportados por elevados valores de *bootstrap*. Corrobora-se assim a proposta expressa em estudos anteriores de revalidar a espécie Ibérica, *H. molleri*. Analisando separadamente as sequências obtidas de elementos da Península Ibérica obtiveram-se dois 'grupos', aqui denominados por 'Norte' e 'Sul', mas sem grande suporte ao nível da árvore filogenética. Os haplótipos pertenecentes ao grupo do 'Norte' distribuem-se na sua maioria a Norte do rio Mondego e no Noroeste de Espanha; os haplótipos do grupo 'Sul' ocupam as áreas a sul do rio Mondego e a costa Atlântica Nordeste da Ibéria. O último grupo apresenta uma diversidade haplotípica mais elevada que o grupo 'Norte', contrariando resultados de estudos anteriores efectuados para esta espécie, mas que estão de acordo com estudos efectuados sobre outros anfíbios ibéricos/portugueses. Ao nível da acústica não se observou este padrão com uma divisão Norte-Sul. Pelo contrário os parâmetros dos cantos de acasalamento revelaram-se homogéneos entre os vários locais

amostrados, excepção feita ao local amostrado na Serra da Estrela (CFO) cujos valores da duração dos 'grupos de chamadas' e o número de chamadas por 'grupo de chamadas' são significativamente maiores que os dos demais locais amostrados. Esta diferença, não reflectida ao nível do ADN mitocondrial, poderia ser explicada pela altitude, tratando-se de um local acima dos 1500 m. Seria importante, no entanto, amostrar outros locais a altitudes semelhantes para comparar os cantos.

Os padrões de diversidade genética de *H. meridionalis* estudados através da sequenciação do gene COI, revelaram a existência de dois clados robustos com elevados valores de *bootstrap*, um incluindo haplótipos da região da Tunísia e Argélia (TA) e outro que se subdivide em três subclados distintos, um de Marrocos Central (CM), e os outros dois subclados que partilham haplótipos com o Norte de África e a Europa. Um deles, denominando por 'Norte de Marrocos' (NM) inclui haplótipos de amostras provenientes do Norte de Marrocos e, na Europa, do Nordeste Ibérico, Sul de França e Itália, e também das ilhas Canárias. O outro subclado denominado 'Sudoeste' inclui haplótipos do sudoeste de Marrocos e do sudoeste da Península Ibérica. Estes resultados estão de acordo com os obtidos em estudos anteriores, e em conjunto com a baixa diversidade genética encontrada nos grupos Ibéricos quando comparados com os Africanos bem como a presença de haplótipos partilhados dos dois lados do Estreito de Gibraltar, vêm ao encontro da hipótese de ter havido uma colonização recente da Península Ibérica. A divergência entre o clado TA e os demais sugere uma divergência já antiga destes grupos, sendo até possível que se trate de um grupo tão diferenciado que possa pertencer a uma subespécie ou até espécie distinta. No subclado NM a presença de haplótipos partilhados aponta para uma colonização mais recente da Ibéria que a do subclado SW, em que não há partilha de haplótipos entre a Ibéria e África. No entanto, em qualquer dos casos, os baixos valores de diversidades haplotípica suportam a hipótese de uma colonização recente, pós abertura do Estreito de Gibraltar (~5.33 Mya, após a Grande Crise Messiânica durante a qual o Mediterrâneo ocidental secou), levando a supor que ou os animais terão usado 'jangadas' naturais ou então teriam sido transportados por humanos. Os cantos de acasalamento dos machos seguem este padrão de baixa diversidade entre populações, observado ao nível do ADN mitocondrial, não se verificando diferenças significativas.

No que respeita ao nível do estudo da interacção das duas espécies, ao contrário do esperado, não foram encontrados elementos relevantes, nomeadamente da ocorrência de híbridos na natureza. Apesar do esforço de amostragem efectuado nas zona sintópicas, a análise de RFLP não revelou a presença de nenhum híbrido F1 ou retrocruzamento. Igualmente, da análise dos parâmetros dos cantos de acasalamento não se encontraram quaisquer indicíos que pudessem apontar nesse sentido. No entanto, a análise dos cantos mostrou que quando em simpatria os machos de *H. meridionalis* alteram alguns dos

parâmetros das suas chamadas de acasalamento. Quer a frequência fundamental quer a frequência dominante apresentam valores médios mais baixos em situações de sintopia, assim como na duração das chamadas que foi, em média, inferior. Será difícil justificar com certeza a razão para estas diferenças, sendo que estudos sobre as preferências fonotácticas de fêmeas e experiências de *playback* com machos poderão ajudar a esclarecer esta matéria.

No seu conjunto, os resultados obtidos realçam a importância da utilização de duplas abordagens como utilizámos, em que vias, em princípio, mais ou menos independentes, são usadas de forma complementar para tentar responder às questões referentes à determinação dos padrões espaciais e temporais da estrutura populacional destas espécies (e dos anuros e outros vertebrados em geral). O que fica ainda por esclarecer obriga à realização, idealmente num futuro próximo, de estudos utilizando de forma efectiva vários marcadores moleculares nomeadamente microssatélites, em particular no caso da *H. arborea/H. molleri* para tentar discernir qual a zona de fronteira entre as espécies e esclarecer melhor a estruturação populacional evidenciada pela existência dos dois grupos, 'Norte' e 'Sul'. O uso de microssatélites e outros marcadores nucleares irá favorecer o estudo ainda mais aprofundado de potenciais casos de hibridação/introgressão, utilizando-se a acústica para explorar o valor que representam os cantos de acasalamento como barreira reprodutiva eficaz entre as espécies.

Palavras chave: Mediterrâneo ocidental, Península Ibérica, Portugal, *Hyla arborea* (=*Hyla molleri*), *Hyla meridionalis*, genética, bioacústica

Summary

The focus of this work is on the species of Hylids (Amphibia, Anura) present in Portugal, *Hyla meridionalis* and *Hyla arborea* (=*H. molleri*), to examine the geographic patterns of genetic and mating calls diversity. The aim is to determine the patterns of variation of genetic and bioacoustic markers between populations, exploring how the current distribution patterns were influenced by the origins, colonization events of both species, and finally their evolutive history. For comparison and biogeographically integrative reasons, information from outside Portugal is used.

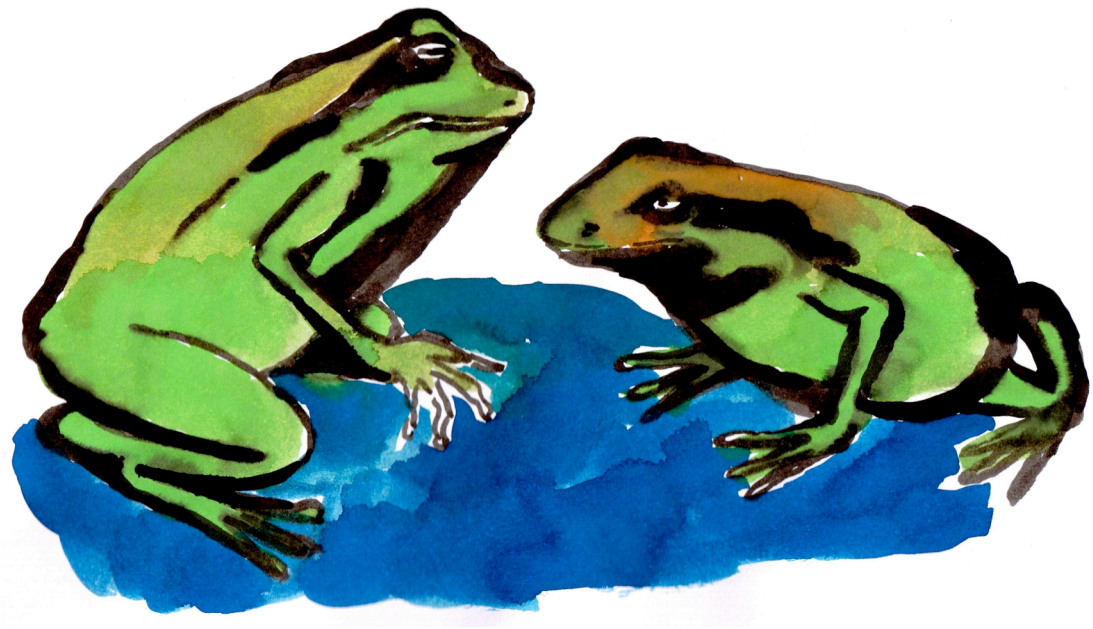
The genetic and bioacoustic analyses revealed different diversity patterns among the studied populations of both species. Iberian *H. arborea* is highly divergent from European taxon, supporting the ressurection of *H. molleri* species. Within Iberia two distinct groups, 'North' and 'South', were identified, having the southern one higher haplotype diversity, a pattern seen in other Iberian amphibians. A different pattern was however seen at the bioacoustic level. Populations did not cluster into groups, and no significant differences were found between populations.

In *H. meridionalis* COI analysis corroborated previous results, with three well differentiated clades identified, all with little genetic diversity within groups. Tunisia and Algeria specimens were highly divergent from all other, suggesting an ancient split between the two lineages. Advertisement calls showed little diversity between populations.

In syntopy, the presence of males of the other species calling apparently has little effect on advertisement calls: only frequencies and call duration of *H. meridionalis* were significantly smaller in sympatry. Being unlikely the hypothesis of reproductive character displacement of mating calls. This, together with the absence of F1 hybrids in the studied sites (although earlier studies have reported their presence) and of any sign of introgression in adults and tadpoles, suggests a rare occurrence of heterospecific mating, and that advertisement calls are an effective premating barrier.

Keywords: Western Mediterranean, Iberian Península, Portugal, *Hyla arborea* (=*Hyla molleri*), *Hyla meridionalis*, genetics, bioacoustic.

Chapter I. Introduction



by Sara Maia

Le biologiste passe, la grenouille reste.
The biologist passes, the frog remains.

Jean Rostand, 'Inquiétudes d'un biologiste' (1967)

Introduction

The general aim of biology is the study of life and its origins, evolution, diversity and distribution. The study of the genetic structure of populations allows us to know more about their evolution history, past and current distributions, colonisation patterns (expansion and retractions) and interaction with other species – in other words, hybridisation phenomena, with various consequences. Acoustic communication is of particular importance in anurans, as advertisement calls are a crucial trait for intraspecific identification (i.e. a pre-mating reproductive isolation barrier). Moreover, in this particular group acoustic traits are closely related to genetics, as, unlike other vertebrates, variations depend significantly on the physical characteristics of sound reception and emission structures (the other vertebrate groups usually depend more on imitation and learning from peers. This makes acoustic traits more difficult to be used as a significant evolutive parameter). These two broad fields interact with other biological sub-disciplines, such as evolution, ecology, population genetics, ethology, neurobiology and physiology. Largely due to technological innovations in genetics and bioacoustics, like the development of Polymerase Chain Reaction (PCR) technology, DNA sequencing, computer-assisted molecular and acoustic analysis and new software for the analysis of complex multivariate data series have allowed biologists to study the complex interactions between genetics and bioacoustics both in isolation or via an integrated approach.

Based on these assumptions, to further explore molecular variation patterns, mating behaviour, species interaction, namely the presence of hybridisation or its avoidance, the treefrogs inhabiting Portugal will be studied using a two-fold approach: one molecular, making use of DNA analysis techniques, and the other acoustic, making use of analysis of bioacoustic data. The stripeless treefrog, *Hyla meridionalis*, and the European treefrog, *Hyla arborea*,[1] are the only extant Hylids in the Iberian Peninsula. Their geographic distributions overlap over a wide area and they are known to interbreed; however, this seems to seldom happen (Oliveira et al. 1991; Barbadillo & Lapeña 2003).

In this chapter, I will describe the specific biogeographical context of the study and the concepts and methods used, and explain in detail the questions addressed in my research. The field and laboratory methods are described in the second chapter, as well as the statistical analyses applied to the data. The third and fourth chapters comprise the results and the

[1] Recently published works suggest that Iberian *H. arborea* should be recognised as *H. molleri* (Stöck et al. 2008; Barth et al. 2011), resurrecting the taxon previously described (Bedriaga 1890). However, when this study began in 2005, this taxon was still acknowledged as *H. arborea*. For consistency reasons I will use the term *H. arborea* across this thesis, and discuss in Chapter 5 why – in my opinion – the *H. molleri* taxon should be resurrected.

discussion, respectively, and the fifth chapter includes the final considerations about the current work, and also addresses remaining questions for future studies.

1. Biogeographical History of the Western Mediterranean and Iberian Peninsula and Amphibian Distribution (Origins and Colonisation)

To understand the geographical distribution of the Hylidae and their evolution in different parts across the globe, it is important to consider the geological dynamics of the Earth's surface and the resulting climatic and ecological changes. Species tend, in general, to move (migrate) to the very extreme of the biogeographical spaces they inhabit. Species evolve through time by adaptive radiation to the environmental conditions of their new habitats. Where geographical boundaries disappear, e.g. through the creation of new land corridors, they tend to migrate and colonise the new spaces. Such land corridors appear as part of the very slow tectonic movements of the Earth's plates, but also as the result of shorter-term crisis and climate change events. In the first case, tectonic movement – the drifting apart or convergence of plates – changes the geology of the biogeographical environment by joining or separating continents, opening or closing seas, and leading mountain chains and islands to emerge. In the second case, changing temperatures and sedimentation events caused by climate change can temporarily modify the morphology of biological corridors, allowing species to pass from one continent to another, or creating separating walls. The appearance of the two frog species studied in this research project, *Hyla meridionalis* and *Hyla arborea*, results from the particular biogeographical history and biological colonisation of the Iberian Peninsula. In this section, I describe the different events that have marked their particular history.

1.1. The Western Mediterranean Region in the Context of Global Tectonic Movements

The western Mediterranean region (the main spatial scope of this study) was formed during the Tertiary period late Oligocene (~28-23 Mya), emerging in the convergence area between the European and the African plates (Fig. 1.1) (Krijsman 2002; Rosenbaum et al. 2002). The geological and climatic history of this area is very complex, but can be inferred from the wider context of global tectonic movements and the particular climatic events marking this area. During the early Oligocene (~33-28 Mya) – the earliest geohistorical period referred to in the evolutionary biology of modern Hylidae species (Duellman & Trueb 1986;

Pleguezuelos 1997) – the continental landmasses of Europe and Asia joined, while the land connection between Asia and North America disappeared. In the same period, temperatures in Europe dropped significantly, and the continent passed from a tropical and sub-tropical climate to a moderate one (Prothero 1994; Pleguezuelos 1997). The newly established geographical connection between Europe and Asia enabled the migration of fauna and flora between the two continents, as evidenced by the presence of amphibians of Asian origin – such as the genera *Bombina*, *Bufo*, *Rana* (*Pelophylax*) and *Hyla* – in Europe. The latter migrated from Asia during the Oligocene or early Miocene (~30 Mya) (Sanchiz & Rocek 1996). Within Europe, the biogeographical history of the western Mediterranean area represents a particular case. The Iberian Peninsula is marked by two major events: the appearance of temporary land corridors between it and Africa and the uplifting of the Pyrenees as a *de facto* (and effective) geographic barrier in the north.

1.2. Temporary Land Corridors between Iberia and Africa

Since the late Oligocene and early Miocene, large parts of the area separating Africa and Europe have been subjected to subduction rollback and wholesale extension (Fig. 1.1 B and C). As a result of the slow convergence between the Eurasian and African plates and the large scale horizontal movements of smaller plates (Jolivet & Faccenna 2000; Rosenbaum et al. 2002, 2010), back-arc basins have formed. This led to the break-up and drifting away of several continental fragments formerly attached to southern France and Iberia. Today, these are scattered throughout the western Mediterranean (e.g. the Betic region in the Iberian Peninsula, the Rif and the Kabylies in North Africa, the Balearic Islands, Sardinia, Corsica and parts of Calabria – Rosenbaum et al. 2002). The Betic and Rif straits were the only links between the Mediterranean and the Atlantic Ocean. These corridors periodically narrowed, creating temporary land connections between Africa and Europe, allowing biotic dispersal.

The earliest known African-European intercontinental connection, as evidenced by vertebrate palaeontology data, was established during the Burdigalian (early Miocene) era (Rögl 1988, 1998). Another such event was observed during the Betic crisis, in the early Miocene (~16-14 Mya), when the orogenic uplift of the Albóran Basin between Iberia and Africa (the area currently forming the Betic and Rif regions) connected the insular Betic region to Africa (forming the present-day Rif Mountains) (Duggen et al. 2003; Weijermars 1991). Moreover, in the late Tortonian (~7.8-7.6 Mya), the depositing of sediments in the eastern Betic region, which occurred as the result of a salinity crisis, created a connection between southern Iberia and the Gibraltar landmasses (Weijermars 1991). Later on, a similar event occurred in the Rif region (Weijermars 1991).

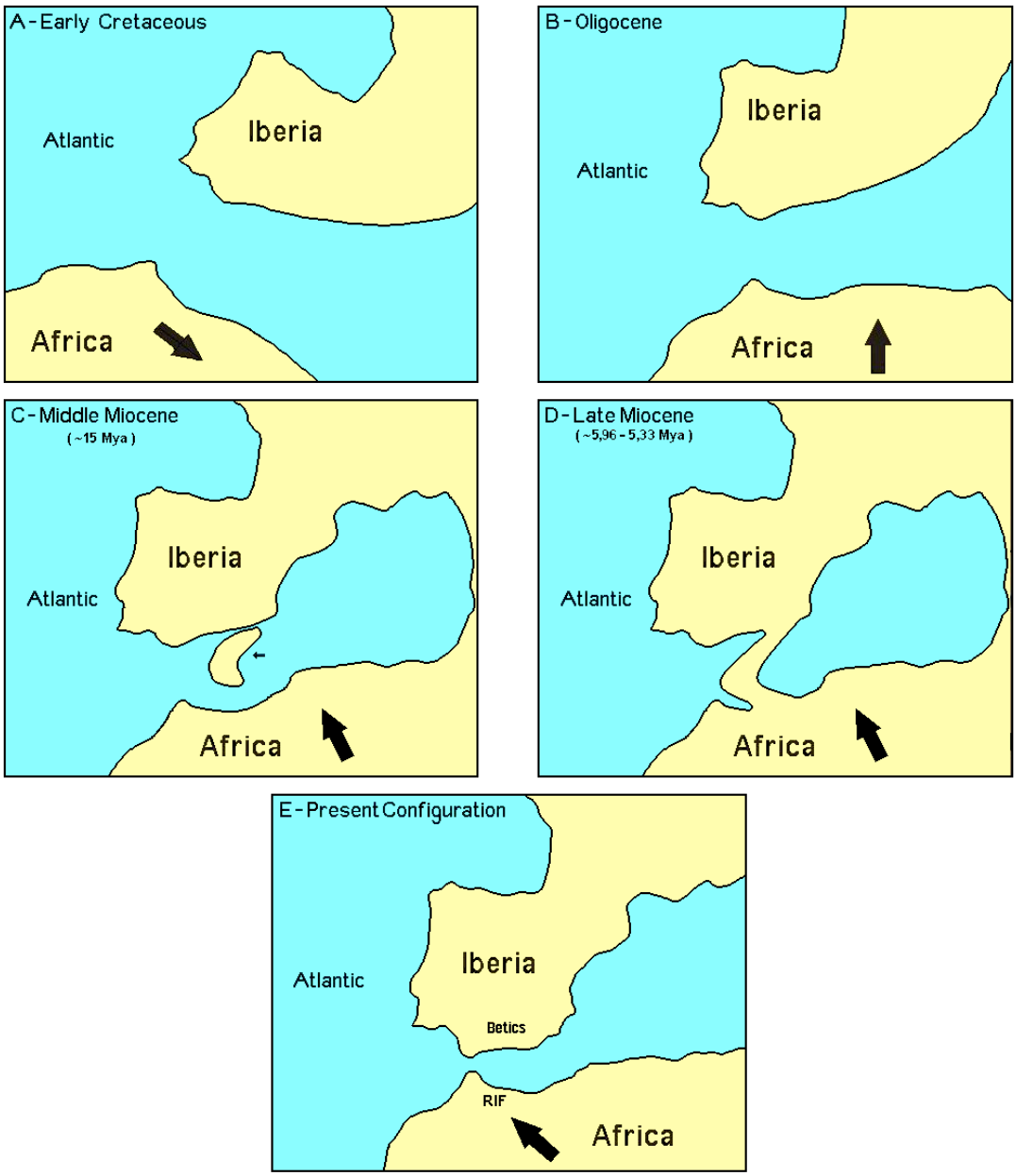


Figure 1.1. Reconstruction of the Western Mediterranean, from the early Cretaceous (~145.5 Mya) to the present.

A. Mesozoic opening of the Tethys Ocean, **B.** Iberia and Africa gradually suffered a counterclockwise rotation, shifting from N-S to near WNW-ESE (in 1.1 E), **C.** establishement by the Betic-Rif land masses of a transient land connection between Iberia and North Africa, **D.** New connection between Iberia and North Africa during the Messinian Salinity Crisis **E.** Present configuration. Adapted from Weijermars (1991), Rosembaum et al. (2002), Duggen et al. (2003) and Duarte et al. (2011).

The most recent catastrophic event in the geological history of the Mediterranean is associated with the Messinian Salinity Crisis (MSC, e.g. Hsu et al. 1977; Krijgsman et al. 1999; Duggen et al. 2003; CIESM 2008), which took place around ~5.96-5.33 Mya, and led to the disappearance of the Betic and Rif gateways connecting the Mediterranean to the

Atlantic. There are different hypotheses to explain this event. Some propose that it was caused by a horizontal shortening associated with crustal nappe movements. Others link it to a tectonic uplift and a global sea drop, caused by a glacial stage (CIEMS 2008). The isolation and hydrographic deficit subsequent to a succession of evaporation events led to a rapid desiccation of the western Mediterranean basin. During this Miocene Mediterranean-desiccation period, a land bridge was formed between Iberia and North Africa, allowing the dispersal of many terrestrial organisms to either side (Fig. 1.1D). Yet, the MSC did not last long. The erosion event ended in the early Pliocene (ca. 5.33 Mya), with an unusually rapid inundation (in geological scale, only about 100 years) of the basin and a reopening of the connection to the Atlantic through the Gibraltar gate.

From then on, the African and Iberian coasts remained separated (Fig. 1.1E). The Strait of Gibraltar became a barrier preventing almost any further biotic dispersal of terrestrial organisms between Europe and Africa, isolating taxa and developing endemic and allopatric units both in the continental masses and in the Mediterranean islands. Since the early Pliocene, the Strait of Gibraltar has changed little, and several studies have looked into its role as a barrier to gene flow between closely related taxa (e.g. Busack 1986; Carranza et al. 2006). The vicariant processes generated by the opening of Gibraltar coincided with the molecular clocks applied to some vicariant taxa present on both sides (Busack 1986), like the painted frogs in the genus *Discoglossus* (Martínez-Solano 2004) and the ribbed newts in the genus *Pleurodeles* (Carranza & Arnold 2004; Veith et al. 2004). On the contrary, the wall lizards from genus *Podarcis* (Harris et al. 2002), the fringe-toed lizard, *Acanthodactylus erythrurus*, the chamaeleons, *Chamaeleo chamaeleon* (Paulo et al. 2002), the gecko, *Tarentola mauritanica* (Harris et al. 2004) and the false smooth snake, *Macroprotodon brevis* (Carranza et al. 2004) are considered human-mediated introductions. Some terrestrial species have proven to be able to cross the strait after its formation, presenting closely related forms on both sides. This has, for instance, been shown in the case of the stripe-necked turtle, *Mauremys leprosa* (Busack 1986; Feldman & Parham 2004).

1.3. The Uplift of the Pyrenees

The second important orogenic event shaping the western Mediterranean, and in particular the Iberian Pensinsula region, was the uplift of the Pyrenees during the Oligocene-Miocene period, eventually leading to the separation of Iberia from the rest of the European continent. During the early Miocene, various episodes of faunal exchanges took place between Asia and Europe, and between Europe and Africa (through Asia Minor). Such exchanges have been described for mammals (e.g. Zhang et al. 2012), but less so for other terrestrial animals, including amphibians and reptiles (e.g. Böhme 2003) – the latter witnessed by the presence in

Europe of the *Chamaeleoninae* group during the Miocene. During the late Miocene (~10 Mya), the Neo-Pyrenees in the north of the Iberian Peninsula uplifted to their current shape and height, and became an actual zoogeographical barrier. This second crucial event marking the biogeographical history of this area was responsible for the vicariant divergence of the species *Alytes obstetricans almogavarii* and *A. obstetricans* (Fromhage et al. 2004; Martínez-Solano et al. 2004), among others.

1.4. Rivers as Zoogeographical Barriers

Another important factor influencing the biogeographical history within the western Mediterranean area is related to the appearance of large rivers. Most of the extant river basins in Iberia and North Africa were formed during the Pliocene. They are geographical barriers hard to transpose by terrestrial organisms, functioning as obstacles to the dispersal of many species, excluding birds and flying insects. Considered to be a relatively low vagility group, the herpetofauna is known to be very influenced by such barriers.

For example, the areas south of the Douro River are well known hotspots of genetic diversity among the herpetofauna taxa, and are important glacial refugia where different species have resisted the harsh glacial conditions and were subjected to differentiation-in-isolation processes. Two of the best-studied examples are the endemic *Chioglossa lusitanica* and *Lacerta schreiberi*, whose populations north of the Douro River would have become extinct during the glacial periods (e.g. Alexandrino et al. 2000; Paulo et al. 2002). Moreover, *Alytes* sp. (Gonçalves 2007) and *Salamandra salamandra* (García-París et al. 2003) show highly differentiated lineages (mtDNA and nDNA) within groups south of the Douro River, resulting from a more recent, secondary colonisation of the area. One other example is the present distribution of *Podarcis bocagei*, mostly north of the Douro, suggesting that it was unable to move south during the last glacial stages when looking for a less extreme habitat (Sá-Sousa 2001). Indeed, similar patterns of genetic structure have been suggested for *Rana iberica*, *Lissotriton boscai* and *Alytes obstetricans*, with different phyloclades being observed north and south of this river (Fonseca et al. 2003; Teixeira et al. 2004; Martínez-Solano et al. 2006).

One other example of a river functioning as a barrier is the Mondego River in central Portugal. *C. lusitanica* allozyme and mitochondrial DNA variation analysis uncovered two genetically distinct groups geographically separated by the Mondego River. Probably resulting from climate changes, these two groups are said to have diverged in the early Pleistocene (Alexandrino et al. 1997, 2000, 2002). The group presently inhabiting the area north of the Mondego River is thought to have its origins from a refuge located between the Mondego and Douro rivers (Sequeira et al. 2005).

1.5. Effects of Climate Change Cycles

The Quaternary Pleistocene (~2.6 Mya to 11,700 years ago) was marked by several climatic oscillations, caused most probably by changes in the Earth's orbit (Hays et al. 1976). Recent studies of the oxygen levels and carbon isotopes, as well as carbon dioxide (CO_2) measures in animal, vegetable and mineral remains found in the seabed, on land and in ice, corroborate the hypothesis that the global climate during this period was dominated by major glacial periods intercalated by short, warmer interglacial periods (reviewed in Hewitt 1996). The Plio-Pleistocene glacial period saw the growth of ice sheets in the northern hemisphere (ca. 2.5 Mya), with other major oscillations occurring during the last 700 000 years, with a cycle of roughly 100 000 years (Webb & Bartlein 1992). Different parts of the globe were differently affected by these climate oscillations, depending on the distance from the Equator, ocean position and currents, and the presence of continental masses and mountain ranges (Hewitt 1996).

These dramatic climate changes have had a great influence on the survival and distribution of most organisms worldwide. During the Pleistocene ice ages, the European mountain regions of Cantabria, Pyrenees, Alps, Transylvania and the Caucasus were all covered by large ice-sheets, while European plains between these mountain regions and the northern ice sheet were covered with tundra and cold steppe (Hewitt 1996; Andersen & Borns 1997; Ehlers & Gibbard 2003). During the ice ages, the areas of the Mediterranean Sea and the North African deserts thus represented biogeographical barriers. Many species of large carnivores, like the Hyaenid *Chasmaporthetes lunensis*, became extinct because the available habitat did not provide sufficient shelter and food to maintain sustainable populations (e.g. O'Regan et al. 2002). The effects of these extinctions are reflected in pollen and fossil data. These indirectly allow for the development of more general models of migration patterns, e.g. how specific fauna and flora responded to climatic change, and therefore the presence of more or less suitable conditions. In this sense, temperate taxa would often migrate south during cold periods, while, inversely, they would expand northwards when the ice retreated during the warmer interglacial periods (Comes & Kadereit 1998; Taberlet et al. 1998; Hewitt 1999; Gómez & Lunt 2007).

Palaeodata (palaeontological and genetic) gathered over the past two decades indicate that some regions in southern Europe remained ice-free, with relatively stable temperatures throughout the oscillating climate of the Quaternary (Huntley & Birks 1983; Bennett et al. 1991; Hewitt 1999, 2001). In particular, the southern European peninsulas of Iberia, Italy and the Balkans had temperate climates that allowed many species to survive (e.g. Bennet et al. 1991; Tzedakis et al. 2002; Ehlers & Gibbard 2003), which were thus called 'Mediterranean

Refugia'. More recent palaeoecological, palaeobotanical and genetic findings also suggest that many species could have survived the glacial maxima outside the area of Mediterranean influence in other ice-free regions located in the north, including central and northern Europe, the southern Urals or even southern Siberia (e.g. Taberlet & Bouvet 1994; Bilton et al. 1998; Babik et al. 2004; Bhagwat & Willis 2008). These non-Mediterranean glacial refugia are often referred to as 'Cryptic Northern Refugia' (Stewart & Lister 2001).

2. Phylogeography and Evolutionary Genetics Advances History

Analysing population genetic structure in relation to historical processes (both short- and long-term) allows for a better understanding of how certain populations have responded to environmental changes, and how these changes have affected contemporary patterns of distribution, genetic variation and population interaction. Phylogeography makes use of present geographic distribution and genealogical relationships to infer the historical biogeography and demography of species, and also what processes – evolutionary and ecological – may have led to a species' current distribution (Avise et al. 1987; Avise 2000).

Population and evolutionary genetics have made significant progress since the late 1960s, with the development of many new molecular techniques. Probably one of the most important practical advances was the Polymerase Chain Reaction, which made it possible to obtain, in a relatively short time and with minor costs, several copies of a certain fragment of genetic material. It also made it possible to sequence genomic portions, or even complete genomes. In the last decade, a diverse array of genetic tools and techniques has been brought to and by the scientific community, allowing the integration of different sources of information, as well as high-resolution genetic studies of population-level processes. DNA-based techniques have become favoured tools in most studies, combined with new statistical methods and approaches.

2.1. Patterns of European Phylogeography: Mediterranean Refugia

Over the last decades, several studies have developed phylogeographic scenarios for European animals and plants (e.g. Gómez-Campo et al. 1984; Doadrio 1988; Moreno Saiz et al. 1998; Ribera 2000; García-Barros et al. 2002). The geographical features and climatic oscillations of the past are now quite well-studied. The phylogeograpic patterns are the consequence of climate changes during the Quaternary, as has been described above: the intercalation of glacial and interglacial periods forced the expansion and retraction of many

species' distributions. In many cases these migrations resulted in geographic isolation of many species in refugia areas, which ultimately led to processes of fragmentation, differentiation and speciation.

Many studies on the western part of Europe stress the relatively high diversity and genetic structure of the southern populations (when compared to the homogeneity of the northern ones) – the so-called contrast between 'Northern-Purity' and 'Southern-Richness' (reviewed in Hewitt 2000). This difference is usually related to the climatic oscillations during the Quaternary since ~2.4 Mya. During the glacial periods of the Pleistocene, the three Mediterranean peninsulas (Iberia, Italy and the Balkans), and, more to the east, the Caucasus as well, constituted refugia for many animals and plants. Their migration to these refugia led to their fragmentation. Many of the originating groups of allopatric populations were, indeed, distributed among different glacial refugia, which, in turn, led to a high genetic differentiation within some groups. In several cases, new subspecies and species emerged (Avise et al. 1998). The three southern-European peninsulas that constituted glacial refugia for the temperate biota subsequently functioned as sources of post-glacial recolonisation of the northern deglaciated areas (e.g. Willis 1996; Taberlet et al. 1998; Hewitt 1999, 2000; Petit et al. 2003; Michaux et al. 2003, 2005; Centeno-Cuadros et al. 2009). The high level of endemism found in animals and plants suggests, indirectly, local, long-term differentiation and speciation (Gómez & Hunt 2007) as a consequence of the survival and isolation of fragmented populations among refugia.

2.2. Iberian Refugia: 'Refugia within Refugia'

The Iberian Peninsula, very likely offered a heterogenous, discontinuous inhabitable area. The peninsula is physiographically complex, with large mountain ranges oriented east-west and a multiplicity of climates, with both Atlantic and Mediterranean influences. Additionally, its large area (around 580 000 km^2) makes it an ideal locale for 'refugia within refugia' (Gómez & Hunt 2007; Weiss & Ferrand 2007). Studies in paleoclimatology have pointed out that the most favourable areas in Iberia were located in the southwest and southeast, likely suitable to xero-thermophilic species that might have used these two disjunct areas as glacial refugia for survival and differentiation (reviewed in Schmitt 2007).

Due to their generally low individual vagility and high environmental requisites, amphibians and reptiles tend to have populations genetically highly structured over short geographical distances, and retain signals of historical events responsible for current species distributions (Zeisset & Beebee 2008). They thus differ from other animal groups (namely birds) and also many plants, whose seeds and pollen can be transported over long distances. Studies regarding amphibians and reptiles in the Iberian Peninsula have come to a consensus

on several recurrent phylogeographic patterns that describe the genetic consequences of the climatic oscillations in the region (Weiss & Ferrand 2007). Different genetic lineages exist in most of the species, suggesting long-term isolation of populations in multiple, distinct glacial refugia. Various degrees of genetic divergence and asymmetrical geographic distribution also exist, usually with the northern populations containing lower diversity as a consequence of recent post-glacial expansions. Furthermore, multiple secondary contact zones have been established with various degrees of admixture.

Complex phylogeographic structures related to Pleistocene climatic oscillations and signatures of rapid post-glacial expansion from refugia have been inferred from genetic data in many amphibian and reptile species – including the golden-striped salamandra *Chioglossa lusitanica* (Alexandrino et al. 2000, 2002; Sequeira et al. 2005), the Schreiber's lizard *Lacerta schreiberi* (Paulo et al. 2001, 2002), the natterjack toad *Bufo calamita* (Rowe et al. 2006) and Bosca's newt *Lissotriton boscai* (Martínez-Solano et al. 2006). The establishment of secondary contact zones is well documented only in *Chioglossa lusitanica* (Alexandrino et al. 2000, 2002; Sequeira et al. 2005, 2006), the lizard, *Lacerta schreiberi* (Godinho et al. 2006, 2008) and the Iberian midwife toad, *Alytes cisternasii* (Gonçalves 2007; Gonçalves et al. 2009).

The Atlantic-Mediterranean refugial area, including Iberia and Maghreb, also show strongly differentiated taxa north and south of the Gibraltar barrier, especially in amphibians from genera *Salamandra* (Steinfartz et al. 2000; García-París et al. 2003), *Pleurodeles* (Carranza & Arnold 2004; Carranza & Wade 2004), *Discoglossus* (García-París & Jockush 1999; Fromhage et al. 2004; Martínez-Solano 2004), *Alytes* (Arntzen & García-París 1995; Martínez-Solano et al. 2004), *Rana* (Plötner 1998) and *Hyla meridionalis* (Busack 1986).

2.3. Molecular Techniques and the Use of Different Molecular Markers

In the last decade, and with the development of newer molecular techniques, the analysis of genetic variation in nucleic acid sequences has become a common approach. The development of molecular techniques that generate taxon-specific markers, permitting genetic analyses of population-level processes such as the study of hybrid zones, has been a major concern among scientists (Parker et al. 1998). Such techniques include Protein Eletrophoresis, Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphisms (RFLP), DNA Sequencing, Single Nucleotide Polymorphisms (SNPs), and, even more recently, the sequencing of complete genomes. Highly variable regions of DNA, such as microsatellites, can sometimes provide a unique 'fingerprint' for each individual. Choosing different regions of the genome that experience different selective pressures (Li et

al. 1985) may lead to different answers, and thus the optimal DNA segment to use for any particular study will depend on its objectives.

Nowadays, combining several molecular markers has revealed itself to be a more secure option, as the use of only one locus or only one type of marker can lead to bias/limited inferences and even to discordant evolutionary patterns for a given species. However, it is also a common misconception that more data are necessarily better which may not be the case if robust conclusions can be taken with less data (Karl et al. 2012). There are many other techniques and molecular markers that can be used, but here I only refer to those that have been used in the present study.

Restriction Analyses

The discovery of restriction endonucleases enzymes, in 1970, was of extreme importance to molecular analysis; since the the early eighties, many RFLP markers have been developed, and have been used to characterise hybrid zones (Harrison & Arnold 1982; Cothran & Zimmerman 1985; Guttman & Karlin 1986; Howard 1986) and type individuals as 'pure species' or hybrids (Kocher & Sage 1986; Chu et al. 1995). Restriction enzymes cleave DNA at specific nucleotide-sequence recognition sites, generating DNA fragments of different sizes. The fragment size variation is the marker itself, called 'Restriction Fragment Length Polymorphism' (RFLP). The choice of the enzyme for a restriction digest should depend on the size and number of desired fragments: too small or too many may be hard to visualise. Also, when discriminating between two or more sets of individuals, like two species, fragments very similar in size and in number may not be as useful for analysing patterns.

The most common and efficient application of RFLP analysis is the selection of a specific gene fragment that is amplified via PCR prior to restriction enzyme digestion. Specific restriction sites are targeted in the nucleotide sequence of these fragments so as to produce species- or type-specific restriction fragments. The separated fragments can then be visualised by ethidium bromide staining to identify the RFLPs. To clarify phenomena such as hybridisation and introgression, nuclear DNA markers have been the most frequently chosen, but mitochondrial DNA markers have also been used, to identify the maternal species.

Polymerase Chain Reaction

Probably the invention of the last century that undoubtedly changed molecular biology and all related fields is the Polymerase Chain Reaction (PCR, Mullis & Faloona 1987), which allowed researchers to quickly amplify, or clone, large amounts of almost any piece of DNA from almost any source. The development of the PCR technique owes much to the discovery,

in 1976, of a thermostable DNA polymerase (Taq), an enzyme originally isolated from a bacteria, *Thermus aquaticus*, which lives in hot environments with temperatures higher than 50ºC. The DNA polymerase isolated from this bacteria is stable at high temperatures, and maintains activity even after DNA denaturation occurs.

One other advantage of PCR is the possibility to use very little bits of biological material, when compared to other earlier techniques. Also, a wide nature-variety of material can be used, from feathers to hairs to tail tissue to saliva or dung, and from fresh, dried or ethanol-preserved material, allowing, for example, the use of many of the long-stored museum biological materials. However, as expected, the technique is not free of difficulties, and sometimes the degree of fidelity of PCR amplification can be a problem (Saiki et al. 1988). Misincorporation of nucleotides, lack of specificity of the primers, or simply contamination of the samples can lead to the amplification of molecules other than those intended, which can sometimes result in interpretative errors.

DNA Sequencing

Developed in the mid-1970s, DNA sequencing methods are today probably the most used molecular techniques. At the time, two different approaches were introduced, but it was the one from Sanger et al. (1977) that turned out to be the forerunner of techniques employed today. Relying on a supervised interruption of in-vitro DNA replication, this method output provides a description of bases at successive nucleotide positions in one of the DNA strands.

In recent years, DNA sequence information has allowed scientists to describe full genomes of organisms as diverse as frogs (e.g. *Xenopus tropicallis* – Hellstein et al. 2010), nematodes (e.g. *Caenorhabditis elegans* – *C. elegans* Sequencing Consortium 1998), humans (*Homo sapiens sapiens* – Gregory et al. 2006), plants (e.g. *Arabidopsis thaliana* – The *Arabidopsis* Initiative 2000), etc.

Mitochondrial DNA

In the last one or two decades, most works of phylogeography and phylogeny were solely based on the analysis of mitochondrial DNA (mtDNA) (Avise 2000, 2007). The mitochondrion, the organelle responsible for generating much of the energy for eukaryotic cellular functions, has a specific circular genome. Mitochondrial DNA has several properties that made it widely used in phylogeographic and phylogenetic studies: its has a relatively high mutation rate compared to nuclear genes (generally 3-10 times higher); it has an almost exclusive maternal inheritance in vertebrates (with exceptions described in Gyllensten et al. 1991 and in Zhao et al. 2004); and it is a haploid genome and lacks recombination (with

limited existence, explored in Rokas et al. 2003; Tsaousis et al. 2005; Guo et al. 2006). Absence of recombination and uniparental inheritance results in a effective population size of mtDNA equal (on average) to a quarter of that of the nuclear genome. Consequently, mtDNA is a more sensitive molecular marker to stochastic phenomena and genetic drift (Moore 1995), having a higher probability of tracking splitting events. Moreover, from a technical perspective, there is a large amount of mtDNA in each cell due to the high abundance of mitochondria, which allows for high quantities of mtDNA for analysis.

However, there are also some caveats worth mentioning (see review in Zhang & Hewitt 2003 and Ballard & Whitlock 2004): the absence of recombination makes it behave like a single locus, limiting analyses to only one of the several possible replicate genealogies of the evolutionary process – this may not produce a representative view of the whole genome (Moritz 1994; Bazin et al. 2006) – and uniparental inheritance can bias the obtained scenarios specially in cases of sex-biased dispersal or reproductive success, introgressive hybridisation, variation in rate of evolution in different parts of the genome, and severe population bottlenecks. The limitation of using just one locus also applies to single nuclear gene genealogies. Thus, phylogenetic inference using multiple loci is always preferable to using a single locus approach

Nuclear DNA

Unlike mtDNA, nuclear autosomal genes are bi-parentally inherited, revealing the history of both females and males. The ability for recombination allows one to infer the level of miscegenation, introgressive hybridisation of differentiated groups being of extreme importance in studies of hybrid zones. Also, for this reason, even genes located in the same chromosome can have completely independent evolutionary histories, which can be exploited by selecting various genes with different evolution rates, which can also bias the observed inferences (Zhang & Hewitt 2003).

Microsatellites

Microsatellites are tandemly repeated sequences of 2-6 bp (Tautz 1993), abundantly distributed across the genome and with very high levels of allelic polymorphism. Their key feature is the hypervariability in species and populations, with an estimated mutation rate of 10^{-2} to 10^{-6} per locus per generation (Ellegren 2000), which is several orders of magnitude greater that a nonrepetitive DNA (ca. 10^{-9}) (Weber & Wong 1993).

Microsatellite loci were discovered in the late 1980s, and soon become widely used. Their use in more recent studies of population genetics has revealed their potential to infer the

occurrence of bottlenecks and or population expansions (e.g. Rowe & Beebee 2001; Jehle et al. 2001; Jehle & Arntzen 2002), as well as to resolve phylogenies depicting deeper branching (e.g. Estoup et al. 1995) – and sometimes even provide more accurate geographic clustering of closely more related populations (e.g. Bowocock et al. 1994)

3. Bioacoustics of Anurans

Biological communication is described by E.O. Wilson (1975):

> is the action on the part of one organism (or cell) that alters the probability pattern of behavior in another organism (or cell) in a fashion adaptive to either one or both of the participants. By adaptive I mean that the signalling, or the response, or both, have been genetically programmed to some extent by natural selection.

Broadly speaking, this means that there is a transfer of information, a message between a sender and a receiver (Simmons 2004), and the sender influences the receiver's behaviour in a certain way. A particular case of biological communication is acoustic communication (i.e. bioacoustics); bioacoustics is a scientific discipline that studies the production, propagation and reception of acoustic signals produced by living organisms, as well as the effects of those signals on the organisms themselves (Sueur 1998; Tubaro 1999).

Among animals, acoustic communication plays a crucial role in many mating systems. In amphibian anurans, males use advertisement calls to attract females and to compete with other males for mates and territory (Wells 1977); thus, acoustic communication has an important role in reproductive success. Most extant species of anurans – around 5000 species of frogs and toads in the world, in which Hylids are included – have well-developed vocal structures capable of producing sounds that serve to attract mates, defend territories or express distress (Duellman & Trueb 1986). There is a strong sexual dimorphism in the structures that produce the sounds (Schneider 1988; Wells 2001), being usually well-developed in males that produce relatively loud signals and weakly developed or absent in females, which, in most species, do not produce any sound. In almost every anuran species, male vocalisations originate in the larynx, when air is forced from the lungs into the mouth and vocal sac (Martin 1971; Rand 2001) and the vocal cords and associated cartilages vibrate, producing sound (Fig. 1.2). This system, inherited from the respiratory mechanism of their ancestors, the Sarcopterygians fishes, is based on positive pressure created during the respiratory cycle, when air is pumped into the lungs by the muscles of the buccal cavity (Gans

1970; Gans 1973; McClelland et al. 1996) When present, female vocalisations are mostly limited to a release call occurring upon tactile stimulation (Bogert 1960; Capranica 1968; Kelley 1982; Boyd 1992). They can also make reciprocal calls to respond vocally to males (Márquez & Verrel 1991; Bush 1997; Tobias et al. 1998), or occasionally initiate the calling (Given 1993; Bush 1997). In extremely rare cases (e.g. *Rana blythii*), females produce loud advertisement calls, whereas the males are mute (Emerson 1992, reviewed in Emerson & Boyd 1999).

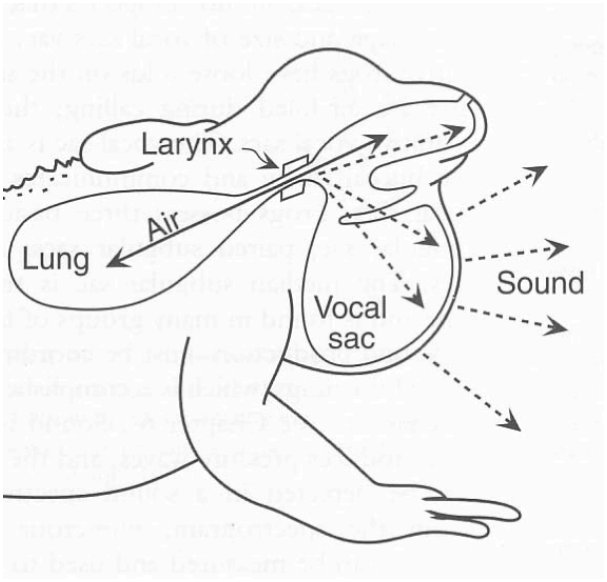


Figure 1.2. Mechanism of sound production in Anurans.
Schematic representation of an anuran showing the structures involved in vocalization and the pathways of air during respiratory cycle and sound production. During the respiratory cycle, the air reaches the lungs by positive pressure created by buccal muscles. The air is then pumped from the lungs through the larynx to the buccal cavity and vocal sac. When the air passes by the larynx it causes the vocal cords and associated cartilages to vibrate, and sound is produced. (Adapted from Duellman 1986).

Most species of anurans have up to five different context-dependent calls, which include an advertisement call, a distinctive agonistic or territorial call, a release call, a distress call and an encounter call (Rand 1988). The advertisement call is particularly important, as it is generally species-specific (Blair 1961), and can be used to announce an occupied territory to other conspecific (i.e. from the same species) and heterospecific (i.e. from a different species) males. It can be also be used – often simultaneously – to attract conspecific females for mating. The latter aspect explains why these calls are sometimes referred to as 'mating calls' (Wells 1977). The species specificity of the advertisement call is an important mechanism for species recognition, acting as a primary prezygotic barrier of reproductive isolation (Blair 1958; Nevo & Capranica 1985). Preventing interbreeding can be of extreme relevance between (closely related) sympatric (and syntopic) species to avoid hybrid – and

then often sterile – offspring. The uniqueness of each species-specific call allows for its use as a taxonomical diagnostic characteristic, and has long been regarded as a valuable taxonomic character (e.g. Blair 1958; Littlejohn & Oldham 1968; Martin 1972; Schneider et al. 1992; Vences & Glaw 1996; Schneider & Sinsch 1999). Studies in *Bufo* and *Rana* genera have revealed the important informative value of the advertisement calls for the analysis of phylogenetic relationships (Schneider & Sinsch 1992; Crocroft & Ryan 1995; Vences & Glaw 1996). Other types of calls, although much less considered, have also been shown to be phylogenetically relevant (e.g. Sullivan & Wagner 1988; Di Tada et al. 2001).

3.1. Chorus and Mating Strategies

During the breeding season, anurans rarely call alone. Both explosive or prolonged breeders (*sensu* Wells 1977) gather together in certain areas, usually close to water bodies with food availability and good reproductive conditions (i.e. temporary ponds, streams, and plants such as Bromeliacea, etc.). They usually focus their efforts on sound-making only when congregated with others (Gerhardt & Huber 2002). Variation in acoustic parameters of the advertisement calls and calling behaviour are, therefore, determinant for their reproductive success (Lopez et al. 1988), since male anurans of many different species typically aggregate at breeding sites during the reproductive season. They form choruses to attract females to mate, and to advertise their territory to other males (Wells 1977), a strategy also common among insects (Alexander 1975). Within this lek mating system, males chose locations in which they will be more protected from predators, and where their advertisement call, thermoregulation and energy cost will be optimised.

In dense choruses, however, it can be particularly difficult for a male to be detected, recognised and chosen by a conspecific female (Wollerman 1999). The high levels of background noise, and consequent overlap of calls in dense mono or multispecific choruses, are almost inevitable. They may mask a male's own advertisement call, and may even alter the female's discrimination ability (Wiley 1994; Wollerman & Wiley 2002; Schwartz & Wells 1983; Schwartz 1987) – a significant cost to the chorus strategy. In addition, the number of conspecific (and even heterospecific) males present in the breeding and chorus area may reduce the probability of a male being found and chosen by a female. To minimise such costs and still benefit from the chorus effect in attracting females, and in reducing individual predation risk (known as the *dilution* or *selfish herd effect*, proposed by Hamilton in 1971 to explain the benefits of animal aggregations), individual vocalising males do not call randomly with respect to the other males (Brush & Narins 1989).

The way males will cope with the chorus effect in their breeding area can follow two main strategies. In the first one – the grouping strategy – males make full use of the group

effect, using call attributes similar to those of their closest neighbours. In the second one – the individual strategy – individual males will emphasise their differences, using call attributes that vary significantly from those of their closest neighbours. The grouping strategy may seem altruistic, but is not. When a male follows this strategy, females are likely to be attracted by a potentially stronger signal, as the whole chorus is using similar calling attributes, engaging in a unison bout of calling, with background noise levels fluctuating in intensity (Schwartz 1991). This may save energy for the calling male, as its attractiveness is helped by the group effect and does not exclusively rely on its self-effort. However, as referred to previously, call interferences may occur, reducing the attractiveness of the signal, and thus of the calling male. In this case, it might be advantageous to the male to be located in the periphery of the chorus. Its advertisement call is then spatially more separated from the rest of the chorus, which potentially reduces the negative effect of interference from other calls and allows the male to reach the female before the others in the centre of the chorus. Female mating choices can, however, largely depend on subtle details related to a male's advertisement call (as well as to visual cues, such as the visibility of the vocal sac, for example) (Hödl & Amézquita 2001; Rosenthal et al. 2004; Gomez et al. 2009). This would be advantageous to males that follow the individual strategy. In this case, each male employs certain calling attributes that will make him sound as different as possible from the others. The difficulty here is remaining attractive to a potential mate. Unlike the first strategy, it is expected that males located closer to the female will have more differentiated calls than males located farther apart.

Summarising the various strategies that have been described: males can respond 1) by avoiding overlapping calls with neighbouring, conspecific and heterospecific males (Greenfield 1994; Narins 1992; Schwartz 1991, 1993, 1994; Narins et al. 2003); 2) by changing call attributes, like the calling rate or the overall complexity of the call (reviewed in Wells 1988 and Narins 1992); 3) by choosing a specific location within the breeding and chorus area (Resetarits & Wilbur 1989, 1991); 4) by using satellite mating strategies (Perrill et al. 1978; Arak 1988; Lucas & Howard 1995; Byrne & Roberts 2004); or 5) by presenting agonistic behaviour towards other males, even actually engaging in physical attacks (Narins et al. 2003). Male reproductive success depends entirely on whether or not each male can attract at least one conspecific female to the chorus area with his calls – by enhancing female phonotaxis – guiding her to the specific site where the male is standing, and actually mate with her before any others can (Bowker & Bowker 1979; Gerhardt 1987).

3.2. Sexual Selection and Variation of the Advertisement Calls

Anuran calls, and particularly advertisement calls, are frequently discrete, periodic and stereotyped in both frequency and duration. Although considerable diversity and complexity of vocal repertoires have been described (e.g. Littlejohn 1977), when compared to vocalisations produced by birds and mammals they remain fairly simple. When gravid females arrive at the breeding sites they are confronted with a cacophony of various species' sounds. Nonetheless, like many other animals, anurans demonstrate a strong ability to recognise conspecifics (reviewed Tibbets & Dale 2007). The efficiency of this acoustic communication system relies on the accurate production of the signals, on the transmission conditions of the environment (e.g. Marten & Marler 1977; Marten et al. 1977; Penna & Solis 1998; Wiley & Richards 1978; Bradbury & Vehrencamp 1998) and on the auditory system, especially tuned for the reception of conspecific calls (Ryan & Wilczynski 1988; Roy 1994; McClelland et al. 1996; Smotherman & Narins 2000). Amphibians have two auditory sensory structures: the amphibian papilla and the basilar papilla. The amphibian papilla is sensitive to lower frequencies (between 100-900 Hz); the basilar papilla is sensitive to higher frequencies (above 1000 Hz) (Feng et al. 1975; Schoffelen et al. 2008). Moreover, the intraspecific variation in call characteristics also allows females to discriminate, based on auditory and neurophysiological mechanisms (Zakon & Wilczynski 1988), between potential conspecific mates (Littlejohn & Michaud 1959; Littlejohn 1961). The differential mating success of males via female conspecific recognition and intraspecific male discrimination – known as sexual selection – may have influenced the evolution of male advertisement calls and female phonotaxis (Endler 1993; Ryan 1991). Furthermore, the examination of call parameters that elicit female phonotaxis shows that particular spectral and temporal features increase the probability of a female approaching a male (e.g. Ryan 1985; Bödner 1996; Gerhardt 1991; Passmore et al. 1992; Jennions et al. 1995; Kime et al. 1998; Wilczynski et al. 1999).

3.3. Mating Calls Characteristics

The acoustic properties of the advertisement calls, even though they are highly stereotyped within a certain species, typically show a characteristic range of variation between individuals, and between and within populations of the same species (e.g. Capranica et al. 1973; Sullivan 1985; Ryan & Wilczinsky 1988, 1991; Wagner 1989; Bee et al. 2001; Castellano et al. 2002). Variation at the individual level has been observed in both frogs and toads (e.g. *Acris crepitans* – Nevo & Capranica 1985; Ryan 1990; Ryan & Wilczinsky 1991; *Alytes* spp. – Márquez 1995; *Physalaemus pustulosus* – Ryan et al. 1996; *Neobatrachus* sp. – Roberts 1997; *Rana clamitans* – Bee et al. 2001 and *Litoria booroolongensis* – Smith & Hunter 2005). While basic properties are nearly invariable at all individual and population

levels, the calls can be categorised according to qualitative properties such as pulsation (i.e. pulsed and non-pulsed) and spectra (i.e. narrow and broadband). Also, the signal's spectral-frequency content and temporal (fine-scale <100ms) properties show different degrees of variation. Despite the continuum in the variation of properties, quantitative variable properties such as frequency, call duration or pulse rate are classified according to the coefficient of variation (CV) (Gerhardt 1991). The latter considers properties with a CV <4% as being static, and properties with a CV> 10% as dynamic. The frequency is said to be intermediate when the CV of a property is between 4% and 10%. Frequency and pulse rate typically show relatively little variation within individuals (CV usually <5%). By contrast, call duration and call rate are highly variable (CV usually> 10%). The individual variation is thus not only influenced by extrinsic factors, such as ecological characteristics of the habitat, environmental temperatures and social interactions, but also by intrinsic factors such as body size and sound-apparatus characteristics (both for reception and emission of sound).

3.4. Ecological Factors

Individual variation of call parameters is closely related to environmental factors. Habitat-specific conditions, such as air humidity, the density of vegetation and micro or macro relief (e.g. rocks or mountains, respectively), affect the transmission of the sound, and thus also influence the call production (Schiotz 1967; Marten & Marler 1977). Accordingly, frogs living in open habitats with scarce or low vegetation tend to have prolonged calls with lower pitches, while those living in densely forested habitats have higher-pitched, discontinuous advertisement calls (Heyer 1971). Males have to make a compromise, here, between the distance of transmission they achieve and the detectability of their location. Low frequencies carry for a greater distance than high frequencies, but the latter are easier to locate (Konishi 1970). Also, the material properties of the calling site are important. Some males produce sounds inside burrows; the sounds' higher frequencies are readily absorbed by the soil, and therefore audible only within short distances. Others use cavities as amplification boxes, increasing the intensity, and thus the transmission distance and efficiency, of their vocalisations (Bailey & Roberts 1981; Lardner & bin Lakin 2002; Márquez et al. 2005). Cavities may also amplify conspecific vocalisations generated externally, facilitating the reception – to the male inside its cavity – of their neighbours' vocalisations in chorusing aggregations (Penna & Solís 1999; Penna 2004; Penna & Márquez 2007). Some species are also known to produce calls under water, taking advantage of the fact that sound transmits better and suffers less attenuation in the water than in the air (Boatright-Horowitz et al. 1999).

3.5. Male Size Effect on Calls: Vocal Apparatus Characteristics

According to Gerhardt (1991), female frogs and toads generally prefer dynamic advertisement call properties. This statement, based on empirical observations, is supported by other studies, reporting that females of various species generally prefer call characteristics based on faster call rates, lower dominant frequencies and higher call intensities (Arak 1983; Ryan 1985; Schwartz 1987; Márquez 1995). These characteristics are often related to male 'quality', vigour or condition, and there is a relationship between call characteristics and anatomic features of the peripheral auditory system (e.g. larynx size), which are correlated with male body size (McLelland et al. 1996). Many studies have analysed the relationship between body size and call characteristics, but not all have demonstrated this relationship. In *Alytes obstetricans* and *A. cisternasii*, dominant frequency is inversely correlated with male size (Márquez 1995). This relationship has been shown in many other species of anurans, like *Physalaemus pustulosus* (Ryan 1980), *Uperoleia rugosa* (Robertson 1986), *Hyla chrysoscelis* (Morris & Yoon 1989) and *Crinia Georgiana* (Smith & Roberts 2003). In *Bufo woodhousei*, *Bufo fowleri* and *Bufo americanus*, frequency is negatively correlated with body size, while pulse rate and duration of calls are not (Sullivan 1982; Zweifel 1968). Other studies have focused on differential male mating success, manifested through size-related variation in spectral parameters of the advertisement calls. Larger males are clearly advantaged for female choice in *Physalaemus pustolosus* (Ryan 1980) and in *Bufo calamita* (Tejedo 1992), but *Eleutherodactylus coqui* males are not (Lopez & Narins 1991).

3.6. Temperature Effect on Calls: Environmental and Male Body Temperatures

Being ectothermic animals, anurans' body temperature is highly dependent on environmental temperatures, which are expected to have an important influence on males' behavioural performance and on the physiological reaction rates that power the muscles involved in sound-production (Prestwich 1994; Navas & Bevier 2001). The effect of environmental temperatures on the anurans' call-parameters have been the main focus of several studies.

The majority of researchers have observed the effects of air and/or water temperature, but not the anurans' body temperature, on call parameters. Higher environmental temperatures are generally expected to correlate with decreased call duration and shortened intervals between calls (Lorcher 1969; Heizmann 1970; Schneider & Eichelberg 1974; Weber 1974; Paillette 1970, 1986). Parameters determined by active neuromuscular mechanisms (e.g. the rate of calling and duration of the calls) seem to be strongly affected by temperature, whereas those resulting from passive properties of the male as sound-emitter (e.g. fundamental frequency characteristics) seem to be less affected (Littlejohn 1977; Prestwich

1994). For example, in *Alytes obstetricans*, Heinzmann (1970) found a positive correlation between temperature and fundamental frequency. However, Castellano & Giacoma (1998) found a significant negative correlation between temperature and fundamental frequency in *Bufo viridis*. At higher temperatures, *Acris crepitans* and *Bufo viridis* significantly increase their call and pulse rates, respectively (Jackson 1952; Castellano & Giacoma 1998). Positive relationships between temperature and pulse rate, dominant frequency and call duration were found in species like *Hyla versicolor* (Blair 1958), *Bufo woodhousei* and *B. fowleri* (Zweiffel 1968). In *Alytes cisternasii*, males produced longer calls at higher temperatures (Crespo et al. 1989). In *Bombina variegata*, Zweifel (1959) found that call rate and frequency have a significant positive correlation with temperature, but call duration is negatively correlated. The variety seen in the responses of the different species to similar temperature patterns thus requires from the researcher an extreme attention to detail when extrapolating the effects of temperature on call characteristics. Though vocal apparatuses are similar in structure, specific differences apply that can alter species behaviour.

4. Species Interaction

The majority of anuran studies focus on only one species, despite the fact that many species co-inhabit the same geographic area (sympatry) and even the same water body (syntopy), participating in the same acoustic environment. When in close geographic contact, and especially when closely related species cohabit, species-specific advertisement calls may not be strong enough to maintain species integrity, and mating efficiency – and thus coexistence – may lead to hybridisation. Hybridisation can be very costly to parental species, reducing their fitness and eventually leading to a more or less strong introgression that can ultimately result in speciation. To maintain the integrity of species, there is a very high cost associated with the avoidance of mistakes in mate choice. Pre- and post-mating isolating mechanisms are extremely important during species interaction, such as mechanical or genetic incompatibilities, strategies to reduce interference in bioacoustic communication (see above) and to maximise the efficiency of mate attraction.

4.1. Character Displacement and Speciation

Grant (1972) has called ‘character displacement’ the ‘process by which a [morphological] character state of a species changes under natural selection arising from the presence, in the same environment, of one or more species similar to it ecologically and/or reproductively’ (p. 44). Character displacement can be divergent or convergent, depending on the direction and

outcome of the selection. Reproductive character displacement or reinforcement are a result either of selection against hybrids or selection in favour of the divergence of the anurans' advertisement call attributes (Howard 1993; Noor 1999). It occurs when sympatric species, relative to allopatric ones, evolve greater differences in male acoustic signals and in female preferences for those signals. Divergent character displacement of advertisement calls is well documented in anurans (e.g. Littlejohn & Watson 1985; Gerhardt 1994), and it has also been documented for female preference in sympatry (Márquez & Bosch 1997).

Controversy arises when debating whether speciation requires complete geographic isolation (allopatric speciation), or if it occurs in populations with overlapping distribution – and therefore between which there is gene flow (both parapatric and sympatric speciation, when distributions partially or almost totally overlap, respectively). In allopatric speciation, the absence of gene flow gradually leads to reproductive isolation and genetic drift, resultant of mutation (Dobzhansky 1951; Coyne & Orr 2004), contrasting with speciation directed by natural selection against hybrids (Dobzhansky 1951; Howard 1993). For example, Hoskin et al. (2005) have demonstrated that reinforcement can drive rapid allopatric speciation in *Litoria genimaculata*.

4.2. Species Hybridisation: The Ambivalent Topic of Species Contact

Hybridisation is defined as the crossing of genetically distinct groups of taxa leading to the production of viable hybrids (Mallet 2005). The study of hybridisation has received attention since the founding of evolutionary theory and taxonomy. Linnaeus (1751) described it in his books of plants, and Darwin (1859) dedicated a chapter to it in *The Origin of Species* to demonstrate that there is no clear and rigid boundary between species. However, hybridisation is still perceived as a breakdown of boundaries between species, thus feeding controversies about the very processes of speciation. Do such processes require complete geographic isolation – allopatric speciation – or not? Can they occur in populations with overlapping distributions – parapatric speciation (even if there is only partial overlap) or sympatric speciation (if they overlap widely)? In this context, hybrids have long been seen as drawbacks to evolution. Mayr (1963, p. 133) contests that 'hybridization plays a major evolutionary role among higher animals', suggesting instead that the majority of hybrids are sterile, or only able to produce genotypes of inferior viability that are eliminated by natural selection where they backcross to the parental species. Darwin (1859, p. 260) also questioned the issue of sterility:

> Now do these complex and singular rules indicate that species have been endowed with sterility simply to prevent their becoming confounded in nature? I think not. For why

> should the sterility be so extremely different in degree, when various species are crossed, all of which we must suppose it would be equally important to keep from blending together? Why should the degree of sterility be innately variable in the individuals of the same species? Why should some species cross with facility, and yet produce very sterile hybrids; and other species cross with extreme difficulty, and yet produce fairly fertile hybrids? Why should there often be so great a difference in the result of a reciprocal cross between the same two species? Why, it may even be asked, has the production of hybrids been permitted?

Today, hybrids are no longer the 'big bad wolf'. Evolutionary biologists now view them as a window through which we can observe some of the most complicated and understudied aspects of speciation. Studying the presence of hybrids, a type of progeny that results from the crossbreeding of genetically distinct individuals, is now used to infer levels of isolation between species, which requires identification of the isolating barriers and of the isolating mechanisms involved. Reproductive isolation is a requirement for speciation, following the Biological Species Concept (BSC) (Dobzansky 1935; Mayr 1942), which says that 'species are groups of actually or potentially inter-breeding natural populations which are reproductively isolated from other such groups' (Mayr 1942, p. 120). The contact between two congeneric populations may or may not result in interbreeding. If interspecies crosses do not occur, in situations of sympatry (i.e. when different taxa distribution overlaps to a certain point) and syntopy (i.e. when different taxa live in strict sympatry sharing – for example, in the same water body), both populations will keep their own independent evolutionary pathway and diverge from one another. However, if, instead, interbreeding prevails, and F1 hybrids are viable and fertile, allowing for further crosses, subsequent generations could backcross. If backcrosses and posterior generations are also fertile to a certain degree, and become more abundant in the contact area than the original wildtypes, the taxa status (either specific or subspecific) of the populations becomes dubious.

The existence of natural hybrids thus provides relevant information in understanding various evolutionary phenomena. Hybrid zones, as areas where interactions between genetically distinct groups of individuals occur, resulting in offspring of mixed ancestry (Harrison 1990; Barton & Hewitt 1985), are natural laboratories for speciation experiments, and have been the subject of many studies over the last 40 years (see Sobel et al. 2009 for an evaluation of citations for the topic 'speciation' from 1978 to 2008). Studying aspects of the parental species biology is crucial to understanding hybridisation and why it happens. Among these aspects, the efficiency or inefficiency of the pre-mating isolating barriers (i.e. the factors affecting the ability of an individual to successfully choose a mate from its own species, including temporal and spatial partitioning of the habitat, etc.), or of the post-mating isolating

barriers (i.e. postzygotic incompatibility and inviability of the hybrids), are particularly important.

Natural hybridisation is well known in amphibians, and has been the focus of many studies for decades (e.g. Volpe 1959; McDonnell et al. 1978; Griffiths et al. 1987; Arntzen & Wallis 1991; Sequeira et al. 2005). With anurans in particular, there are many examples of successful hybridisation (e.g. in *Hyla* sp. – Fortman & Altig 1974; in *Bufo* sp. – Colliard et al. 2010; and in *Rana* sp. – Mezhzherin et al. 2004). A well-studied case is the water-frog complex in Europe, *Rana esculenta* (now *Pelophylax esculentus* – Frost et al. 2006), a hybridogenetic frog originated from *R. ridibunda* (now *Pelophylax ridibundus* – Frost et al. 2006) and *R. lessonae* (now *Pelophylax lessonae* – Frost et al. 2006). *R. esculenta* typically live as diploid hybrids with one of the parental species, with which they mate and generate viable hybrid offspring. However, *R. esculenta* can also be found in pure hybrid populations that achieved their reproductive independence by way of triploid hybrids (e.g. Schultz 1969; Hotz et al. 1992; Borkin et al. 2004; Christiansen 2005; Christiansen & Reyer 2009; Arioli et al. 2010). Other examples have been observed among toad species, such as the two American toads, *Bufo microscaphus* and *Bufo woodhousii* (Sullivan & Lamb 1988, etc), and between *Bufo valliceps* and *Bufo fowleri*, whose hybrids are sterile but participate actively during the breeding season, and mate with females of either parental species (Volpe 1956, 1959). The Hylidae family is not an exception. Long-term hybridisation has been described between *Hyla cinerea* and *Hyla gratiosa* (Mecham 1960; Gerhardt 1974; Gerhardt et al. 1980; Schlefer et al. 1986; Lamb et al. 1990), and evidence of introgression has been found, suggesting that at least some F1 hybrids are fertile, crossing with other hybrids and backcrossing with the parental species. There seems to be a prevalence of crosses between males of *H. cinerea* and females from *H. gratiosa* (Lamb & Avise 1986). Fertile hybrids are also common between *Hyla chrysoscelis* and *Hyla versicolor* from North America (Gerhardt 1994), and between *Litoria ewingi* and *L. paraewingi* in Australia (Watson 1972).

5. Combining Approaches: Geographic Variation in Genetic Structure and Advertisement Calls

Mating behaviour, in general, in many organisms can be partially acquired by learning, determined by genetics or the product of a gene-environment interaction (Dawson & Ryan 2009). Variations in acoustic properties depend highly on the physical structures of the internal ear and the vocal apparatus (larynx size, vocal sac, etc.), and so learning and social influence are less crucial when compared with other vertebrates, such as birds and mammals,

where acquisition of acoustic signals by learning and imitation processes have been widely studied. Anurans usually have a lower capacity for call dispersion relative to birds and mammals, and the development of species-specific typical signals and their recognition is neither masked by learning or imitation, nor by brain processing (Zann 1990; Simpson & Vicario 1990). Moreover, the production of distinctive calls with intermediate parameters of those of the parental species in some hybrids (e.g. hybrids of *Hyla cinerea* and *H. gratiosa* – Mecham 1960; hybrids of *Scaphiopus bombifrons* and *S. hammondii* – Gerhardt et al. 1980; and of *Hyla arborea* and *H. meridionalis* – Oliveira et al. 1991) further supports the strong genetic basis – in this group of vertebrates – of the production and reception of the advertisement calls. Consequently, call parameters have long been accepted as inherited directly, rather than transferred culturally (Crespo 1993); nevertheless, caution should be taken, as, for example, *Physalaemus pustulosus* males' early experience was shown to lead to an alteration of the advertisement-call properties (Dawson & Ryan 2009). Males reared in isolation produced shorter calls, and males reared in other groups kept the species-typical calls.

The genetic population structure of certain species may be associated with a geographic gradient, by phenomena of isolation by distance, the presence of geographical barriers or even for climatic reasons, for example. Also, the acoustic properties of the anurans' advertisement calls commonly exhibit geographic variation (reviewed in Gerhardt 1994; Wilczynski & Ryan 1999). In various bird species, call structures also vary intraspecifically as a function of geographical distribution (e.g. in white-crowned sparrows – Marler & Tamura 1962).

Regional variations have been recognised in several species of anurans: in the North American pacific treefrog, *Hyla regilla* (Snyder & Jameson 1965); in cricket frogs, *Acris spp*.(Nevo 1969); in the green treefrog, *Hyla cinerea* (Asquith et al. 1988); in Central American Hylid frogs (Duellman 1970); in neotropical poison-dart frogs, *Dendrobates* (Myers & Daly 1976); in European and southwestern Asian toads, *Bufo viridis* (Nevo & Schneider 1976); in *Pelodytes punctatus*; in Portugal (Paillete et al. 1992); and in the southern Australian Booroolong frog, *Litoria booroolongensis* (Smith & Hunter 2005) (reviewed in Gerhardt 1994 and Wilczynsky & Ryan 1999). However, when analysing geographic variation in anuran calls, special attention should be paid to the existence of different types of calls (Littlejohn 1976). Only calls of the same type should be considered for comparison, i.e. advertisement calls should not be compared to agonistic calls. Also, the effect of body size, temperature and habitat characteristics (e.g. vegetation, geographic relief, humidity) on call parameters should not be ignored in the analysis, as these variables have been shown to significantly affect calls, ignoring them could lead to incorrect conclusions.

The increasing number of studies on the genetic structure of species and phylogeography patterns, facilitated the study of call variation across species' biogeographical range (e.g. in warblers, *Phylloscopus trochiloides* – Irwin et al. 2001), showing a correlation (sometimes clear) between genetic and acoustic variation along the same range. Regional intraspecific variation in the anurans' call characters can be correlated with different habitats (e.g. Ryan & Wilckzynski 1991), but have proved to be related to genetic variation, under the assumption that acoustic variation should retain information about the phylogeography of the species (Wycherley et al. 2002). In some studies a correlation between genetic and behavioural variation was clearly observed. In the dendrobatid, *Colostethus palmatus*, endemic to the Colombian Andes, Bernal et al. (2005) examined whether call variation and RAPD products were correlated along a geographic transect. They found evidence for acoustic and genetic differentiation patterns between opposite slopes of the Cordillera Oriental, suggesting the existence of different lineages.

Another example of concordance between genetic and call variation is observed in the European pool frog, *Rana lessonae*, which has a wide distribution range in Europe, extending from France to Russia, including the Scandinavian countries and the UK (AmphibiaWeb 2012). Microsatellite analysis (Zeisset & Beebee 2001) of this species strongly suggests the existence of a distinct clade, geographically restricted to Scandinavia and Britain. This pattern was also found with regard to call parameters (Wycherley et al. 2002). One other positive case of this relationship has been described in canyon treefrogs (*Hyla arenicolor*), where Klymus et al. (2010) found that call differentiation was better explained by the three clade nuclear topology than that of the mitochondrial, with two distinct clades. This pattern does not reveal strong divergence among the clades, and, as the authors noted, the degree of call divergence may not be sufficient to promote isolation of the nuclear clades if they become sympatric.

However, this is not always the case. For the Amazon parrot, *Amazona auropalliata*, there is no correspondence between dialects and population genetic variation, as analysed via mtDNA and microssatelites, suggesting that regional vocalisations are maintained by selective pressures that promote some kind of learning of local call-types by juveniles and immigrants (Wright & Wilkinson 2001; Wright et al. 2005). As in other vertebrates, geographic variation of calls in anurans appears to correlate with genetic differentiation, but this is not always the case. In some cases, geographic distance is a better predictor of acoustic variation than genetic distance. In Túngara frogs (*Physalaemus pustulosus*), Ryan et al. (1996) examined call variation in relation to allozyme variation and geographic distances. The data revealed two genetically different lineages, which were further supported by later phylogenetic analysis based on mitochondrial COI sequence data (Weigt et al. 2005). Though the call parameters varied significantly among populations, those differences were better

explained by geographic distance than by the allozyme dissimilarity. These results were also corroborated by Pröhl et al. (2006), with microsatellite markers. Another study, with a South American frog, *Leptodactylus fuscus*, wanted to determine whether the call variation coincided with the previously known marked genetic differentiation (Wynn & Heyer 2001). Analysis revealed no concordance between call variation and genetic variation with geographic distance (Heyer & Reid 2003).

All these findings open the debate of whether the anurans' calls can only be transmitted genetically (i.e. a strong genetic determinism when compared with other vertebrates), meaning that their local adaptive variations are exclusively the result of phenotypic plasticity, or whether there can be some level of cultural transmission or brain processing. Consequently, the recently expanded discipline of bioacoustics focuses on the analysis of the relationship between population genetics and the biogeography of genetic lineages of related species, and of populations of the same species, in building genealogies. Equally interesting is to investigate species that are known to hybridise, as intermediate characteristics often seen in hybrids' progeny can give clues to the resolution of this enigma.

6. Natural and Evolutionary History of Hylidae Rafinesque, 1815

6.1. Taxonomy

Among the amphibians, the Hylidae is the second largest family, representing more than 13% of all known ~6,798 amphibian species (AmphibiaWeb 2012). With around 901 species distributed within 55 genera (AmphibiaWeb 2012), it is divided among three subfamilies: Pelodryadinae, Phyllomedusinae and Hylinae (recently revised by Faivovich et al. 2005 and Wiens et al. 2005). Colloquially known as treefrogs, most Hylid species are arboreal, and display several traits presumably adapted to arboreal habits (e.g. expanded adhesive toepads and intercalary phalangeal elements).

Hylids are in the suborder Neobatrachia, of the order Anura, sometimes called the 'advanced' or 'higher' frogs, which includes 95% of living Neobatrachian species. The Neobatrachia contain two well supported clades, the Ranoidea (the firmisternal frogs, including five families: Hyperolidae, Mantellidae, MicroHylidae, Ranidae and Racophoridae) and the Hyloidea (all other neobatrachians, which include Bufonidae, Hylidae, Leptodactylidae and Pseudidae families) (Darst & Cannatella 2004; Hoegg et al. 2004) (Fig. 1.3, Hyloidea in green). The Hyloidea group is primarily a New World clade, even though they can be found in Europe and in the Australopapuan region. It includes nine families:

Hylidae, Leptodactylidae, Bufonidae, Centrolenidae, Pseudidae, Dendrobatidae, Brachycephalidae, Myobatrachinae and Limnodynastinae. Morphologically, the Hyloidea is a poorly consistent group (Ford & Cannatella 1993), but it appears to be a monophyletic lineage based on molecular data (Hay et al. 1995; Ruvinsky & Maxson 1996; Feller & Hedges 1998; Darst & Cannatella 2004).

Since the 1970s, many authors have tried to clarify the Hylidae phylogeny in particular. Duellman (1970) arranged the family into four subfamilies: Amphignathodontinae (marsupial treefrogs), Hemiphractinae (helmeted treefrogs), Hylinae (classical treefrogs, including the Australian and American groups) and Phylomedusinae (leaf-breeding treefrogs). Amphignathodontinae was later synonymised with Hemiphractinae by Trueb (1974), a classification that was used until recently (see Faivovitch et al. 2005 and Wiens et al. 2005). The Australian Hylids were moved to their own subfamily, Pelodryadinae, based on evidence from Tyler (1971) and Duellman (1977). Ruvinsky & Maxson (1996) illustrated the polyphyly of the Hylidae by analysing relationships among all the Neobatrachia, based on mitochondrial 12S and 16S sequences. Their neighbour-joining tree showed that the monophyletic bufonids-group nested within the dendrobatids (Hemiphractinae). Polyphyly of Hylids has since been revealed, based on morphological data and molecular data; in these studies, no evidence of a relationship between Hemiphractinae and the other subfamilies was found (e.g. Salducci et al. 2002; Haas 2003; Daarst & Cannatella 2004).

With the goal of reviewing Hylid systematics, Faivovich et al. (2005) presented a major work of phylogenetic analysis for 228 species that included approximately 5100 base pairs from five nuclear and three mitochondrial genes, and a small data set from foot musculature. Wiens et al. (2005) used less molecular and more morphological evidence in their phylogeny, using 169 Hylid taxa, based on a combined data set of two mitochondrial genes and two nuclear genes, as well as 144 morphological characters. Later, Wiens et al. (2006) reconstructed a phylogeny for 124 Hylid species based on up to 10 genes (four mitochondrial and six nuclear; 7390 base pairs combined). The results of these three studies agree on many aspects. All three stress that the Hemiphractinae subfamily is not related to the other three Hylid subfamilies. This subfamily was subsequently removed from the Hylidae, now restricted only to the Hylinae, Pelodryadinae and Phyllomedusinae. The first two studies propose a new place for the Hemiphractinae: as part of the subfamily Leptodactylidae (Faivovich et al. 2005) – as previously suggested by other authors (Haas 2003; Darst & Cannatella 2004) – or as a separate family (Wiens et al. 2005). The three studies also agree on a major clade containing Pelodryadinae and Phyllomedusinae as sister groups. Moreover, they suggest that the monophyly of the Hylinae subfamily is supported by two synapomorphies (Duellman 2001), comprising several major clades, including some that were unrecognised in previous taxonomies. The grouping of these clades differs slightly among the

three studies. While all three agree that the Cophomantini clade is a sister group to all other Hylinae (i.e. the Boana clade – Wiens et al. 2005; the Hylini clade – Faivovich et al. 2005; the middle American clade – Wiens et al. 2005; the Lophiophylini or the Phrynohyas clade), no clear agreement exists with regard to the other clades. Faivovich et al. (2005) and Wiens et al. (2005) recognise Dendropsophini, including *Scinax*, *Sphaenorhyncus*, *Xenophyla*, *Dendropsophus* (formerly known as 30-chromosome *Hyla*) and the former family Pseudidae (*Lysapsus* and *Pseudis* genera). However, Wiens et al. (2006) include the genus *Dendropsophus*, *Xenophyla*, *Lysapsus* and *Pseudis* in the Dendropsophini clade, and the genus *Scinax* and *Sphaenorhyncus* in the Scinaxini clade.

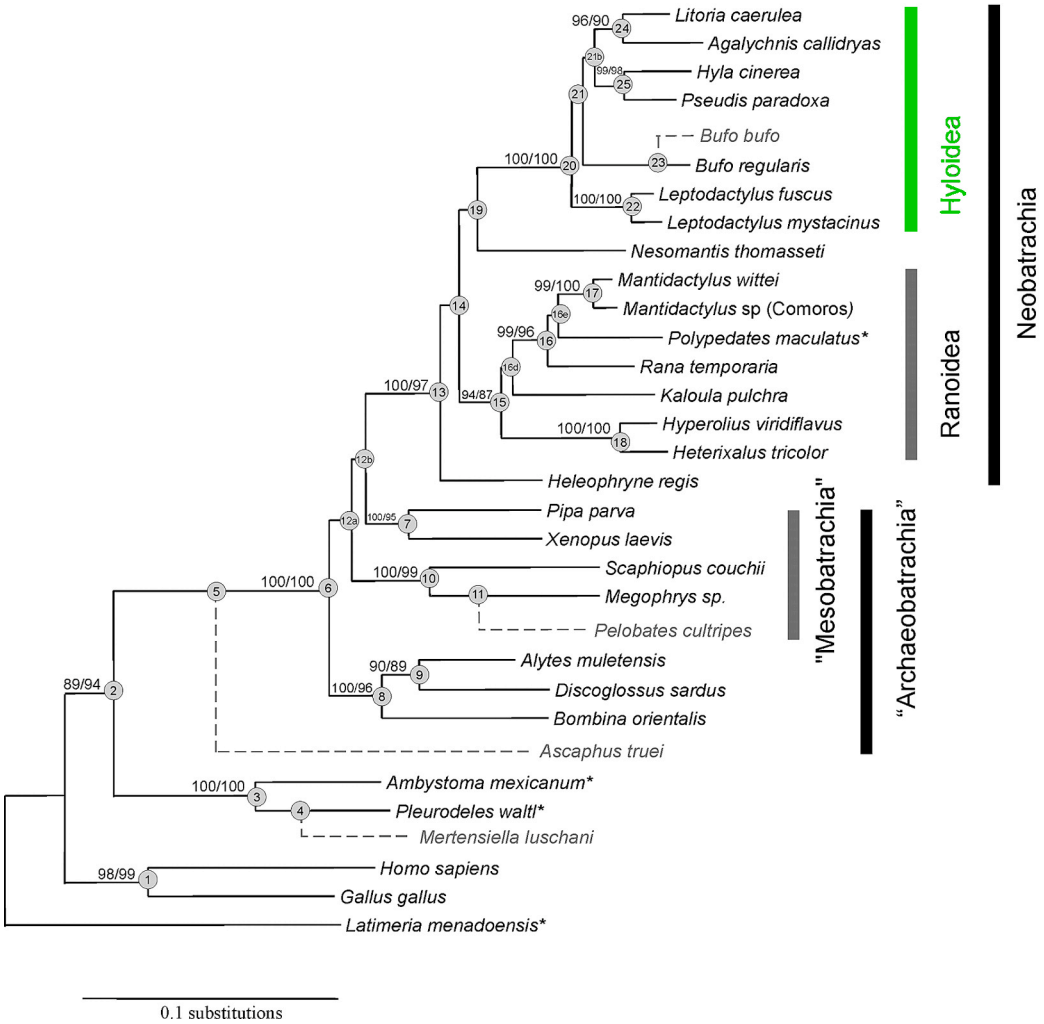


Figure 1.3. Phylogenetic tree of anurans.

Phylogenetic tree obtained from ML analyses of combined data sets (Rag-1, Rag-2, and rhodopsin). Nodes are numbered; values shown are the ML and MP bootstrap values from the nucleotide data set. Hyloidea group in green. Asterisks indicate hybrid sequences, composed of sequences from closely related species. Grey dashed lines indicate taxa that were added to the tree based on the analysis of only a subset of these sequences (adapted from Hoegg et al. 2004).

The most comprehensive phylogeny for Hylids, proposed recently by Wiens et al. (2010), is based on the maximum likelihood analysis of 362 putative Hylid species, including all genera in the family. The new likelihood-based phylogeny is, in general, better-resolved and supported than the previous ones, and its major input is an improved estimate of the Hylidae phylogeny, corroborating the previous major hypothesese of Faivovich et al. (2005) and Wiens et al. (2005, 2006). It is, therefore, suggested to divide the family Hylidae into three subfamilies: the Pelodryadinae, the Phyllomedusinae and the Hylinae. The first two are sister groups, and the third one, the Hylinae subfamily, is formed by three major clades: the Cophomantini, the Dendropsophini and the former Pseudidae family. However, despite this increased understanding regarding the Hylidae phylogeny, the authors agree that additional work is still needed to solve the problem of the relationships among the Hylidae taxa. European Hylids all belong to the Hylinae subfamily, and are included in a well-supported subclade (see Fig. 6 in Wiens et al. 2010).

6.2. Distribution

Hylids are present in almost all major continents, including North and South America, the West Indies, the Australo-Papuan Region, temperate Eurasia, extreme northern Africa and the Japanese Archipelago. They have been introduced into New Caledonia, New Hebrides (Vanuatu), Guam and New Zealand (Fig. 1.4), and comprise the majority of the species distributed in the Neotropics (Duellman & Trueb 1986; AmphibiaWeb 2012). The Pelodryadinae occur in Australia and Papua New Guinea, the Phyllomedusinae in Mexico through Central and South America; the Hylinae subfamily, with the widest distribution, occurs in North, Central, and South America, Eurasia and North Africa.

The New World Hylids show a typical latitudinal diversity gradient characteristic of many other vertebrates, such as birds (Blackburn & Gaston 1997), mammals (Kaufman 1995), freshwater fishes (Lappalainen & Soininen 2006) and Atlantic Ocean species (MacPherson 2002; Pianka 1966; Mittelbach et al. 2007). Many occur in tropical Central America (n ~ 162; Mexico to Panama) and tropical South America (n ~ 456; latitude ≤ 30ºS), with fewer in temperate North America (n ~ 28; United States and Canada) and South America (n ~ 22; latitude≥ 30ºS) (AmphibiaWeb 2012).

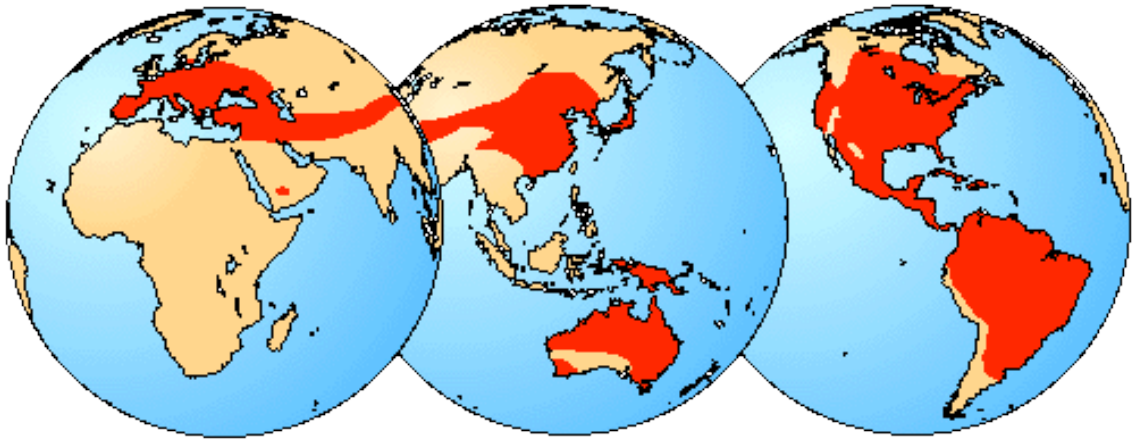


Figure 1.4. World distribution of Hylids.

Distribution map of the living members of the family Hylidae (in red) Notice that Northern Morocco, Tunisia and Algeria are not marked in red even though *H. meridionalis* is present there. (source: Tree of Life web project. http://www.tolweb.org/Hylidae).

6.3. Colonisations

Hylids are thought to have originated in the tropical regions, and to have spread to temperate regions more recently (Wiens et al. 2006). Based on morphological and biochemical data, the three Holarctic genera (e.g. *Acris*, *Pseudacris* and *Hyla*) were thought to have arisen from a single invasion of South American Hylids into North America (Anderson 1991). There would have been an expansion through California, eastwards to eastern North America and westwards across Beringia into Eurasia (Anderson 1991; Kuramoto 1991; Duellman 2001). In each of the colonised regions, Hylids would have undergone in-situ speciation, giving rise to groups of endemic species in North America (East and West) and Eurasia. More recent studies based on DNA sequence data, however, support a different Holarctic Hylid scenario (Faivovich et al. 2005; Smith et al. 2005; Wiens et al. 2005, 2006; Lemmon et al. 2007; Hua et al. 2009; Moen et al. 2009; Wiens et al. 2010): the Hylini clade may have had its ancestral area in Central America, and several colonising events would have taken place from there.

Two independent colonisations of North America are thought to have occurred by the *Acris-Pseudacris* clade, around ~30-43 Million years ago (Mya), and by the North American *Hyla*, ~33 Mya. The split between European and Asian *Hyla* would have occurred ~23-28 Mya, with the European Hylids diversifying at about ~21-25 Mya, whereas the Asian clade diversification would have been later, around ~18-22 Mya. The first colonisation of Asia by *Hyla* probably occurred around ~18-19 Mya, and its recolonisation from North America to the montane Middle America would have taken place at ~13.5-14.3 Mya. The *Hyla* species from Europe and Asia each form a monophyletic group. The second invasion of Asia would have had originated in the New World, as is corroborated by the hybridisation of *H. japonica*

with the western North American *H. eximia* group species (Faivovich et al. 2005; Smith et al. 2005; Wiens et al. 2006; Hua et al. 2009). Also supporting the idea of the double invasion of Asia by New World *Hyla* is the placement of *H. immaculata* and *H. tsinlingensis* into the Eurasia *Hyla* group (Hua et al. 2009). The paraphyletic Eurasia *H. arborea* group (which also includes *H. meridionalis*), forming a sister taxon with the eastern North American *Hyla* (i.e. a non-monophyletic group), could have had a western North American origin; however, Faivovich et al. (2005) disagree, arguing that a western North American origin would have required a shift in the distribution of the current eastern North American species group, an eastern North American-European vicariance or an assumed dispersal event, which is unlikely to have happened.

Among all genera of the Hylinae subfamily, only the *Hyla* genus is found outside the New World, and includes the *H. arborea* and the *H. japonica* groups, with 16 species currently recognised (Faivovich et al. 1995; Smith et al. 2005; Wiens et al. 2005; Stöck et al. 2008): *Hyla annectans* (Jerdon, 1870); *Hyla arborea* (Linnaeus, 1758); *Hyla chinensis* Günther, 1858; *Hyla hallowellii* Thomson, 1912; *Hyla immaculata* Boettger, 1888; *Hyla intermedia* Boulenger, 1882; *Hyla meridionalis* Boettger, 1874; *Hyla sanchiangensis* Pope, 1929; *Hyla sarda* (De Betta, 1853); *Hyla savignyi* Audouin, 1827; *Hyla simplex* Boettger, 1901; *Hyla tsinlingensis* Liu and Hu, 1966; *Hyla ussuriensis* Nikolsky, 1918; and *Hyla zhaopingensis* Tang and Zhang, 1984. Still excluded from this list is *H. molleri* (ancient subspecies of *H. arborea*), from Iberia, which has very recently been suggested for resurrection (Barth et al. 2011; Stöck et al. 2012). Palearctic species of *Hyla* have few morphological differences; in fact, their similarity in shape, size and colouration has made their classification difficult (Schneider 1974; Maxon & Wilson 1975; Maxson 1978; Hedges 1986; Nishioka et al. 1990). Molecular and acoustic (i.e. mating calls) data have been valuable in distinguishing among European and Asian species (e.g. Paillete 1969; Schneider 1974; Nevo & Yang 1979; Kuramoto 1980; Park et al. 1996; Gvozdík 2010). Immunological data were used to estimate the divergent time among Palearctic *Hyla* (Riehl et al. 1995): Asian *Hyla* are thought to have first diverged from the European *Hyla* ~24 Mya. Results from Riehl et al. (1995) suggest, as well, that *Hyla meridionalis* had diverged from *H. arborea* ca. 8 Mya, and that the European *H. arborea* and the Middle Eastern *H. savignyi* were more closely related to each other than to *H. meridionalis*, with an estimated time of divergence at ~6 Mya. These relationships had already been suggested by Schneider (1974) based on acoustic data.

6.4. The Holarctic Hylids

Middle American and Holarctic (from the Northern Hemisphere) Hylinae species were suggested to be monophylethic based on biogeographic evidence (Duellman 1970, 2001), and included the genera *Hyla*, *Acris*, *Anotheca*, *Duellmanohyla*, *Plectrohyla*, *Pseudacris*, *Pternohyla*, *Ptychohyla*, *Smilisca* and *Tripion*. Early studies divided the Holarctic Hyline treefrogs into several groups of species, based on external morphology, skin colour and pattern, osteology, karyotypes, mating calls, potential for interspecific hybridisation and larval characteristics (e.g. Blair 1959; Jameson et al. 1966; Duellman 1970; Ralin 1970; Jameson & Richmond 1971; Bogart 1973; Gaudin 1974; Trueb & Tyler 1974). Though most primitive Hylids, and almost all *Hyla*, have 26 chromosomes, and related genera, like *Acris*, have 24 chromosomes, many Neotropical *Hyla* have 30. Some species are polyploid, like the well-known tetraploid *H. versicolor*, with 48. Based on the karyotype, Bogart (1973) divided the *Hyla* genus into two major groups: 2n=24 and 2n=30. Immunological distances are lower among Holarctic species, suggesting closer inter-relationships compared with Neotropical or Australian Hylids (Hedges 1986).

Maxson & Wilson (1975) were the first to follow a phylogenetic approach for the systematics of the Holarctic Hylines, using albumin and morphological data. Their classification, based on organismal and albumine similarities (tables 1.1 and 1.2), agreed in many ways, suggesting, for example, that *Osteopilus*, *Pternohyla* and *Smilisca* are not as similar to Holarctic *Hyla* as the *Hyla* are among each other, and that the *Pseudacris* genus is more similar to Holarctic *Hyla* than to the other genera.

Table 1.1. Organismal classification of Holarctic Hyline treefrogs.
Phylogenetic classification of Holarctic Hylines based morphological data (adapted from Maxson & Wilson 1975).

	Genus or species
Group 1.	*Acris*
Group 2.	***Hyla*** and *Pseudacris*
Group 3.	*Limnaoedus ocularis*
Group 4.	*Osteopilus septentrionalis*
Group 5.	*Pternohyla fodiens*
Group 6.	*Smilisca baudini*

The discrepancies between organismal and molecular classifications found by Maxson & Wilson (1975) emphasise the occurrence of convergent morphological evolution. Phylogenetic analysis of the albumin data shows that *Hyla regilla* and *H. wrightorum*, though morphologically very similar, belong to two distinct protein lineages. These results were

corroborated by Case et al. (1975), whose data for protein-variation also suggested that *H. eximia* and *H. wrightorum* were more closely related than the latter is to *H. regilla*, *Limnaoedus* sp. and *Pseudacris* sp. The *Acris* group is another striking example: the cricket frog has been placed in a separate genus for its morphological, ecological and behavioural distinctness when compared with other Hylines. *Acris* is non-arboreal but semi-aquatic, and is osteologically distinct from all the other North American Hylines. It is the only genus among Holarctic Hylines to have 22 instead of 24 pairs of chromosomes; however, at the molecular level, *Acris* is a member of the Holarctic assemblage of *Hyla* lineages (Maxson & Wilson 1975).

Table 1.2. Classification of Hyline treefrogs based on albumin data.
Classification of Hyline treefrogs according to albumin similarities (adapted from Maxson & Wilson 1975).

		Genus or species
Group 1	Subgroup A	*Hyla andersoni*, *H. arenicolor*, *H. avivoca*, *H. chrysoscelis*, *H. cinerea*, *H. euphorbiacea*, *H. eximia*, *H. femoralis*, *H. gratiosa*, *H. squirella*, *H. Wrightorum*
	Subgroup B	***Hyla arborea*** (France, Israel and Japan)
	Subgroup C	*Acris crepitans*, *A. gryllus*
	Subgroup D	*Hyla crucifer*
	Subgroup E	*Pseudacris brachyphona*, *P. brimleyi*, *P. clarcki*, *P. feriarum*, *P. nigrita*, *P. ornata*, *P. streckeri*, *P. Triseriata*
	Subgroup F	*Hyla regilla*, *H. cadaverina*
	Subgroup G	*Limanoedus ocularis*
	Other Subgroups	*Anotheca spinosa*, *Hyla elaeochroa*, *H. pseudopuma*, *H. staufferi*, *Plectrohyla matudai*
Other Groups		*Hyla bifurca*, *H. bogotensis*, *H. crepitans*, *H. fasciata*, *H. favosa*, *H. lanciformis*, *H. leucophyllata*, *H. marmorata*, *H. parviceps*, *H. phlebodes*, *H. rhodopepla*, *H. rubra*, *H. sarayacuensis*, *H. triangulum*, *Litoria aurea*, *L. booroolongensis*, *L. caerulea*, *L. ewingi*, *L. verreauxi*, *Osteocephalus verrucigerus*, *Osteopilus septentrionalis*, *Phrynohyas venulosa*, *Pternohyla fodiens*, *Smilisca baudini*, *S. phaeota*, *Tetraprion jordani*

Based on the rate of albumin divergence observed in the Hyline frogs, Maxson & Wilson's (1975) calculated a rate of evolution of 1.7 units per million years, which supports the possible divergence of the Australian and American Hylines when the last land bridge existed between Australia and South America, across Antarctica, around 75 Mya (Table 1.2). A calculated divergence time of ~65 million years, between the North American and South American *Hyla* species, also corresponds to the disappearance of the land bridge between

North and South America, which reappeared ~5 Mya. Moreover, Paleartic *Hyla* (the Paleartic region includes northern Africa and all Eurasia, excluding southeast Asia and the Indian continent) are thought to have derived from a lineage that diverged from those leading to the North American Hylines, about 40-Mya, which entered into Eurasia from North America around that time. The oldest known Hylid record for Europe was described by Sanchiz (1998). From the early Miocene (~17-23 Mya) lignite deposits in Austria, the discovered remains were found to be similar to the living *H. arborea* and *H. meridionalis*, and were assigned to *Hyla* spp. fossils from later periods (i.e. those from the late Miocene, and from the Pliocene to the Holocene) have been assigned to *Hyla* spp., *Hyla arborea* and to some other extant *Hyla* species.

In the late 1980s, with the available technique of starch gel electrophoresis, Hedges (1986) examined the phylogenetic relationships of 30 taxa of Holarctic Hylid frogs. He identified two major clades: one grouping together *Hyla regilla*, *H. cadaverina*, *H. crucifer*, *Limnaoedus oculars* and all the species of the genus *Pseudacris* recognised at the time; the other clade including all the remaining species of the Holarctic genus *Hyla*. However, the location of *Acris crepitans*, *A. gryllus* and *Hyla meridionalis* remained unclear. The two groups defined by Hedges (1986) were also supported by previous analysis based on albumin immunological distance, karyotypical data, mating calls, morphological characters related to toe-pads and ecological characters. In the same study, the author proposed taxonomic changes in order to produce a classification of Holarctic Hylid frogs in agreement with the phylogenetic relationships that he had found. Accordingly, *Hyla cadaverina*, *H. regilla*, *H. crucifer* and *L. ocularis* should be placed in the *Pseudacris* genus.

Later on, Hedges' (1986) allozyme and morphological data were reanalysed – firstly by Highton (1991), who corroborated most of his main results, including the polyphyly of *Hyla* genus, and secondly by Crocroft (1994), who proposed to bring the *crucifer*, *cadaverina* and *regilla* taxa back into the *Hyla* genus. The discussion about these taxa has far from reached a consensus, and da Silva (1998) agreed to return these species to the *Pseudacris* genus, arguing, nonetheless, that their phylogenetic position was consistent with the placement in either genus. Moriarty & Cannatella (2004) strongly support the monophyly of the *Pseudacris* genus, and definitely (until now) included *crucifer*, *ocularis*, *cadaverina* and *regilla* – as primarily proposed by Hedges (1986). According to Hedges, the calibration of the molecular clock for the Holartic Hylids suggests that *Pseudacris* diverged from a *Hyla*-like ancestor in the early Tertiary – probably between the Eocene (~50 Mya) and the mid-Oligocene (~27 Mya).

In summary, the speciose and polyphyletic genus *Hyla* has been dismantled and redefined (Faivovich et al. 2005) as a smaller group of North American, Central American

and European Hylids. This major review of the group has been supported in subsequent studies (Smith et al. 2005, 2006, 2007; Wiens et al. 2005, 2006, 2010; Hua et al. 2009), but has not yet completely cleared up the relationships within *Hyla*, in which several ambiguities still exist. From the 353 of the former '*Hyla*' species, 297 were placed in 15 different genera. Among these, four have resurrected names (e.g. *Dendropsophus* [former 30-chromosome *Hyla*], *Exerodonta* [*Hyla sumichrasti* group], *Hyloscirtus* [*Hyla armata*, *H. bogotensis* and *H. larinopygion* groups] and *Hypsiboas* [the Gladiator frogs group]). A further four are currently recognised (*Aplastodiscus*, *Plectrohyla*, *Ptychohyla*, *Hyloscirtus*) and seven are new (*Bokermanohyla Myersiohyla*, *Isthmohyla*, *Megastomatohyla*, *Charadrahyla*, *Bromeliohyla*, *Ecnomyohyla*). The *Hyla* genus is now restricted to *H. femoralis* and the *H. arborea*, *H. cinerea*, *H. eximia* and *H. versicolor* groups, whose contents have also been adjusted. The latest works combining the analysis of mitochondrial and nuclear genes support the monophyly of the *Hyla* genus (Smith et al. 2007a, b; Hua et al. 2009), identifying five major species groups: *H. arborea*, *H. japonica*, *H. cinerea*, *H. versicolor* and *H. eximia*. In all studies, *H. meridionalis* is included in the *H. arborea* group, but not without questioning their relationship; this means that *H. meridionalis* appears to be more closely related to the Asian *Hyla* than to the European ones.

6.5. The Iberian Hylids: *Hyla meridionalis* and *Hyla arborea*

Distribution, Biology and Phylogeography

Currently, there are two species of Hylids recognised in the Iberian Peninsula: *H. meridionalis* (stripeless treefrog) and *H. arborea* (European treefrog).[2] Both species also occur outside the Iberian Peninsula and have been the focus of several studies there and beyond (e.g. Paillette 1967, 1967a; Schneider 1982; Rodríguez-Jiménez 1986; Etxezarreta & Rubio 1998, 2002; Friedl & Klump 2002; Sillero 2010). Their geographic distribution is well known. *Hyla meridionalis* is found in the northwest of Africa – especially the humid regions of the Mahgreb, including the Middle and High Atlas – in southern Europe (Iberian Peninsula, south of France and northwest of Italy); it was introduced into the Canary Islands and Menorca (Balearic Islands) (Pleguezuelos et al. 2002; Crespo 2008) (Fig. 1.5). It survives at altitudes of 2650 m (Schleich et al. 1996).

[2] As mentioned before, the resurrection of the *Hyla molleri* taxum has recently been proposed in the Iberia Peninsula (Stöck et al. 2008; Barth et al. 2011) as a different species from the congeneric *Hyla arborea* occupying the rest of Europe.

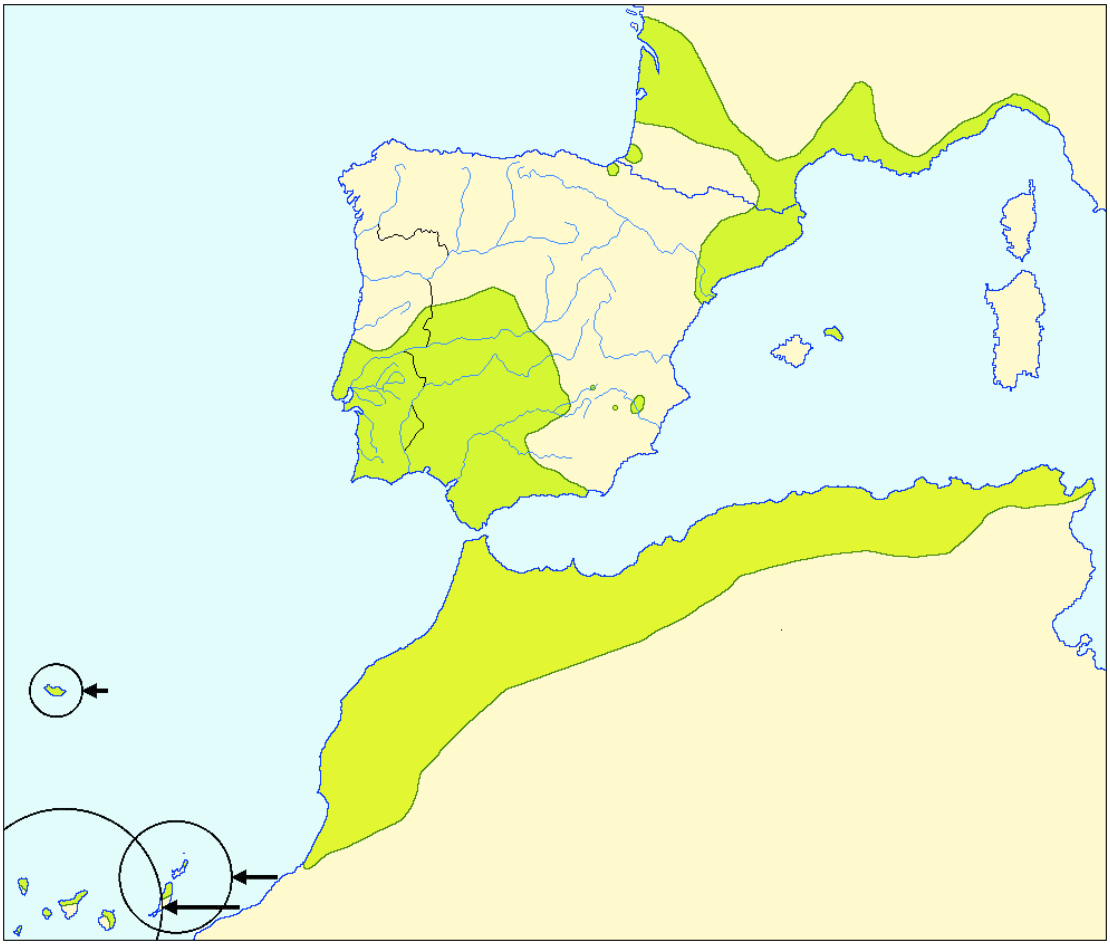


Figure 1.5. *Hyla meridionalis* geographic distribution.

The areas in green correspond to the areas of occurrence of the species. Canary islands and Madeira islands were brought closer to the African continent (in black circles) (source: IUCN [International Union for Conservation of Nature], Conservation International and Nature Serve, 2011).

Hyla arborea is found almost all over Europe, from the Atlantic coast of Portugal to the south of Sweden, in the Ukraine, Azerbaijan and another 33 European countries. It is absent from the eastern and southern parts of the Iberian Peninsula, southern France and most of the Scandinavian area. It is reported to have been introduced to the United Kingdom as well as to Madeira, but in both areas it is now extinct (Barbadillo et al. 1999; Pleguezuelos et al. 2002; Oliveira & Pargana 2008; Rosa & Pargana 2008; AmphibiaWeb 2012) (see maps of geographic distribution range, Fig. 1.6).

The distribution of these two species in the Iberian Peninsula has been systematically observed over the past four decades (Fig. 1.7). These data were recently compiled in the Spanish and Portuguese edition of the *Atlas of Amphibians* (Pleguezuelos et al. 2002 and Loureiro et al. 2010, respectively), which provides a more detailed picture at the regional and local scale. Accordingly, *H. meridionalis* occupies the southern, southwestern and northeastern regions of Iberia, with a few reported isolated populations in the southwestern

part of the Mondego River valley, near Figueira da Foz (Portugal) (Oliveira et al. 2002), in San Sebastian (Basque Country, Spain – probably resulting from human introduction) and in the Atlantic French Pyrenees region of the Landas and Hasparne (Paillete 1989). *H. arborea* occurs in the western and northern regions of the Iberian Peninsula, and is absent from the eastern and southern parts (for a more detailed distribution see Pleguezuelos et al. 2002; Loureiro et al. 2010; AmphibiaWeb 2012).

Figure 1.6. *H. arborea* geographic distribution.

The areas in green correspond to the areas of occurrence of the species (source: IUCN [International Union for Conservation of Nature], Conservation International and Nature Serve, 2011).

Their distribution range largely overlaps in southern France, Spain and Portugal (Paillette 1967; Pleguezuelos et al. 2002), with many areas of strict syntopy both in Spain (e.g. in the Tiétar Valley, the Sierra Morena, in Badajoz and Guipúzcoa – Pleguezuelos et al. 2002) and in Portugal (e.g. Porto de Mós, Loures, Santiago do Cacém and the São Mamede region – Crespo 1972; Malkmus 1982, 1995; Oliveira et al. 1991; Tejedo & Reques 2002; in the lower Mondego River valley – Oliveira et al. 2002; Sillero & Carretero 2007; Moreira Pers. Obs) (see map of Iberian and Portugal distribution of the species, figs. 1.7 and 1.8).

Hyla meridionalis' geographic distribution extends across a greater area than *H. arborea*, with more arid environmental conditions, such as the southern Iberian Peninsula and Morocco. The habitat conditions maybe reflected in their ecology and physiology, i.e. some traits may be more adapted to such hot and dry environments. For example, the reproductive season usually starts and finishes earlier than *H. arborea*'s reproductive season, and in Morocco is shortened to only about two months (Pargana et al. 1998; Friedl & Klump 2002), making them exploit the availability of water and avoid the drier periods. Also, in Hylids,

skin pigmentation can be related to physiological characteristics, namely as an adaptation to the environment conditions and to improve thermoregulatory efficiency (Vences et al. 2002).

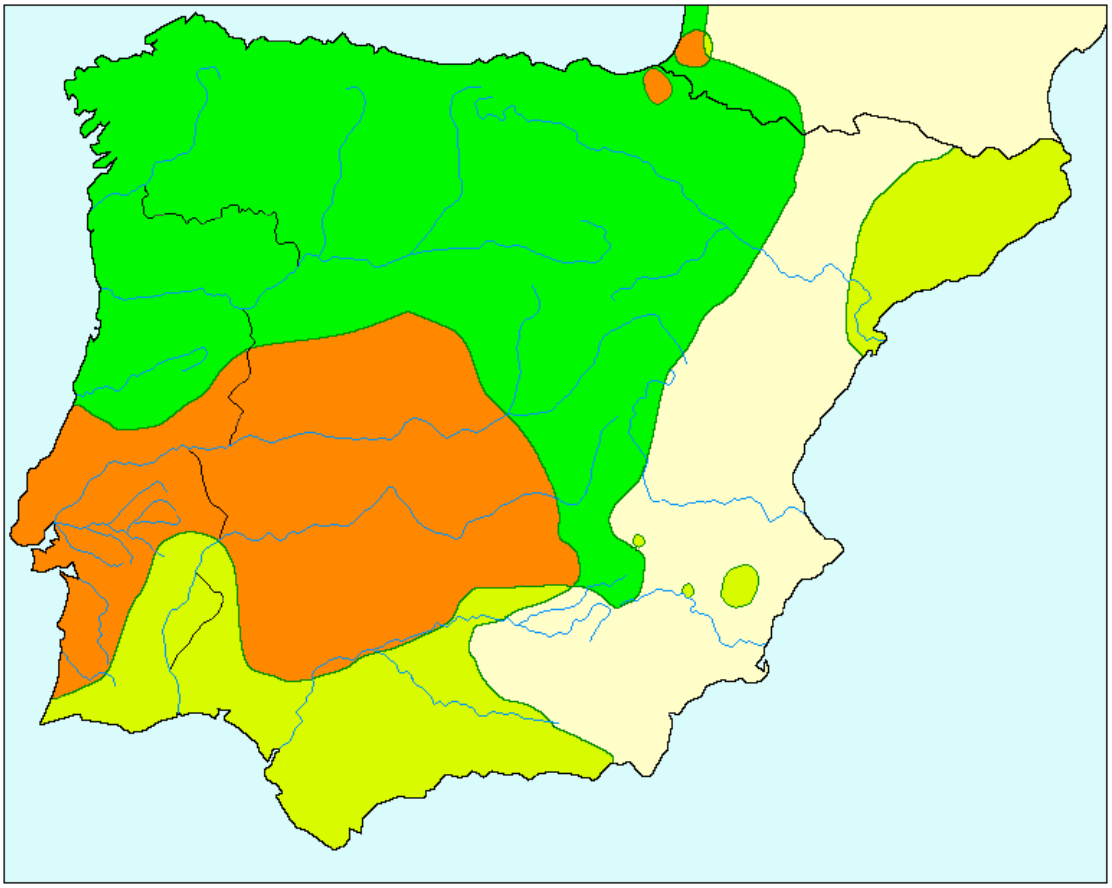


Figure 1.7. Iberian distribution of *Hyla meridionalis* and *H. arborea*.

Map of Iberian Peninsula *H. meridionalis* distribution range, *H. arborea*, and overlaping sympatric area (adapted from IUCN, Conservation International and Nature Serve; Pleguezuelos et al. 2002; Loureiro et al. 2010. Rivers of Iberia from mapsof.net).

H. meridionalis, with a more arid distribution area than *H. arborea*, exhibit black-coloured testes, whereas *H. arborea* males have yellow ones; this colouration could be an adaptation to protect them from stronger U.V. light. Lateral stripes, which are usually absent in the stripeless treefrog (*H. meridionalis*), are, however, present in some individuals on the Canary Islands (Dufresnes et al. 2011), but the authors could not conclude why these unusual phenotypes occur, suggesting it might be age-related (they observed it mostly in juveniles) or a microevolution of skin patterns in the archipelago. Physiological and ecological research is needed to understand these variations and the role of darker versus lighter pigmentation of the testes in these two species.

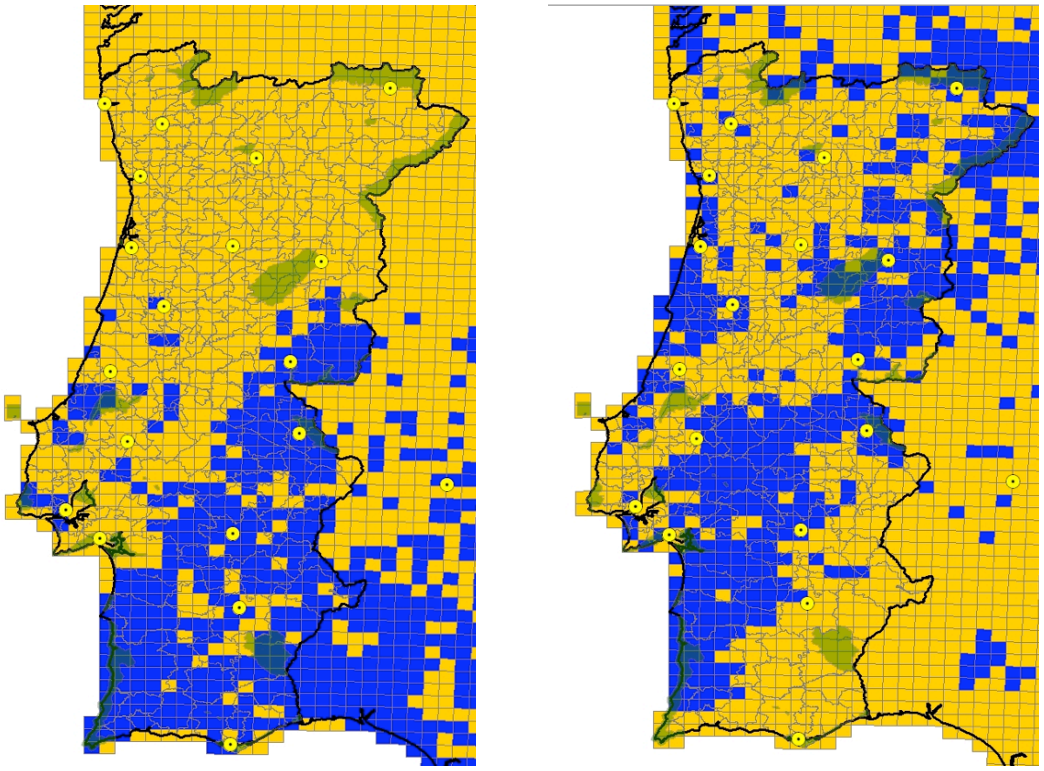


Figure 1.8. Distribution of *Hyla meridionalis* and *Hyla arborea* in Portugal.
Distribution of *H. meridionalis* (left) and *H. arborea* (right) in Portugal by quadricles of 10x10 km. Yellow with dark central dot-circles correspond to main cities. Blue squares mean presence, and yellow squares absence of the species (source: Loureiro et al. 2010).

Morphologically, the two Iberian species are very similar. They are usually recognisable by the lateral band extending from the nostril to the hind limbs and the inguinal loop; generally, both traits are present in *H. arborea* and absent in *H. meridionalis* (see photos, Fig. 1.9). However, the presence and shape of the lateral band should not be taken as absolute criteria to distinguish the two species, since occasionally individuals of *H. arborea* do not have the full-length band (Pinston & Craney 1991 and Dufresnes et al. 2011 reported *H. meridionalis* individuals with stripes in Alegranza, in the eastern Canary Islands). Moreover, variations in band colour, length and width have been observed among the two species. Also, variation of the inguinal loop in *H. arborea* is quite high (see photos of some extreme examples found in Portugal, Appendix 1, Moreira Pers. Obs.); in some cases, these can be very dark and conspicuous, while in others it is barely noticeable. Another example of a potential band-reading error is the intermediate phenotype found in the hybrids described in the literature (Fig. 1.10).

Figure 1.9. Males from *Hyla meridionalis* and *H. arborea*.

H. meridionalis (left) and *H. arborea* (right) males with inflated vocal sac (black arrows) during calling activity in Portalegre, Portugal. The *H. arborea* male has the characteristic lateral band and inguinal loop of the species (black arrows) (photos: C. Moreira).

H. arborea have low size-dimorphism between sexes for individuals of same age, with a female-male SVL ratio of ~1.12 in Spain (Márquez & Tejedo 1990) and slightly lower, ~1.02, in Portugal (Friedl & Klump 1997). On the other side, among *H. meridionalis*, females are generally larger than males (no ratio available), and both males and females are on average larger than *H. arborea* individuals.

Hyla arborea and *H. meridionalis* have a prolonged breeding season (*sensu* Wells 1977), which depends greatly on water availability and environmental temperatures (mainly air temperature) (Paillette 1967, 1969; Blackenhorn 1972; García et al. 1987; Díaz-Paniagua 1992). In central Europe, *H. arborea* breed from early March to late July (Friedl & Klump 2002) and *H. meridionalis* from late February to late June or early July. In North Africa, they have a shorter season, breeding from March to April (Schleich et al. 1996). In Portugal, in the region of S. Mamede, Portalegre, Pargana et al. (1996) reported that chorusing activity starts in February and lasts until April for *Hyla meridionalis*, and May for *H. arborea*, but longer breeding seasons, lasting until June, have also been described for both species (Moreira 2003). The two species' breeding seasons thus show significant temporal overlapping. Looking for better breeding areas, treefrogs can move from one pond (or any other type of water body) to another within one breeding season (reviewed in Fog 1993), depending on habitat quality and availability. In *H. arborea*, some individuals studied had migrated more than 40 km (Anderson et al. 2004).

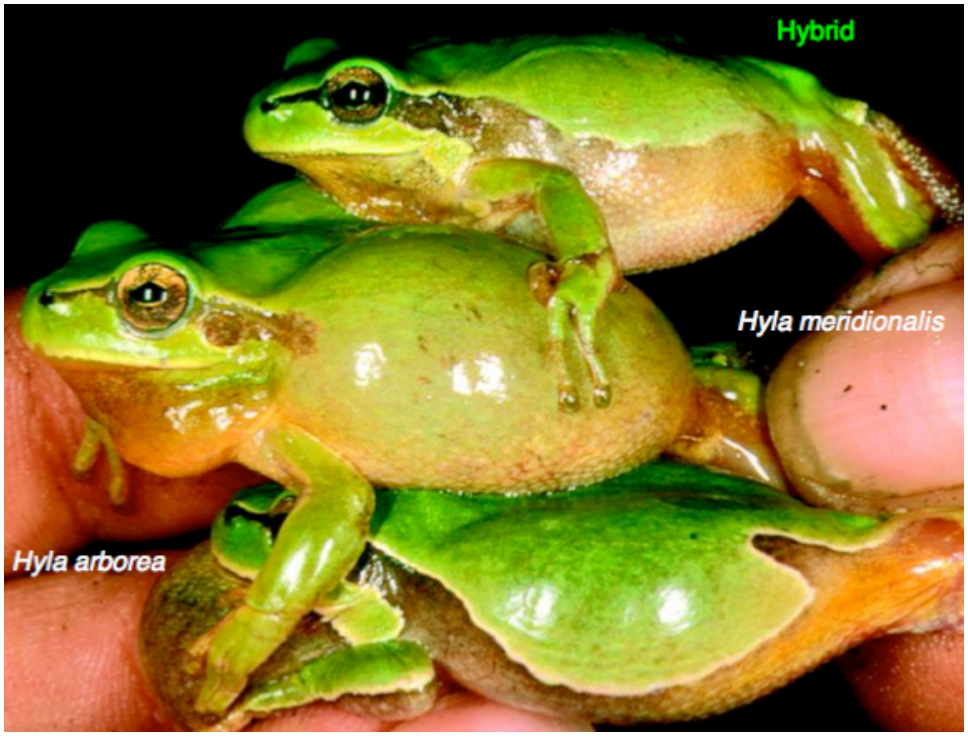


Figure 1.10. *Hyla meridionalis* and *H. arborea* hybrid male.
Hybrid male, *H. meridionalis* and *H. arborea* males (from top to bottom) from Candeleda, Ávila (Spain) (adapted from Barbadillo & Lapeña 2003). The lateral band and the inguinal loop are present in the *H. arborea*, absent in the *H. meridionalis* and intermediate in the hybrid.

For a long time, these two species were thought to be one species – *Hyla arborea* – with a wide geographic distribution in the Paleartic region and very subtle morphological differences. Six subspecies of *H. arborea* were initially identified: *H. a. arborea*, *H. a. molleri*, *H. a. kretensis*, *H. a. sarda*, *H. a. schelkownikowi* and *H. a. meridionalis* (Boettger 1874; Mertens & Wermuth 1960). Subsequently, some of these subspecies were accepted as distinct species, such as *Hyla meridionalis* (Paillette 1967; Schneider 1968) and *H. savigny* (Schneider & Nevo 1972). Though *Hyla meridionalis* had been considered a valid species previously (Boscá 1880; Héron-Royer 1884; Chaplin 1950; Chaplin & Lester 1954), it was not until *H. arborea* and *H. meridionalis* were shown to be well-differentiated, through the work of Paillette (1967) and Schneider (1968) in bioacoustics and of Crespo (1972) and Schenkel-Brunner & Kothbauer (1978) in morphology and immunology, that it gained its current species status. Hedges (1986) also found that *H. arborea* and *H. meridionalis* were not closely related. Additionally, Rosa & Oliveira (1994), using allozyme electrophoresis data and artificial hybridisations attempts, further corroborated the idea that these taxa have undergone substantial genetic divergence (Nei's genetic distance D=0.725). Based on immunological data, the divergence time is of ~8 million years (Riehl et al. 1995). This accentuated divergence between *H. meridionalis* and *H. arborea* and the other species of the

H. arborea group has been consistently recognised in many studies (e.g. Kuramoto 1984; Hedges 1986; Highton 1991; Rosa & Oliveira 1994; Riehl et al. 1995; Rosa 1995; Smith et al. 2005; Faivovich et al. 2005; Stöck et al. 2008).

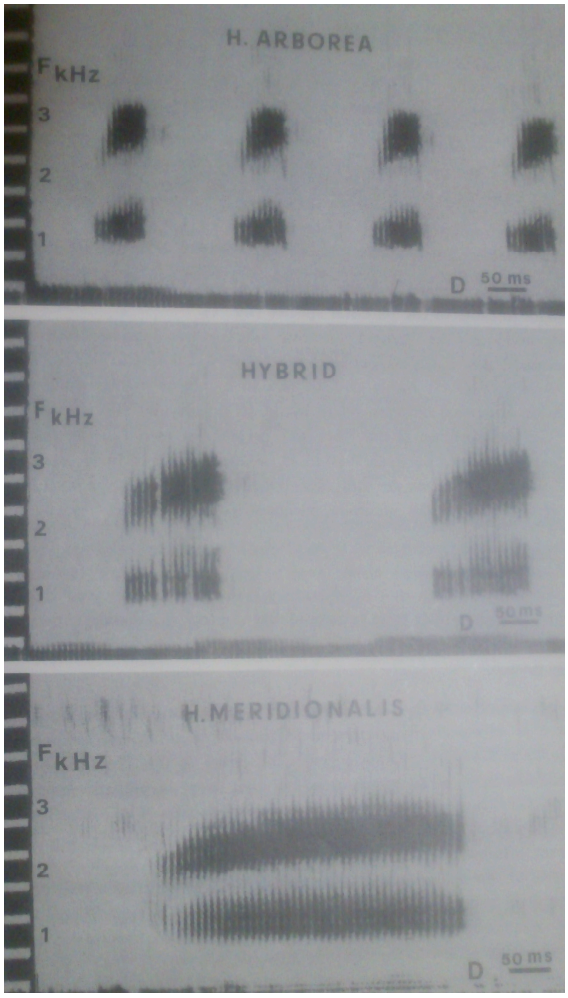


Figure 1.11. Advertisement calls of *Hyla arborea*, *H. meridionalis* and their F1 hybrid.
Sound spectrograms of the of the advertisement calls of *Hyla arborea* (top), *H. meridionalis* (bottom) and their presumptive natural hybrid. F – frequency and D – duration. (Adapted from Oliveira et al. 1991)

Nevertheless, natural hybridisation between these two species exists (Oliveira et al. 1991; Barbadillo & Lapeña 2003). The first natural hybrid, which was described by Oliveira et al. (1991), was detected by its 'unusual tree-frog mating calls, intermediate between those of the *H. arborea* and *H. meridionalis*' in a pond in Alpalhão, Northern Alentejo, Portugal (Fig. 1.11). The authors analysed the mating-call structure, the allozyme variation (eight loci) and the structure of the testes. They found that the hybrid was most probably an F1, with intermediate characteristics of the mating calls, heterozygosity of all the discriminative loci and testes that were atrophied, indicating sterility. Natural hybrids were also reported in

individuals found in Spain (Barbadillo & Lapeña 2003). The authors observed four interspecific amplexus, all of them from a male of *H. arborea* with a female of *H. meridionalis*. They captured seven hybrid males, from the Castilla-León, Castilla-La Mancha and Extremadura autonomous communities in Spain, and subsequent histological analysis of the testes revealed that all of them were sterile. The sterility of the natural hybrids seemed to corroborate the lack of evidence of introgression in the frogs captured in sympatric areas (Rosa & Oliveira 1994; Rosa 1995), meaning that F2 descents or beyond would very rarely or never occur. Even though natural hybrids appear to be relatively rare, and those captured were always identified as being of F1 descent, it is not uncommon to watch heterospecific amplexus (i.e. a male and a female from different species) (Crespo, pers. comm.).

In the last five years, several phylogeographic studies regarding *Hyla arborea* and *H. meridionalis* have been published. Stöck et al. (2008) studied the phylogeography of the *Hyla arborea* group around the Mediterranean (Fig. 1.12), and Gvozdíc et al. (2010) focused on the Middle Eastern *Hyla*. Recuero et al. (2007) presented a biogeography analysis for *H. meridionalis* (Fig. 1.13). Within the *H. arborea* species group, Stöck et al. (2008) identified three strongly divergent mitochondrial lineages, well supported by a highly conserved nuclear gene (RAG1-fragment). Yet they are not necessarily the closest relatives of each other under the mitochondrial topology (but not the nuclear), rendering *H. arborea* a paraphyletic species group. Their results also supported the status of *H. sarda* and *H. intermedia* as valid species: *H. intermedia* is reproductively isolated in the zone of parapatry from *H. arborea*, with no F1 and F_2 hybrids nor backcrosses found, even though introgressed alleles were observed in both species, testifying to the occurrence of introgressive hybridisation in the past (Verardi et al. 2009); *H. sarda* exhibits well-described morphological and bioacoustic differences from *H. arborea* (Schneider 1974; Lanza 1983; Rosso et al. 2001, 2004; Castellano et al. 2002). Also, from the results of Stöck et al. (2008), *H. intermedia* was found to have two well-supported subclades, based on mitochondrial DNA data: a southern Apennine Peninsula and Sicilian subclade, and a Switzerland subclade, which have a direct correspondance to the most southern and northern main mitochondrial lineages recognised by Canestrelli et al. (2007a).

Stöck et al.'s (2008) unnamed clade from Switzerland, based on the allozyme data of Canestrelli et al. (2007b), did not diverge enough from the central-south group to be considered a valid species; Nei's distance was 0.07, whereas Verardi et al. (2009) found a value of Nei's distance of 0.55 between *H. arborea* and *H. intermedia*. The geographic morphological variation between the Middle Eastern Hylids *H. savignyi* and *H. arborea* is less significant than within their conspecific populations from their most distant regions; it took Gvozdík et al. (2008) to suggest that this similarity could be driven by a convergent response to the environment.

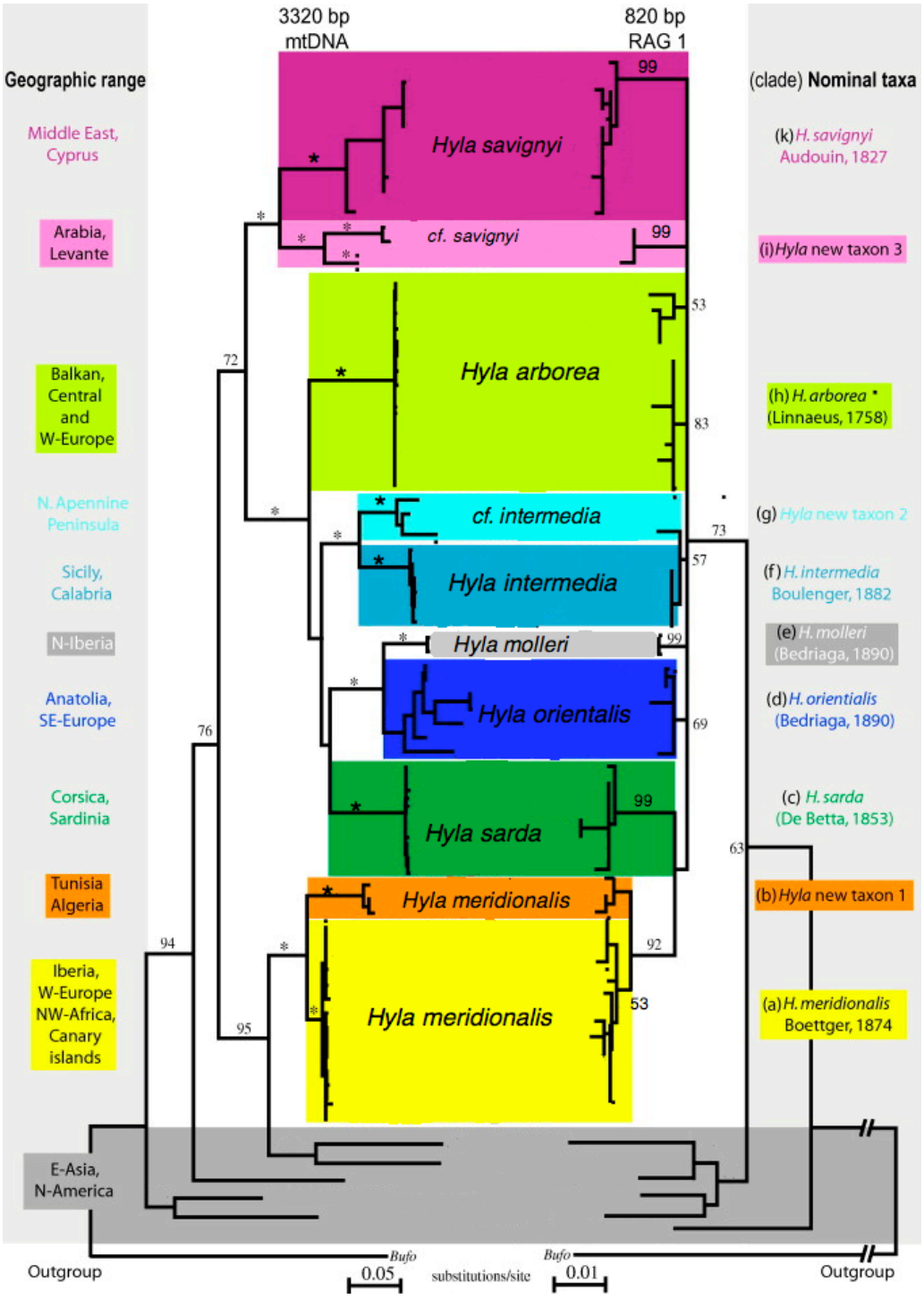


Figure 1.12. ***Hyla arborea*** **species group phylogeny.**

Tree derived from maximum likelihood analysis of mitochondrial DNA COI, Lys, ATP6, ATP8, COIII, Cyt *b* genes and nuclear DNA RAG1 gene, with bootstrap values for *Hyla arborea* group. (adapted from Stöck et al. 2008) (see original paper for details).

Also, the lineages defined by mitochondrial and nuclear data (Stöck et al. 2008; Gvozdík et al. 2010) have no correspondence with any morphological and/or acoustic identified

subgroups (e.g. Scnheider & Nevo 1972; Gvozdík et al. 2008; Kaya & Simmons 1999). Moreover, there are no obvious morphological and/or acoustic differences between the pairs Iberia *molleri* and Central European *arborea*, and *orientalis* and *arborea* (Scnheider 1974, 2002). Nevertheless, strong evidence of mitochondrial data (but not nuclear, once again) for *Hyla orientalis* (from Asia Minor and Eastern Europe) and *Hyla molleri* (from the Iberian Peninsula) have seen them resurrected as valid species by Stöck et al. (2008). Barth et al. (2011) also assert that *H. molleri* (Iberian Peninsula) has a separate status from *H. arborea* (central Europe) and, based on COI and 16S rRNA sequences, they have observed within the *H. molleri* Iberian populations a low genetic variation along with low phylogeographic structure.

Hyla meridionalis, whose phylogenetic relationships with the other *Hyla* species have been difficult to unveil, has recently had its biogeographical history more clarified by Recuero et al. (2007) (Fig. 1.13). Across the various published phylogenetic works, *H. meridionalis* has been placed closer to the Asian taxa than to the other European *Hyla* (e.g. Maxson & Wilson 1975; Smith et al. 2005; Stöck et al. 2008). Recuero et al. (2007) studied the patterns of genetic variation of *H. meridionalis* across its geographic range, and identified three mitochondrial, well-differentiated clades, but all with little within-clade genetic diversity: one distributed in southwestern Iberia, the High Atlas, Anti-Atlas and the Massa River in Morocco; a second restricted to the Medium Atlas Mountains; and a third extending from northern Morocco, northeast Iberia, southern France and the Canary Islands (where the species has been introduced). A fourth group, highly divergent from all other lineages, included the Tunisian, suggesting that an ancient split may have occurred between ~2-12 Mya (during the Pliocene) – this being more likely the older scenario. Stöck et al. (2008) found two major clades that coincided with Recuero et al.'s (2007) groups: the Tunisian group, also including new samples from Algeria; the other group – which combined both the first and second groups identified by Recuero et al. (2007) – from Iberia and northern Morocco, as described above. The central Moroccan clade revealed by Recuero's et al. (2007) was not identified in their study.

The low haplotype diversity and the simultaneous presence of the same haplotypes on both sides of the Gibraltar Strait are consistent with the allozyme and mitochondrial DNA data previously published by Busack (1986) and Busack & Lawson (2008). This consistency suggests dispersal events after the last opening of the Strait of Gibraltar (around 5.3 Mya), a pattern also seen in other terrestrial vertebrates (e.g. the snake *Macroprotodon brevis* – Carranza et al. 2004; the rodent *Crossidura russula* – Cosson et al. 2005). Moreover, Recuero et al. (2007) found higher haplotype diversity within the Moroccan than within the European populations, and Busack & Lawson (2008) found the same pattern for morphological

differentiation, suggesting a founder effect in the studied Spanish populations (again a similar pattern to the one seen in *M. brevis* – Carranza et al. 2004).

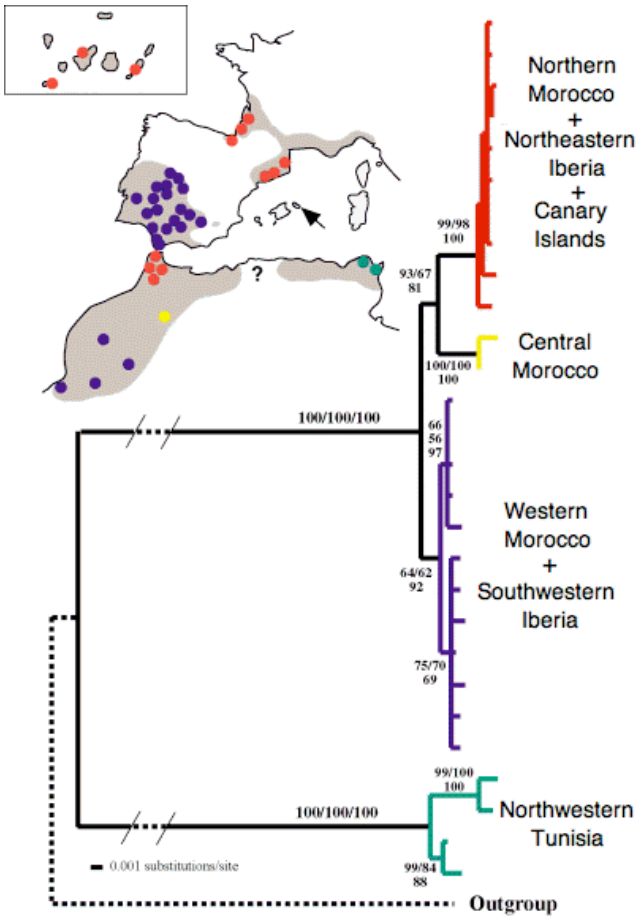


Figure 1.13. ***Hyla meridionalis*** **phylogenetic tree.**
Maximum likelihood tree of *H. meridionalis* showing the different lineages found in the phylogenetic analysis and map of the distribution of the respective haplotypes and lineages (adapted from Recuero et al. 2007).

This means that the south-west of Europe and the Canary Islands would have been recently colonised from Moroccan individuals of *H. meridionalis*. The colonisation of southwest Europe is thought to have occurred in two events: 1) from Northern Morocco to the Mediterranean coast of France; 2) from the western coast of Morocco to Southern Iberia, in both cases either via natural means or by human-mediated transport (Recuero et al. 2007).

Mating Behaviour and the Advertisement Calls of Males of the Iberian Hylids

Both species have a lek mating strategy, i.e. the males aggregate around water bodies, forming choruses during the mating period that can be heard from long distances (at least 1-2 km for large choruses, often with 50 males calling at the same time – Moreira, pers. comm.).

Males of both species are territorial during the mating period: *H. arborea* males have an exclusive calling area (Brzoska & Schneider 1982), and *H. meridionalis* males, although described as calling within 5 cm of each other (observation at an artificial reservoir), frequently attempt to clasp neighbour males (Gerhardt & Schneider 1980). Occcasionally, males interact aggressively, either by physical contact, such as jumping against other males, or by emitting aggressive calls. The males of both species have similar mating behaviour, calling from the water near shore, often anchored to emergent vegetation. Usually, *H. arborea* males stay close to shore (Márquez & Tejedo 1990), whereas *H. meridionalis* frequently call from the middle of the ponds (even when the ponds are wider than 5 m – Moreira pers. obs.). In *H. arborea*, the amplexi are formed strictly on female initiative (Friedl & Klump 2005), while in *H. meridionalis* this does not seem to be the rule (Gerhardt & Schneider 1980; Moreira, pers. comm.).

Daily calling activity is highly influenced by air temperature and light. In Germany, the temperature thresholds for nightly calling for *H. arborea* are a mean of 4.5ºC of air temperature and 9ºC-22ºC of water temperature, and an average light intensity of 171 lux (Schneider 1993; Friedl & Klump 2002). For *H. meridionalis*, Schneider (1974) describes calling activity at 6ºC. Calling activity usually starts a couple of hours before sunset and continue sporadically until dawn, with an activity peak around midnight-1 a.m. (Schneider 1974; Moreira 2003, unpubl.). The chorus is usually initiated by one or two males, and then followed by several others, with occasional breaks in the activity until a new set of calls begins (Paillette 1969a, b). Their reproductive season overlaps for most of its length, from February to April or May (Pargana et al. 1996), for *H. meridionalis* and *H. arborea*, respectively; the calling season can extend until June for both species (Moreira, unpubl.). Besides sporadic observations in the field, to my knowledge there are no studies about reproductive phenology of these two species – for example, knowing if the occurrence of amplexi and clutches in the two species happens simultaneously, or if there is a delay for one of the species (in relation to the other). Other crucial phenomena that should be taken into account is if the gonads develop at the same time, or if, again, one species is delayed in relation to the other. The occupation of temporal and physical space by the individuals when the two species are in syntopy is of extreme importance to understand the possibility of hybridisation.

The advertisement calls, as discussed earlier, are often species-specific. These two *Hyla* species are not exceptions; their calls are different temporally and in frequency. Interestingly, the one hybrid described in the literature (Oliveira et al. 1991) had advertisement calls intermediate between those of the parental species (Fig. 1.11). The fundamental frequency band was between 1.0-1.5 KHz and the dominant frequency band between 2.0-2.5 KHz; call duration ranged between 120-140 ms, and the number of pulses

per call between 14-16, with an average pulse rate of 115 pulses per second. Call parameters in both species are fundamentally influenced by temperature and body size. Manz (1975) found a positive correlation between temperature and the frequency of contraction of laryngeal muscles in *H. arborea*, and a negative correlation between call duration and air temperature (Schneider 1974). Also, in the same work Schneider (1974) sampled *H. arborea molleri* (at that time *H. arborea* from the Iberian Pensinsula was considered a subspecies) from Portugal and found no influence of air temperature on the number of pulses per call, but, conversely, call duration and intercall interval were strongly affected by temperature: decreasing with rising temperatures. In *H. meridionalis* (at the time also considered a subspecies of *H. arborea*) males from France, Schneider (1968) found that when air temperature rises, the number of pulses per call, call duration and intercall duration all decrease significantly.

Hyla meridionalis mating calls (Fig. 1.14) differ markedly from *H. arborea* calls (Fig. 1.15) in that they consist of longer calls with more pulses. The calls consist of a group of pulses rising slowly to a peak, slowing considerably afterwards and increasing again slightly at the end of the call.

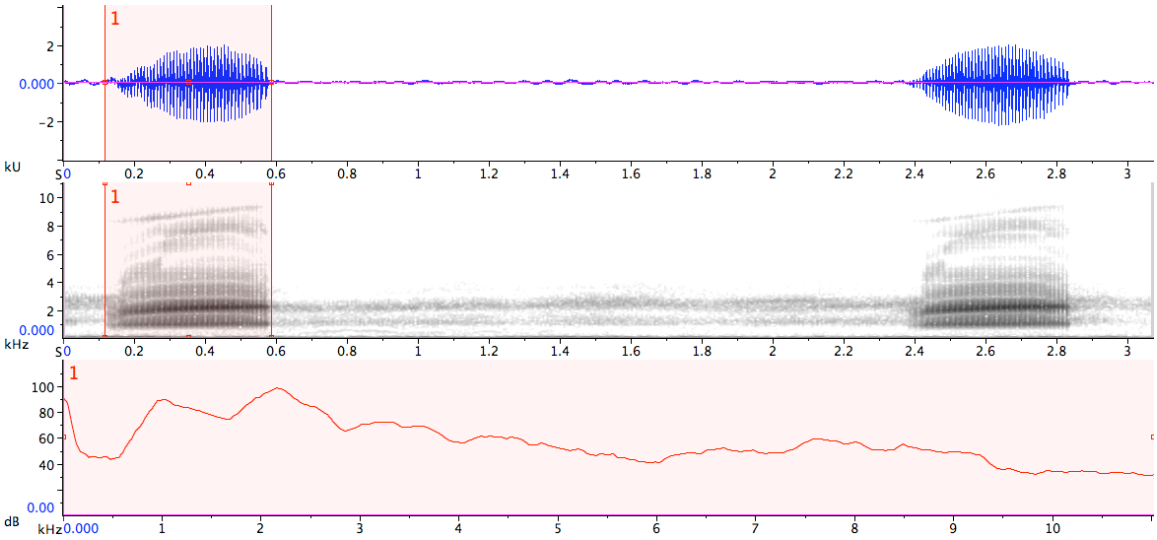


Figure 1.14. *Hyla meridionalis* advertisement call.

Advertisement call of *Hyla meridionalis* male from Grândola (Southern Portugal, GRA). It is visible two calls within a time frame of ~3 seconds. Male temperature = 15.6ºC; air temperature = 12.5ºC; and substrate temperature = 16.2ºC. Body measurements: SVL = 3.8 cm and mass = 3.9 g. First row is the waveform (amplitude by time), second is the audiospectrogram (frequency by time) (audiospectrogram parameters: Raven software, sampling rate = 44.1 kHz, 16 bits, 1024 FFT points) and the third represents average spectrum of the selected period (intensity by frequency).

For individuals from France, Schneider (1968) has described a call with an average of 41.95 pulses at 10ºC and 36.85 pulses at 20ºC; call duration and intervals between calls vary with temperature, from 0.5 sec at 10ºC to 0.25 sec at 20ºC, and from 0.25 sec at 6ºC to 0.82 sec at 19ºC. Eekhout et al. (2003) analysed the calls of a population in the Mendizorrotz Mountains of the Basque Country, Spain, and another population in Portalegre, Portugal, and found significant differences in call duration and in the number of pulses per call. The differences could be explained as geographic variation or be an artefact due to the higher background noise in the Basque Country recordings. Nonetheless, it may also be indicative of a potential natural call variation among the populations.

The advertisement calls of *Hyla arborea* (Fig. 1.15) are produced in call series or call bouts. Each call consists of a group of pulses; these rise slowly in amplitude until a maximum is reached, and then fall sharply until the end of the call (Schneider 1968). In central Europe, the *H. arborea* call is very typical, and usually comprises nine pulses with very low variation: normally in a series of 15-30 consecutive calls, sometimes up to 50 calls (Schneider 1993). The fundamental frequency of these calls ranges from 900 Hz to 1200Hz, and the dominant frequency (higher energy frequency) from 1900 to 2500 Hz. In Coimbra, Schneider (1974) recorded males within a range of temperatures from 8-18.5ºC – the average number of pulses per call was between 9.7 -11.9. He noticed an influence of the ambient temperature on the call parameters: the call duration was 0.107 sec at 8ºC and 0.605 sec at 18.5ºC, and the average duration of the inter-call intervals was 0.170 sec at 10.5ºC and 0.1095 sec at 18.5ºC.

Call parameters in both species, although more explored for *H. arborea* (Castellano et al. 2002 and Friedl & Klump 2002), are known to vary within and between individuals and among populations. Usually, larger males produce lower pitched and lower frequency calls (Castellano et al. 2002 and Friedl & Klump 2002). Márquez et al. (2005) compared the sound-pressure level (SPL) of mating calls among both species and found that *H. meridionalis* had higher SPL values (i.e. were more intense) than those of *H. arborea*. Also, they compared sympatric and allopatric populations of each species and found that *H. arborea* had more intense calls in allopatry. This could be a male strategy to save energy, as calling louder (or more intensively) requires, in principle, higher energetic expenditure. At the individual or population level, the call parameters can be classified into static and dynamic (see Gerhardt 1991 and description above). Based on these criteria, Friedl & Klump (2002) and Castellano et al. (2002) analysed *H. arborea* advertisement calls and classified the duration of call groups and number of calls per call group as dynamic call properties (i.e. more variable, see description above). The call rate within a call group, call duration, number of pulses per call, pulse period and fundamental and dominant frequencies were classified as static call properties (i.e. less variable).

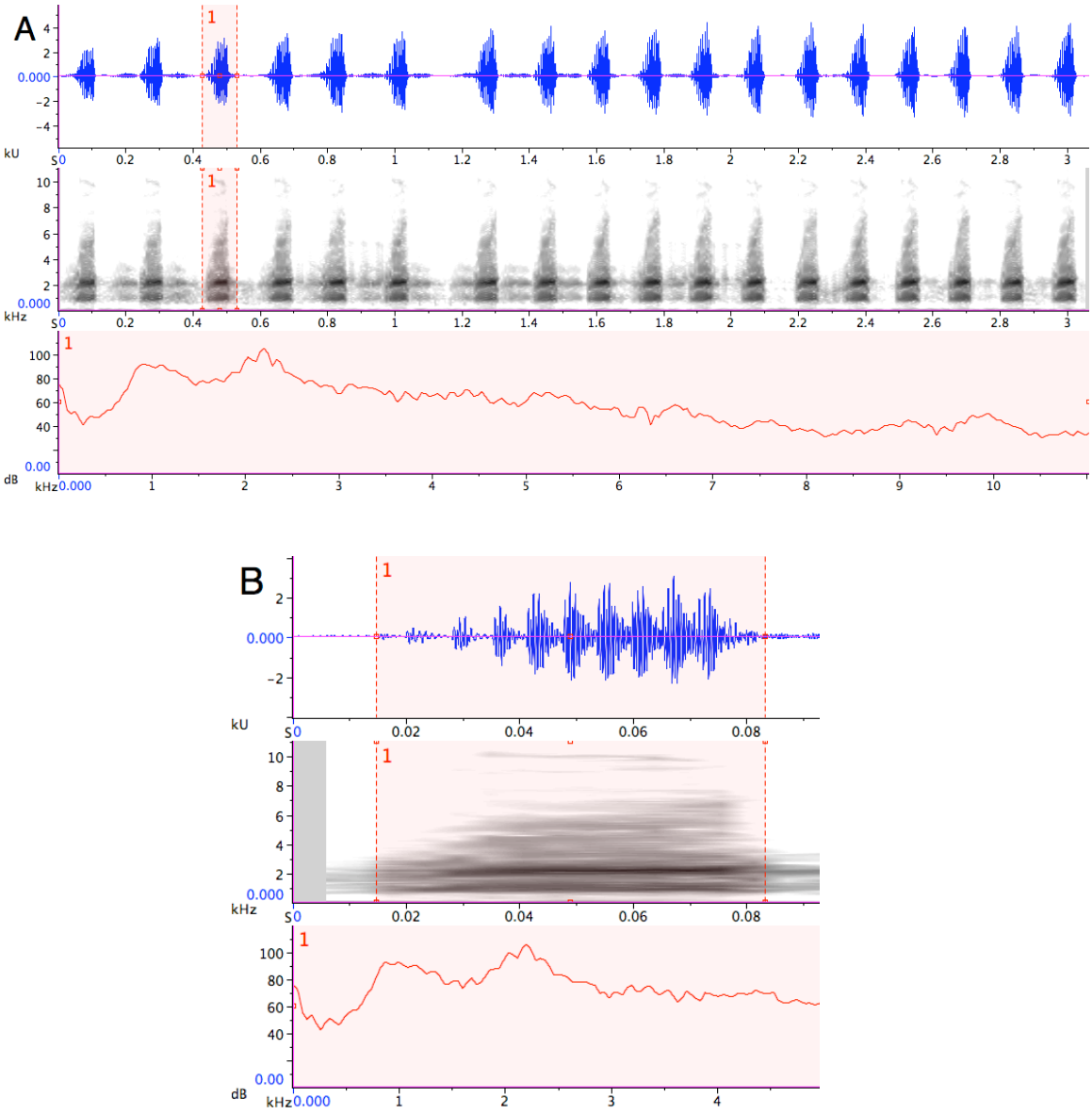


Figure 1.15. *Hyla arborea* advertisement call.

A. Group of 18 calls. **B.** One call selected from A. where the pulses that constitute the call are visible in the waveform. Advertisement call of *Hyla arborea* male from Covelães (Northern Portugal, COV). It is visible 18 calls within a time frame of ~3 seconds. Male temperature = 18.6ºC; air temperature = 14.2ºC; and substrate temperature = 22.0ºC. Body measurements: SVL = 3.6 cm and mass = 3.7 g. In both **A.** and **B.** First row is the waveform (amplitude by time), second is the audiospectrogram (frequency by time)

Choosing a mate often depends on females' preferences for certain call parameters. Females typically prefer calls from conspecific males; *Hyla meridionalis* females of a Tenerife population discriminated in favour of conspecific calls (Gerhardt & Schneider 1980), though the authors have reported one female responding to a *H. arborea* male call (instead of responding to a *H. meridionalis* one). Paillette (1967) found that *H. meridionalis* males responded to playbacks of the calls of *H. arborea*, but the responses were weaker than when

responding to conspecific calls. One could argue that the similarity in the spectral properties of the calls could lead to a misjudgement of the potential mate, and that temporal parameters are more important for the choice made. Female phonotaxis preferences were also tested: *H. meridionalis* females on Tenerife seem to prefer calls with high temperature characteristics (with differences up to 6ºC, Schneider 1982), showing a preference for calls with lower call duration, lower number of pulses per second (pulse rate) and shorter intercall intervals. Unlike those of *H. meridionalis*, *Hyla arborea* females have not yet been subjected to playback mate-choice tests in laboratory.

Friedl (2006) argues that the use of playback tests in laboratory may not represent the reality of mate choice; natural anuran breeding assemblages are often composed by multispecies choruses, where the background noise plays an important role in masking the conspecific and heterospecific chorus (e.g. Ehret & Gerhardt 1980; Gerhardt & Klump 1988; Narins & Zelick 1988; Wollerman 1999). Friedl (2006) states that laboratory playback tests do not show that female preferences have any selective pressure effect on male calling behaviour in natural populations, and Márquez (1990) stresses that these kinds of phonotaxis tests are not useful tools for the study of female mate choice, at least in *Alytes obstetricans* and *A. cisternasii*, as they might not clearly expose female natural choices. Moreover, it has been shown that chorus attendance by females is a better predictor of male mating success than any other call parameter – even when females show a preference for some particular call parameters (Cherry 1993; Pröhl 2003). This has been demonstrated in *H. arborea*, where lek attendance was positively related to male mating success (Friedl & Klump 2005; Jaquiéry et al. 2009), but did not correlate to offspring fitness, which was in fact correlated with male attractiveness (i.e. number of matings obtained per night of calling) (Jaquiéry et al. 2009). Friedl & Klump (2005) failed to prove a relationship between male mating success and body size. Friedl (2006) did not find any evidence for a directional selection based on dominant frequency, which, though negatively correlated with body size (Castellano et al. 2002), is not statistically significant in most of the studied populations (3 out of 9: see Castellano et al. 2002 for more details). In his field study, Friedl (2006) provided the first rough evidence of stabilising selection on static call properties – number of pulses per call and, maybe, call duration in *H. arborea*. One other factor, recently explored for female mate choice, is the use of visual cues. Gomez et al. (2010) conducted experiments under controlled light conditions with *H. arborea* females and realised that the use of colour vision may play a role in the selection of a mate.

7. Objectives of the Thesis

To my knowledge, and despite the increasing attention paid to the European Hylids, their phylogeographic histories are, in many aspects, controversial, as has been explained already. Very few studies have focused on the Iberian *Hyla* populations, particularly within Portuguese borders. Not that the political boundaries of the region have a direct influence on species assembly, but studying local population characteristics and dynamics allows for better global understanding, especially facilitating the development of better conservation policies at a national level. Moreover, and as discussed above, the Iberian Peninsula was an important refugium for the fauna and flora of the Quaternary during the glacial periods, meaning that a better understanding of the evolutionary history of the Iberian Peninsula – and in the particular case of the Portuguese Hylids – will certainly tell us more about the origin and evolution of the Iberian lineages.

The works cited, including those on both of the two focus species in the present study, *Hyla arborea* and *H. meridionalis*, have only used one approach, and not the double and integrative genetic and acoustic approaches as in the present study. The recently published work on the eastern Mediterranean Hylids (Gvozdík et al. 2010) is an exception, as it does discuss the genetic variation of members of the Hylidae family in Europe and the geographic variation of their mating calls. However, most authors often exclude or under-represent the Iberian and (in particular) Portuguese populations in their sampling, and fail to follow a multi-character approach.

The only work focusing on the genetic differentiation of *H. arborea* (=*H. molleri*?) and *H. meridionalis* specifically in Portugal is the PhD of Rosa (1995), in which the author obtained a general picture of the genetic differentiation within and between the two species, although, once again, his sampling was very limited. Based on protein electrophoresis, he observed a low level of genetic variation within *H. meridionalis* populations and found no correlation between genetic distance and geographic distance among *H. arborea* populations, and, even though there was considerable differentiation among populations, he could not explain the observed pattern with certainty.

Historically, within Iberia, there were three Hylid taxa, *H. arborea arborea* Linnaeus, 1758; *H. a. molleri* from Coimbra in central Portugal described by Bedriaga (1890), and *H. arborea meridionalis* described by Böttger (1874). The status of the Iberian ***Hyla arborea*** has been in debate since then. Morphologically and bioacoustically the first two cannot be told apart (Boulenger 1898), and it is typically assumed that the subspecies *H. a. arborea* occupies the area of central Spain (and the rest of Europe), and *H. a. molleri* northwestern Spain and northern Portugal (e.g. Rosa & Oliveira 1994; Márquez 2002; Oliveira & Pargana 2008). As

for *H. meridionalis*, it has an advertisement call clearly distinct from *H. arborea*, and also morphologically the two species quite different (see description above). Recently, Stöck et al. (2008) and Barth et al. (2011) have tried to clarify the relationships within the *arborea* species group, suggesting the resurrection of the species status for the Iberian treefrog *Hyla molleri*, formerly with the subspecies status, *H. arborea molleri*. Thus, Iberian *Hyla arborea* not only is expected to be highly genetically divergent from the Central European *Hyla arborea*, supporting the recent suggestion that the *H. molleri* taxon be reevaluated (Stöck et al. 2008 and Barth et al. 2011), but it may also show a relatively strong population genetic struture as it has been described for other amphibian populations within Portugal (e.g. *Chioglossa lusitanica* – Sequeira et al. 2006) and also a significant level of divergence within the Iberian territory (Rosa 1995), eventually, corresponding to the two subspecies aforementioned.

Recuero et al. (2007) studies on the phylogeny of ***H. meridionalis*** give an indication of the genetic structure of the biogeographic patterns of the species. Biogeographic data suggest an African origin of the species ancestor and a wider geographic distribution, currently greatly more-restricted and fragmented. Environmental differences among the different habitats occupied by *H. meridionalis*, and the expansion processes thought to have been used (namely, an invasion from the South of Iberian Peninsula), could lead to the expectation of an Iberian South-North geographic gradient with higher meridional diversity levels, both at the genetic and bioacoustic levels. However, recent works suggest a very low differentiation of mitochondrial DNA within Iberian populations, probably due to a very recent invasion of the Iberian territories from northern Africa either by natural means (by rafting) or by artificial human action (Recuero et al. 2007). Even though Recuero's work reflected a large scale pattern, with little representation of Portuguese populations, if the suggested recent invasion had indeed taken place, one would expect a low genetic diversity within Portugal, with a south-north decrease.

However, the genetic differences are not always reflected bioacoustically and morphologically (Paillette 1969; Schneider 1974) and so the same variation patterns may not be observed. Furthermore, assuming a relatively strong genetic determinism and a dependence on morphological traits of the call parameters of both sound production and reception apparatuses (discussed above), it would be expected that there would be a correlation between genetic variation levels and bioacoustic ones. If, on the contrary, the patterns show no correlation, then the variation seen among population-call parameters would be more likely be a consequence of environmental plasticity.

When investigating ***species interaction***, previous studies have described the occurrence of F1 hybrids between *H. arborea* and *H. meridionalis* (Oliveira et al. 1991; Barbadillo & Lapeña 2003). In both works the advertisement calls were said to be the initial

identification tool, as they sounded intermediate between the two parental species advertisement calls (see description in Introduction and in Oliveira et al. 1991). Moreover, the observation of heterospecific amplexi in nature is not a rare event; their occurrence has been reported by Oliveira et al. (1991), Barbadillo & Lapeña (2003) and Crespo (pers. comm.). The presence of hybrids in sites where the two species are present is thus expected. Also, in sympatric areas, and in particular in syntopic areas where the two species cohabit and use the same water bodies during reproductive periods, it is expected that males adopt either one of two strategies in their acoustic behaviour: 1) as their advertisement calls are clearly distinct (see description above) they increase the differences between the call parameters that are more closely related (e.g. frequencies) by altering the call parameters (character displacement), or make use of different spatial and acoustic niches, so avoiding other species and reducing interference (temporal, spatial); 2) make no alteration in behaviour, relying on females' ability to choose the conspecific, and not the heterospecific, male. Therefore, testing for the existence of character displacement the tested null hypothesis H0 would be no alteration in call characteristics in sympatry.

Relating to genetic analysis of species interaction, the interest is in evaluating the degree of hybridisation/introgression both in adults and tadpoles, inferring details of the efficiency of the premating isolation barriers of the two species, and thus expecting: 1) a process of population 'fusion'; 2) differentiation of the two species if the hybrids are subjected to negative selective pressures; or 3) a situation of positive hybrid selection – 'hybrid vigour' – that could lead to a new species distinct from the parental ones.

In the effort to contribute to the betterment of knowledge about both Iberian Hylid species, *Hyla arborea* and *H. meridionalis*, and attempting to validate (or not) previous hypotheses formulated in the context of their phylogeny and phylogeography, we used a double, complementary and integrative exploratory analysis drawn from two relatively independent sources of data: molecular genetic and bioacoustic. We also evaluated the coincidence (or not) of the variation patterns between these two approaches, and the efficacy of using this double approach in such studies with anurans. In this context, we tried to gather more relevant information about geographic origins and colonisation patterns (historical and recent events, expansion and contraction movements, and glaciar refugia) and evolutive and biogeographic consequences of species interaction.

More specifically, the intended objectives are the following:

1. Examine the geographic patterns of genetic diversity of *Hyla meridionalis* and *Hyla arborea* in the Iberian Peninsula, using different molecular markers, increasing the number of sampled sites within Iberia (compared to previous studies), reexamine the

European and North African *Hyla* species phylogeographic patterns and analyse the phylogeographic patterns on a smaller scale;

2. Investigate the geographic patterns of bioacoustic diversity of *Hyla meridionalis* and *Hyla arborea*, by analysing the advertisement-call parameters of males from the same sites sampled in Portugal;
3. Analyse the behaviour of the two species from a genetic and bioacoustic perspective in sympatric and allopatric (as a reference) situations using this integrated approach: attempting to understand the degree of hybridisation and introgression between the two species and the level of any eventual character displacement in the mating calls.

Chapter II. Materials and Methods

by Sara Maia

coaxar de rãs é toda a melodia
que a noite tem no seio –
- versos dos charcos
e dos juncos podres,
calmamente, com luar no meio.

Eugénio Andrade, 'nocturno' in *mãos e frutos*

Material and Methods

The present work was run between 2005 and 2011, and included two distinct but complementary parts: fieldwork and laboratory procedures. Fieldwork included various sampling locations with either one or both species in Portugal, *Hyla meridionalis* and *H. arborea*: for audio recording of the male advertisement calls and body measurements (body mass and snout-vent-length – SVL); collecting of tissue samples from all the recorded males and other non-audio-recorded individuals (non-audio-recorded males, females and tadpoles); and capturing the individuals used in laboratory crosses. For the laboratory procedures I used Western Kentucky University's (Bowling Green, KY, USA), CIBIO's (Vairão, Portugal) and IGC's (Oeiras, Portugal) laboratory products and facilities.

The field-sampled individuals were collected from 53 locations along the Portuguese distribution range of both species (figs. 1.7 and 1.8). In addition, I used nine specimens from six different populations preserved at the Tissue and DNA Collection of the Museo Nacional de Ciencias Naturales (CSIC) of Madrid (Spain), five samples sent by Paul Arens, from the Netherlands, 27 samples from Portuguese populations preserved at the Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO), two samples sent by Wouter de Vries, from Sevilla and five samples sent by Helder Duarte and Juan Paco Beltran, from Doñana. In total, I obtained tissue samples from 209 individuals of *Hyla meridionalis*, 129 individuals of *Hyla arborea* and 50 initially unidentified tadpoles, and recorded advertisement calls and body measurements from males of 126 *H. meridionalis* and 58 *H. arborea*.

1. Fieldwork Procedures: Specimen Collection and Sampling Strategy

1.1. Sampling Sites

The choice of the sampling sites depended on their accessibility, and, more importantly, in their coverage of the species' geographic range in Portugal. Sites were also chosen to ensure that both allopatry and sympatry (including situations of strict sympatry, syntopy) conditions were represented. Some of the sampling locations were chosen according to those used in previous studies (e.g. Pargana et al. 1996; Loureiro et al. 2008), or based on information provided by colleagues in the field or by locals indicating the presence of one or both species. Additional sample sites were chosen by targeting areas with high probability of having water

bodies (e.g. ponds, streams and dams) where it would be more likely to observe individuals of either species, using both detailed military maps (scale 1:50 000) and GoogleEarth. After locating potential water bodies, several geographic references were noted, such as GPS coordinates, main access roads and reference points, to help identify the site in the field. Other sampling sites were found through local prospecting, or by travelling both on foot and/or by car within areas exhibiting yet unknown water bodies or humid areas. At dusk and throughout the night, acoustic surveillance was also conducted to detect calling activity at the sites.

Each site was visited prior to sampling to confirm the existence of either one or both species, and to ensure adequate sampling conditions (e.g. access to ponds/streams), as proper conditions can change over time (e.g. temporary ponds or small streams could have dried or been modified by human action, or simply become inaccessible due to the presence of cattle, dense vegetation, or fences and/or locked gates). An acoustic surveillance of the previously located/chosen sites was also done at night, when the males are more active acoustically, usually through a tour by car, to evaluate the calling activity of the site. Permission for access to sites inside private lands was requested and granted by the owners prior to each sampling event (when possible). The geographical location of all sampling sites (both genetic and bioacoustic samples used) is shown in figs. 2.1, 2.2 and 2.3 is listed in Tables 2.1, 2.2 and 2.3.

To fulfill all the objectives of this work it was necessary to collect acoustic and morphological data, as well as tissue samples from individuals of *Hyla meridionalis* and *H. arborea* – mainly during the reproductive season of each species, i.e. from March to July. During the rest of the year, occasional visits to field sites were made to register levels of activity. The methodologies used for collection and analysis of acoustic data, as well as for tissue collection and genetic analysis, will be described separately, even though, at least during fieldwork, data and tissue collections happened simultaneously on many occasions. During fieldwork, special attention was paid to the identification of hybrid individuals in nature using acoustic (advertisement calls with intermediate characteristics of those of the parental species) or morphological traits. As previously described, the hybrids found in nature (Oliveira et al. 1991; Barbadillo & Lapeña 2003) were identified by their advertisement calls presenting characteristics intermediate between those of the parental species, and also by the presence of a lateral band.

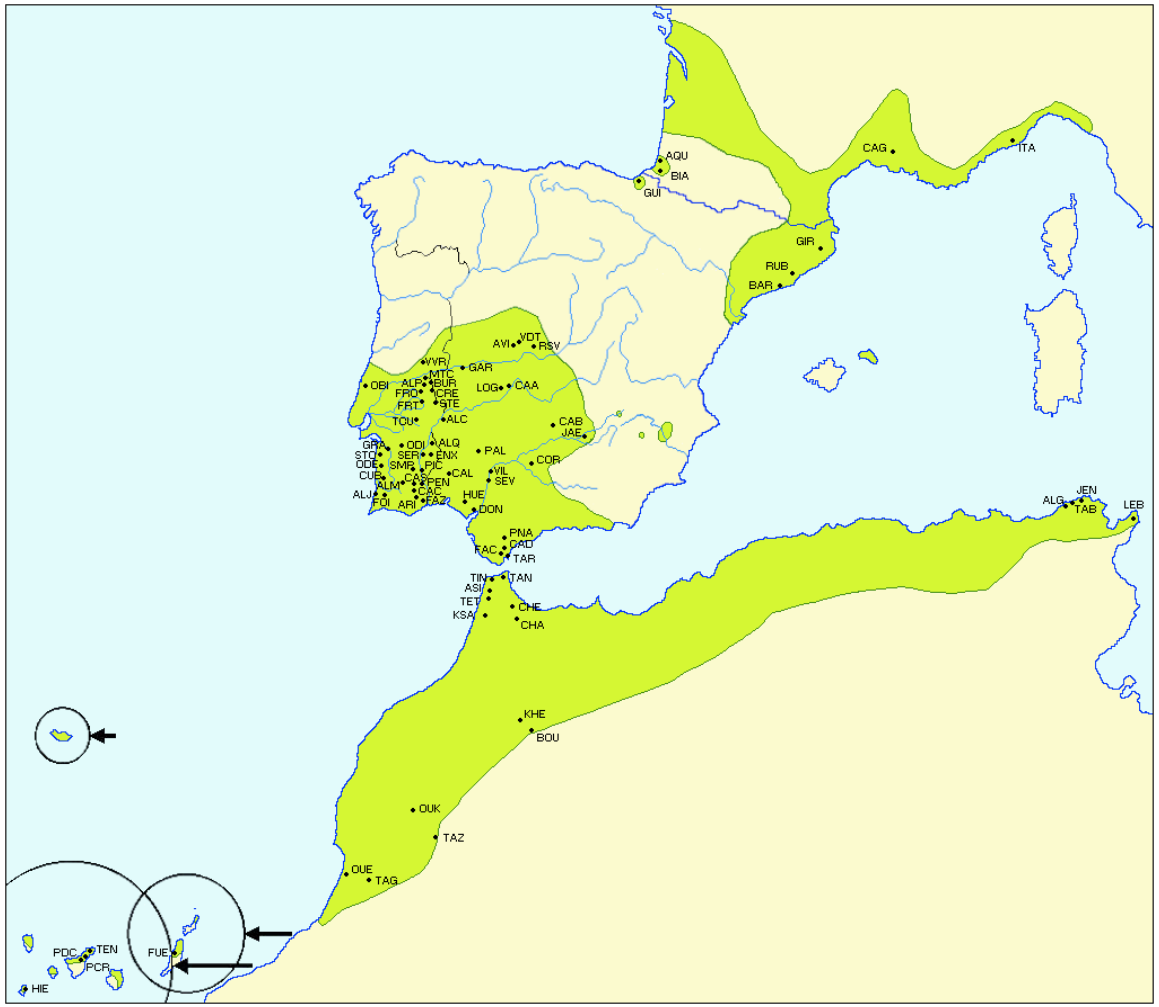


Figure 2.1. Sampled sites of *Hyla meridionalis* for the molecular genetic analysis.

Distribution of *H. meridionalis* (green) and location of all sampled sites for tissue collection and audio recordings. Population acronyms are as shown in table 2.1.

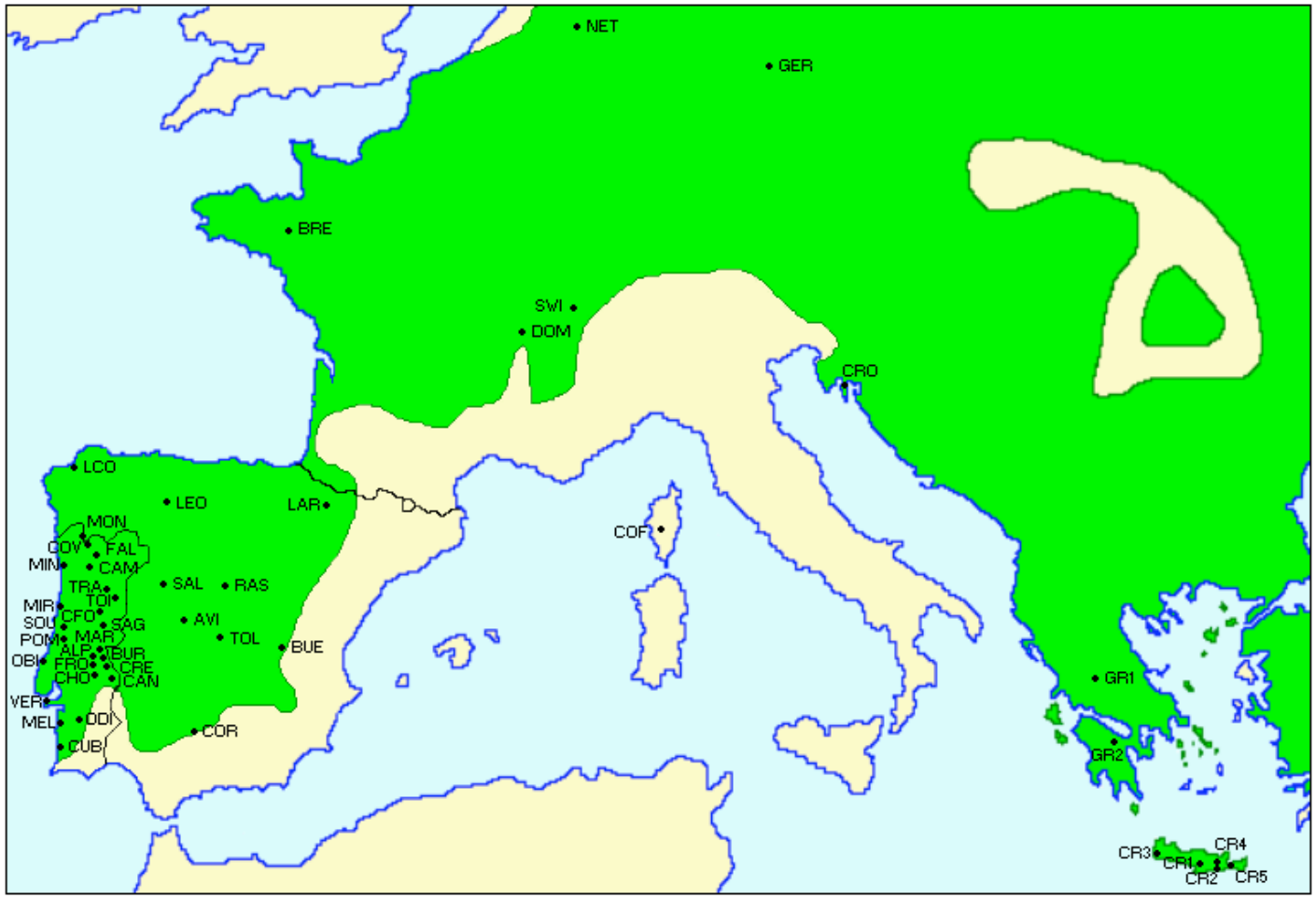


Figure 2.2. Sampled sites of *Hyla arborea* for the molecular genetic analysis.

Distribution of *H. arborea* (green) and location of all sampled sites for tissue collection and audio recordings. Population acronyms are as shown in table 2.2.

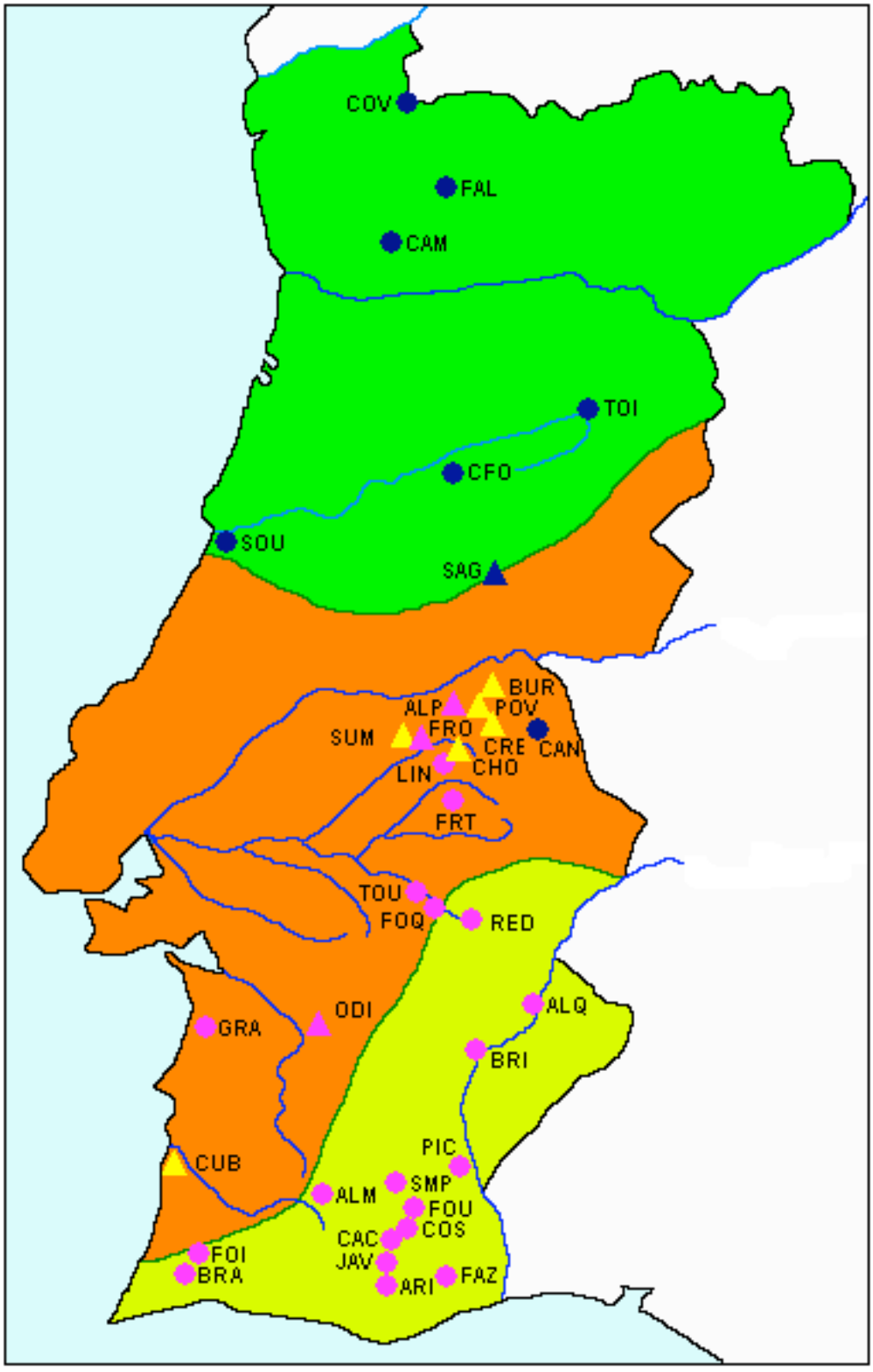


Figure 2.3. Sampled sites of *Hyla arborea* and *H. meridionalis* for the bioacoustic analysis.
Circles indicate sites where there was only one species present, *H. arborea* (●) and *H. meridionalis* (●); triangles indicate sites where the two species were present: (▲) if they were both recorded, (▲) if only *H. arborea* was recorded and (▲) if only *H. meridionalis* were recorded. Distribution in Portugal of *H. meridionalis* , *H. arborea* , and overlaping sympatric area .

Table 2.1. *Hyla meridionalis* COI samples information.

Locality information, sample size and haplotypes for *Hyla meridionalis*. GenBank Accession numbers are provided for sequences obtained from GenBank respectively. * samples provided by a third party, not captured by myself. [??] GPS coordinates are not exact as they were not provided by the donor of the sample(s), being approximate to the known location. Red and blue colours of haplotypes code stand for south and north lineages, respectively.

Country	Locality	Code	*n*	Haplotypes	GPS	GenBank Accession number
Portugal	Almodovar	ALM	9	**H7, H22, H29**	37°30'25.79"N 8°8'37.38"W	–
	Alpalhão, Nisa	ALP	9	**H7**	39°28'20.58"N 7°38'6.84"W	–
	Arimbo, São Brás de Alportel	ARI	2	**H30, H37**	37°11'5.45"N 7°52'8.39"W	–
	Arrifana, Aljezur	ALJ	2	**H7, H22**	37°17'51.78"N 8°50'27.24"W	–
	Brunheiras, Odemira	ODE	5	**H7, H22, H23**	37°43'34.14"N 8°42'30.72"W	–
	Póvoa de São Miguel, Alqueva	ALQ	3	**H7**	38°15'51.84"N 7°19'48.60"W	–
	Buraco, Póvoa e Meadas	BUR	19	**H7, H10**	39°27'39.54"N 7°29'27.54"W	–
	Cachopo, Tavira	CAC	4	**H29, H30, H31**	37°20'52.01"N 7°49'13.01"W	–
	Castelhanos, Alcoutim	CAS	4	**H29**	37°28'41.08"N 7°47'7.50"W	–
	Crespo, Portalegre	CRE	14	**H7, H23**	39°22'45.84"N 7°28'31.86"W	–
	Cuba, Odemira	CUB	6	**H7, H22, H24**	37°38'4.62"N 8°42'21.66"W	–
	Enxoé, Serpa	ENX	1	**H29**	37°59'49.37"N 7°28'1.22"W	–
	Faz Fato, Tavira	FAZ	1	**H7**	37°11'18.41"N 7°35'20.59"W	–
	Flôr da Rosa, Crato	FRO	1	**H7**	39°18'46.08"N 7°40'45.48"W	–
	Fóia, Monchique	FOI	2	**H7, H32**	37°18'58.18"N 8°35'35.97"W	–
		FOI2	3	**H7, H29**	37°18'36.00"N 8°35'45.60"W	FJ226804 FJ226803 FJ226802
	Fronteira	FRT	2	**H7**	39° 4'23.28"N 7°36'59.05"W	–
	Grândola	GRA	7	**H7, H29, H34**	38°10'14.76"N 8°35'17.28"W	–
	Lagoa de Óbidos, Óbidos	OBI	1	**H39**	39°22'43.78"N 9°12'49.14"W	–
	Odivelas, Ferreira do Alentejo	ODI	9	**H7, H25, H26, H27, H33**	38°10'50.97"N 8° 9'28.05"W	–
	Penedos, Mértola	PEN	3	**H7**	37°30'20.29"N 7°47'55.00"W	–
	Picoitos, Mértola	PIC	8	**H7, H29**	37°36'52.50"N 7°36'39.46"W	–
	Santa Eulália	STE	2	**H28**	39° 3'20.29"N 7°16'12.32"W	–

Country	Locality	Code	*n*	Haplotypes	GPS	GenBank Accession number
	Santo André, Santiago do Cacém	STO	1	**H23**	38° 3'26.36"N 8°45'24.29"W	–
	São Miguel do Pinheiro, Mértola	SMP	7	**H7**, **H32**	37°33'6.22"N 7°51'1.08"W	–
	Serpa, Beja	SER	2	**H7**, **H29**	37°56'57.16"N 7°31'16.08"W	–
	Tourinha, Évora	TOU	1	**H7**	38°41'5.32"N 7°44'53.04"W	–
	Vila Velha de Ródão	VVR	1	**H36**	39°44'5.79"N 7°35'51.35"W	–
	Monte Claro, Portalegre	MTC	1	**H7**	39°31'58.10"N 7°43'8.61"W	DQ996413
Spain	Doñana*	DON	5	**H7**, **H8**, **H38**	36°59'24.36"N 6°27'2.28"W	–
	Cádiz	CAD	1	**H7**	36°17'59.67"N 5°43'12.47"W	FJ226806
	PNAlcornocales, Cádiz	PNA	2	**H35**	36°26'33.73"N 5°27'35.57"W	–
	Valle del Tiétar, Ávila*	VDT	1	**H7**	40°12'46.68"N 5°5'47.13"W	–
	Sierra Norte, Sevilla* ??	SEV	2	**H7**	37°32'57.67"N 5°49'16.14"W	–
	Puerto de la Cruz, Tenerife	PDC	2	**H3**	28°24'0.00"N 16°31'48.00"W	FJ226807 FJ226808
		PCR	1	**H2**	28°22'58.61"N 16°33'2.96"W	Recuero et al. 2007
	Tenerife*??	TEN	1	**H3**		–
	Tarifa, Cádiz	TAR	1	**H9**	36° 0'43.12"N 5°36'25.98"W	DQ996416
	El Rocio, Huelva	HUE	1	**H10**	37° 7'59.00"N 6°29'14.43"W	DQ996414
			1	**H7**		DQ996413
			1	**H8**		DQ996415
	Real de San Vicente, Toledo	RSV	1	**H7**	40° 8'6.83"N 4°41'11.81"W	Recuero et al. 2007
	Logrosán, Cáceres	LOG	1	**H7**	39°20'5.22"N 5°29'2.14"W	
	Garrovillas, Cáceres	GAR	1	**H7**	39°42'56.38"N 6°32'57.90"W	DQ996412
	Cañamero, Cáceres	CAA	1	**H7**	39°22'35.44"N 5°21'33.55"W	
	Pallares, Badajoz	PAL	1	**H7**	38° 7'3.18"N 6°8'41.34"W	
	Alconchel, Badajoz	ALC	1	**H7**	38°31'1.24"N 7°4'2.76"W	
	Aldeaquemada, Jaén	JAE	1	**H7**	38°23'55.43"N 3°22'0.18"W	
	Calañas, Huelva	CAL	1	**H7**	37°38'15.59"N 6°54'9.22"W	Recuero et al. 2007
	Candeleda, Ávila	AVI	1	**H7**	40°8'59.98"N 5°13'60.00"W	DQ996413
	Villanueva del Río, Sevilla	VIL	1	**H7**	37°36'53.65"N 5°40'22.23"W	Recuero et al. 2007
	Córdoba, Córdoba	COR	1	**H7**	37°53'0.00"N 4°46'0.00"W	Recuero et al. 2007
	Facinas, Cádiz	FAC	1	**H7**	36° 8'23.53"N 5°42'1.54"W	Recuero et al. 2007

Country	Locality	Code	*n*	Haplotypes	GPS	GenBank Accession number
	Facinas, Cádiz		1	H9	36° 8'23.53"N 5°42'1.54"W	DQ996416
	Cabezarrubias, Ciudad Real	CAB	1	H7	38°37'0.25"N 4°10'59.15"W	Recuero et al. 2007
	Donostia, Guipúzcoa	GUI	1	H2	43°18'59.67"N 1°59'1.09"W	Recuero et al. 2007
	Vega del Río Palmas, Fuerteventura	FUE	1	H2	28°23'10.94"N 14°5'31.41"W	Recuero et al. 2007
	San Andrés, El Hierro	HIE	1	H2	27°45'60.00"N 17°56'60.00"W	Recuero et al. 2007
	Garraf, Barcelona	BAR	1	H2	41°15'0.00"N 1°54'0.00"E	Recuero et al. 2007
	Rubí, Barcelona	RUB	1	H2	41°28'59.49"N 2°1'59.03"E	Recuero et al. 2007
	Santa Coloma de Farnés, Girona	GIR	1	H2	41°51'59.99"N 2°39'59.98"E	Recuero et al. 2007
Morocco	Oukaimeden, Marrakesh Province	OUK	1	H11	31°12'20.99"N 7°51'51.00"W	FJ226842
			1	H11	31°12'15.45"N 7°51'39.89"W	DQ996417
	Tetouan Province	TET	1	H4	35°27'57.60"N 6°1'58.80"W	FJ226801
	Tanger Wilaya	TAN	1	H42	35°50'52.80"N 5°33'46.80"W	FJ226799
	Chechauen, Chaouen	CHA	1	H3	35°10'15.60"N 5°6'10.80"W	FJ226798
	Middle Atlas Mountains, Boulemane Province	BOU	1	H11	32°40'58.83"N 4°45'0.01"W	FJ226800
	Tagounit, Taroudant	TAG	1	H11	29°48'33.00"N 9°2'51.00"W	DQ996420 DQ996419
			1	H13		DQ996418
			1	H15		DQ996421
	Oued Massa, Tiznit	OUE	1	H14	29°53'21.76"N 9°35'30.35"W	DQ996422
	Taznakht, Ouarzazate	TAZ	1	H11	30°41'43.00"N 7°16'13.00"W	Recuero et al. 2007
			1	H12		DQ996423
	Ait Oufella, Khenifra	KHE	1	H6	32°56'35.05"N 5°3'36.42"W	DQ996411 DQ996410
	Ksar el Kebir, Larache	KSA	1	H1	35° 3'2.56"N 5°54'27.51"W	DQ996407
			1	H5		DQ996409
	Asilah, Tanger	ASI	1	H2	35°27'51.43"N 6°2'20.00"W	Recuero et al. 2007
			1	H4		DQ996403
	Chefchaounen, Chaouen	CHE	1	H2	35°10'7.24"N 5°16'3.25"W	DQ996404
			1	H3		DQ996401
	Tingis, Tanger	TIN	1	H2	35°47'4.62"N 5°48'46.63"W	Recuero et al. 2007
			1	H4		DQ996402

Country	Locality	Code	*n*	Haplotypes	GPS	GenBank Accession number
	Morocco* [??]	MOR1	1	H3		–
		MOR2	1	H11		–
Tunisie	Lebna, Nabeul	LEB	1	H16	36°46'53.07"N 10°59'16.98"E	DQ996428
			1	H18		DQ996429
			1	H17		DQ996424
	Tabarka, Jendouba	TAB	1	H18	36°57'15.97"N 8°45'27.73"E	DQ996429
			1	H19		DQ996426
			1	H20		DQ996425
			1	H21		DQ996427
		JEN	1	H18	36°56'24.00"N 8°45'36.00"E	FJ226809
Algerie	Parc National d'El-Kala	ALG	2	H40, H41	36°49'1.20"N 8°25'1.20"E	FJ226793 FJ226794
Italie	Liguria, Badalucco	ITA	4	H3	43°54'36.00"N 7°50'23.99"E	FJ226795 FJ226796 FJ226797 FJ226840
France	Tarnos, Aquitaine	AQU	1	H2	43°31'59.94"N 1°27'59.61"W	
	Biarritz, Aquitaine	BIA	1	H2	43°29'0.03"N 1°34'0.05"W	
	Camargue, Tour du Valat	CAG	1	H3	43°31'11.99"N 4°42'0.00"E	FJ226841
	TOTAL		**209**			

Table 2.2. *Hyla arborea* COI samples information.

Locality information (include GPS coordinates), sample size and haplotypes for *Hyla arborea*. GenBank Accession numbers are provided for sequences obtained from GenBank respectively. * samples/sequences either from other researcher or from genbank. [??] GPS coordinates are not exact as they were not provided by the provider of the sample(s), being approximate to the known location. Red and blue colours of haplotypes code stand for south and north lineages, respectively.

Country	Locality	Code	*n*	Haplotypes	Coordinates	GenBank Accession number
Portugal	Alpalhão, Nisa	ALP	2	H17, H22	39°28'20.58"N 7°38'6.84"W	–
	Campeã, Vila Real	CAM	4	H1, H11, H12	41°17'33.19"N 7°52'35.46"W	–
	Buraco, Póvoa e Meadas	BUR	14	H17, H19, H21, H25, H26	39°27'39.54"N 7°29'27.54"W	–
	Crespo, Portalegre	CRE	7	H17, H19, H21, H28	39°22'45.84"N 7°28'31.86"W	–
	Cantarinhos, Serra São Mamede	CAN	2	H22, H27	39°18'43.74"N 7°23'23.52"W	–
	Covão do Forno, Seia	CFO	8	H21	40°22'20.57"N 7°38'9.69"W	–
	Covelães, Montalegre	COV	8	H1, H3, H4, H5, H12	41°48'53.04"N 7°54'56.34"W	–
	Cuba, Odemira	CUB	9	H21, H31, H32, H33, H34, H35, H36	37°38'4.62"N 8°42'21.66"W	–
	Falperra, Vila Pouca de Aguiar	FAL	3	H1	41°30'26.04"N 7°39'51.84"W	–
	Flôr da Rosa, Crato	FRO	2	H21, H23	39°18'46.08"N 7°40'45.48"W	–
	Lagoa de óbidos, Óbidos	OBI	1	H14	39°22'43.78"N 9°12'49.14"W	–
	Melides, Grândola	MEL	5	H21, H30	38° 8'20.07"N 8°44'33.49"W	–
	Odivelas, Ferreira do Alentejo	ODI	4	H21, H37	38°10'50.97"N 8°9'28.05"W	–
	Santa Águeda Dam, Gardunha	SAG	2	H21	40° 0'22.78"N 7°28'55.56"W	–
	Soure, Coimbra	SOU	8	H1, H12, H16, H18, H19, H20	40° 3'24.18"N 8°37'32.22"W	–
	Pombal*[??]	POM	2	H13	39°54'49.41"N 8°37'39.18"W	–
	Ribeira do Chocanal, Crato	CHO	1	H17	39°16'46.98"N 7°38'29.58"W	–
	Marvão, Beirã	MAR	1	H24	39°27'3.60"N 7°21'36.00"W	FJ226829
	Mindelo*[??]	MIN	1	H12	41°18'51.76"N 8°42'55.61"W	–
	Mira, Aveiro	MIR	2	H12, H13	40°28'47.58"N 8°44'13.59"W	–
	Toito, Guarda	TOI	2	H6, H12	40°37'29.30"N 7°7'4.55"W	–
	Tourém, Montalegre	MON	3	H1, H5	41°54'47.54"N 7°53'58.40"W	–
	Trancoso*[??]	TRA	2	H7	40°46'42.39"N 7°20'56.98"W	–

Country	Locality	Code	*n*	Haplotypes	Coordinates	GenBank Accession number
	Verdizela, Seixal	VER	4	**H21**, **H29**	38°34'33.90"N 9° 8'38.90"W	–
Spain	Valle del Tiétar, Ávila*[??]	VDT	1	**H21**	40°12'46.68"N 5°5'47.13"W	–
	Buenache de Alarcón, Cuenca*	CUE	1	**H1**	39°39'0.01"N 2°10'12.01"W	–
	Cordóba*[??]	COR	1	**H21**	37°53'5.02"N 4°46'44.95"W	–
	Embalse de Cecebre, La Coruña*[??]	LCO	2	**H2**, **H15**	43°16'48.58"N 8°17'50.43"W	–
	Larúes, Huesca, Aragon*	LAR	1	**H1**	42°31'11.99"N 0°51'0.01"W	–
	Leon*	LEO	2	**H8**, **H10**	42°35'59.55"N 5°34'18.31"W	–
	Parque Natural de Peñalara, Rascafría, Madrid*[??]	MAD	1	**H9**	40°54'8.20"N 3°52'57.51"W	–
	Salamanca*	SAL	1	**H17**	40°55'48.00"N 5°40'12.00"W	FJ226828
	Toledo*	TOL	1	**H21**	39°52'12.00"N 4° 1'48.00"W	–
Croatia	Insel Cres, Lubenice	CRO	1		44°52'48.00"N 14°19'48.00"E	FJ226837
France	Corsega*	COF	5		42° 2'22.57"N 9°0'46.41"E	–
	Dombes	DOM	1		46° 0'36.00"N 4°56'24.00"E	FJ226777
	Bretagne, Monterfil	BRE	1		48° 2'49.20"N 1°58'15.60"W	FJ226825
Germany	Papitz, Saxony	GER	1		51°22'48.00"N 12°14'24.00"E	FJ226836
Greece	Greece*	GR1	2		39° 4'27.15"N 21°49'27.52"E	–
	Peloponnes, Stymphalian Lake, Kinia	GR2	1		37°50'60.00"N 22°27'0.00"E	FJ226783
Crete	Kroussonas	CR1	1		35°14'2.50"N 24°58'58.70"E	FJ226778
	Lassithi Plateau	CR2	1		35°10'60.00"N 25°28'0.00"E	FJ226779
	Kaloudiana Kissamos	CR3	1		35°29'13.20"N 23°41'24.00"E	FJ226833
	Lassithi Plateau	CR4	1		35°11'31.20"N 25°25'51.60"E	FJ226834
	Thrypti Plateau	CR5	1		35° 4'48.00"N 25°52'12.00"E	FJ226835
Switzerland	Lavigny	SWI	1		46°30'7.20"N 6°25'8.40"E	FJ226772
The Netherlands	Roeterinksbroek, Gelderland, Achterhoek area*	NET	1		52° 9'37.29"N 6°29'6.64"E	–
	Vleer, Gelderland, Achterhoek area*	NET	2		52° 8' 44.22"N 6° 38' 40.34"E	–
		TOTAL	**129**			

Table 2.3. Bioacoustic Sampled Locations in Portugal.

Location and GPS coordinates, Species and number of males whose Advertisement Calls were recorded. Populations are organized top to bottom according to their location along a north-south axis. In the species column, 'Ha' stands for *H. arborea* and 'Hm' for *H. meridionalis*. Rows in grey indicate syntopic populations, but not always with samples from the two species. See also map Fig. 2.3.

Locality	Code	Species	*n*	Coordinates
Covelães, Montalegre	COV	Ha	3	41°48'53.04"N 7°54'56.34"W
Falperra, Vila Pouca de Aguiar	FAL	Ha	5	41°30'26.04"N 7°39'51.84"W
Campeã, Vila Real	CAM	Ha	3	41°17'33.19"N 7°52'35.46"W
Toito, Guarda	TOI	Ha	2	40°37'29.30"N 7°7'4.55"W
Covão do Forno, Seia	CFO	Ha	3	40°22'20.57"N 7°38'9.69"W
Soure, Coimbra	SOU	Ha	11	40° 3'24.18"N 8°37'32.22"W
Santa Águeda Dam, Gardunha	SAG	Ha	4	40° 0'22.78"N 7°28'55.56"W
Alpalhão, Nisa	ALP	Hm	4	39°28'20.58"N 7°38'6.84"W
Buraco, Póvoa e Meadas	BUR	Ha/Hm	3/5	39°27'39.54"N 7°29'27.54"W
Rib. Póvoa e Meadas	POV	Ha/Hm	3/3	39°27'7.16"N 7°32'0.71"W
Crespo, Portalegre	CRE	Ha/Hm	5/3	39°22'45.84"N 7°28'31.86"W
Cantarinhos, Serra São Mamede	CAN	Ha	3	39°18'43.74"N 7°23'23.52"W
Flôr da Rosa, Crato	FRO	Hm	2	39°18'46.08"N 7°40'45.48"W
Sume	SUM	Ha/Hm	4/3	39°20'3.18"N 7°47'48.00"W
Ribeira do Chocanal, Crato	CHO	Ha/Hm	3/3	39°16'46.98"N 7°38'29.58"W
Ribeira de Linhais, Crato	LIN	Hm	7	39°15'9.42"N 7°38'32.52"W
Fronteira	FRT	Hm	4	39° 4'23.28"N 7°36'59.05"W
Tourinha, Évora	TOU	Hm	6	38°41'5.32"N 7°44'53.04"W
Foros do Queimado, Évora	FOQ	Hm	3	38°39'47.94"N 7°42'32.19"W
Redondo	RED	Hm	10	38°37'3.94"N 7°36'1.82"W
Póvoa de São Miguel, Alqueva	ALQ	Hm	5	38°15'51.84"N 7°19'48.60"W
Odivelas, Ferreira do Alentejo	ODI	Hm	3	38°10'50.97"N 8°9'28.05"W
Grândola	GRA	Hm	4	38°10'14.76"N 8°35'17.28"W
Bringes, Serpa	BRI	Hm	4	38° 5'33.95"N 7°33'14.16"W
Cuba, Odemira	CUB	Ha/Hm	6/5	37°38'4.62"N 8°42'21.66"W
Picoitos, Mértola	PIC	Hm	4	37°36'52.50"N 7°36'39.46"W

Locality	Code	Species	*n*	Coordinates
São Miguel do Pinheiro, Mértola	SMP	Hm	5	37°33'6.22"N 7°51'1.08"W
Almodovar	ALM	Hm	4	37°30'25.79"N 8°8'37.38"W
Rib. da Foupana, Faro	FOU	Hm	4	37°24'41.04"N 7°48'21.18"W
Corte Serranos	COS	Hm	5	37°22'57.84"N 7°49'1.44"W
Cachopo, Tavira	CAC	Hm	7	37°20'52.01"N 7°49'13.01"W
Javali, São Brás de Alportel	JAV	Hm	2	37°14'6.95"N 7°53'18.00"W
Bravura	BRA	Hm	6	37°14'9.72"N 8°40'48.36"W
Fóia, Monchique	FOI	Hm	6	37°18'58.18"N 8°35'35.97"W
Faz Fato, Tavira	FAZ	Hm	4	37°11'18.41"N 7°35'20.59"W
Arimbo, São Brás de Alportel	ARI	Hm	5	37°11'5.45"N 7°52'8.39"W

1.2. Male Advertisement Call Recordings

Collection of acoustic data began at dusk and lasted until male calling activity ended or became irregular and rare (usually between 1-3 am). Males were located by their calling activity, and each species was immediately identified based on the calling features (see description above), as *H. meridionalis* advertisement calls differ markedly from those of the *H. arborea* in that they consist of more pulses per call and lower call rates (i.e. fewer calls per minute). The advertisement calls were recorded with a directional Sennheiser M67/K6 super-cardioid spot shotgun condenser microphone, protected with a Sennheiser windshield and either a Marantz PMD 660 digital solid recorder or a Sony Hi-MD NZH1 digital recorder.

Sounds were recorded with an uncompressed algorithm using 16-bit linear Pulse Code Modulation (PCM) recFormat with mono channel, to files with .wav extension, with a sampling rate of 44.1 kHz and a bit rate of 705.5 kbps. The tip of the microphone was at a distance not less than 50 cm from the individual being recorded, and held steady to maintain the same distance from the male during the whole time of recording. No vegetation or other obstacles existed between the microphone and the male frog. The distance to the emitter of sound – the male treefrog – affects the amplitude of the sound and, in extreme cases, its frequency, which can bias the sound analyses when these parameters are used (Wiley & Richards 1978; Richards & Wiley 1980; Michelsen & Larsen 1983; Ryan 1988; Kime et al. 2000). Considering that a male frog is an omnidirectional sound source, the sound of the advertisement call produced will radiate as a sphere, and, according to the inverse-square law, sound-pressure level decreases linearly with the distance from the source, while intensity drops with the square of the distance (i.e. these parameters are usually measured on decibel

(dB) scale, resulting in a decrease at a rate of 6 dB per doubling of distance). This effect is referred to as excess attenuation. Excess attenuation is frequency dependent, i.e. higher frequencies are usually more attenuated than lower frequencies. Attenuation can also be habitat dependent, due to the presence of solid barriers such as vegetation; one of the most significant factors is the distance between the sound source and the ground. Calls transmitted at ground level suffer greater degradation than those transmitted above the ground, and this is even more exaggerated when including scattering from dense vegetation (Morton 1975; Marten & Marler 1977; Wiley & Richards 1978; Kime et al. 2000). For each male, the recording level was adjusted to obtain the best signal-to-noise ratio and avoid distortion, and kept constant over the recording session. Whenever possible, a minimum of five consecutive calls was recorded for each male.

A minimum of four males per sampling site were audio recorded, captured for body measurements and for tissue collection, with some exceptions (see tables 2.3 for details). We could not record four males at 8 sites for *H. meridionalis* and 8 sites for *H. arborea*, and in a few cases our presence at the calling sites was disturbing (e.g. male acoustic activity decreased after capture of the first male). This conflict between the collection of data for acoustic, biometric and genetic purposes could not, unfortunately, be avoided, but it was reduced as much as possible, since each male had to contribute with all types of data. In places where removing males from the chorus had a high impact (i.e. caused the cessation of activity), sampling was interrupted, and continued later or on a different night. On some occasions, capturing the first male only after a second male had been recorded was the strategy adopted. To ensure that the 'correct' males were captured, the individuals were kept under visual surveillance, using a flashlight, during the 'waiting' period.

Immediately after audio-recording, and before capturing the male, male body temperature and that of the calling-site substrate (all types of vegetation, dirt, rocks, etc., except water) were measured with an infrared thermometer, a Raytek Raynger ST, to the nearest 0.1ºC. Water and air temperatures were measured with a mercury thermometer (to the nearest 0.5ºC) in the vicinity of the frog – as close as possible to the male's location for water and about 1 m from the male for air.

After each male's recording session, the specimen was captured and kept in individually labelled plastic bags until all sampled males per site had been recorded and captured, in order to avoid repeated recordings of the same individual. To prevent contamination among individuals and sampling sites, each bag was used only once. For each captured male, body mass was obtained with a Pesola scale to the nearest 0.1 g, and the snout-vent length (SVL) was measured to the nearest 1 mm by gently pressing the individual ventral side flat against a plastic ruler (Fig. 2.4). Prior to field measurements, for accuracy purposes 13 males were measured three times each and measurement errors were calculated; no

statistically significant differences were found between the obtained measurements (results not shown), suggesting that the method chosen to measure SVL is acceptable and trustable. All males were released at the end of each sampling session in the same water body where they were captured. No males died during sampling.

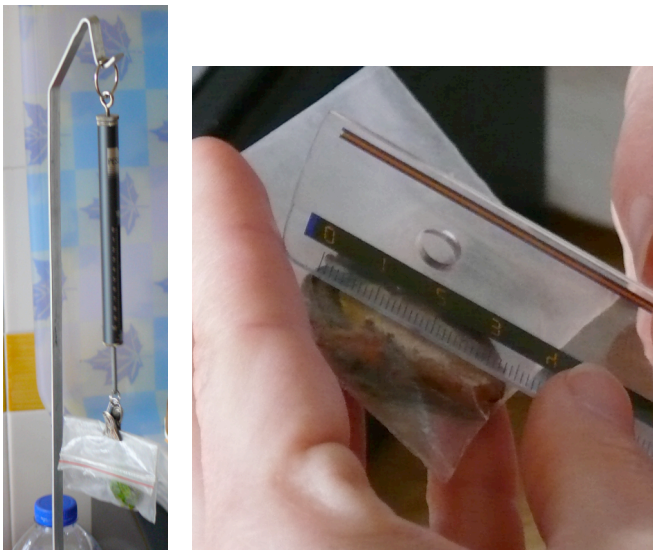


Figure 2.4. Measuring treefrogs' body size.

Individuals captured during field work had their SVL and mass recorded. Individual mass was taken with a *Pesola* scale (on the left) and SVL with a plastic ruler (on the right). Males are inside the plastic bags. (Photos by J. Moreira).

1.3. Tissue Sample Collection

Tissue samples were collected from 181 *H. arborea* and 180 *H. meridionalis* adult males and females, and 50 tadpoles from both species.[3] All the recorded males were used for tissue-sample collection. A total of 334 males (166 of *H. meridionalis* and 168 of *H. meridionalis*) and 27 females (13 of *H. arborea* and 14 of *H. meridionalis*) were captured. Since females do not produce calls, specific surveys were done for their detection and capture. Some adults were captured solely for tissue collection (females and males without being audio-recorded). Tadpoles were captured using a sweep-net technique, in the margins and middle of the water bodies. Adults were toe-clipped and tadpoles were tail-tip-clipped with a stainless steel nail clipper. All samples were preserved in Eppendorf tubes with 96º ethanol, at room

[3] Tadpoles when captured were not identified to species (only to genus), nor to their development stages according to the Gosner staging table (Gosner 1960). For the purpose of this work, stages of development were not that important, and, for practical reasons of fieldwork efficiency I did not identify them. Species identification was attempted but proved to be hard and not clear, which again, for fieldwork efficiency, would pose a time problem; also, it was not that important. Tadpoles from allopatric areas were considered to belong to the species there present, and those in sympatric areas were all typed using RFLPs.

temperature. For safety and contamination reasons, all cutting equipment used to collect tissue samples was cleaned with ethanol between individuals and bleached between sampling sites.

1.4. Ethical Note

All treefrogs captured during this study were toe- or tail-clipped because tissue samples were needed for posterior DNA analysis. A few recent studies (e.g. Pidancier et al. 2003; Poschadel & Möller 2004; Berset-Brandli et al. 2006, 2007, 2008, 2008a; Broquet et al. 2006, 2009; Angelone & Holderegger 2009; Jaquiéry et al. 2009; Rovito 2010; Barth et al. 2011) used buccal swabs with *H. arborea* as a less invasive technique to collect DNA samples, instead of toe-clipping. I attempted this technique in the field, but found it much more difficult to execute, as individuals become stressed and force is required to open their mouths (relatively small when compared to other anuran species such as *Bufo bufo* or *Pelophylax perezi*). Based on observations from animals kept in captivity for a period of around two months, I was able to observe that clipped toes healed quickly and did not affect the individual's behaviour. In the field, and in particular with males, toe-clipped individuals seemed unaffected in their reproductive behaviour, as they returned to calling shortly after being released. For all these, I opted to use the more invasive but, in my opinion, more effective technique of toe-clipping. All individuals were captured under a permit issued by Instituto de Conservação da Natureza e Biodiversidade (ICNB) for this specific project.

2. Laboratory Procedures

2.1. Molecular Techniques

DNA Extraction

Genomic DNA (gDNA) was extracted from ethanol-preserved tissues using a standard high-salt protocol, according to Sambrook et al. (1989), or using the DNeasy tissue kit (QIAgen), following the manufacturer's instructions, with modifications. Samples were previously soaked in nanopure water for at least three hours to remove ethanol residues from the tissue, and the gDNA was eluted in a 150 μl of AE Buffer (QIAgen). The DNA extraction yield was quantified using a Nanodrop spectrophotometer (ng/μl) immediately after extraction. All DNA extraction products were stored at -20ºC until further analysis.

DNA Amplification

Fragments of three mitochondrial genes, Cytochrome Oxidase I (COI), 12S rRNA and 16S rRNA (COI, 12S and 16S) and 36 microsatellite loci were used for the differente molecular analyses employed on this work (see details below).

Mitochondrial DNA (COI, 12S and 16S). Using a combination of two primers, the Amp-P3F and the Amp-P3R, previously described by San Mauro et al. (2004) (see Table 2.4 for primer sequence), it was possible to amplify a fragment of 838 bp of the gene Cytochrome Oxidase I (COI). Polymerase chain reactions (PCR's) were performed in a total volume of 12.5 μl, including 1 unit of Taq polymerase (Promega 5 U/μl), 2.5 μM of each primer, 0.4 μM of dNTP's, 1.5 mM of $MgCl_2$ and 67 mM of reaction buffer (Tris-HCl, pH 8.3, Promega). The PCR protocol consisted of a initial denaturation at 94ºC for 5 minutes followed by 35 cycles at 94ºC for 45 seconds, annealing at 50ºC for 45 seconds, extension at 72ºC for 45 seconds and a final extension at 72ºC for 5 minutes. Primers used in both amplification and sequencing were 12sa and 12sb for the 12s rRNA (Kocher et al. 1989) and 16SL1 and 16SH1 for the 16S rRNA (Palumbi et al. 1991). PCR was carried out in a 12.5 μl, following conditions described above for COI, except that annealing temperature for 12S rRNA was 53ºC and 51ºC for 16S rRNA.

Microsatellites. Thirty six microsatellite loci isolated and characterised for *H. arborea*, 15 microsatellite loci (WHA 1-9, WHA 1-20, WHA 1-25, WHA 1-54, WHA 1-60, WHA 1-61, WHA 1-67, WHA 1-103, WHA 1-104, WHA 1-133, WHA 1-140, WHA 5-22A, WHA 5-29, WHA 5-57 and WHA 5-201) isolated by Arens et al. (2000), eight loci (Ha A-103, Ha A-110, Ha A-136, Ha A-139, Ha D-104, Ha D-106, Ha D-110 and Ha H-116) from Berset-Brändli et al. (2008) and 11 loci (Ha A-11, Ha A-119, Ha A-127, Ha A-130, Ha B-5R3, Ha B-12, Ha D-3R3, Ha D-115, Ha E-2, Ha H-107 and Ha H-108) from Berset-Brändli et al. (2008[a]). The microsatellite loci were amplified and scored (see Table 2.5 and cited works for the specific primers and PCR profiles). Microsatellite loci were first amplified using regular primers, and, when successfully amplified, microsatellite loci were amplified using fluorescently labelled primers with 6-FAM. Amplified products were run against LIZ 500 size standard on an ABI Prism 3130 XL (Applied Biosystems) automated DNA sequencer. Alleles were visualised and scored with GENEMAPPER 3.7 (Applied Bio-systems).

DNA Sequencing

All amplicons from mtDNA genes were sequenced. Microsatellites were not sequenced. A sodium acetate and ethanol-precipitation standard method was used to purify PCR products before sequencing. Sequencing reactions were performed for both strands using the ABI PRISM BigDye Terminator Cycle Sequencing protocol, following the manufacturer's instructions. Sequences were obtained using an ABI PRISM 377 and ABI PRISM 3130*xl* sequencers (Applied Biosystems). The primers used for sequencing were the same as those for PCR amplification.

RFLP (Restriction Fragment Length Polymorphism) Assay at Species Identification

The aim of this analysis was to check for the presence of individual hybrids in natural sympatric-syntopic zones using the PCR-RFLP technique. This technique is relatively easy to use toidentify F1 hybrids and backcrosses, requiring only a few markers (Boecklen & Howard 1997).

To develop the RFLP attempt, a subset of four *H. arborea* and four *H. meridionalis* individuals from allopatric populations (i.e. not overlapping geographically) was selected. Five DNA regions were chosen, including one mitochondrial (Cytochrome Oxidase Subunit I, COI) and fragments of four nuclear genes (Pro-Opiomelanocortin, POMC2; Proto-Oncongene Cellular Myelocytomatosis, C-myc2; Recombination-Activating Gene 1, RAG1, and Tyrosinase Precursor Exon 1, TYR1). All DNA regions had previously been used in amphibian studies, and specific primers were available for each of them (Table 2.4).

The five aforementioned fragments were amplified via PCR using gene-specific primers. For the mitochondrial gene COI, a combination of two primers, the Amp-P3F and the Amp-P3R, previously described by San Mauro et al. 2004 (see Table 2.4 for primer sequence), were used. The PCR temperature protocol was as described above. The same PCR protocol described for COI was used for both POMC2 and C-myc2 amplification, using the specific primers POMC1 and POMC2 (Wiens et al. 2005) and C-mycex2d (Wiens et al. 2005) and C-myc1U (Crawford 2003), respectively. For the RAG1 gene, the primers RAG-1Hf and RAG-1HR were specifically designed for this study by Sequeira (unpubl). For the Tyr1 the primers Tyr1C and Tyr1G described by Bossuyt and Milinkovitch (2000) were used. Amplifications were performed in a total volume of 12.5 μl, including 1 unit of Taq polymerase (Promega 5 U/μl), 2.5 μM of each primer, 0.4 μM of dNTPs, 1.5 nM of $MgCl_2$ and 67 mM of reaction buffer (Tris-HCl, pH 8.3, GoTaqPromega).

Table 2.4. Primers used in this study.

Gene fragment, primers names and sequences.

Gene Fragment	Primers	Primers sequence	Reference
COI	Amp-P3F	5' – CAA TAC CAA ACC CCC TTR TTY GTW TGA TC – 3'	San Mauro et al. 2004
	Amp-P3R	5' – GCT TCT CAR ATA ATA AAT ATY AT – 3'	
12s rRNA	12sa	5' – CTG GGA TTA GAT ACC CCA CTA – 3'	Kocher et al. 1989
	12sb	5' – TGA GGA GGG TGA CGG GCG GT – 3'	
16S rRNA	16SL1	5' – CGC CTG TTT AAC AAA AAC AT– 3'	Palumbi et al. 1991
	16SH1	5' – CCG GTC TGA ACT CAG ATC ACG T– 3'	
POMC2	POMC1	5' – GAA TGT ATY AAA GMM TGC AAG ATG GWC CT – 3'	Wiens et al. 2005
	POMC2	5' – TAY TGR CCC TTY TTG TGG GCR TT – 3'	
C-myc2	C-mycex2d	5′ – TCA TTC AAT GGG TAA GGG AAG ACC– 3'	Wiens et al. 2005
	C-myc1U	5′ – GAG GAC ATC TGG AAR AAR TT – 3'	Crawford 2003
RAG1	RAG-1Hf	5' – CCA TGA AAT CCA GTG AGC TCC – 3'	Sequeira (unpubl).
	RAG-1HR	5' – CAK AGC AAC TCT GGG CAC TC – 3'	
Tyr1	Tyr1C	5' – GGC AGA GGA WCR TGC CAA GAT GT – 3'	Bossuyt & Milinkovitch 2000
	Tyr1G	5' – TGC TGG GCR TCT CTC CAR TCC CA – 3'	

Table 2.5. Characterization of 36 microsatellite loci for the European tree frog *Hyla arborea*.

Reported are: locus name; repeat motif, sequences for forward (F) and reverse (R) primers and GenBank Accession No

Locus	Repeat	Primer sequences (forward, reverse)	GenBank Accession no
WHA1–9	$(CA)_{20}$	5′-CGTTTGGACGTGATGCTG-3′ 5′-GAGGAGTTTCTTCACAAGGGG-3′	AJ403985
WHA1–20	$(GT)_{18}$	5′-GTCCCTTCCTGAATAAGTGTCG-3′ 5′-CCATTCCCTCCTGGCTTT-3′	AJ403986
WHA1–25	$(GT)_{20}$	5′-AAGAATCTGCCGCAAAGAAG-3′ 5′-TAGGAAGGGACAGGAGGTCA -3′	AJ403987
WHA1–54	$(CA)_{19}$	5′-CCTGGTCATGCTACACGCTA-3′ 5′-GACAACAACCCCCAATAATCC -3′	AJ403988
WHA1–60	$(GT)_{22}$	5′-TAGGTCATGTATAGCCTGTT-3′ 5′-TCTGTTTACTTCAGGGGT-3′	AJ403989
WHA1–61	$(TG)_7$ ... $(AT)_6$	5′-CAGGTCCAAGCTCTCTCCC-3′ 5′-GCACATTCACATTATATAGACAACACA-3′	AJ403990
WHA1–67	$(CA)_{21}$	5′-GCTTTACACATGGGGGTAT-3′ 5′-CACTCCTTTTAGAGTATGTTGTTG-3′	AJ403991
WHA1–103	$(GT)_{21}$	5′-CAAAGTGACAATGTGGGGTCTCAT-3′ 5′-ATAGCATCAAATCCAGCCGTAGG-3′	AJ403992
WHA1–104	$(GT)_{22}$	5′-ACTTGGGACAGCCAGTATGTTTT-3′ 5′-TGAGCTGGTGGGTATAACCTAAC-3′	AJ403993
WHA1–133	$(CA)_{24–1}$	5′-ATGCCCCTCATAGAACACATACAA-3′ 5′-GGGCTGCGGGTACAGTAGTG -3′	AJ403994
WHA1–140	$(GT)_{25}$	5′-ATGTGCCATAGAAATGAAGG-3′ 5′-AGGCTTGCTGCTATTATGTC -3′	AJ403995
WHA5–22A	$(CAA)_6$ ... $(CAG)_6$	5′-TTACAGCAACAGCAAATGG-3′ 5′-ATCAGGGACTGGGTCTGT-3′	AJ403996
WHA5–29	$(ACC)_{74–20}$	5′-TTCATCCATTCTCATCTCTTCTCA-3′ 5′-ACATGGGGCCCTTCTACC-3′	AJ403997
WHA5–57	$(GGT)_6$	5′-TTGTCCTGACATGCACACCT -3′ 5′-CGTGTCTAACCCCAGCTCAT-3′	AJ403998
WHA5–201	$(CAC)_{13–1}$ ... $(CAC)_{8–1}$	5′-TCATGGACTGTCGTCATGGT-3′ 5′-AGGTAAATGGAATCTGGGTGTG-3′	AJ403999
Ha-A11	$(CA)_{14}$	5’- CCTCCCTCACTCTGCTGAC -3’ 5’- CAATCCCCGAAAAACATTG -3’	EU029094
Ha-A119	$(CT)_5(CA)_6TA(CA)_{14}$	5’- CAACTTCCCCCTCTGTTC -3’ 5’- GCTGAGTGTGAGTGTGTTTG -3’	EU029095
Ha-A127	$(TG)_{14}$	5’- CTCTGGGTTGCACTACTTAGTC -3’ 5’- TTCAGGGCTAATTCTTTGTATG -3’	EU029096
Ha-A130	$(CA)_{10}$ ... $(CA)_{13}$	5’- ATTGCTCACACATACACACAGG -3’ 5’- GCAGTCACAACTCATTTTGATG -3’	EU029097
Ha-B5R3	$(TC)_{13}$	5’- CCCCTTTAGAGTCGCCATAC -3’ 5’- AGCCATCTTGTGGTCAGTCA -3’	EU029098
Ha-B12	$(TC)_{21}$	5’- AATGGTATCTCGGTGGTATCC -3’ 5’- TTGAAAAATCTCTCCCTACAGC -3’	EU029099
Ha-D3R3	$(TATC)_{21}$	5’- ATCACCATCCCTGCATTAC -3’ 5’- CGACATGATAGATGTGAGATAA -3’	EU029100
Ha-D115	$(TAGA)_{16}$	5’- GTTTTTCGATTCCTGGATAAC -3’ 5’- TGGGAGTTTTCAAAAGTGAC -3’	EU029104
Ha-E2	$(CAA)_7$	5’- ACAACTTCCAACTGGAGTCAAC -3’ 5’- CCTTAGTGGGAGCTGTAATCAC -3’	EU029103
HaH107	$(GT)_7$ ... $(TATG)_4$	5’- CACCCTGGTAGGGAAATTC -3’ 5’- GGCAAATGGGGATGAGTA -3’	EU029101
HaH108	$(TACA)_{10}$	5’- GGGGGTGAGTAAGGGTTAAATC -3’ 5’- GCCACTGTATAGTCCCTCCCTA-3’	EU029102
Ha A-103	$(GT)_{17}$	5’-GCCTAGAAATGTGCAGTGATC-3’ 5’-CAATTCACACCCAAATCAGAT -3’	EU525921
Ha A-110	$(CA)_{15}$	5’-AAGGGTTAAATCACCTATCC-3’ 5’-ACGCAAAAAACATCTGTG -3’	EU525922
Ha A-136	$(CA)_{15}$	5’-CCACTGTAAGTAAAATGTGTGC-3’ 5’-TAAAATCCACCAAGAAACCTAC-3’	EU525923

Locus	Repeat	Primer sequences (forward, reverse)	GenBank Accession no
Ha A-139	$(GT)_{21}$	5'-GTTTCCAGATAGCGAAAACTG-3' 5'-CACTGCTCCCAGTATCAGAA-3'	EU525924
Ha D-104	$(TAGA)_7$	5'-FAM-GCTGGCTGACTTATTCTTTG-3' 5'-TCTTCTCTCCACGGTCTTC -3'	EU525925
Ha D-106	$(TAGA)_9$	5'-FAM-CACCATAGCTGTATAGCTCTCC-3' 5'-CAAAGATTAAGGCTGTGTTCA -3'	EU525926
Ha D-110	$(TAGA)_{42}$	5'-AACTGCATGTTCATGTTTCAC-3' 5'-CCTGACTTCTTAAATGTGCTTT -3'	EU525927
Ha H-116	$(TACA)_{17}$	5'-AATGGGGGTGAGTAAGGGTTA-3' 5'-CAGGTCCTGACACTGTGACAC-3'	EU525928

Thermal cycling conditions included an initial denaturation at 94ºC for 5 minutes, followed by 35 cycles at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 20 seconds and a final extension at 72ºC for 4 minutes. After PCR amplification, fragment size was checked via electrophoresis separation on a 2% agarose gel and staining with ethidium bromide. Sequencing reactions of the amplicons were performed for both strands, following the ABI Big Dye Terminator Cycle Sequencing protocol and using the same sets of primers used for PCR amplification. Sequencing was done on an ABI PRISM 377 sequencer following the manufacturer's instructions.

Table 2.6. Restriction enzyme reactions.

Reactions protocols for each of the diagnostic restriction endonuclease assays.

	COI	RAG 1	Tyr	POMC 2	C-myc 2
Restriction Enzyme	Sfan I	BSA I	Bsh NI	BsrI	BsrI
Buffer	Tango	NE3	Tango	B	B
Temperature (ºC)	37	56	37	65	65
Duration (H)	3	4	3	Overnight	overnight
Enzyme volume (μl)	0.15	0.5	0.3	0.15	0.15
Buffer volume (μl)	0.75	1	1	0.75	0.75
H_2O volume (μl)	4.1	6.5	5.7	4.1	4.1
PCR product volume (μl)	5	2	3	5	5

Diagnostic restriction endonuclease assays were designed using BioEdit Sequence Alignment Editor v7.0.9.0 software (Hall 1999). DNA sequence alignment from each gene fragment were used to choose a restriction endonuclease, targeting the nucleotide polymorphisms that distinguish the two parental species, *H. meridionalis* and *H. arborea*. For a COI fragment of 838 base pairs (bp), SfaNI identifies three recognition sites (80, 324 and 434 bp) for *H. meridionalis*, and two recognition sites (70 and 764 bp) for *H. arborea*. For Pomc2 fragment of 554 bp and for C-myc2 fragment of 408 bp, BsrI identifies two recognition sites (42 and 512 bp) for *H. meridionalis*, three recognition sites (42, 148 and 405 bp) for *H. arborea*, four recognition sites (31, 59, 132 and 186 bp) for *H. meridionalis*, and

two recognition sites (31 and 377 bp) for *H. arborea*. BSAI identifies two recognition sites (86 and 567 bp) for *H. meridionalis* and three recognition sites (86, 195 and 372 bp) for *H. arborea* in a RAG1 fragment of 653 bp. And finally, for a fragment of Tyr1 with 488 bp, BshNI identifies two recognition sites (95 and 393 bp) for *H. meridionalis* and three recognition sites (95, 109 and 284 bp) for *H. arborea*. Enzyme digestion reactions conditions for each assay are shown in Table 2.6. Electrophoresis separation of digested DNA fragments were run on 2% agarose gels, stained with ethidium bromide (0.5 µg/ml) and visualised under UV light.

The five RFLP assays were run for all samples from sympatric sampling sites. Samples from allopatric sampling sites were also typed for quality control. Finally, 12 F1 hybrid samples obtained in laboratory crosses (see detailed description below) were also used as constrols. PCR and enzyme-restriction protocols were carried out as described above.

2.2. Species Hybridisation in the Laboratory: Interspecific Crosses

Artificial hybridisation attempts were made to obtain a reference pattern using Restriction Fragment Length Polymorphism (RFLP), and therefore test the accuracy of hybrid identification for all the samples collected in the field, so that if any hybrid of F1 generation was captured in nature this would guarantee that it would be correctly identified. A total of 8 crosses (four interspecific and four intraspecific as controls) were done with the following combinations: two crosses of ♂*Hyla meridionalis* x ♀*Hyla arborea* (from FRO and CRE); two crosses ♂*Hyla arborea* x ♀*Hyla meridionalis* (from ALP and FRO); two crosses ♂*Hyla arborea* x ♀*Hyla arborea* (from BUR and SOU) and two crosses ♂*Hyla meridionalis* x ♀*Hyla meridionalis* (from FRT and BUR). For these crosses, eight males and eight gravid females (i.e. containing ovocytes, see Fig. 2.5) were captured in six different sites. Females and males used in each cross originated from the same localities, in order to reduce the probability of spreading epidemic diseases like chytridiomycosis, which is responsible for many amphibian deaths (e.g. St-Amour et al. 2010). For each combination attempted, one cross was a result of induced ovulation and the other by 'natural' means. In the first case, ovulation was induced through intraperitoneal injection of 400 IU of chorionic gonadotropin, and sperm was obtained by dissecting the two testicles from a male and macerating them in amphibian Ringer's solution (following Rosa & Oliveira 1994 and Silva unpubl.). After the ovocytes' release, sperm suspension was pipetted into each clump of ovules collected on Petri dishes. After fertilisation (confirmed through microscopy), eggs were transferred to an aquarium with spring water where embryos were raised. In the second case, couples were placed together in small (10x25 cm) aquariums overnight (about 7-8

hours), and the resulting eggs were moved to a new aquarium with spring-clean water to avoid chloride intoxication and reduce the presence of fungus and parasites.

Figure 2.5. *Hyla arborea* gravid female.

Female captured in CUB sampling site (southwest Portugal). Ovocytes are visible inside the ventral cavity (arrow). See Table 2.2. for population acronym. Photo by J. Moreira

The tadpoles were maintained and fed with fish pellets. Dead embryos were removed regularly and stored individually in ethanol 96º at room temperature for genetic analysis. As the goal of this experiment was not to test for hybridisation viability and survival rates, but only to get known hybrid patterns for posterior field control of the occurrence of this phenomenon, no extra-special care was taken to guarantee full metamorphosis of the tadpoles. After each usage, all the aquariums were bleached for a minimum of ten minutes in a bath of 70% bleach-water (Jonhson et al. 2003).

Tissue samples from the adults used in these heterospecific crosses were collected only after the couple had been in amplexus and laid eggs, so that toe-clipping would not affect the success of the crosses. The collected toes were stored individually in ethanol 96º for genetic analysis. All adults used in the crosses were released in the same area where they were caught.

3. Data Analysis

3.1. Molecular Analysis of Mitochondrial DNA sequences

Mitochondrial DNA diversity variation and phylogenetic analyses

All protein-coding sequences obtained from the amplification of the fragment of COI for both species (see above) were edited, prior to alignment, using BioEdit, and inspected by eye against the original chromatogram. The program DNAsp v.5.10.01 (Librado & Rozas 2009) was employed to look for the presence of insertions, deletions and potential stop codons that would reveal a non-functional protein, as this allows the protein-coding region to be translated into amino-acid sequences. No evidence of non-functional protein was found. Nucleotide sequences were then compiled and aligned using the BioEdit v7.0.9.0 software (Hall 1999) and a clustal X algorithm with default parameters. Most alignments were straightforward, not requiring further handling. A subsequent ends-cut was performed, as the quality at the sequence extremes was very low in some cases. All the new nucleotide sequences reported here will be registered in the GenBank Nucleotide Database.

To better cover each species range, 13 additional COI sequences of *Hyla arborea* (from Portugal, Croatia, France, Germany, Switzerland, Spain and Greece) available in GenBank (see Table 2.2 for respective GenBank accession numbers) were added to the analysis. Likewise, 51 additional COI sequences of *H. meridionalis* available in GenBank (see Table 2.1 for complete list) were included in the analyses, and corresponded to locations in Spain, France, Morocco, Tunisia and Portugal.

For both species' data set, the following genetic diversity statistics were calculated in DNAsp v.5.10.01: the nucleotide diversity index (π, Nei 1987; i.e. the probability that two randomly chosen homologous nucleotides differ in the sample), the number of haplotypes (H), the haplotype diversity (h), the average number of nucleotide differences among sequences (k), population mutation parameter (Θ) (Watterson 1975) and the number of segregating sites (S). The software MEGA v4.0 (Tamura et al. 2007; Kumar et al. 2008) was used to evaluate the haplotype sequence divergence (p-uncorrected distance) among haplogroups within each species.

Phylogenetic analyses of the mitochondrial sequence data set were performed using Maximum Likelihood (ML) and Bayesian Inference (BI). For both analyses sequence evolution was first applied as a best fit model, as a traditional approach. The best-fit substitution model for the COI fragment (model of molecular evolution) was chosen with jModelTest v.0.1.1 (Posada 2008) under the Akaike Information Criteria (AIC; Akaike 1974), following Posada & Buckley (2004). Afterwards, and following recent studies (e.g. Brandley

et al. 2005; Wiens et al. 2010) a partitioning scheme was also performed, based on intron and exon regions and codon positions in the protein-coding COI mitochondrial gene, using an independent model for each partition. Standard Bayes Factors (BF; Nylander et al. 2004) were used to determine the most appropriate strategy, applying Kass & Raftery's (1995) conventions (2LnBF> 10 considered to offer 'very strong' support for a particular partitioning model).

ML analyses were accomplished using RAxML GUI software (Silvestro & Michalak 2010), a graphical front-end for RAxML-VI-HPC (Randomized Axelerated Maximum Likelihood; Stamatakis 2006). ML with the thorough bootstrap option was run ten times from starting random seeds, to generate 1000 nonparametric bootstrap replicates. General time-reversible model (GTR) with gamma rate heterogeneity was used for all ML analyses. BI analyses were conducted in MrBayes v. 3.0b4 (Ronquist & Huelsenbeck 2003) using two replicate searches of $20x10^6$ generations each, sampling every 1000 generations (i.e. retaining one tree every 1000 generations). All searches consisted of three 'heated' and one 'cold' Markov Chain Monte Carlo (MCMC). For the analysis of COI sequences by codon position, the nucleotide frequencies were considered fixed for the first and the second positions, and the Dirichlet process model was used for the third position (Bofkin & Goldman 2007).

Historical Demography

The demographic histories of *Hyla meridionalis* and *H. arborea* were inferred using different complementary approaches, making use of the partitions suggested by the phylogenetic trees and network haplotype structures. First, the model of sudden demographic expansion was tested by using mismatch distribution analyses. Mismatch distribution analyses are useful to describe the frequency of pairwise substitution differences among individuals (Slatkin & Hudson 1991), which can reveal aspects of the demographic history of the studied populations by inferring population expansion events. Theoretical distributions, based on assumption of constant population size, and the sudden expansion model are compared to the observed data. This is the case of populations subject to recent bottleneck events and/or rapid expansion with high levels of migration between neighbouring demes, where a unimodal mismatch distribution would be expected (Rogers & Harpending 1992; Hudson & Slatkin 1991; Rau et al. 2003; Excoffier 2004; Martínez-Solano et al. 2006). The distribution is usually multimodal for samples drawn from populations under demographic equilibrium. The tau value (τ), reflecting the location of the mismatch distribution crest, here provides an approximate estimate of the time when rapid population expansion started.

Second, to test for deviations of DNA sequence evolution from selective neutrality under an 'infinite-site' model (Waterson 1975), as expected under a demographic expansion scenario, we used Tajima's *D* (1989) and Fu's *Fs* (1997) tests. Tajima's *D* test is used to infer the presence of natural selection from DNA polymorphisms, based on the difference between two estimates – the number of segregating sites (k) and the average number of pairwise differences (π) – of the amount of variation. Tajima's *D* is expected to be nearly zero for a constant-size neutral equilibrium population. It will tend to be positive if the populations are undergoing balancing selection with decreasing population size, and will be negative in a population with increasing size (Tajima 1989; Innan & Stephan 2000). The Fu's *Fs* statistics provides a test for population growth; recent population expansion or genetic hitchhiking will carry negative values as evidence for an excess number of alleles when compared to the number expected in a stationary population, while positive values of *Fs* will be evidence for deficiency of alleles as expected from a recent population bottleneck, or from selection (Fu 1997). Fu's *Fs* appears to be more sensitive to population expansion than Tajima's *D* (Ramos-Onsins & Rozas 2002). Estimation and testing were done by bootstrap resampling (10,000 replicates) using DNAsp software. Also, population size changes were evaluated using the R2 test, which is a more robust test to detect population expansions in small sample sizes (Ramos-Onsins & Rozas 2002). Pairwise mismatch distribution analysis (pairwise nucleotide differences) (Rogers & Harpending, 1992), Tajima's *D* and Fu's *Fs* and R2 tests were carried out on DNAsp to test for population expansion in the total data and main lineages. Significance values of all statistical tests were computed using the coalescent simulator implemented in DNAsp software by comparing estimated values against a distribution generated from 10,000 random samples, under the hypothesis of selective neutrality and population equilibrium, with no recombination (Hudson 1990).

Haplotype Network

Genealogical relationships among haplotypes were estimated using a network-approach, using phylogenetic algorithms with migration, and using proper models of sequence evolution (Salzburguer et al. 2011). Phylogenetic reconstructions among mitochondrial haplotypes were estimated using a maximum-likelihood approach using the software PHYML 3.0 algorithm (Guindon et al. 2010). The program was run using the default options, with a best-fit model for the locus as selected by Kakusan4 (Tanabe 2007). A haplotype network was constructed with the Haplotype Viewer program (Salzburguer et al. 2011) using the previously generated trees.

Isolation-by-Distance

To examine isolation by distance in data from spatial genetic surveys, scatter plots with geographic distance on the *x*-axis and genetic distance on the *y*-axis were constructed. Only sampling sites with more than five individuals were used in this analysis. Genetic distance was measured as the nucleotide divergence between pairs of sampling sites; after accounting for nucleotide diversity within populations (Da), nucleotide divergence among populations was estimated using DNAsp. Geographic linear distances between sampling sites were measured using the GoogleEarth rule tool.

To assess whether there was a statistically significant relationship between the genetic distance and geographic distance matrices, a non-parametric Mantel's test (Manly 1991) of matrix association between genetic distance and Log10 geographic distance – using IBDWS v.3.22 (Jensen et al. 2005) with default settings – was used. This test calculates the regression coefficients between dependent-distance and independent-distance matrices; by a randomisation procedure it provides a probability estimate of the null-hypothesis of not-significant-relationship between the two distances. Additionally, a reduced major axis (RMA) regression was used to calculate the slope and intercept of the isolation-by-distance relationship. RMA regression is preferred in this program, according to the authors, as it 'is more appropriate than standard linear regression when both the dependent and the independent variables are measured with error' (p. 2). A steeper slope is expected for shorter dispersal-distance relationships, although the relationships may not be linear. An effectively well-mixed population should show no relationship between geographic and genetic distances, because thorough mixing would randomise location with respect to genotype.

3.2. Sound Analyses of Advertisement Calls

The recordings were downloaded to an Apple computer (a PowerBook G4 and MacBook Pro), and calls were analysed using Raven software (Cornell Laboratory of Ornithology, Ithaca, NY, USA; version 1.2.1). 3-5 consecutive calls per male were analysed, except in the cases where males did not engage in long call sequences.

The nomenclature used here for call variables follows that described by Schneider (1974) and Castellano et al. (2002). Advertisement calls of the two species differ in component structures (see figs. 2.6 and 2.7), and thus different sets of parameters were measured per call. Frequency measurements were the same for both species. A power spectrum was calculated for each call, using a Fast Fourier Transform (FFT) analysis with a frame length of 1024 points (overlap 75%, Hamming's sampling window with a bandwidth of 56 Hz), and the following frequency measurements were taken:

i. fundamental frequency – FF (Hz);
ii. dominant frequency – DF (Hz, the frequency with maximum intensity);
iii. fundamental frequency maximum relative amplitude (dB);
iv. dominant frequency maximum relative amplitude (dB);
v. difference of amplitude between dominant and fundamental frequencies – ADF.

The temporal call parameters measured varied between the species, due to the species-specific structure of the calls. For the advertisement calls of *Hyla arborea*, seven parameters were measured (Fig. 2.6):

i. call group duration – CGD (*s*, duration of a series of calls);
ii. number of calls per call group – CCG (number of calls);
iii. call duration – CD (*ms*, duration of a series of pulse groups);
iv. intercall duration – ICD (*ms*, duration of the interval between calls within the same call group);
v. call rate – CR (number of calls per call group duration, i.e. number of calls per second);
vi. number of pulses per call – PC;
vii. pulse rate – PR (number of pulses per call duration, i.e. number of pulses per ms).

For *Hyla meridionalis*, however, only four temporal parameters were measured, as follows (Fig. 2.7):

i. call duration – CD (s);
ii. intercall duration – ID (s);
iii. number of pulses per call – PC;
iv. and, pulse rate – PR (number of pulses per second).

Intercall group duration for *H. arborea* was not used, as some males emitted their calls sporadically and not in sequence. This propensity could give an erroneous idea of temporal distribution of calls.

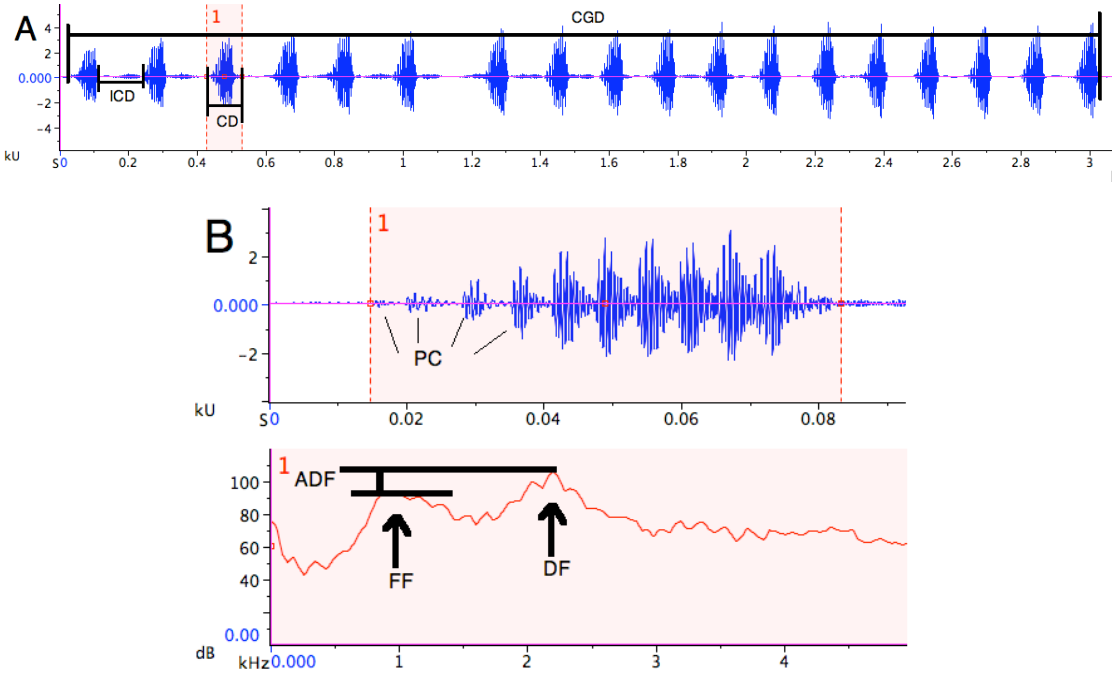


Figure 2.6. Parameters measured in the advertisement calls of *Hyla arborea*.

Schemes (A and B) of an oscillogram and mean power spectrum of *Hyla arborea* advertisement calls. The parameters that were measured for each call are indicated in the figures. For call parameters acronyms see above.

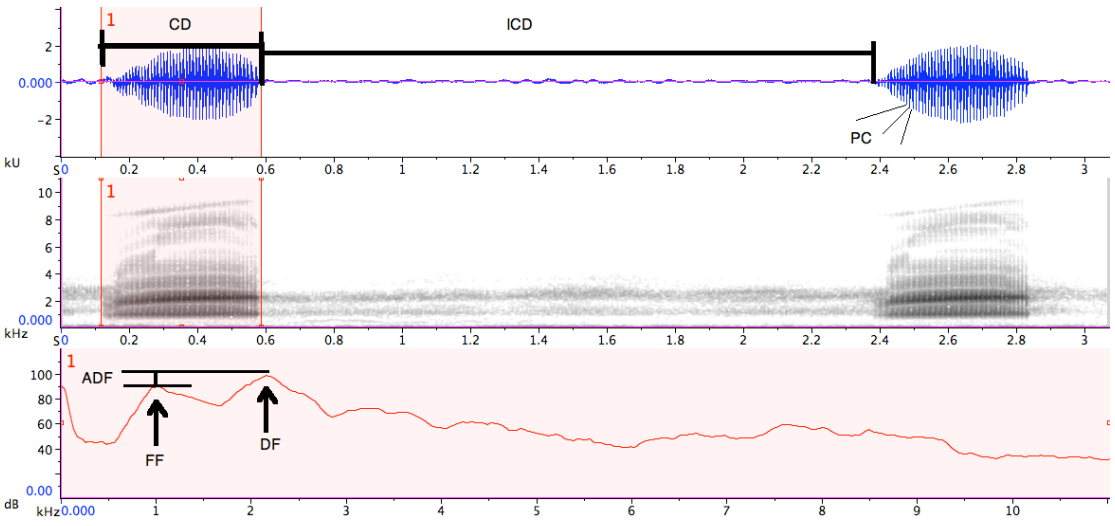


Figure 2.7. Parameters measured in the advertisement calls of *Hyla meridionalis*.

Schemes of an oscillogram and mean power spectrum of *Hyla meridionalis* advertisement calls. The parameters that were measured for each call are indicated in the figures. For call parameters acronyms see above.

Coefficients of Variations in Call Parameters

To evaluate within-individual and between-individual variation in call structure I calculated the coefficients of variation (CV) of all call properties (CV=standard deviation/mean x 100). All call parameters were classified as static if CV <5% (parameters change relatively little from call to call), dynamic if CV> 12% (parameters greatly vary from call to call) or intermediate if 5% <CV <12% (Gerhardt 1991; Gerhardt & Huber 2002).

H. arborea males produce a series of calls – a call group – while *H. meridionalis* males produce single calls at a time. Therefore, I quantified the within-individual call variation in *H. arborea* by calculating the CV of all call properties at two different levels: first, within-call-group (i.e. for each call group recorded per male) and second, between-call-group (i.e. average per male per call group), always both for within population and globally for the species.

Effect of Temperature and Body Size

The effect of temperature (air, substrate and body) and of body size (i.e. SVL and mass) on male call variation was evaluated using multiple-regression analyses in JMP 9.0.3 (SAS 2010). Call parameters were used as dependent variables and regressed against temperature (air, substrate and male) and body size (SVL and mass) independent variables. Location was not used at first, as it would necessarily impact if temperature or size had an effect. The acoustic parameters that were significantly correlated with temperature and/or size were adjusted by regressing out the significant effect. Temperature- or size-independent residuals from univariate regression analysis of each call parameter against male temperature were used for all subsequent analyses.

Since within each population the range of temperatures and body size was limited, potentially reducing the statistical power of the regression analysis, I chose to exclusively analyse the overall (between-population) effects of temperature and body size, using all the individuals from all populations as points in the regression, i.e. each individual represents a point in the regression.

Call Variation among Populations

One-way Analysis of Variance

To study the call variation among populations I considered the temperature- and body-size-adjusted acoustic properties. One-way Analyses of Variance (ANOVA) were used to test for differences in the mean value of call variables between different populations. When significant differences were found, a *post hoc* analysis of Tukey-Kramer Honestly Significant

Difference test (Tukey-Kramer HSD) for pairwise comparisons was used to determine which populations were significantly different from each other. Tukey's test is more appropriate than other *post hoc* tests, such as LSD and Scheffe, when all pairs of means are being compared, and its power remains high even with unequal sample sizes per group (Day & Quinn 1989).

Discriminant Function Analysis

To estimate the distinctiveness of calls within the various sampling locations, a Discriminant Function Analysis (DFA) was conducted. Discriminant analysis is a statistical test with great power to identify and interpret differences between data groups (i.e. sampling sites), and to attribute membership to items according to linear combinations of several descriptors (Sokal & Rohlf 2000). In the first step, DFA generates canonical Discriminant Functions (DF) that represent linear combinations of the original variables that maximally separate items in a multidimensional signal space (Nelson & Marler 1990). Then, an algorithm assigns the items (males in this case) to one of the groups (sample sites) based on the combination of discriminant scores generated during the first step. The percentage of misclassified items indicates how effective the DF is in identifying group differences (Nelson & Marler 1990).

Principal Component Analysis

Principal Component Analysis (PCA) was carried out to provide a visual, multivariate comparison of the difference in calls between sampling sites of each species, and to overcome problems associated with intercorrelation between call properties. All call parameters were used for the analysis (adjusted or not for temperature and body size depending on previous analysis, see above).

We extracted only factors with eigenvalues ≥ 1 (Aspey & Blankenship 1977). Because in many variables with medium values, correlations among the factor loadings (around 0.5 in absolute value) made the interpretation of factors complicated, a Varimax rotation was used. These strategies should be done with caution, as they may lead to different interpretations (Preacher & MacCallum 2003). The aim of the rotation methods is to increase the contrast among correlations by rotating the factors, thus making their interpretation much easier. The VARIMAX rotation is an orthogonal rotation (i.e. a change of the coordinates used in the PCA that maximises the sum of the variances of the squared loadings), while preserving an essential property of the PCA: the factors remain orthogonal after the rotation. This results in all coefficients being larger or near zero, with fewer intermediate values. As a criterion in interpreting the rotated factor pattern, an item was considered to load on a given component if the factor loading was 0.60 or greater (in absolute value).

Chapter III: Results

by Sara Maia

Nothing has really happened until it has been described.
So you must write many letters to your family and friends, and keep a diary.

Virginia Woolf

Results

For practical reasons, the results obtained for each species from the genetic and bioacioustic approaches used in this study are presented separately. Species interaction results will be described at the end.

1. Molecular Genetic Analyses

Mitochondrial DNA *(COI, 12S and 16S).*

12S and 16S – A total of 24 individuals from four different populations (12 *H. meridionalis*: 4 CRE, 4 CUB and 4 SMP; 12 *H. arborea*: 4 CRE, 4 CUB, 4 COV) were sequenced. Sequences obtained for genes 12S and 16S in both species were little informative. No further sequences were made, and so no further analyses.

1.1. *Hyla arborea*

COI Variation and Phylogenetic Analyses

A fragment of 738 bp of the mtDNA COI gene was obtained from 131 individuals of *H. arborea* (all sequences will be deposited in GenBank) and from two individuals of *H. meridionalis* (outgroup). The ingroup alignment revealed 48 haplotypes defined by 189 polymorphic sites (25.5%), of which 178 were parsimony informative. The nucleotide composition of the fragment was AˆT-rich (A, 26.4%; T, 32.2%), and variations consisted of exclusively transition/transversion substitutions (Ti:Tv=6.6). No insertion or deletion were detected.

For the COI alignment, the analysis with a distinct evolutionary substitution model by codon position was preferred (2lnBF=78.66), following Kass and Raftery (1995). For both ML and BI, the substitution model used was HKY+I. ML, and BI methods produced identical results (Fig. 3.2). Both showed a well-supported monophyletic clade for *H. arborea* that is further subdivided into two well-supported clades corresponding to Iberian-only and non-Iberian European populations. These two separate groups, with clear, distinct geographic distributions – one restricted to the Iberian Peninsula and the other spread across the rest of Europe (hereafter referred to as Iberian and European Groups, respectively) – will be treated separately in the following analyses.

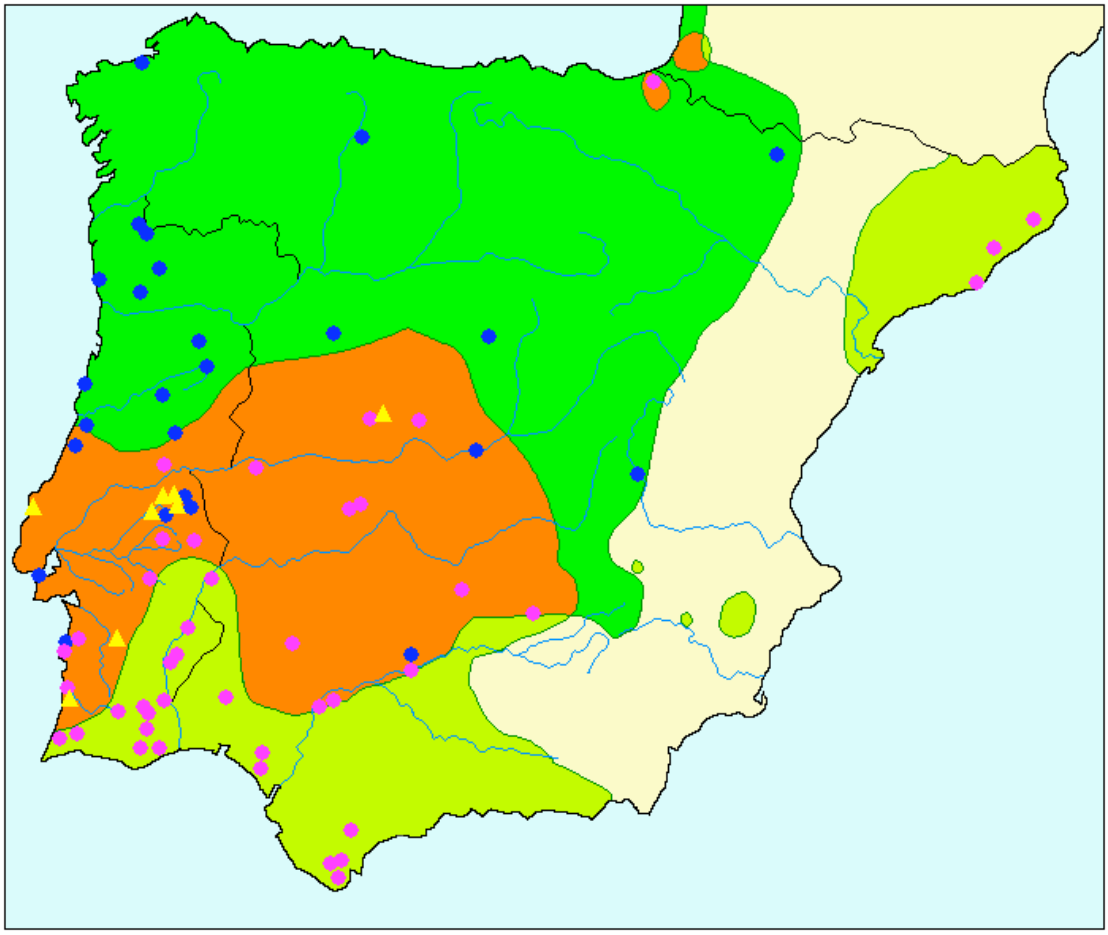


Figure 3.1. Sample sites used in COI analysis within Iberian Peninsula.
Distribution of all sites from where samples/sequences were collected for the molecular genetic analysis. Sites with samples only from *H. arborea* (●), *H. meridionalis* (●) and with samples from both species (▲). Distribution in Iberian Peninsula of *H. meridionalis*, *H. arborea*, and overlaping sympatric area.

Within the European clade, Corsica (H44, H45, and H46) forms a separate clade from continental Europe and Crete in the Bayesian tree (1.0 posterior probability), but not in the ML topology (Fig. 3.2). Within the Iberian clade, haplotypes recovered from sites mostly located south of Mondego River and the northeast Atlantic coast of Iberia (in red) form a clade nested within haploptypes recovered from sites located mostly to the north of the Mondego River and northwest of Spain (in blue), though the split is not very well supported (0.64 posterior probability). The genetic divergence between the European and Iberian groups is of 0.125 (p-distance). Within the Iberian group, the P-uncorrected distances between northern and southern groups is 0.009.

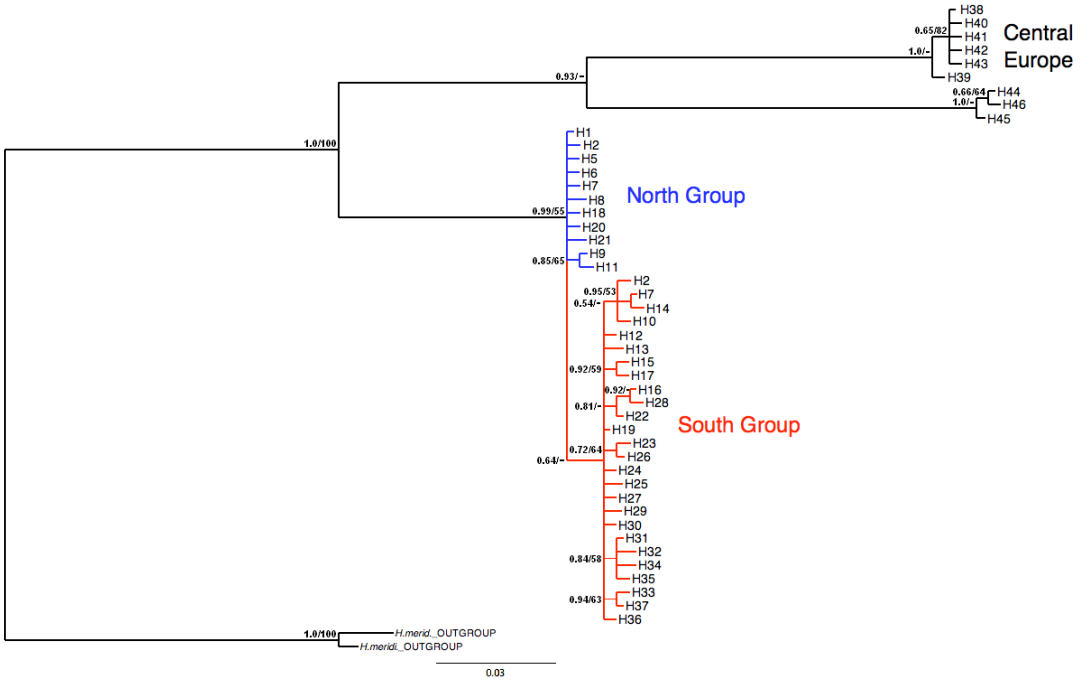


Figure 3.2. ML phylogenetic tree for COI for *Hyla arborea*.

Tree derived from Maximum Likelihood analysis of 740 bp of the mitochondrial COI sequences in *Hyla arborea*. Bayesian posterior probabilities and bootstrap values for ML are given near the branches, respectively. Values under 50 are represented by '–.'. Blue and red haplotypes correspond to the northern and southern groups identified in the Iberian haplotype genealogy.

The haplotype genealogy from ML of the mtDNA COI gene region of the Iberian *H. arborea* clade revealed two clearly divergent groups of haplotypes (Fig. 3.3). Four mutational steps separate the two groups, both showing a star-like topology with a central dominant haplotype and several derived low-frequency haplotypes. The geographic distributions of the two groups are shown in Fig. 3.2. The dominant haplotype in the northern lineage (H21, 50%) is shared by seven populations, while the one in the southern lineage (H1, 40.24%) was found in 12 populations. All other populations exhibit only haplotypes of either one of the lineages, with the exceptions of SOU, TOI, CAM, COV and LCO (Table 2.2). The southern lineage is widely represented in the southern range of the species and in northern Atlantic Iberia, while the northern lineage is found north of the Mondego area (SOU) in Portugal and in the northeast of Spain (north of Cuenca and Madrid, CUE and MAD, respectively) (Fig. 3.4).

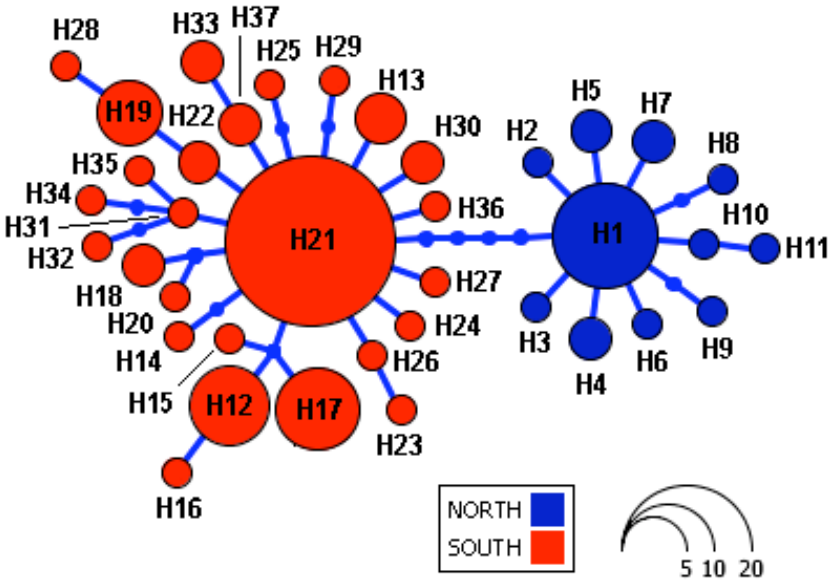


Figure 3.3. Mitochondrial COI genealogy of Iberian *H. arborea*.

Haplotype genealogy from Maximum Likelihood of COI gene tree performed in software Haploviewer. The circle area of each haplotype, coded as an H followed by a number (see Table 2.2), is proportional to its frequency (see scale on the bottom right).

A total of 44 COI variable sites and 37 haplotypes were detected when analysing the Iberian geographical subgroup separately (n=108) (Table 3.1). Overall, the average values of haplotype diversity and nucleotide diversity were 0.883±0.024 and 0.00498±0.00033, respectively, suggesting that haplotype diversity is high and nucleotide diversity is relatively low**.** More than half of the haplotypes (62.16%, 23 haplotypes) were singletons (haplotypes represented by a single sequence in the sample), but shared haplotypes were differentially distributed in space. Of the remaining 14 haplotypes, eight were shared among two or more populations while the remaining six were found in multiple individuals but from the same population (Table 2.2). Six populations had only one haplotype present (shared or not with other populations). The most abundant haplotype (H21) was shared by 33 specimens from 12 sites located exclusively south of the Mondego River, while the second most abundant haplotype (H1) was shared by 13 individuals from seven sites spread widely throughout the Iberian Peninsula, with a concentration in the northern Portuguese sites and the far east Spanish ones (see Table 2.2 and Fig. 3.4). Within the Iberian group, high levels of haplotype diversity were detected within each population, showing a high level of genetic diversity in *H. arborea*. In contrast, very low levels of nucleotide diversity were observed (Table 3.1).

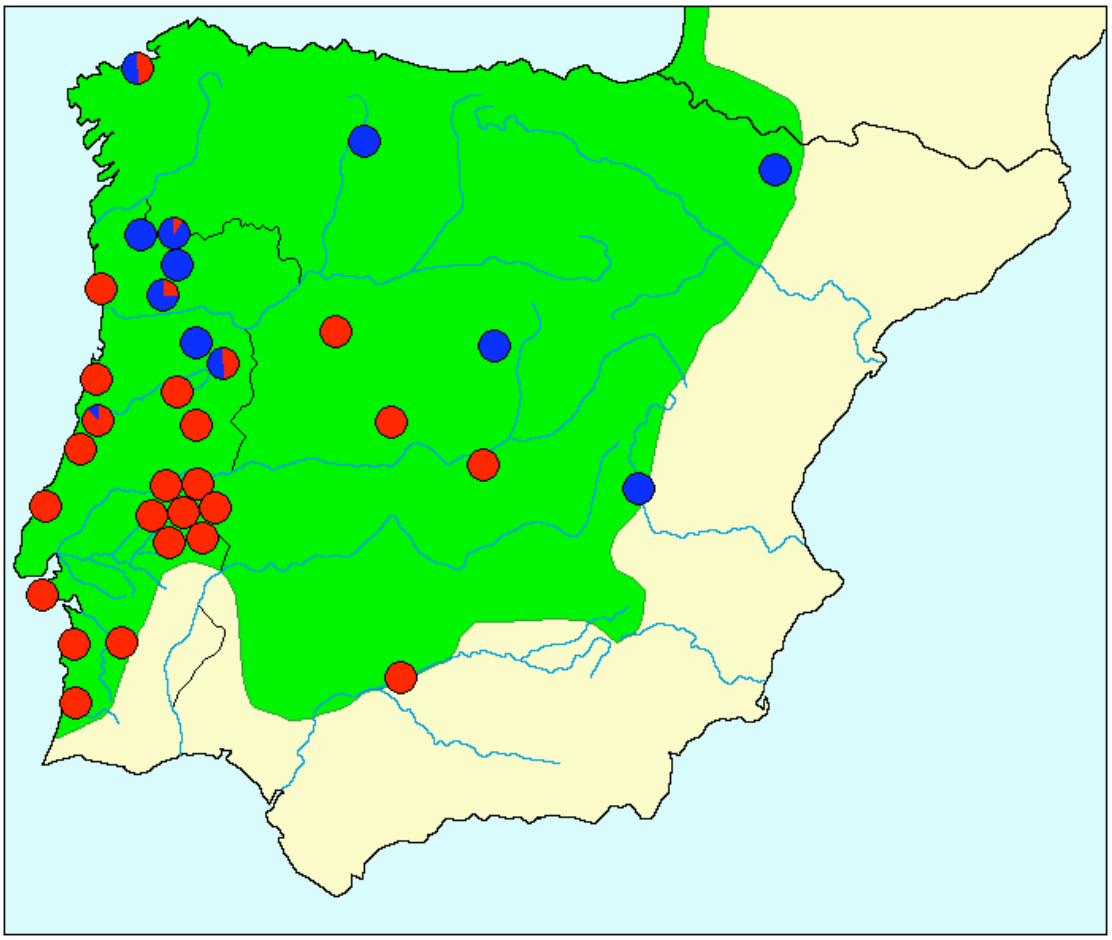


Figure 3.4. COI haplotype distribution map of *Hyla arborea* in the Iberian Peninsula.

H. arborea distribution in Iberian Peninsula in green. Populations sampled for the molecular genetic analyses are marked as circles. Red and blue indicate presence of haplotypes from the southern and northern lineages, respectively.

Table 3.1. Genetic diversity estimates and neutrality tests for *Hyla arborea*.

Sample sizes (*n*), number of haplotypes (*h*), haplotype diversity (*Hd*), nucleotide diversity (π), average number of nucleotide differences (*k*), population mutation parameter (Θ) and Tajimas's *D* and Fu's *Fs* and R^2 statistics for each group of *Hyla arborea* Iberian clade.

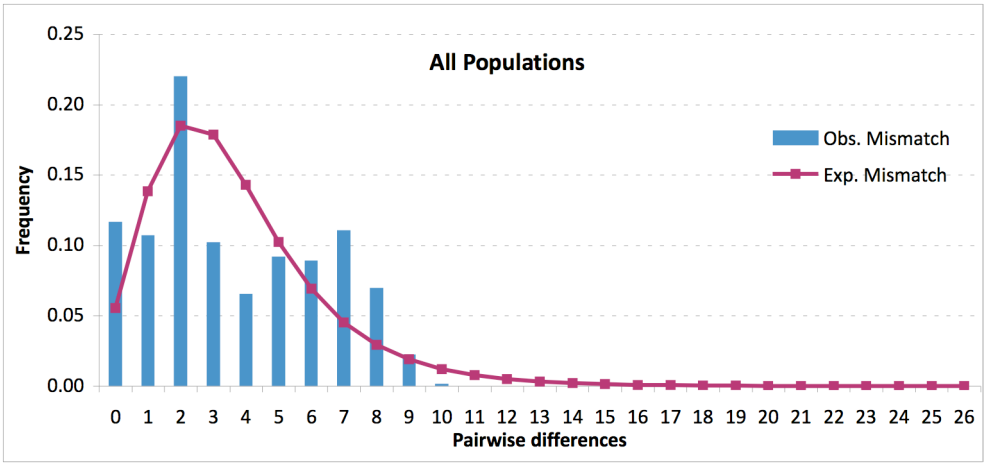
Lineages	N	No. of haplotypes (H)	S	Haplotype diversity (h)	Nucleotide diversity (π)	Average nr of nucleotide differences (k)	Population mutation parameter (Θ)	Tajima's D	Fu's Fs	R^2
South	82	26	31	0.821	0.0027	1.987	0.00842	-2.128*	-21.507**	0.0300**
North	26	11	12	0.751	0.0016	1.283	0.00425	-2.076*	-7.976**	0.0562**
Total	108	37	44	0.883	0.00498	3.693	0.01135	$-1.752^{n.s.}$	-24.800**	0.0388*

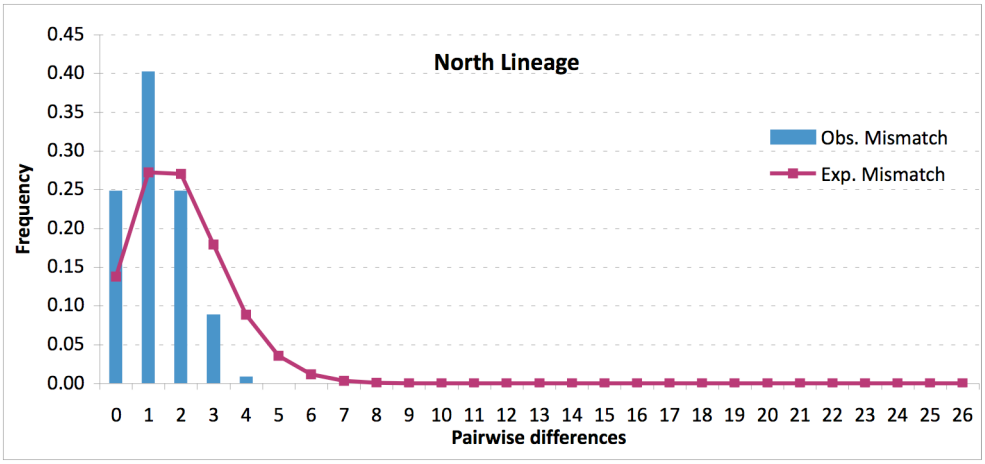
Note: Statistics abbreviated as n.s. (non significant); * for 0.05<p<0.01; ** for p<0.01.

Genetic diversity estimates and neutrality tests within Iberian clades of *H. arborea* are shown in Table 3.1. The southern lineage exhibited higher haplotype diversity (H=26, h=0.821) when compared to the northern lineage (H=11, h=0.751).

Historical Demography

Mismatch distributions were bimodal for the total Iberian data set, but were unimodal for north and south Iberian lineages independently, in agreement with a model of population expansion (Fig. 3.5). Fu's *Fs* tests and Tajima's *D* statistics for the southern and northern lineages separately, and for the Iberian samples together, were negative and significant ($p<0.05$), conforming to a model of recent population demographic expansion for the species (Table 3.1). The R^2 statistics was highly significant for both northern and southern lineages ($p<0.01$), re-enforcing the scenario of population expansion. The observed *tau* values (τ), as estimates of the age expansion parameter, were 1.987 and 1.206 of mutational time for South and North lineages, respectively.





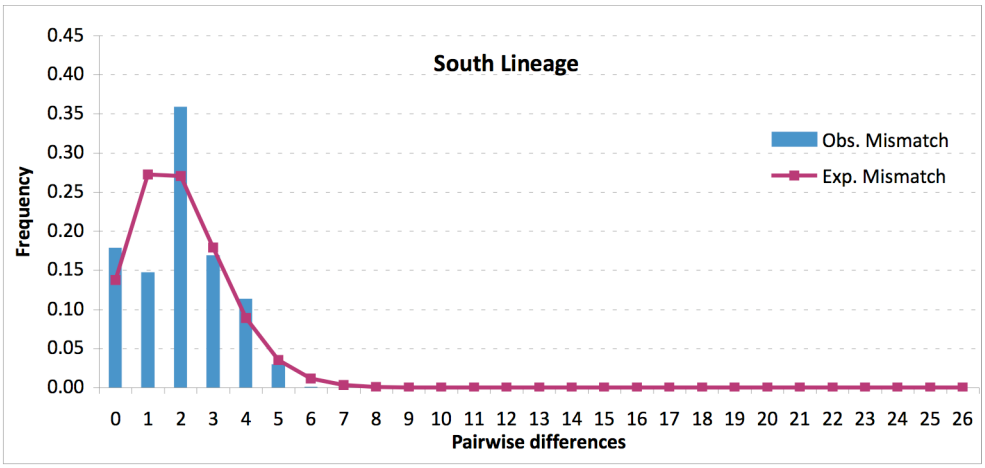


Figure 3.5. Mismatch distributions of *Hyla arborea* COI samples.

Mismatch distribution of pairwise comparisons of COI region haplotypes data set for *Hyla arborea* for the Iberian populations (included in a single group) (top figure), treating the northern (middle figure) and southern (bottom figure) lineages separately. The observed frequencies of pairwise differences are shown in bars (blue), and the expected frequencies of pairwise differences between haplotypes under a sudden expansion model are indicated in solid lines (purple).

Isolation-by-Distance

Genetic distances (Da) and geographic distances between pairwise sampling sites with >5 individuals are given in Table 3.2 and graphically presented in Fig. 3.6. To test if populations geographically distant are more genetically different than populations closer to each other, genetic distance between all population pairs was calculated, and its relationship with the corresponding geographic distance between populations tested using a Mantel test (Jensen et al. 2005). No significant relationship occurred between genetic and geographic distance (r=0.5875, P=0.9941, 10,000 randomisations), with the regression model (reduced major axis regression) explaining 34.5% (r^2=0.345) of the variance between them.

Table 3.2. Genetic and geographic distances among *Hyla arborea* populations.

Genetic (Da, below diagonal) and geographic distances (Km, in bold above diagonal) between pairwise sampling sites of *H. arborea* with more than five individuals.

	BUR	CRE	CFO	COV	CUB	MEL	SOU
BUR (n=14)		**9.15**	**101.88**	**263.46**	**228.71**	**183.41**	**117.81**
CRE (n=7)	0.004		**111.39**	**273.35**	**221.42**	**176.66**	**123.88**
CFO (n=8)	0.021	0.039		**161.95**	**317.73**	**266.19**	**90.87**
COV (n=8)	0.524	0.547	0.513		**468.83**	**414.97**	**203.58**
CUB (n=9)	0.051	0.069	0.03	0.536		**55.44**	**269.38**
MEL (n=5)	0.034	0.052	0.014	0.527	0.044		**213.34**
SOU (n=8)	0.031	0.044	0.048	0.388	0.079	0.062	

Note: For an explanation of population acronyms, see Materials and Methods, Table 2.2; *n* refers to the number of individuals in each population.

There are two clearly separated groups: the one on the upper-right quadrant corresponds to the COV sampling site, whereas the one on the bottom-right quadrant includes all other sampling sites (i.e. BUR, CRE, CFO, CUB, MEL and SOU). All these sites belong to the southern lineage, except for COV and SOU, which share haplotypes with both northern and southern lineages.

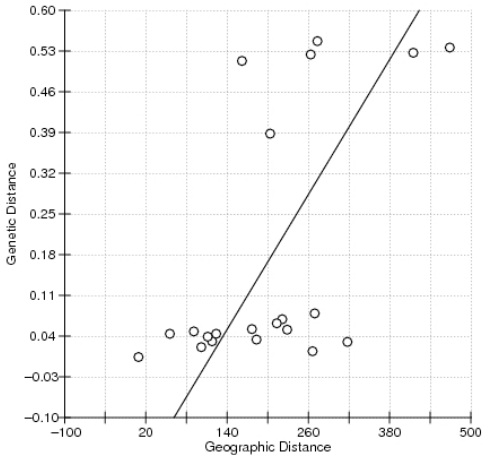


Figure 3.6. Genetic distance vs. Geographic distance in *Hyla arborea*.
Genetic distance based on COI sequences vs. log of linear geographic distances for all possible pairwise combinations of *H. arborea* populations (with more than five specimens sequenced). No significant correlation is observed: regression analysis y=-0.2191+1.932x10^{-3} x, r=0.5875, p=n.s.

1.2. *Hyla meridionalis*

COI Variation and Phylogenetic Analyses

A sequence alignment of 731 bp from the mtDNA COI fragment was obtained from 209 individuals of *Hyla meridionalis* from 82 different localities across the species distribution range (some of the sequences were gathered from GenBank, see Table 2.1). Comparisons among all COI sequences revealed 42 haplotypes, with 82 polymorphic sites (11.22%), of which 67 were parsimony informative (Table 3.3). The nucleotide composition of the fragment was AˆT-rich (A, 27.7%; T, 34.1%), and variations consisted of transition/transversion substitutions only (Ti:Tv=12.6). No insertions or deletions were detected.

Haplotype and nucleotide diversity of the species was relatively high (h=0.763; π=0.00996). More than half of the haplotypes (54.76%, 23 haplotypes) are represented by

only one individual (singletons), with the most abundant haplotype (H7) being shared by 99 individuals from 39 populations in the Iberian Peninsula. The second most abundant haplotype (H29) was shared by 14 specimens from eight populations, followed by haplotype H2, shared by 12 specimens from 12 different populations, and haplotype H3, shared by 11 specimens from six populations. ODI has the highest haplotype diversity, including five haplotypes in nine specimens, of which three haplotypes are exclusive to this population.

A distinct model of nucleotide substitution for each codon position was preferred (2lnBF=2107.18), following Kass & Raftery (1995). For Bayesian inference, the TIM1+G substitution model was used. In the Bayesian tree (shown in Fig. 3.7), two well-supported matrilineal clades were recovered: one clade including Tunisia and Algeria (TA, in green), and a second clade sub-divided into three different clades, called the 'Central Morocco' group (CM, in blue), the 'Northern Morocco' group (NM, in yellow) and the 'Southwestern' group (SW, in red and WM, in orange, respectively) (for ease of comparison the names used here follow the same used in Recuero et al. 2007). The TA group includes the samples from Tunisia and Algeria, presenting two clades not geographically structured: H40 and H41 correspond to specimens from Algeria, whereas the other six haplotypes are from samples from Tunisia. The CM group includes a sample from the Moroccan Medium Atlas. The NM group includes samples from northern Morocco (Rif Mountains and the Tingitane region of Morocco), and all samples from southern France, northeast Iberia, the Canary Islands and northwest Italy (Liguria region, not in the map Fig. 3.9). The 'Southwestern' group includes samples from southwestern Iberia, from Portugal and Spain, and from western Morocco (High Atlas, Anti-Atlas, Massa River area and one sample from the area of the Medium Atlas, next to the CM group). Within the Southwestern group, two clear distinct geographic areas are identified even though not statistically well-supported: Iberia and Morocco, which are marked with different colours (SW, in red and WM, in orange, respectively).

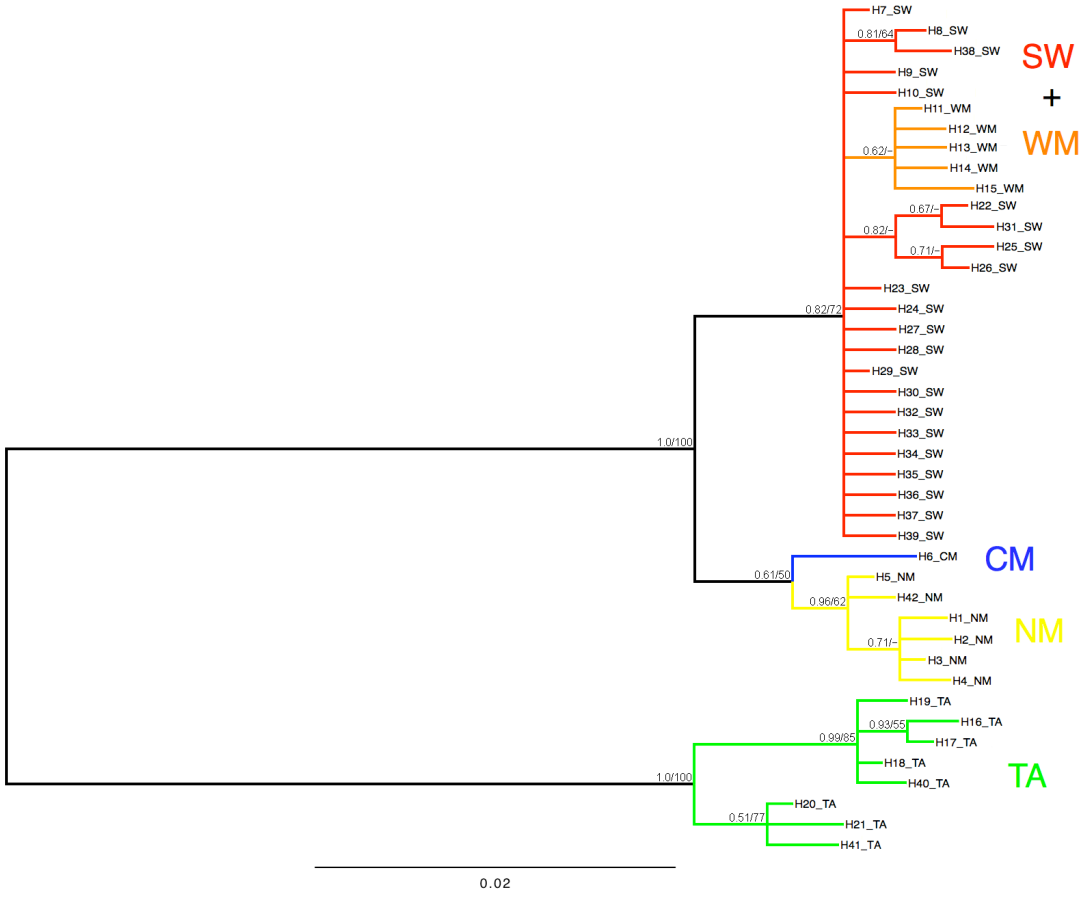


Figure 3.7. Bayesian phylogenetic tree for COI for *Hyla meridionalis*.

Tree derived from Bayesian analysis of 731 bp of COI mitochondrial sequences in *H. meridionalis*. Bayesian posterior probabilities and bootstrap values for ML are given near the branches, respectively. Values under 50 are represented by '–'. Colours correspond to the geographic clades identified (same colour code used in the network): green, Tunisia and Algeria; red, southwest Iberia; orange, western Morocco; blue, central Morocco and yellow, northern Morocco, NE Iberia and the Canary Islands.

The existence of the four main genetic geographic groups (even though not all statistically well-supported) is also evident from the mtDNA haplotype genealogy (Fig. 3.8). 43 mutational steps separate the Tunisia and Algeria group from the remaining sampling locations (i.e. SW, WM, NM and CM). The group corresponding to the geographic region of SW Iberia (red) and W Morocco (orange) revealed a star-type topology; the WM group is derived from the central haplotype, without sharing any haplotypes. SW+WM group dominated in terms of number of haplotypes, number of populations, and in number of specimens. NM group is clearly separated from the SW+WM group by six mutational steps, though sharing the geographic area of Morocco.

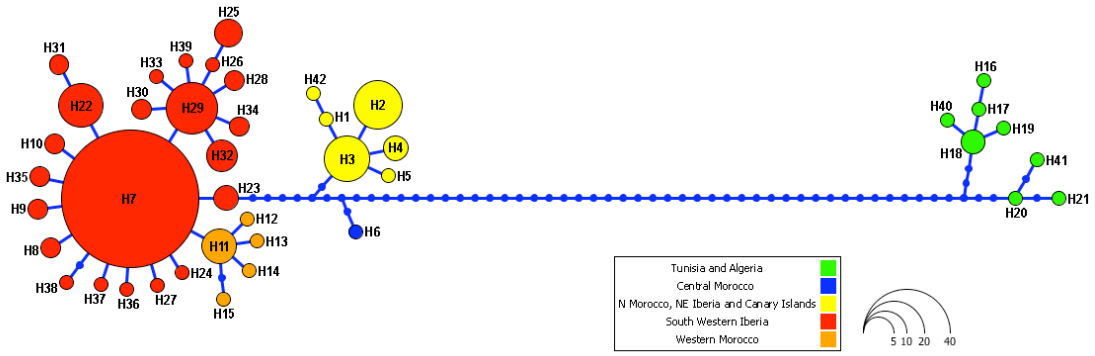


Figure 3.8. Mitochondrial COI genealogy of *Hyla meridionalis*.
Haplotype genealogy from Maximum Likelihood of COI gene tree performed in software Haploviewer. The circle area of each haplotype, coded as an H followed by a number (see Table 2.1), is proportional to its frequency (see scale on the bottom right).

Within the SW group, the sequences obtained from specimens originating in Spain showed lower number of haplotypes relative to Portuguese samples: only six out of the 22 found in Iberia, sharing only two with Portugal (H7 and H10). The other four haplotypes (H8, H9, H35 and H38) are geographically restricted to southern Spain regions of Doñana, Huelva and Cádiz. In Portugal, *H. meridionalis* had 17 haplotypes. The two ramified branches that come out of H7 haplotype, the H22 and H29 subgroups, are geographically concentrated in the area of the Algarve mountainous system (known as 'Serra Algarvia', that extends from west to east, separating the two regions of Algarve and Alentejo) and the southwestern coast of Portugal (know as 'Sudoeste Alentejano'), only including samples from populations where *H. arborea* is absent – even if in potentially sympatric areas – with the exception of H39, which corresponds to the OBI sample, where *H. arborea* was also detected (table 2.1 and 2.2 and figs. 2.1 and 2.2).

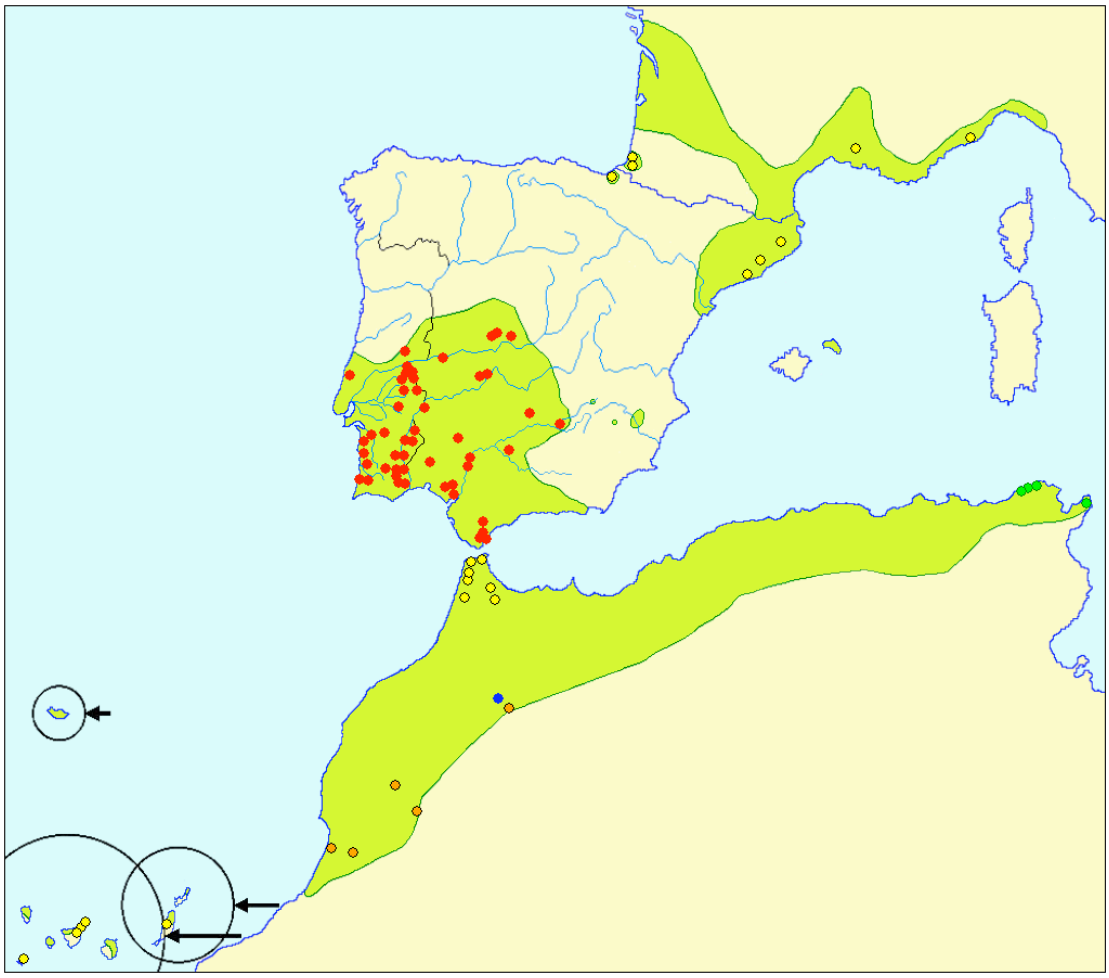


Figure 3.9. COI haplotype distribution map of *Hyla meridionalis* in the Iberian Peninsula and Northern Morocco.

Representation of the obtained clades in the COI mtDNA analyses. *H. meridionalis* geographic distribution in green. Red circles correspond to individuals/sites from SW clade (●); yellow circles to northern Morocco, northeast Iberia and the Canary Islands (NM clade, ●); orange circles to western Morocco (WM subgroup, ●), green circles to Tunisia and Algeria (TA clade, ●) and the blue circle to central Morocco (CM clade, ●) (the same colour code is used in the phylogenetic tree and in the haplotype genealogy, see figs. 3.6 and 3.7).

Within the geographic groups, haplotype diversity ranged from 0.601 (SW) to 0.933 (TA), whereas nucleotide diversity was comparatively low (reference to the global value), ranging from 0.00137 (WM) to 0.00617 (TA) (Table 3.3). Within the SW+WM group, the WM group had higher haplotype and nucleotide diversity values. The lineage with highest diversity values was by far the group of Tunisia and Algeria (Table 3.3). The average number of nucleotide differences when all populations are considered together was 7.284, while within each group means were between 0.961 and 4.511 (SW and TA, respectively, Table 3.3).

Table 3.3. Genetic diversity estimates and neutrality tests for *Hyla meridionalis*.

Sample sizes (*n*), number of haplotypes (*H*), number of polymorphic (segregating) sites (*S*), haplotype diversity (*h*), nucleotide diversity (π), average number of nucleotide differences (*k*), population mutation parameter (Θ), Tajimas's *D*, Fu's *Fs* and R^2 statistics for the groups formed by Moroccan and Iberian *Hyla meridionalis* (Central Morocco is not considered, as it is only constituted by one sequence).

Lineages	n	No. of haplotypes (*H*)	S	Haplotype diversity (*h*)	Nucleotide diversity (π)	Average nr of nucleotide differences (*k*)	Population mutation parameter (Θ)	Tajima's *D*	Fu's *Fs*	R^2
TA	10	8	13	0.933	0.00617	4.51111	0.00629	-0.08338 n.s.	-2.276 n.s.	0.16952**
CM	1	1	–	–	–	–	–	–	–	–
NM	29	6	4	0.695	0.00131	0.96059	0.00139	-0.14744 n.s.	-1.816 n.s.	0.13682**
SW	159	22	20	0.601	0.00132	0.96394	0.00485	-1.9964*	-21.595**	0.08752**
WM	10	5	5	0.667	0.00137	1,000	0.00242	-1.74110*	-2.260**	0.20697**
SW+WM	169	33	34	0.733	0.00341	2.494	0.00815	-1.69581 n.s.	-29.239**	0.08454**
Total	209	42	82	0.763	0.00996	7.28358	0.01896	-1.45021 n.s.	-8.810n.s.	0.08056**

Note: Statistics abbreviated as n.s. (non significant); * for 0.05<p<0.01; ** for p<0.01. 'Total' includes Central Morocco group, but this will not be considered separately as it only has one sample; southern Iberian (SW) clade; western Morocco (WM) subgroup; northern Morocco, northeast Iberia and Canary Islands (NM) clade and central Morocco (CM) clade.

The TA group was clearly the most divergent, with P-uncorrected distances equal to and above 6%. All other genetic distances were below 1.5% (between WM and CM, Table 3.4). These results validate the option of using the TA group as an outgroup in the phylogenetic analysis.

Table 3.4. Pairwise *P*-uncorrected distances for COI in *Hyla meridionalis*.

Pairwise *P*-uncorrected distances for COI sequences of *Hyla meridionalis* between geographic groups, given as a percentage.

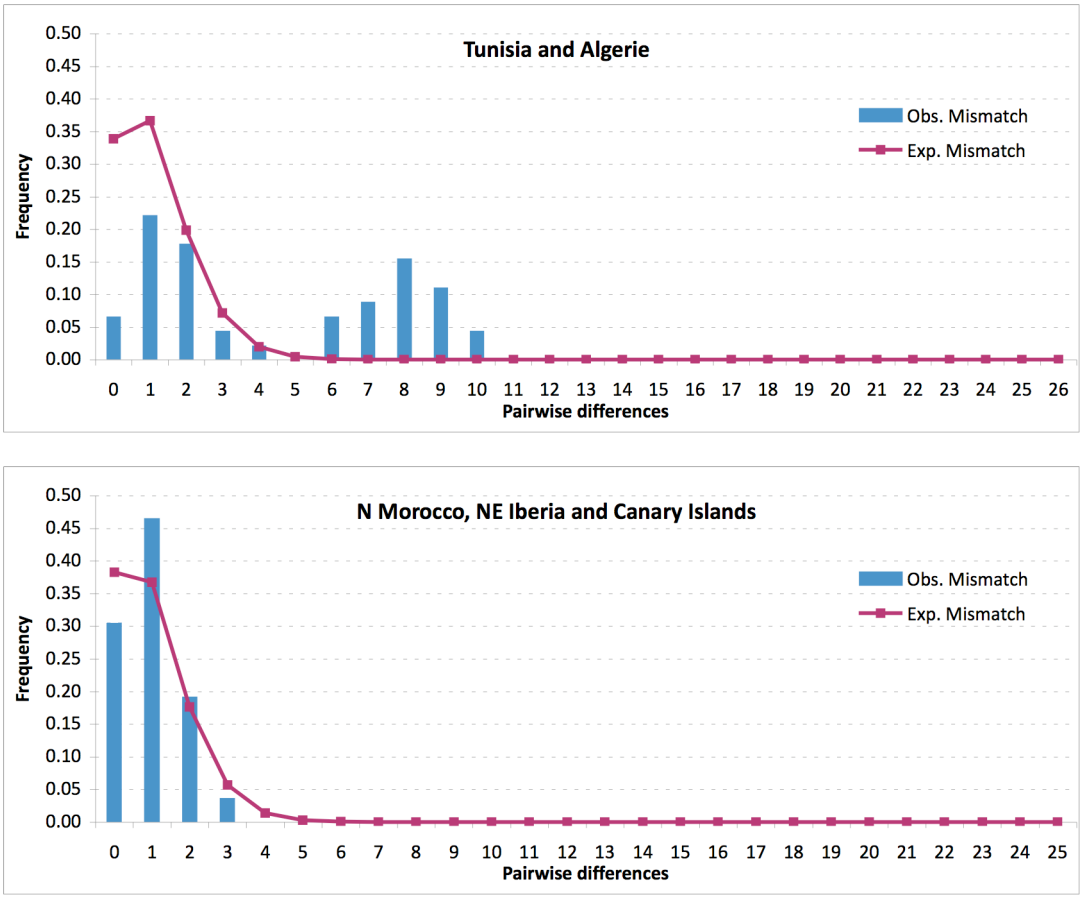
	TA	NM	CM	SW	WM
TA	-				
NM	6.2	-			
CM	6.0	0.9	-		
SW	6.1	1.2	1.4	-	
WM	6.2	1.3	1.5	0.3	-

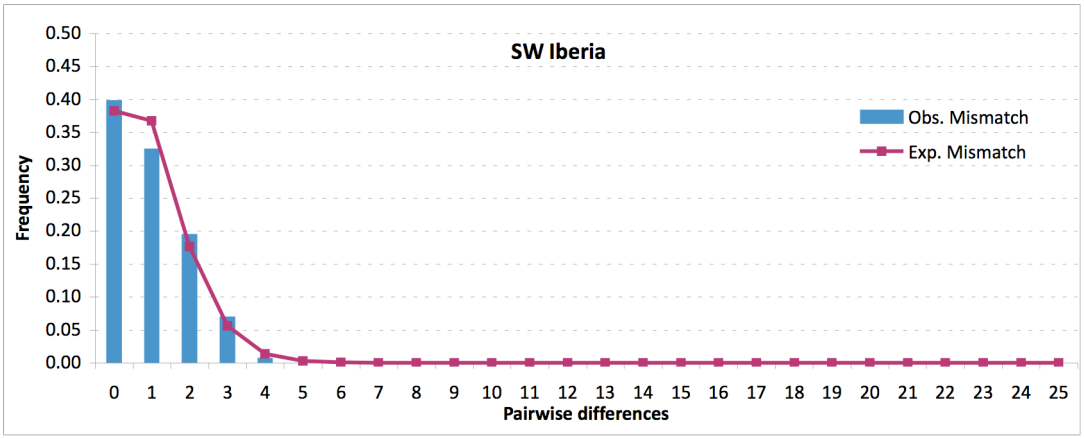
Note: Southern Iberian (SW) clade; western Morocco (WM) subgroup; northern Morocco, northeast Iberia and Canary Islands (NM) clade and central Morocco (CM) clade.

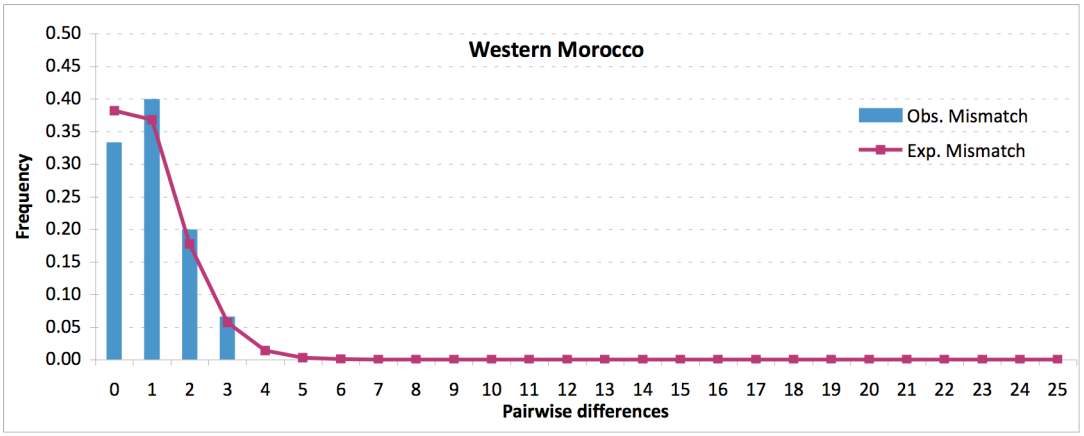
Historical Demography

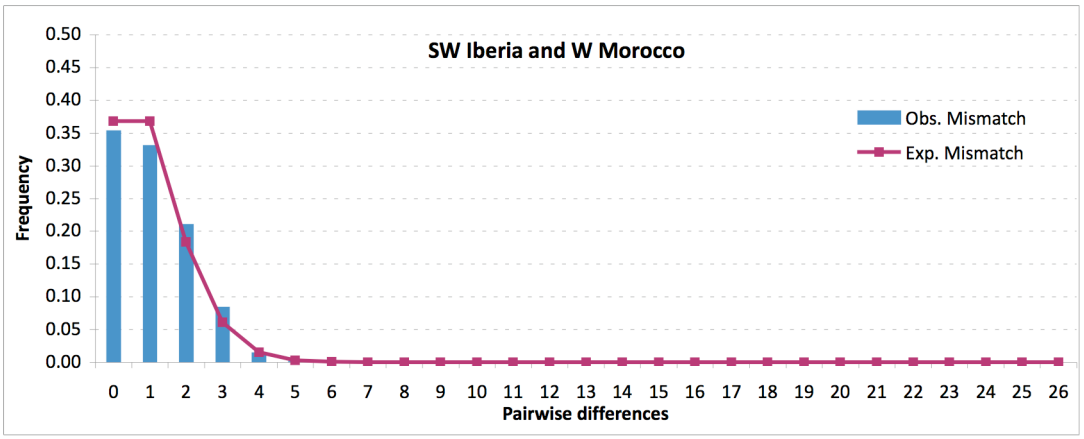
The mismatch distributions for the entire data set (all populations) and for TA independently showed multimodal curves, and rejected a model of sudden population expansion (Fig. 3.10). The multimodal shape for all populations is consistent with the presence of various divergent clades, as well as the bimodal shape for TA. In contrast, mismatch distributions for all the other geographic groups, including the combination of SW and WM, were unimodal, and fit the model of sudden population expansion (Fig. 3.10).

The statistics employed to investigate departures from constant population size showed low (R2) or negative (Fu's *Fs*, Tajima's *D*) values (see Table 3.3) that are consistent with a recent population expansion. Among these, Fu's test and R^2 are the most powerful tests to detect population expansions (Ramos-Onsins & Rozas 2002); however, the first (Fu's) was not statistically significant for TA and NM groups, though it was for the second (R^2). This lack of statistical power is also reflected in the non-significance of Tajima's *D* and Fu's *Fs* tests for the all data set computation.




SW Iberia
Obs. Mismatch
Exp. Mismatch
Frequency
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Pairwise differences


Western Morocco
Obs. Mismatch
Exp. Mismatch
Frequency
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Pairwise differences


SW Iberia and W Morocco
Obs. Mismatch
Exp. Mismatch
Frequency
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
Pairwise differences

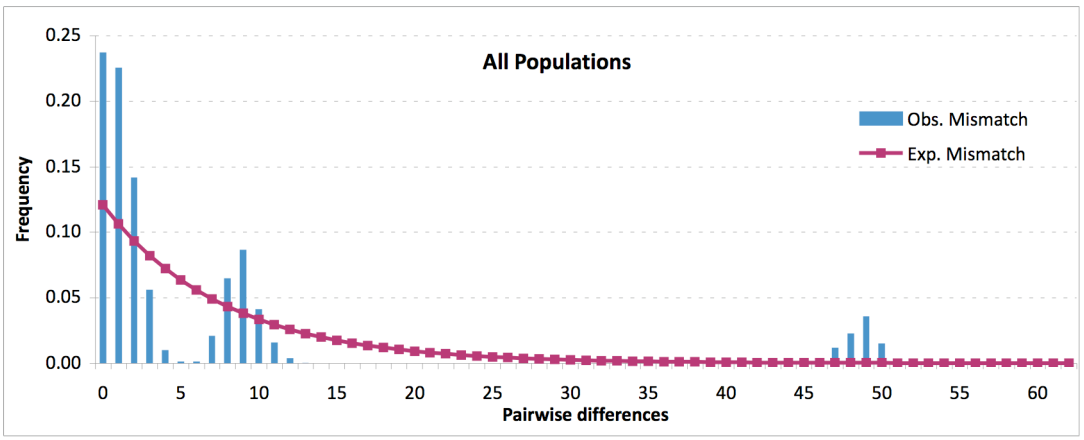

All Populations
Obs. Mismatch
Exp. Mismatch
Frequency
0.25
0.20
0.15
0.10
0.05
0.00
0 5 10 15 20 25 30 35 40 45 50 55 60
Pairwise differences

Figure 3.10. Mismatch distributions of *Hyla meridionalis* COI samples.

Mismatch distribution of pairwise comparisons of COI region haplotypes data set for *H. meridionalis* for the four geographic regions (TA, NM, SW, WM), the combination of SW and WM and, finally, with all regions together, respectively. The observed frequencies of pairwise differences are shown in bars (blue), and the expected frequencies of pairwise differences between haplotypes under a sudden expansion model are indicated in solid lines (purple).

Isolation-by-Distance

Genetic distances (Da) and geographic distances between pairwise sampling sites are given in Table 3.5. To test if populations further apart are more genetically distinct than populations closer to each other, genetic distance between all population pairs was calculated and its relationship with the corresponding geographic distance between populations tested using a Mantel test (Jensen et al. 2005).

Table 3.5. Genetic and geographic distances among *H. meridionalis* populations.

Genetic (Da, below diagonal) and geographic distances (Km, in bold above diagonal) between pairwise sampling sites of *H. meridionalis* with more than five individuals.

	ODE	BUR	CUB	ALP	CRE	ODI	ALM	SMP	PIC	FOI	DON	GRA
ODE (n=5)		**219.85**	**10.35**	**215.07**	**212.71**	**69.69**	**55.47**	**78.18**	**97.62**	**46.63**	**216.05**	**50.53**
BUR (n=19)	0.041		**228.71**	**12.6**	**9.15**	**153.65**	**224.24**	**214.24**	**205.23**	**256.82**	**289**	**171.93**
CUB (n=6)	-0.014	0.055		**224.19**	**221.42**	**77.12**	**51.23**	**75.72**	**96.3**	**36.36**	**211.91**	**60.55**
ALP (n=5)	0.041	0	0.055		**17.28**	**150.44**	**222.5**	**213.92**	**206.15**	**253.43**	**294.36**	**166.27**
CRE (n=10)	0.036	0	0.055	0		**145.89**	**215.88**	**205.52**	**196.28**	**249.03**	**279.99**	**165.32**
ODI (n=9)	0.068	0.118	0.071	0.118	0.118		**74.66**	**74.85**	**79.17**	**103.17**	**200.57**	**37.38**
ALM (n=9)	0.02	0.015	0.029	0.015	0.015	0.038		**26.4**	**48.56**	**45.1**	**160.8**	**83.37**
SMP (n=7)	0.119	0.078	0.133	0.078	0.078	0.092	0.041		**22.26**	**70.76**	**138.91**	**94.47**
PIC (n=8)	0.041	0	0.055	0	0	0.095	0.004	0.059		**93**	**124.04**	**105.74**
FOI (n=5)	0.055	0.014	0.068	0.014	0.014	0.058	-0.008	-0.002	0		**193.75**	**94.89**
DON (n=5)	0.055	0.014	0.068	0.014	0.014	0.131	0.029	0.092	0.014	0.027		**229.72**
GRA (n=5)	0.087	0.046	0.1	0.046	0.046	0.059	0.009	0.034	0.026	-0.003	0.059	

Note: For an explanation of population acronyms see Materials and Methods, Table 2.1

No significant relationship was established between genetic and geographic distance (r=-0.0369, P=0.4331, 10,000 randomisations), with the regression model (reduced major axis regression) explaining less than 1% (r^2=1.364x10^{-3}) of the variance between them.

No clear distinctive clusters appear. All sample sites aggregate in a single cluster, reflecting the lack of genetic divergence between them. Sampling sites used in the analysis were ODE, BUR, CUB, ALP, CRE, ODI, ALM, SMP, PIC, FOI, DON and GRA, all part of the SW Iberia haplotype group (red in maps and haplotype network) (Fig. 3.11).

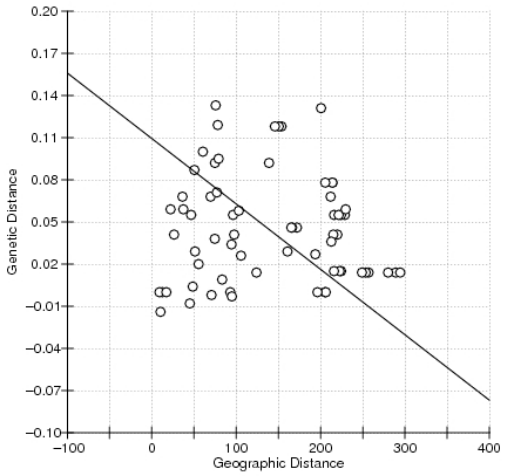


Figure 3.11. Genetic distance vs. Geographic distance in *Hyla meridionalis*.
Genetic distance based on COI sequences vs. log of linear geographic distances for all possible pairwise combinations of *H. meridionalis* populations (with more than five specimens sequenced). No significant correlation is observed: regression analysis y=0.1094-4.65x10^{-4} x, r=-0.0369, p=n.s.

Microsatellites

Of the 34 loci tested, eight (WHA1-9, WHA1-20, WHA1-201, WHA5-22A, WHA5-61, WHA5-133, Ha A-11, Ha D-115 and Ha E-2) were successfully amplified for both species, and four only amplified for *Hyla arborea* (WHA1-67, Ha B-12, Ha A-127 and Ha D-3R3). Most loci were polymorphic, but a few were monomorphic for both species (WHA1-20, Ha A-11 and WHA5-61). Ha D-115 was polymorphic for *H. arborea* but monomorphic for *H. meridionalis*, and apparently with an allele not shared with *H. arborea*. WHA5-22A was polymorphic for both species, and one of the alleles was unique for *H. meridionalis*. No further analyses were done with the microsatellite results, as both PCR and scoring protocols were not accurate.

2. Sound Analyses of Advertisement Calls

184 males from 36 locations were chosen to cover a wide range of each species' distribution range, and to be representative of both allopatric and sympatric conditions (see map, Fig. 3.12). In some of the locations within previously reported potentially sympatric areas, only one of the species was detected (e.g. GRA; see Table 2.3 and Fig. 3.12). It also happened that only males from one of the species were recorded in syntopic sites where the two species were detected (i.e. where the two species share the same water body), either because the non-recorded species was acoustically inactive, or its activity was too weak to allow for recordings. In all such cases the sites were visited more than once to increase the chances of success.

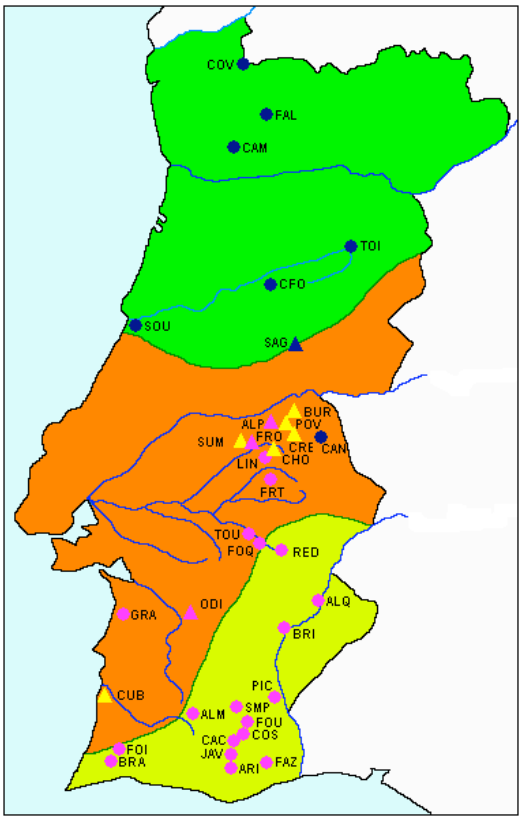


Figure 3.12. Distribution of acoustic sample sites of *Hyla meridionalis* and *H. arborea* in Portugal. Symbols in the map represent sample sites where males were audio-recorded: *Hyla arborea* (●) and *Hyla meridionalis* (●) in allopatric and sympatric populations with the two species detected (▲) or with only one species (▲). Geographic distribution of species is indicated by ■ for *H. arborea* allopatric area, ■ for *H. meridionalis* allopatric area and ■ for sympatric area of both species.

Males were only included in the analysis if acoustic data, temperatures (bodily and environmental) and body measurements were collected. In some cases, where the choruses were too dense, it was not possible to clearly identify the focus-male in the audio-recordings,

and therefore these were not considered for analysis. All the recordings analysed, and the values of body size, temperature and acoustic variables associated with the analyses, are included in Appendices 2, 3, 4 and 5. Calls used for analyses will deposited in the sound library of Fonoteca Zoologica from MNCN, CSIC, Madrid, Spain.

2.1. *Hyla arborea*

Advertisement calls of a total of 58 males from 14 different locations were analysed, of which seven locations were syntopic with *H. meridionalis* (see Table 3.3 and Fig. 3.12). As already mentioned, *H. arborea* advertisement calls are produced exclusively by males, and consist of a series of pulsed calls (Schneider 1974) (Fig. 2.7); the nomenclature used here for call variables follows the ones described by Schneider (1974) and Castellano et al. (2002).

Coefficients of Variation of Call Parameters

Within-Individual Variation

Individual variation was quantified by calculating the coefficients of variation (CV) of all call properties. First, CVs were calculated in terms of within-call-group for each sample site and globally for the species. Here, the coefficients of variation within each individual and in each calling session were calculated, and the average (and CV) of the 58 coefficients of variation of the 58 males was calculated. Second, variability was calculated in terms of within-individual variation, again per sample site and for species.

Table 3.6. Within-call group coefficient of variation of *Hyla arborea*.

CV calculated for call acoustic properties at call group level (n=273 call groups from 58 males).

Call Parameters	Coefficient of Variation (%)	
	Mean	SD
Call duration (CD)	5.17	2.31
Intercall duration (ICD)	28.19	13.40
Nr of pulses per call (PC)	7.46	2.76
Pulse rate (PR)	6.17	1.79
Fundamental frequency (FF)	4.76	2.78
Dominant frequency (DF)	1.91	1.05
Difference of amplitude between dominant and fundamental frequencies (ADF)	21.64	9.85

The most variable properties within the *H. arborea* call group were intercall duration (mean CV=28.19%) and the difference of amplitude between dominant and fundamental frequencies (mean CV=21.64%). In contrast, all other properties had values that either fit the static criteria (CV <5%) or the intermediate criteria (5% <CV <12%), and so can be considered stereotyped properties (Table 3.6). Among sampled sites there was great variation

in CV mean values (see Table 3.7), in particular for amplitude call properties. However, in most sites there was coherence in whether a certain property was more variable or more stereotyped, following Gerhardt's (1991) classification criteria.

Table 3.7. Population within-call group coefficient of variation of *Hyla arborea*.

CV calculated for *H. arborea* call acoustic properties (n=273 call groups from 58 males) within each sampled site.

	CD	ICD	PC	PR	FF	DF	ADF
COV (n=16)	4.59±1.17	32.30±23.24	5.90±1.43	4.84±1.01	6.50±1.94	1.96±0.34	19.97±3.55
FAL (n=26)	5.20±2.07	21.81±5.32	7.63±2.98	6.53±2.82	3.75±2.12	1.87±0.59	18.61±6.00
CAM (N=16)	5.78±2.22	26.60±4.41	7.83±1.81	5.98±1.17	4.24±2.09	1.89±0.50	19.10±6.16
TOI (n=7)	3.37±0.65	32.27±12.35	5.69±1.01	4.98±0.53	2.82±1.19	2.28±0.86	23.81±13.84
CFO (n=9)	4.65±2.48	31.50±10.17	5.40±2.03	4.94±1.05	7.48±2.50	1.80±0.17	16.63±6.64
SAG (n=15)	7.84±4.30	21.50±6.93	11.71±4.53	7.67±1.80	3.26±1.42	2.43±0.82	38.82±16.27
SOU (n=68)	4.35±1.29	27.56±16.60	7.16±1.43	6.27±1.35	6.42±3.30	1.81±0.53	20.34±7.01
BUR (n=10)	5.00±1.80	32.57±10.99	6.57±2.09	6.20±1.60	2.90±0.35	1.85±0.51	17.86±3.83
POV (n=12)	5.56±2.20	31.07±11.19	7.37±1.81	6.65±1.24	2.04±0.45	1.11±0.19	12.57±4.36
CRE (n=31)	6.38±2.99	26.45±8.39	8.10±3.36	6.09±1.95	4.93±2.57	2.63±2.78	19.84±7.33
CAN (n=14)	4.66±1.22	35.31±14.39	7.06±2.56	7.29±2.94	3.89±3.59	2.31±1.47	39.59±13.02
SUM (n=15)	5.78±2.20	25.34±9.51	8.71±3.97	5.71±1.77	3.28±1.14	1.70±0.37	20.80±6.51
CHO (n=16)	5.12±2.55	32.71±13.99	6.43±1.70	5.63±1.25	3.56±0.97	1.52±0.56	20.97±5.23
CUB (n=30)	5.13±2.17	28.56±12.37	7.51±2.78	6.24±1.57	4.83±2.23	1.73±0.47	20.12±4.78

Note: values in the table are CV± standard deviation Populations are organised top to bottom according to their location along a north-south axis. For call parameters acronyms see Table 3.6 and for population acronyms see Table 2.2.

Within individuals, call variation showed similar patterns to the within-call-group variation. Call group duration, number of calls per group, intercall duration, amplitude of fundamental frequency mean, amplitude of dominant frequency and difference of amplitude between dominant and fundamental frequencies were the most variable properties (with mean CV> 12%). In contrast, fundamental and dominant frequencies were the stereotyped properties (with mean CV <5%) (see Table 3.8).

Table 3.8. Within-individual coefficient of variation of the *Hyla arborea*.

CV calculated for all call acoustic properties by male (n=58 males).

Call parameters	Coefficient of Variation (%)	
	Mean	SD
Call group duration (CGD)	26.15	13.63
Number of calls per call group (CCG)	25.85	13.57
Call duration (CD)	5.63	1.93
Intercall duration (ICD)	31.17	10.33
Number of pulses per call (PC)	8.05	2.53
Call rate (CR)	6.49	7.02
Pulse rate (PR)	6.59	1.46
Fundamental frequency (FF)	4.74	2.39
Dominant frequency (DF)	2.15	0.96
Difference of amplitude between dominant and fundamental frequencies (ADF)	24.22	9.79

There was great variation in CV mean values among sampled sites (see Table 3.9), but no sampled site seemed to be different from the others.

Effects of Temperature and Body Size in Call Parameters

Hyla arborea body size was measured in terms of mass and SVL in 58 males (Fig. 3.13). SVL ranged from 3.0 to 4.2 cm with a mean (± SD) of 3.52±0.28 cm, and mass ranged from 2.3 to 5.3 g with a mean of (± SD) of 3.54±0.78 g. We found significant differences in body size among sampled populations (see Appendix 2 for descriptive statistics for each location) in both SVL ($F_{13,44}$=4.3573, p<.0001, n=58) and mass ($F_{13,44}$=3.6911, p=0.0006, n=58). SUM had, on average, the smallest recorded males; males from SOU had the lowest value for mass; CHO had males with the lowest SVL. The largest males, on average, were recorded in CFO, with the heaviest males in TOI and the highest SVL in POV. Mass and SVL were positively correlated (r=0.7428, p<0.0001, n=58); therefore, mass was used as the body-size measure throughout the rest of the analyses, as, according to the literature, mass is highly correlated with the size of the vocal apparatus (McClelland et al. 1996, 1998), which has a significant effect on call properties.

Table 3.9. Population within-individual coefficient of variation of the *Hyla arborea.*

CV calculated for all call acoustic properties per male within each sampled site (n=58 males).

	CGD	CCG	CD	ICD	PC	CR	PR	FF	DF	ADF
COV (n=3)	17.75±10.93	18.05±12.22	5.06±1.09	41.62±25.73	6.23±0.67	6.22±2.63	5.18±0.61	6.61±2.07	2.15±0.41	23.30±6.70
FAL (n=6)	25.19±11.94	24.25±12.68	5.53±1.08	23.07±2.95	8.54±1.39	4.30±1.26	7.53±1.56	4.16±1.54	2.26±0.71	21.75±2.77
CAM (n=3)	31.32±13.97	31.39±13.43	5.97±2.61	27.25±2.65	8.26±1.63	3.83±1.56	6.45±0.66	4.56±1.69	2.03±0.23	23.16±6.16
TOI (n=2)	25.43±19.55	25.58±17.72	3.69±0.43	31.75±11.63	5.71±0.54	3.05±0.87	5.01±0.32	2.72±1.38	2.63±0.64	30.46±13.55
CFO (n=3)	31.6±13.51	41.52±11.58	5.22±2.52	31.21±4.69	6.05±1.87	19.35±28.98	5.42±1.25	7.68±2.81	1.94±0.39	17.85±7.32
SAG (n=4)	27.62±13.94	27.29±18.80	8.89±2.75	23.57±7.50	13.01±3.27	4.80±4.29	8.36±1.24	3.72±1.06	2.67±0.46	39.44±17.65
SOU (n=11)	18.12±8.79	19.27±9.89	4.70±0.55	32.45±13.84	7.44±0.69	5.71±3.41	6.37±0.70	6.71±2.83	2.09±0.77	22.51±6.89
BUR (n=3)	31.49±3.92	31.54±1.59	5.49±1.00	33.65±9.56	7.11±0.62	5.57±6.45	6.55±0.14	2.94±0.27	2.13±0.69	18.67±2.43
POV (n=3)	31.56±19.52	27.09±23.27	6.31±0.98	34.98±6.24	7.66±1.42	5.61±2.71	7.18±0.55	2.13±0.25	1.12±0.07	14.47±3.35
CRE (n=7)	38.70±20.85	33.53±18.85	6.84±3.06	29.94±4.65	8.53±3.18	9.10±2.76	6.17±1.57	4.76±2.54	3.03±2.52	23.81±10.20
CAN (n=3)	25.37±18.05	20.69±15.56	4.75±1.01	39.15±3.15	7.40±2.80	7.81±3.29	7.73±3.06	3.97±2.77	2.68±1.17	40.39±14.54
SUM (n=4)	23.30±7.65	21.94±6.20	5.90±2.09	28.20±5.98	8.89±3.61	7.11±6.03	5.92±1.33	3.31±0.82	1.78±0.17	22.46±6.41
CHO (n=3)	24.08±13.40	23.94±9.63	5.07±2.57	34.11±7.33	6.60±0.90	5.65±0.85	5.85±0.65	3.70±0.37	1.74±0.55	25.25±4.87
CUB (n=6)	27.33±17.20	27.92±15.48	5.46±1.38	31.60±10.85	8.72±3.16	5.16±2.28	7.21±1.86	4.71±2.02	1.76±0.44	21.93±4.82

Note: values in the table are CV± standard deviation. Populations are organized top to bottom according to their location along a north-south axis.. For call parameters acronyms see Table 3.8 and for population acronyms see Table 2.2.

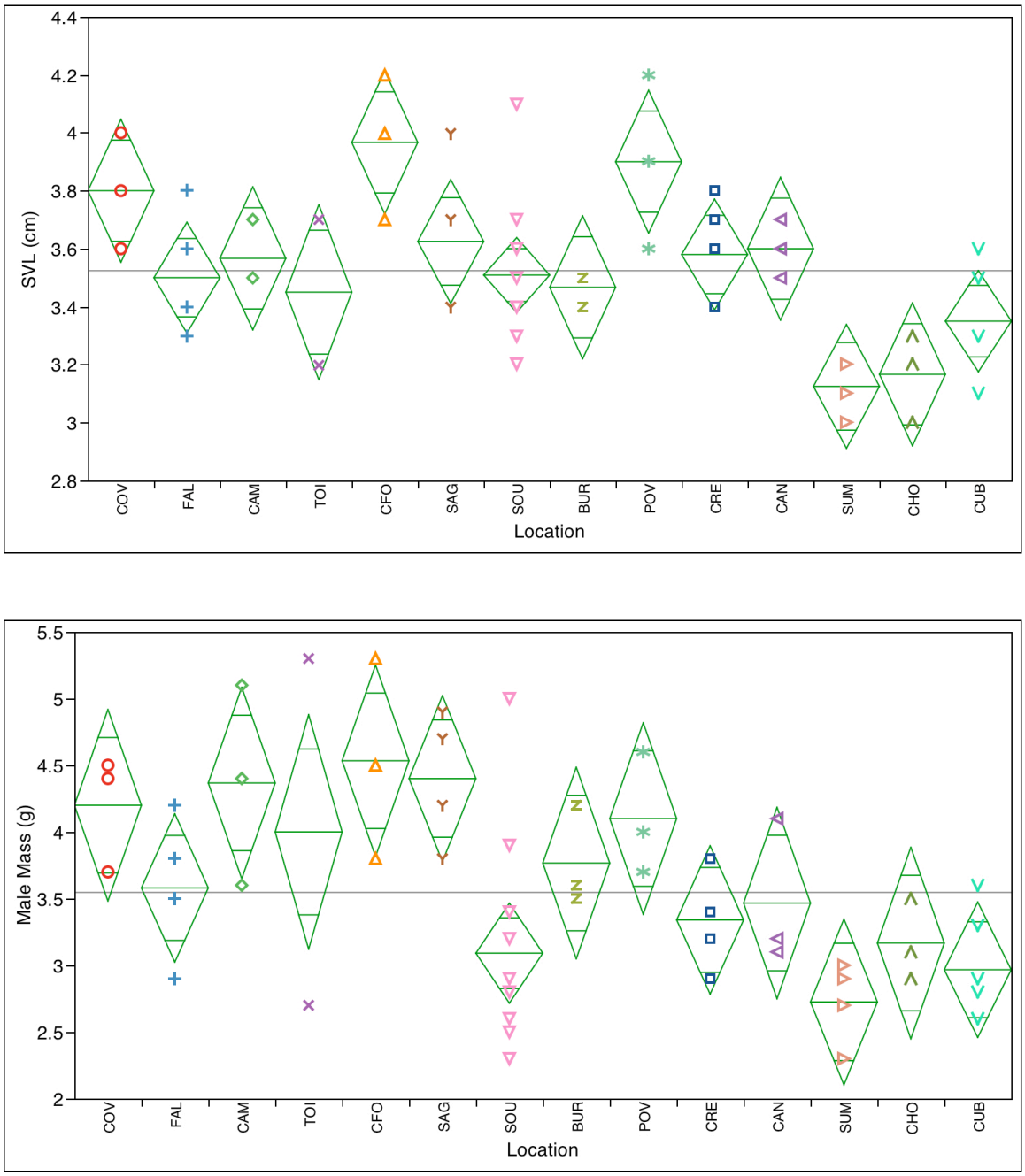


Figure 3.13. Body size of *Hyla arborea* calling males in sampled populations in Portugal.

Boxplots of SVL (on top) and mass (bottom) of *H. arborea* males in each population. The middle line of the diamond represents the group mean, and the top and bottom extremes represent the corresponding 95% confidence interval. The horizontal line in the graphic represents the global mean of all pooled samples. Populations are organised left to right according to their location along a north-south axis. For an explanation of population acronyms see Materials and Methods, Table 2.2.

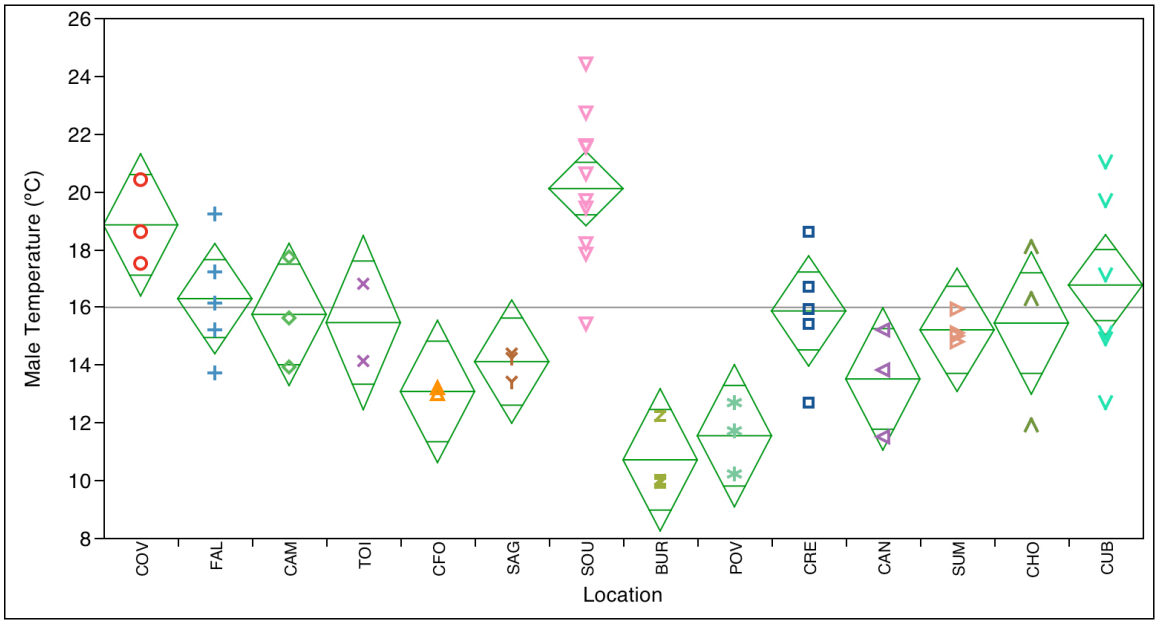
Temperature of males was significantly positively correlated with air and substrate temperatures (Table 3.10). Air and male temperatures will be used to test for effect-model on call-characteristics variation.

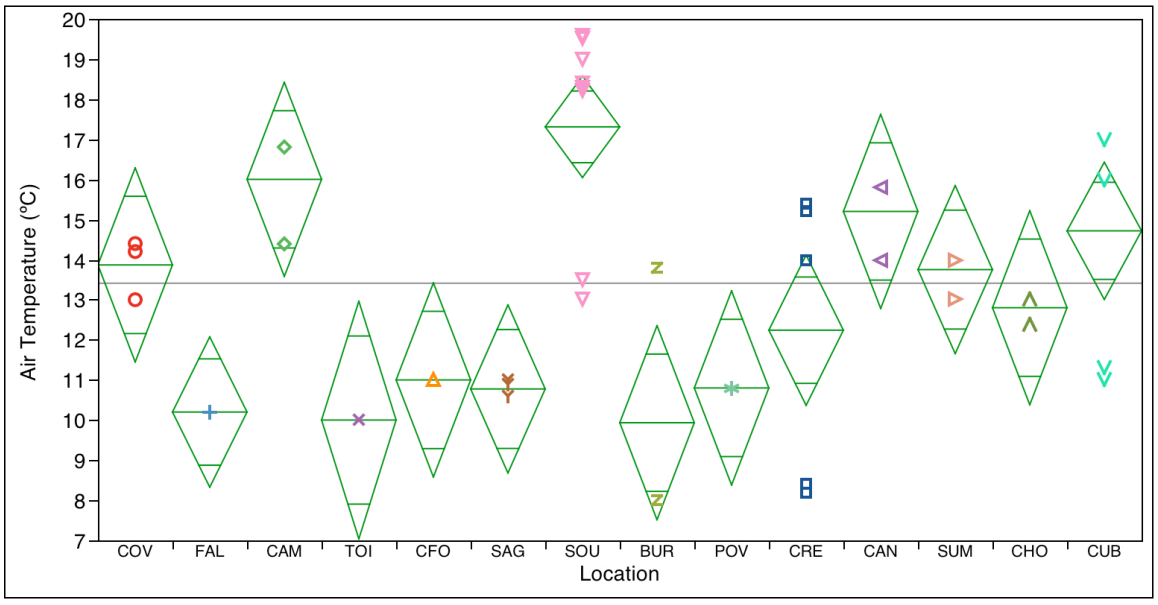
Table 3.10. Temperature correlation matrix for *Hyla arborea* sampled populations.

Pairwise correlation values for air, substrate and male temperature measurements.

	Air Temperature	Substrate temperature	Male Temperature
Air Temperature (n=58)	–		
Substrate temperature (n=54)	0.5359*	–	
Male Temperature (n=58)	0.6967*	0.7561*	–

Note: * p<0.01; *n* refers to the number of individuals.





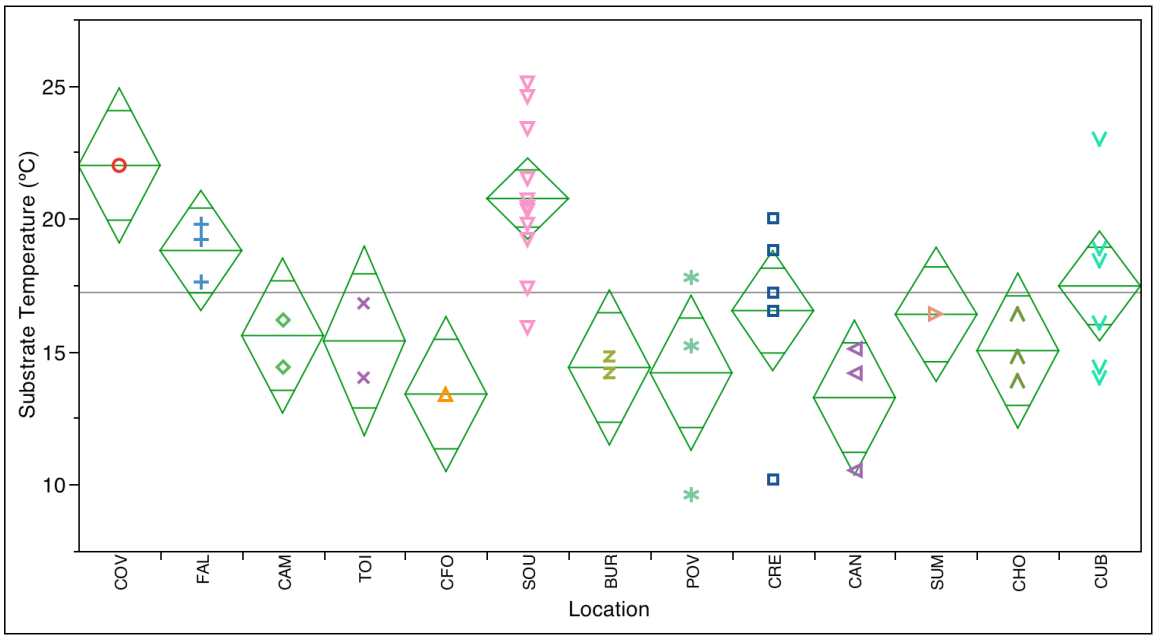
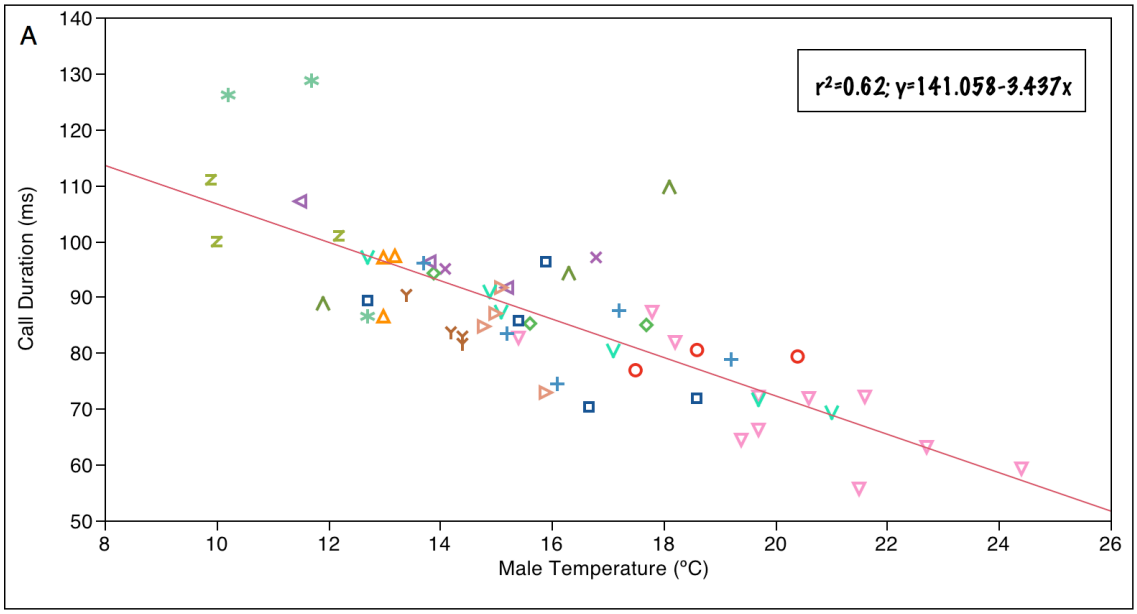


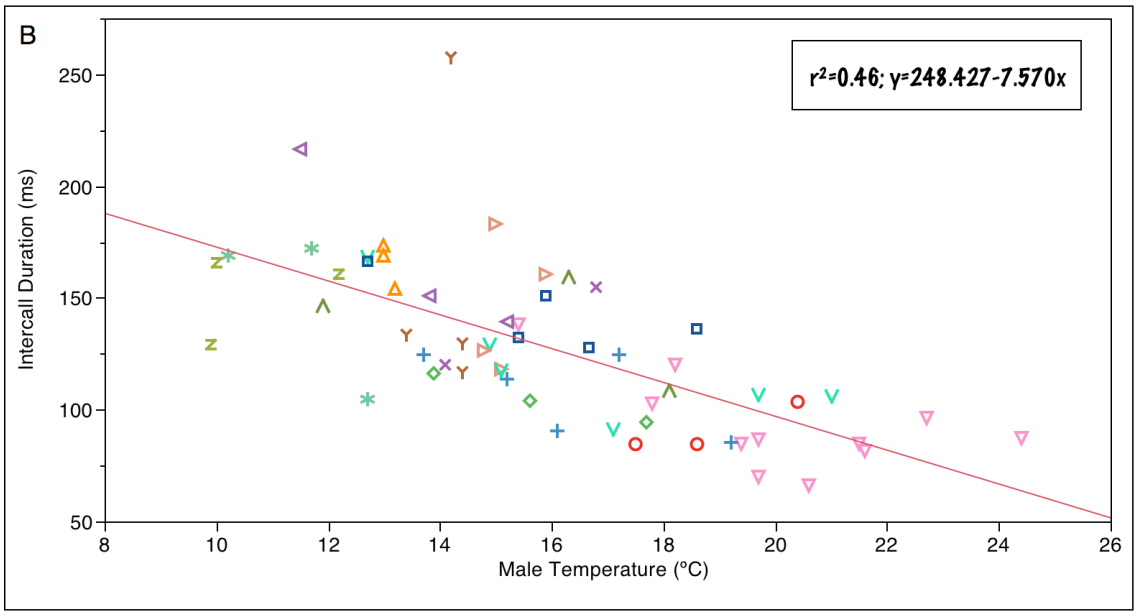
Figure 3.14. Temperature distribution in *Hyla arborea* sampled populations in Portugal.
Boxplots of male (top), air (middle) and substrate (bottom) temperatures measured for each recorded male of *H. arborea* in each population. The middle line of the diamond represents the group mean, and the top and bottom extremes represent the corresponding 95% confidence interval. The horizontal line in the graphic repents the global mean of all pooled samples. Populations are organised left to right according to their location along a north-south axis. For an explanation of population acronyms, see Materials and Methods, Table 2.2.

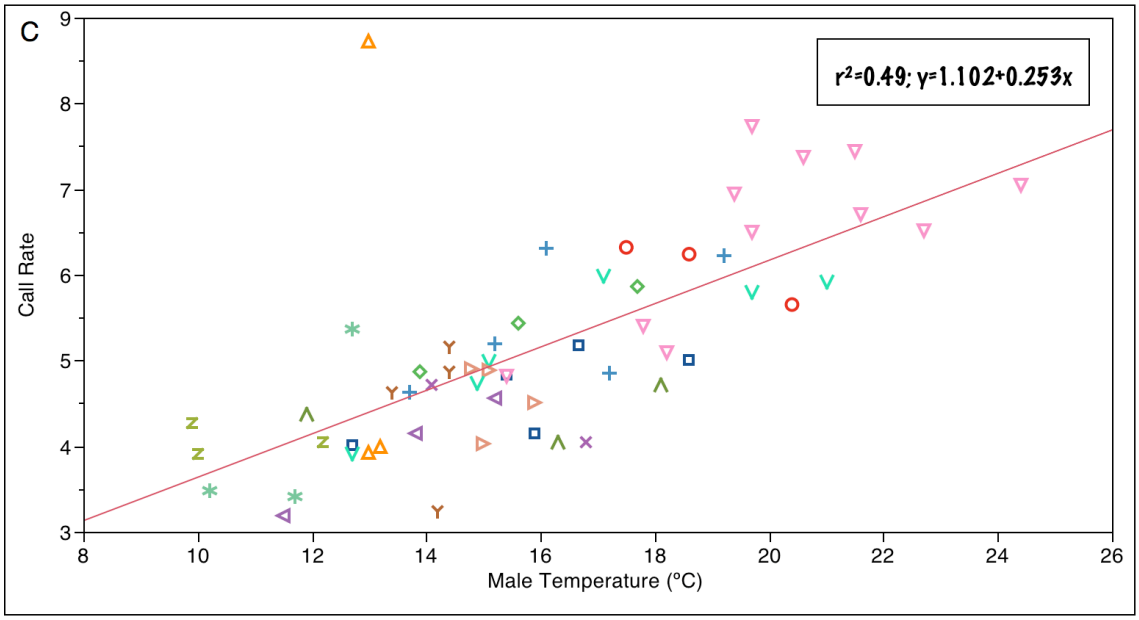
Linear multiple regression models were performed to examine the influence of air and male temperatures and male mass and SVL on each of the call variables (e.g. CD, ICD, PC, PR, FF, AF, DF, AD and ADF). We considered all populations as a single group on the assumption that any putative effect of temperature and/or body size on call characteristics variation would be similar across populations, as all the individuals belong to the same taxon.

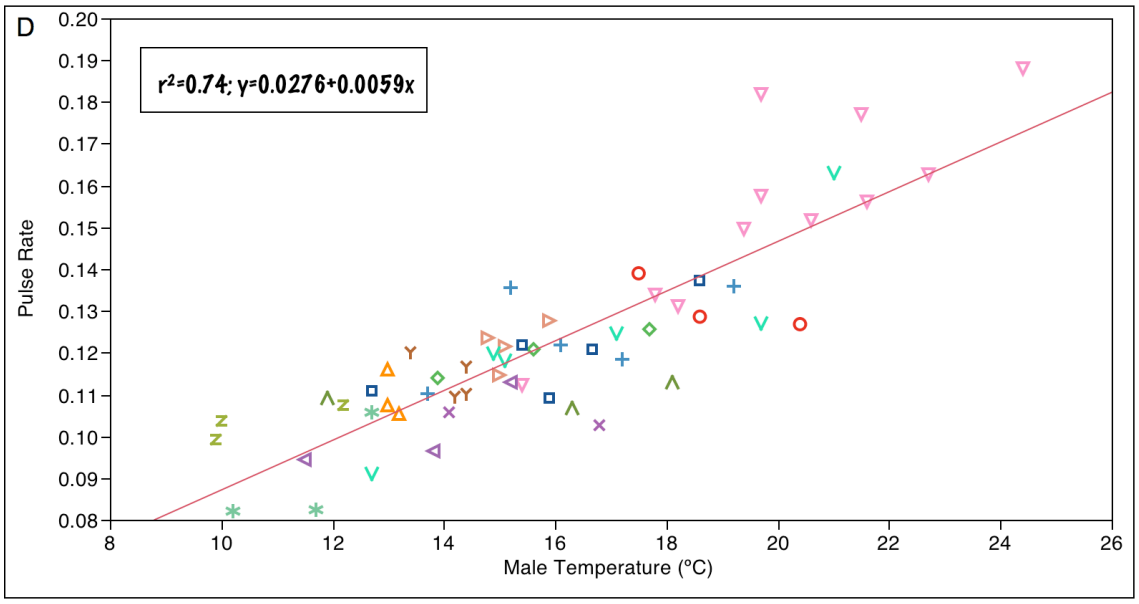
Four regression models revealed a highly significant influence of male temperature on the temporal variables (all $p<0.001$): CD, ICD, call rate and pulse rate. Also, male mass was found to have a highly significant influence on spectral variables (the two regression models had $p<0.001$): fundamental and dominant frequencies. A linear regression was performed for the call variables affected by either one of the factors (male temperature or mass), and the residuals were obtained and used in subsequent analyses. Male temperature explained around 75% of the observed variation in pulse rate and 62% of the observed variation in call duration; only 49% and 46% of the variation in call rate and intercall duration were explained by male temperature. When regressing male mass vs. fundamental and dominant frequencies, the amount of variation in the dependent variables that could be explained by the independent variable was lower – 43% and 26% for dominant and fundamental frequencies, respectively. Call and intercall durations decreased as male temperatures increased, while call and pulse rates

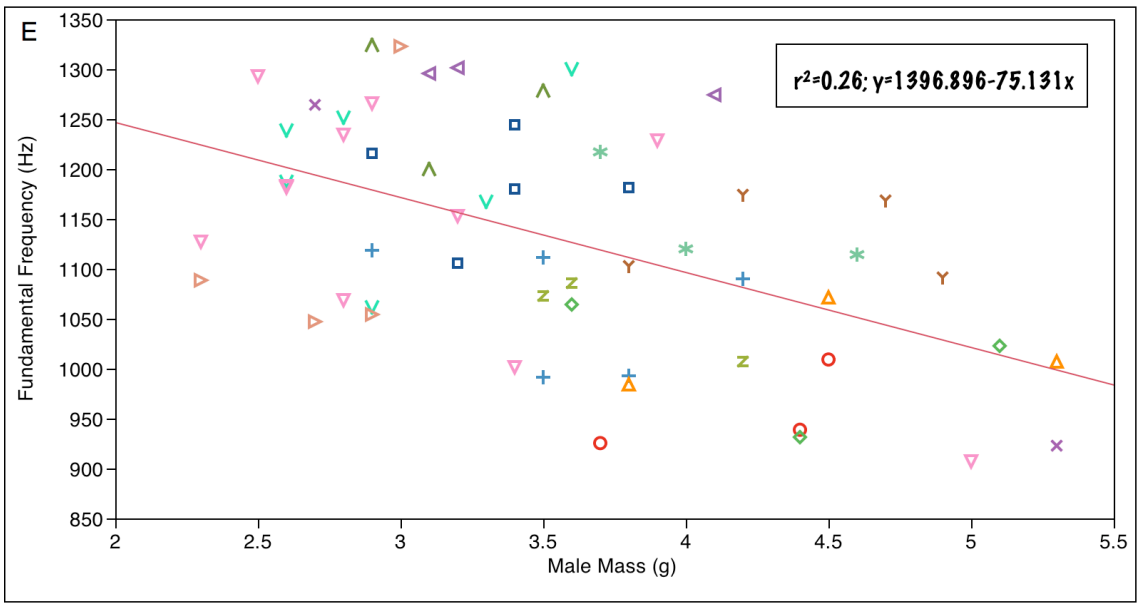
increased along with male temperature decrease. For fundamental and dominant frequencies, heavier males had lower frequencies, i.e. their calls were lower pitched.






C
r²=0.49; y=1.102+0.253x
Call Rate
Male Temperature (°C)


D
r²=0.74; y=0.0276+0.0059x
Pulse Rate
Male Temperature (°C)


E
r²=0.26; y=1396.896-75.131x
Fundamental Frequency (Hz)
Male Mass (g)

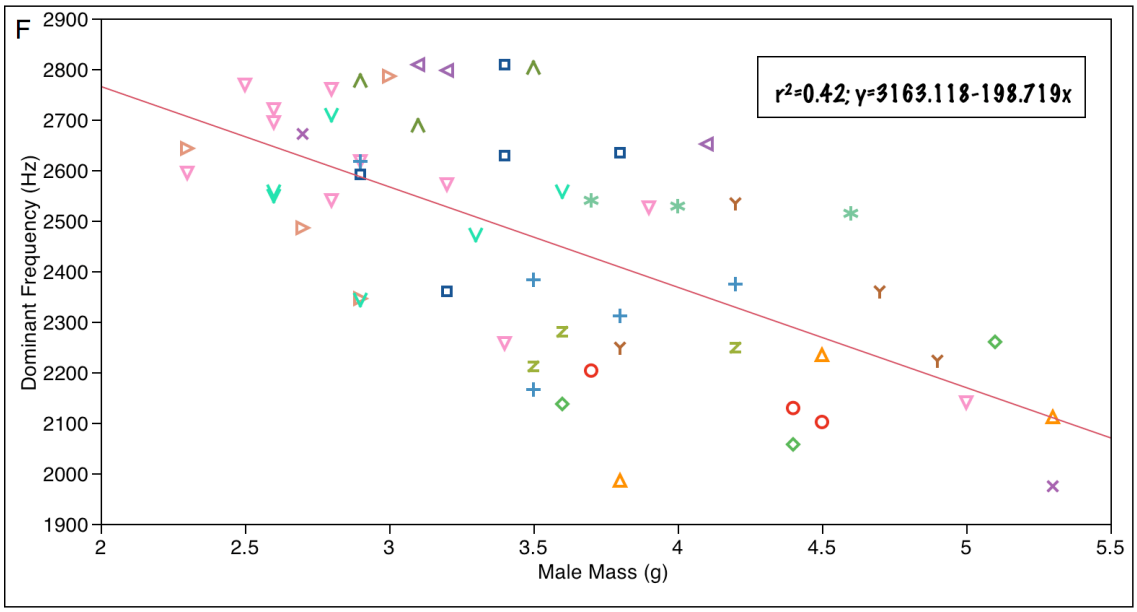


Figure 3.15. Relationship between call parameters and male temperature or male mass in *Hyla arborea* sampled populations.

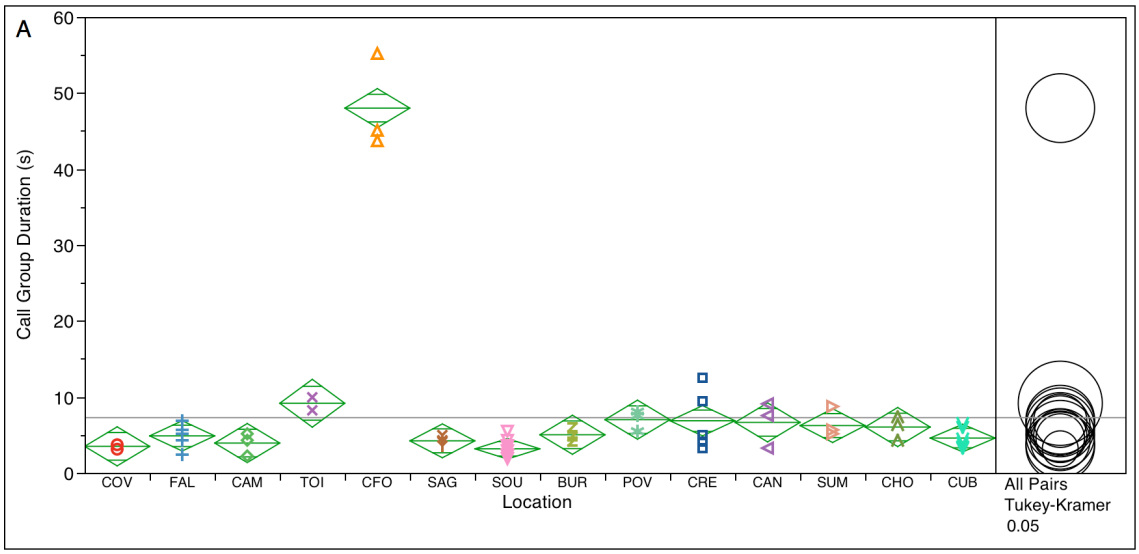
Variation of call duration (A), intercall duration (B), call rate (C) and pulse rate (D) with male temperature and fundamental (E) and dominant (F) frequencies with male mass, according to the pooled data for all the males for all the populations from Portugal. Coefficients of determination (r^2) and linear-regression equations are indicated in each graph, for each of the variable pairs.

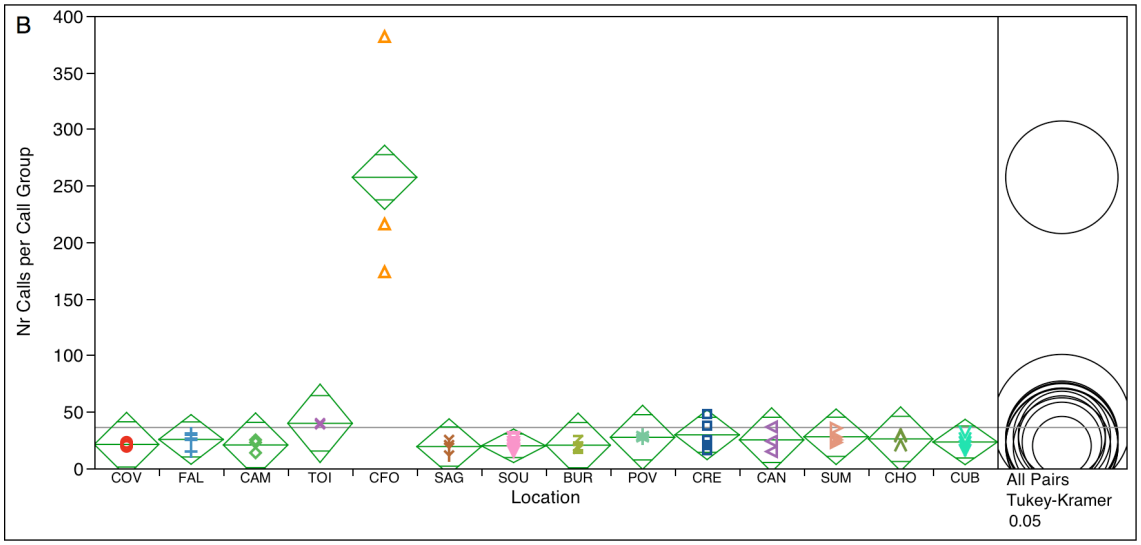
For all call parameters affected by male temperature or body size, residuals will be used in subsequent analyses (ANOVA, DFA and PCA). In such cases, either the term 'adjusted' or 'residual' will follow the call-parameter name.

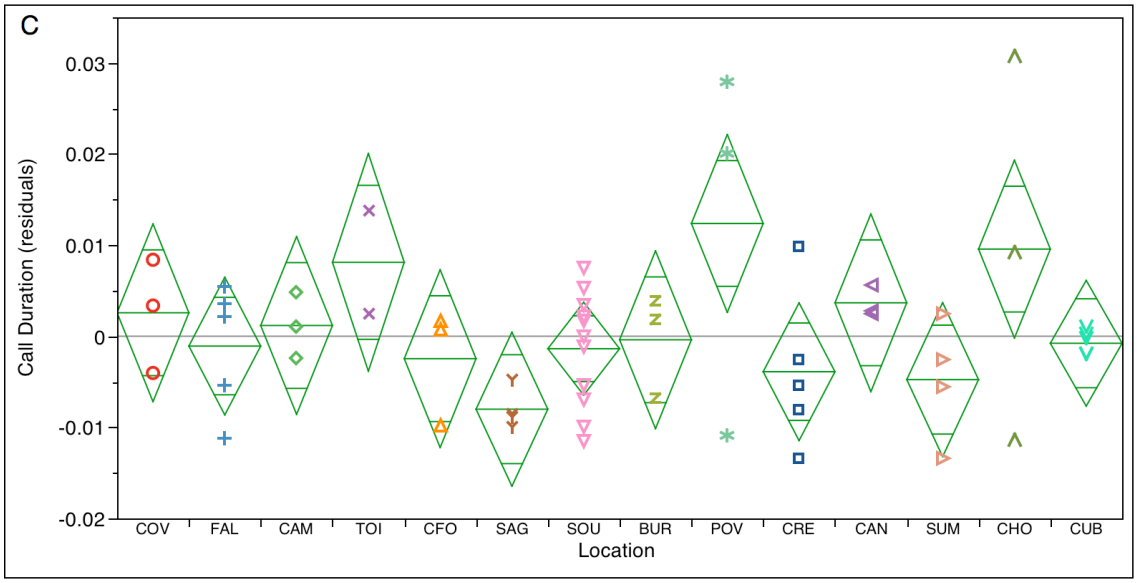
Call Variation among Populations

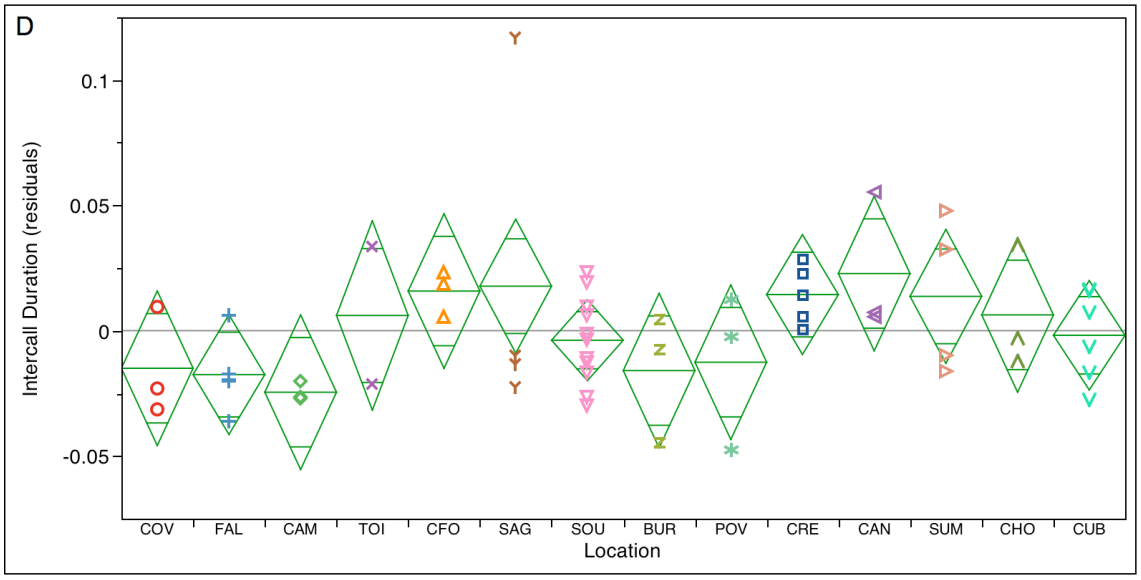
ANOVA

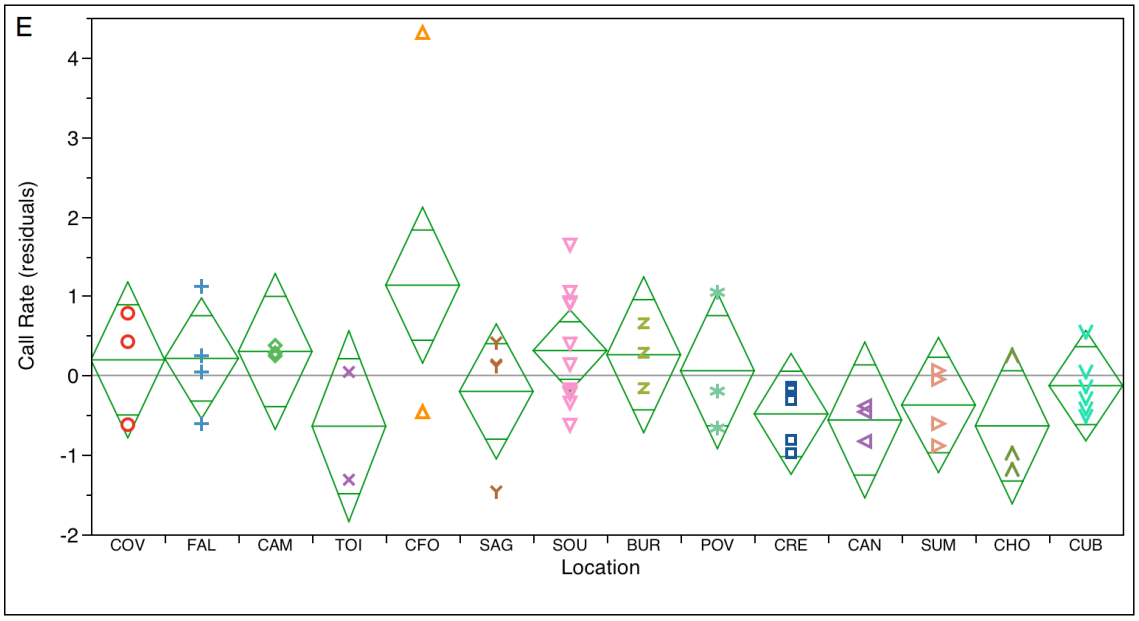
No significant differences ($p>0.05$) were found among *H. arborea* populations for all the temporal variables in a within-call group, i.e. number of pulses per call, call and intercall durations, call rate, and pulse rate. Highly significant differences were found for temporal variables of call group, i.e. call-group duration (ANOVA $F_{13,44}=84.8873$, $p<.0001$, $n=58$) and number of calls per call group (ANOVA $F_{13,44}=20.3043$, $p<.0001$, $n=58$). The spectral variables i.e. fundamental frequency (adjusted) (ANOVA $F_{13,44}=3.0575$, $p=0.0028$, $n=58$), dominant frequency (adjusted) (ANOVA $F_{13,44}=3.4178$, $p=0.0011$, $n=58$), amplitude of dominant frequency (ANOVA $F_{13,44}=2.8168$, $p=0.0052$, $n=58$) and difference of amplitude between dominant and fundamental frequencies (ANOVA $F_{13,44}=2.7607$, $p=0.0060$, $n=58$) all revealed highly significant differences.

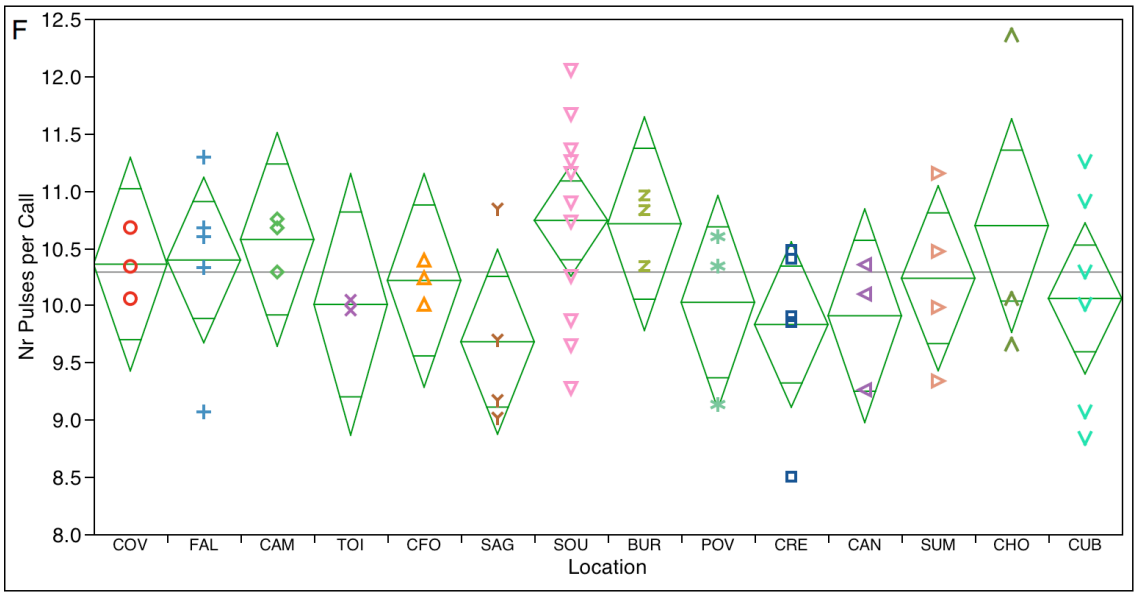

A
Call Group Duration (s)
60
50
40
30
20
10
0
COV
FAL
CAM
TOI
CFO
SAG
SOU
BUR
POV
CRE
CAN
SUM
CHO
CUB
Location
All Pairs
Tukey-Kramer
0.05

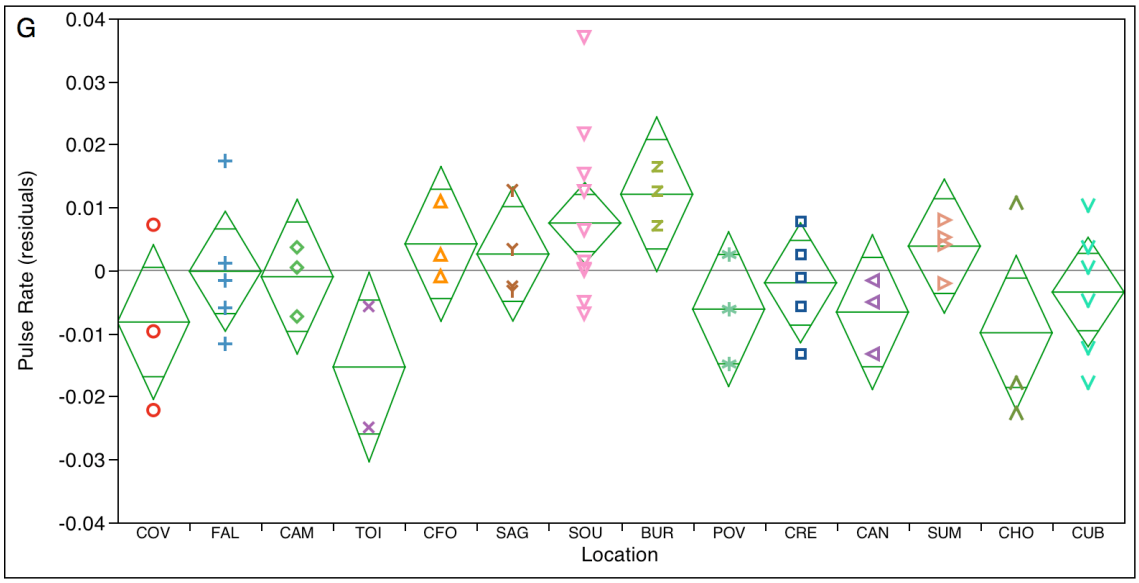

B
Nr Calls per Call Group
400
350
300
250
200
150
100
50
0
COV
FAL
CAM
TOI
CFO
SAG
SOU
BUR
POV
CRE
CAN
SUM
CHO
CUB
Location
All Pairs
Tukey-Kramer
0.05

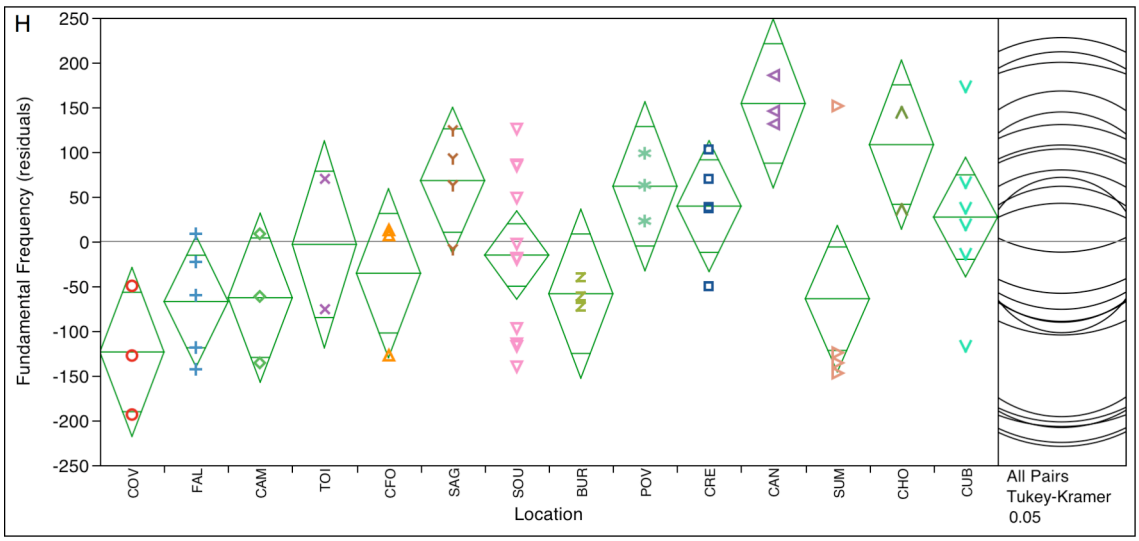

C
Call Duration (residuals)
0.03
0.02
0.01
0
-0.01
-0.02
COV
FAL
CAM
TOI
CFO
SAG
SOU
BUR
POV
CRE
CAN
SUM
CHO
CUB
Location

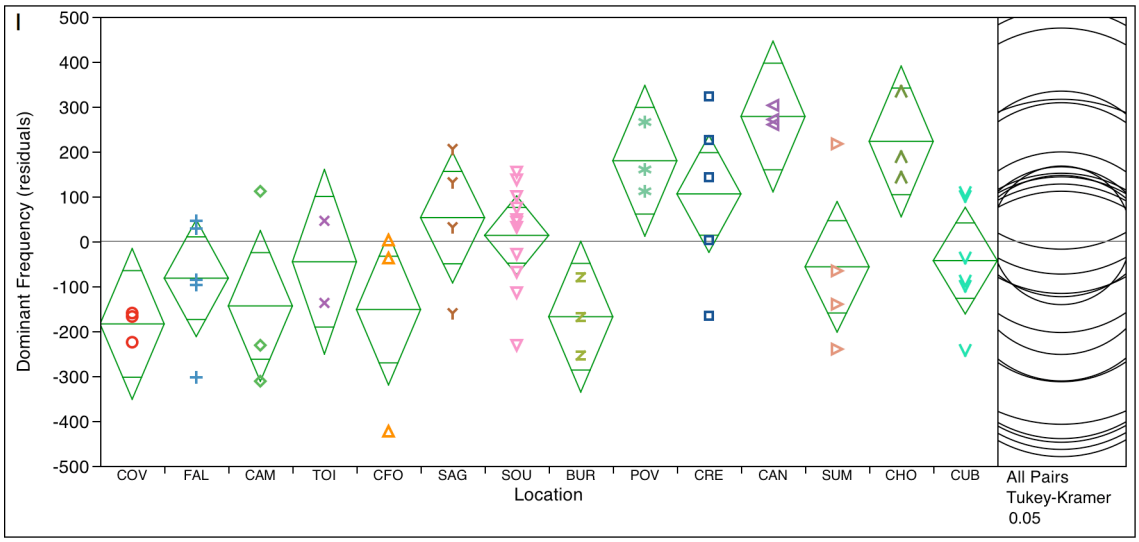

D
Intercall Duration (residuals)
0.1
0.05
0
-0.05
COV FAL CAM TOI CFO SAG SOU BUR POV CRE CAN SUM CHO CUB
Location


E
Call Rate (residuals)
4
3
2
1
0
-1
-2
COV FAL CAM TOI CFO SAG SOU BUR POV CRE CAN SUM CHO CUB
Location


F
Nr Pulses per Call
12.5
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
COV FAL CAM TOI CFO SAG SOU BUR POV CRE CAN SUM CHO CUB
Location


G
Pulse Rate (residuals)
0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
-0.04
COV FAL CAM TOI CFO SAG SOU BUR POV CRE CAN SUM CHO CUB
Location


H
Fundamental Frequency (residuals)
250
200
150
100
50
0
-50
-100
-150
-200
-250
COV FAL CAM TOI CFO SAG SOU BUR POV CRE CAN SUM CHO CUB
Location
All Pairs
Tukey-Kramer
0.05


I
Dominant Frequency (residuals)
500
400
300
200
100
0
-100
-200
-300
-400
-500
COV FAL CAM TOI CFO SAG SOU BUR POV CRE CAN SUM CHO CUB
Location
All Pairs
Tukey-Kramer
0.05

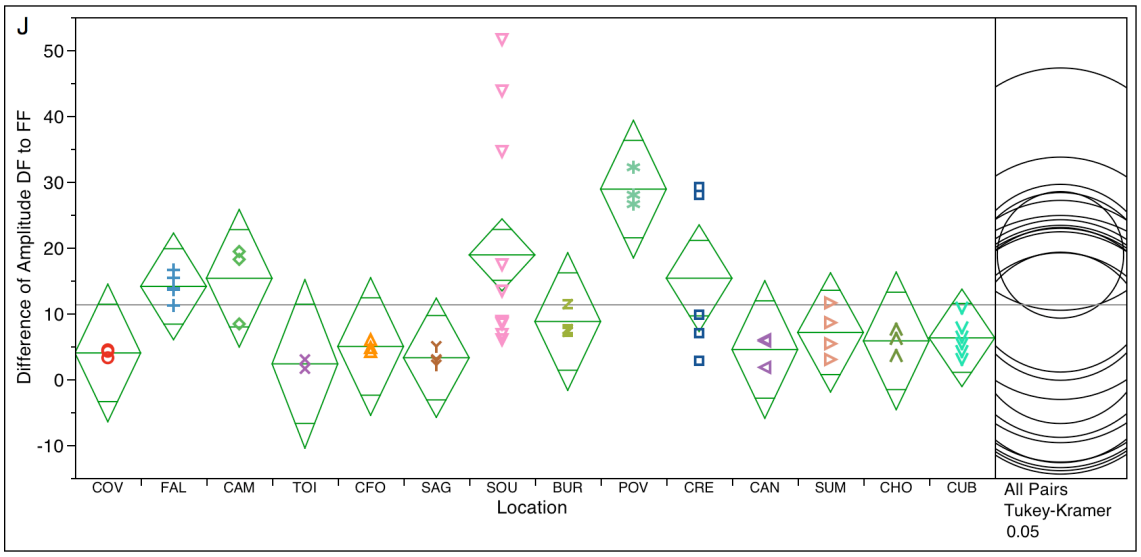


Figure 3.16. Call parameters distribution in *Hyla arborea* sampled populations in Portugal.
Boxplots of the temperature-independent and mass-independent residuals of the acoustic variables analysed for *H. arborea* for each population. Representation of one-way analysis (green diamonds) and Tukey-Kramer multiple comparisons (circles of the right windows, when suitable) outputs. The middle line of the diamond represents the group mean, and the top and bottom extremes represent the corresponding 95% confidence interval. The horizontal line in the graphic represents the global mean of all pooled samples. Populations are organised left to right according to their location along a north-south axis. For an explanation of population acronyms see Materials and Methods, Table 2.2.

For all the ANOVA significant tests a Tukey-Kramer HSD test was done *a posteriori*. For the call group duration (Fig. 3.16A) and number of calls per call group (Fig. 3.16B), the T-K HSD test revealed significant differences between the higher mean values of CFO and all other populations. CFO seems to be an outlier when it relates to call group duration and number of calls per call group, having clearly much higher values than any other sample population. For multiple comparisons, T-K HSD tests showed significant differences of fundamental frequency (Fig. 3.16H) between CAN and both FAL and COV, and of dominant frequency (Fig. 3.16I) between CAN and CAM, CFO, BUR and COV. Amplitude difference between dominant and fundamental frequencies (Fig. 3.16J) showed significant differences between POV and both SAG and CUB.

Discriminant Function Linear Analysis

Discriminant Function Linear Analysis was used to determine if calls would be grouped in the correspondent population of origin according to their characteristics. DFA was highly significant (Wilks' Lambda= 0.0005595 aproxim. F=3.4845; df=130, 296; $p<0.0001$), and the two canonical functions that were extracted (Table 3.11) classified 67.24% of calls correctly according to their population of origin. The first canonical function explained 85.08% of the

between-populations call differences: it was significantly positively correlated to call-group duration and negatively correlated with pulse rate (see Table 3.11). The second canonical function explained only 6.12% of the differences among populations, and was positively correlated with call duration and pulse rate and negatively correlated with the number of pulses per call.

Table 3.11. DFA on call parameters among populations of the *Hyla arborea*.

Standardised coefficients, percentage of variance explained by the individual functions and canonical correlations of the discriminant functions extracted. Analyses performed for the individuals of *H. arborea* sampled for advertisement calls.

Call parameters	Discriminant Functions	
	1	2
Call-group duration (CGD)	1.204	0.369
Nr of calls per call group (CCG)	-0.170	-0.784
Call duration (CD)	-0.777	2.338
Intercall duration (ICD)	0.413	-0.194
Nr of pulses per call (PC)	0.863	-1.478
Call rate (CR)	0.855	0.981
Pulse rate (PR)	-0.883	1.061
Fundamental frequency (FF)	-0.174	0.274
Dominant frequency (DF)	-0.109	0.302
Difference of amplitude between dominant and fundamental frequencies (ADF)	0.209	0.478
Cumulative Variance Explained (%)	85.08	91.20
Canonical Correlations	0.986	0.846

DFA indicated that CFO population was distinct from all other populations and POV samples also seem to create a distinct group from the main overlapping group, including all other populations (Fig.3.17; see map Fig. 2.2 for geographic reference). Although some of the call variables differed significantly among populations, as indicated by highly significant ANOVA tests (see above), DFA analysis was not able to separate populations in the space defined by the first two components, with an accuracy of only 67% in the classification.

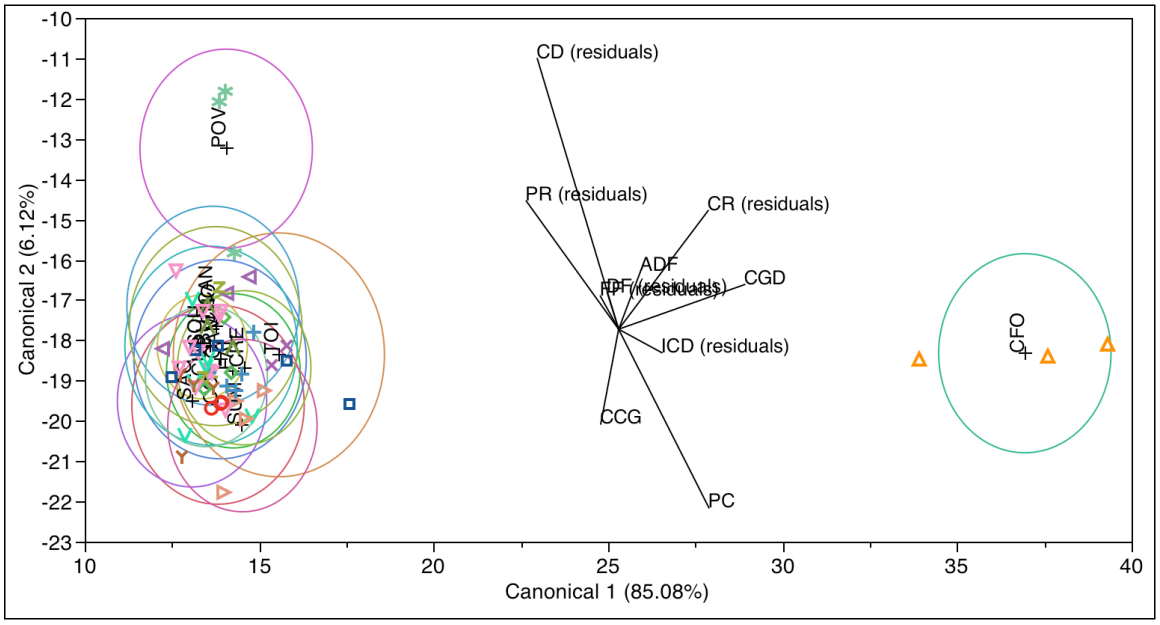


Figure 3.17. Canonical coefficients of the first two canonical functions extracted in *Hyla arborea*.
Two-dimensional space generated by discriminant function analysis of advertisement calls of *H. arborea* populations sampled in Portugal (n=58 males). Canonical factor 1 accounts for 85.08% of the variation (canonical correlation=0.986) while canonical factor 2 explains 6.12% more of the variation (canonical correlation=0.846). The confidence ellipses are centred on the centroid of each group.

Principal Component Analysis

A Principal Component Analysis was conducted with all the valid audio-recorded males, from all populations, to evaluate whether population groups were separating along certain call characteristics. The PCA on call characteristics produced four components with an eigenvalue greater than 1, which accounted for 77.51% of the variation (Table 3.12). Components were interpreted from unrotated loadings, because rotation resulted in interchanging variables loadings from one component to the other (i.e. rotated component 1 reflected call group temporal variables and rotated component 2 reflected the spectral variables, inverting the order of the unrotated components). Component 1 was associated mainly with spectral variables, such as fundamental and dominant frequencies (0.45 and 0.42, respectively), as well as with call rate (-0.44). Components 2 and 3 reflected the temporal variables. Call-group related variables loaded high on component 2 (e.g. call group duration and number of calls per call group=0.63 for both). Component 3 reflected call-specific temporal variables (e.g. call duration=0.67 and number of pulses per call=0.61, respectively). Lastly, dominant frequency and pulse rate loaded high on component 4 (e.g. 0.56 and 0.42, respectively).

Table 3.12. Principal Component Analysis of the advertisement call properties of *Hyla arborea*.
The correlation (eigenvectors) between the four principal components derived from PCA and the original variables are presented. Eigenvalues and cumulative variance explained are also shown. Analyses were performed for the 58 males of *H. arborea* with advertisement calls recorded.

Call parameters	Principal Components			
	1	2	3	4
Call-group duration (CGD)	-012734	**0.62562**	0.10934	-0.00525
Nr of calls per call group (CCG)	-0.18325	**0.63090**	0.11481	0.10906
Call duration (CD)	0.27270	-0.02940	**0.66822**	0.06331
Intercall duration (ICD)	0.35996	0.27013	-0.14969	-0.09918
Nr of pulses per call (PC)	-0.11556	-0.16842	**0.60695**	0.39513
Call rate (CR)	**-0.44147**	0.14033	-0.03561	0.31424
Pulse rate (PR)	-0.39023	-0.09294	-0.22575	**0.42020**
Fundamental frequency (FF)	**0.44916**	0.16450	-0.17221	0.38471
Dominant frequency (DF)	**0.42123**	0.04557	-0.15318	**0.56146**
Difference of amplitude between dominant and fundamental frequencies (ADF)	-0.08232	-022478	-0.17944	0.28204
Eigenvalues	2.8681	2.1093	1.5444	1.2289
Cumulative Variance Explained (%)	28.681	49.775	65.219	77.508

The PCA on the advertisement call variables of *H. arborea* revealed two groups of males (Fig. 3.18), CFO and the rest of the populations. In agreement with the DFA results, CFO population (represented in the figure by, △ top left quarter of Fig. 3.18) grouped away from the remainder populations along the axis, explained by call-group-related variables.

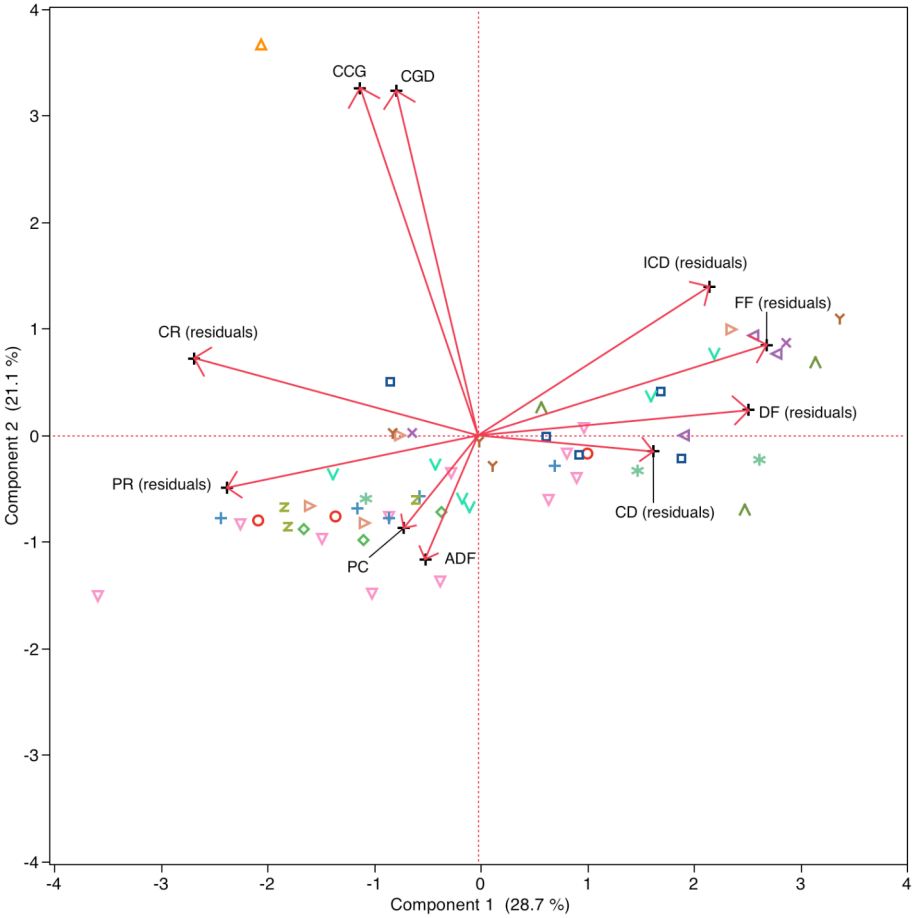


Figure 3.18. PCA scatterplot of the first two principal components.

PCA ordination graph showing data for *H. arborea* populations and variables of male advertisement calls in relation to the first two axes of multidimensional space, describing variables related to call variables of *H. arborea* advertisement calls. For variable component loadings, see Table 3.12 above.

2.2. *Hyla meridionalis*

The advertisement calls of 126 males from 28 different locations (see Table 2.3 and map Fig. 3.12) were analysed. *Hyla meridionalis* males' advertisement calls are single-noted and have pulsed structure (Fig. 2.7), and, as mentioned above, the nomenclature for call characteristics used follows Paillette (1967a) and Schneider (1978).

Coefficients of Variation of Call Parameters

Within-Individual Variation

Call variation at a within-individual level was quantified by calculating the coefficients of variation of all call properties for all pooled individuals (tables 3.13 and 3.14). First, the CV of the species-call acoustic properties was calculated. Second, the CV was calculated at a population level as an average of males within the sampled site. The most variable call property

of *H. meridionalis* males was intercall duration (CV=26.32%), whereas all other call properties were either intermediate (5% <CV <12%) or highly stereotyped, with CV values always below 3.5% (see Table 3.13).

Table 3.13. Within-individual coefficient of variation of the *Hyla meridionalis* call acoustic properties.

CV calculated for all call acoustic properties by male (n=126 males).

Call parameters	Coefficient of Variation (%)	
	Mean	SD
Call duration (CD)	3.39	1.65
Intercall duration (ID)	26.32	15.49
Number of pulses per call (PC)	3.28	1.55
Pulse rate (PR)	2.03	0.96
Fundamental frequency (FF)	1.35	1.36
Dominant frequency (DF)	1.41	1.30
Difference of amplitude between dominant and fundamental frequencies (ADF)	13.62	11.75

Within each population-call variation, TOU had the highest mean CV for both number of pulses per call and call duration, while the same was found in FRT for dominant frequency, in FAZ for intercall duration and pulse rate, in JAV for fundamental frequency; finally, COS had the highest mean CV values for the difference of amplitude between dominant and fundamental frequencies (Table 3.14). Lowest mean CV values were obtained in ODI for call duration and pulse rate, in CHO for intercall duration and difference of amplitude between dominant and fundamental frequencies, in BRI for number of pulses per call, in ARI for fundamental frequency, in ALQ for dominant frequency. Among populations there is a high variation for CV mean values, in particular for intercall duration and difference of amplitude between dominant and fundamental frequencies.

Effects of Temperature and Body Size

Body size of *Hyla meridionalis* males measured as SVL ranged from 2.4 to 4.3 cm with a mean (± SD) of 3.66 (±0.28) cm, and mass ranged from 2.3 to 6.3 g with a mean (± SD) of 3.88 (±0.83) g (see Appendix 4 for descriptive statistics for each location). Body size (both measures) differed significantly among localities (ANOVA SVL: $F_{27,98}$=3.9291, p<.0001, n=126; mass: $F_{27,98}$=5.0450, p<0.0001, n=126) (Fig. 3.19). Mass and SVL are highly correlated (r=0.8186, p<0.001, n=126), and so mass was chosen as a proxy for body measurement in subsequent analysis.

Table 3.14. Population within-individual coefficient of variation of the *H. meridionalis.*

CV calculated for all call-acoustic properties per male within each sampled site (n=126 males)

Location	n	CD	ID	PC	PR	FF	DF	ADF
ALP	4	3.07±2	28.91±2.37	3.22±0.14	1.51±1.27	0.64±6.8	1.33±7.23	11.88±5.19
POV	3	3.09±1.47	50.6±2.06	4.21±0.34	1.93±1.03	1.18±2.67	0.82±1.98	14.3±16.9
BUR	5	2.93±1.06	24.25±2.12	3.44±0.65	1.64±0.96	0.43±4.62	0.7±4.69	8.35±4.6
CRE	3	3.5±1.06	30.21±2.24	4.47±0.45	2.98±1.03	0.6±16.32	0.81±13.52	33.82±21.87
SUM	3	3.68±1.52	26.82±0.47	3.6±0.6	2.61±1.03	1.19±1.92	1.34±2.79	14.85±12.72
FRO	2	3.17±1.13	32.67±1.08	3.5±0.31	0.9±1.25	0.88±6.6	1.25±7.05	7.38±7.12
CHO	3	2.5±1.21	17.6±0.69	2.6±0.38	1.11±0.95	0.55±5.99	1.97±3.03	4.37±1.44
LIN	7	3.27±0.79	25.82±0.92	2.98±0.87	2.15±1.18	2.08±6.39	2.06±6.56	12.93±9.4
FRT	4	2.88±1.05	21.6±2.06	3.39±0.53	1.75±2	2.91±2.13	3.8±1.79	15.42±5.36
TOU	6	5.64±1.22	30.32±1.28	5.11±1.62	2.44±1.25	0.8±10.12	1.69±7.6	11.07±5.64
FOQ	3	4.21±2.29	24.38±1.68	3.35±0.86	1.96±0.64	1.84±6.58	2.38±8.95	25.79±16.31
RED	10	3.56±1.69	26.68±1.73	3.65±0.64	2.29±1	0.94±8.37	1.21±8.26	10.88±7.68
ALQ	5	3.21±2.17	26.15±1.19	2.84±1.96	2.52±1.37	0.61±6.62	0.66±8.87	10.08±9.39
GRA	4	3.08±1.32	26.31±1.2	2.78±0.58	1.76±1.55	1.29±2.3	1.35±3.83	7.85±3.88
ODI	3	2.14±1.4	18.64±0.5	2.58±0.99	1.09±1.19	0.69±5.67	0±2.48	8.91±1.09
BRI	4	2.25±0.51	15.37±0.51	2.42±0.64	1.45±1.44	1.25±1.83	0.84±3.71	12.13±4.17
CUB	5	4.12±1.36	20.46±1.26	3.93±0.37	1.15±0.97	1.72±3.54	1.07±3.19	8.28±3.73
PIC	4	3.03±0.82	29.02±1.23	3.56±0.76	2.63±0.84	2.68±3.88	1.48±1.02	10.3±4.5
SMP	5	3.78±2	20.48±0.45	2.9±0.92	2.76±1.76	3.09±1.34	1.16±1.66	14.39±15.5
ALM	4	2.85±1.36	20.78±0.79	2.64±1.12	2.14±0.47	1.84±5.31	1.38±5.55	41.92±59.68
FOU	4	2.93±1.92	18.37±1.85	2.49±1.03	1.77±1.27	0.63±0.9	2.47±2.09	20.25±21.56
COS	5	3.44±1.37	33.13±1.79	2.84±0.87	1.92±0.91	0.99±3.91	1.33±4.89	8.26±4.28
CAC	7	3.45±3.61	27.1±3.05	3.25±0.46	1.68±1.3	1.21±6.03	1.44±9.56	15.12±13.64
FOI	6	3.04±1.43	25.23±1.22	2.69±1.33	2.11±0.87	1.1±13.79	0.88±19.81	23.61±23.37
BRA	6	3.3±2	16.08±1.61	3.45±0.54	1.64±0.62	1.7±5.65	1.26±4.55	14.97±3.61
JAV	2	4.32±1.34	26.36±2.21	2.67±0.33	2.85±5.31	3.76±30.02	3.64±12.24	8.1±8.77
ARI	5	4.49±0.29	29.01±0.63	3.31±0.83	2.36±0.51	0.23±2.84	1.02±2.38	80.22±160.74
FAZ	4	2.56±0.75	54.26±1.54	3.22±1.26	3.07±0.42	2.02±4.42	1.53±5.48	14.43±6.55

Note: Populations are organized top to bottom according to their location along a north-south axis. For an explanation of population acronyms, see Materials and Methods, Table 2.1; and for call parameters acronyms see Table 3.13.

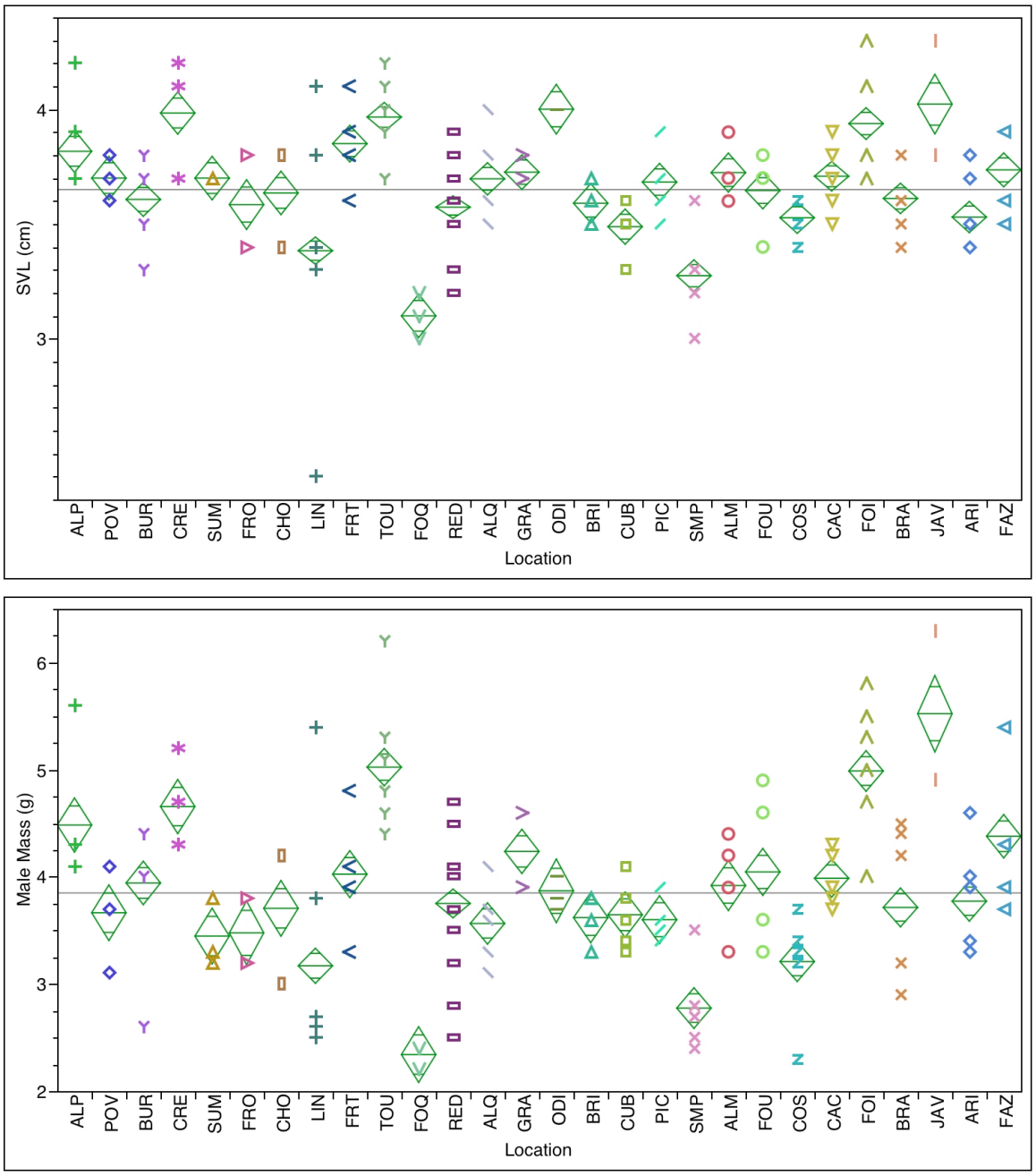


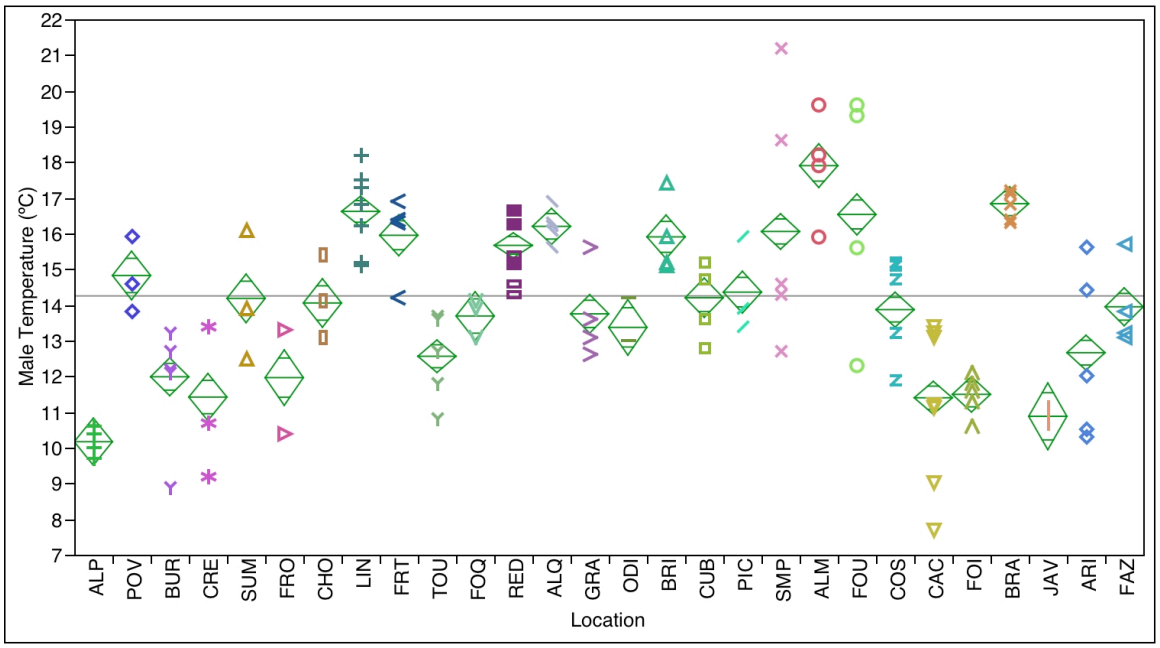
Figure 3.19 Body size distribution of calling males of *Hyla meridionalis* sampled populations in Portugal.

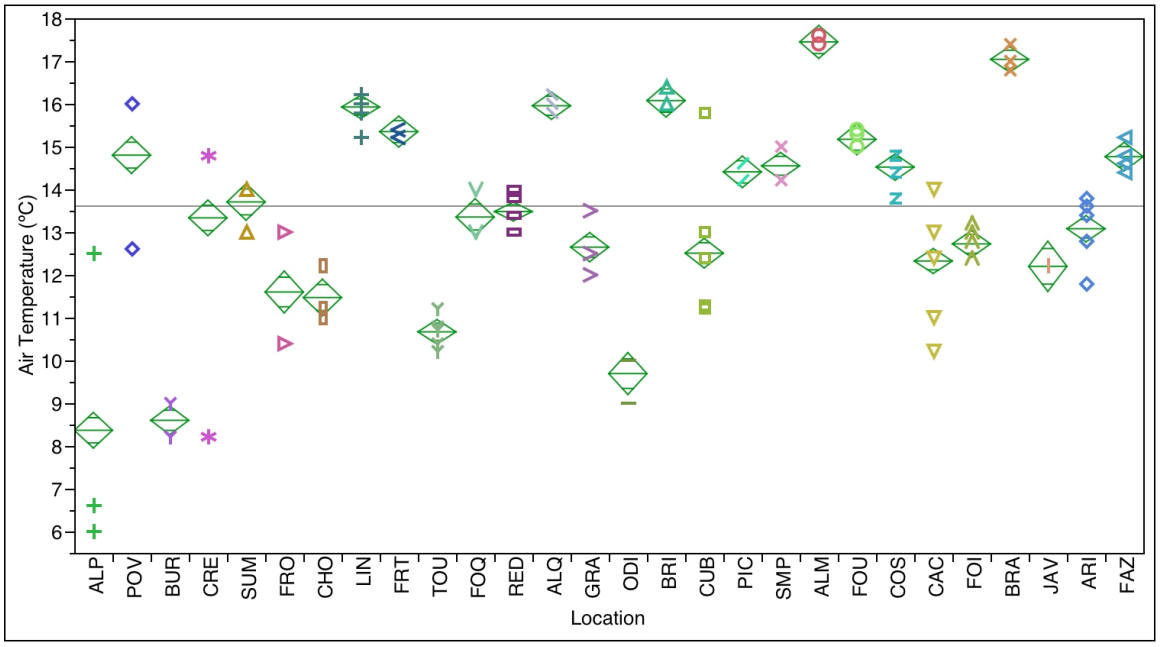
Boxplots of SVL (top) and mass (bottom) for *H. meridionalis* males in each population. The middle line of the diamond represents the group mean, and the top and bottom extremes represent the corresponding 95% confidence interval. The horizontal line in the graphic repents the global mean of all pooled samples. Populations are organized left to right according to their location along a north-south axis. For an explanation of population acronyms, see Materials and Methods, Table 2.1.

Air, substrate (water or soil, vegetation, rock) and male body temperatures were measured in all populations with audio-recorded males. Air temperatures ranged from 6.0 to 17.6ºC with a mean (± SD) of 13.55ºC (±2.43); substrate temperatures ranged from 8.5 to

21.8ºC with a mean (± SD) of 14.71ºC (±2.71), and male body temperatures ranged from 7.7 to 21.2ºC with a mean (± SD) of 14.18 ºC (±2.50) (see Appendix 4 for descriptive statistics for each location).

All three temperature-variables varied significantly among sampled populations. For mean values of male temperature ($F_{27,98}$=8.2240, p<.0001, n=126), ALP registered the lowest and ALM the highest (Fig. 3.20, top). For air temperature ($F_{27,98}$=18.5683, p<.0001, n=126), also the lowest mean values were registered in ALP and the highest again in ALM (Fig. 3.20, middle). For substrate temperature ($F_{26,93}$=9.2319, p<.0001, n=120), the lowest mean values were registered in JAV, and the highest values were in ALM, just like the male and air temperatures (Fig. 3.20, bottom).





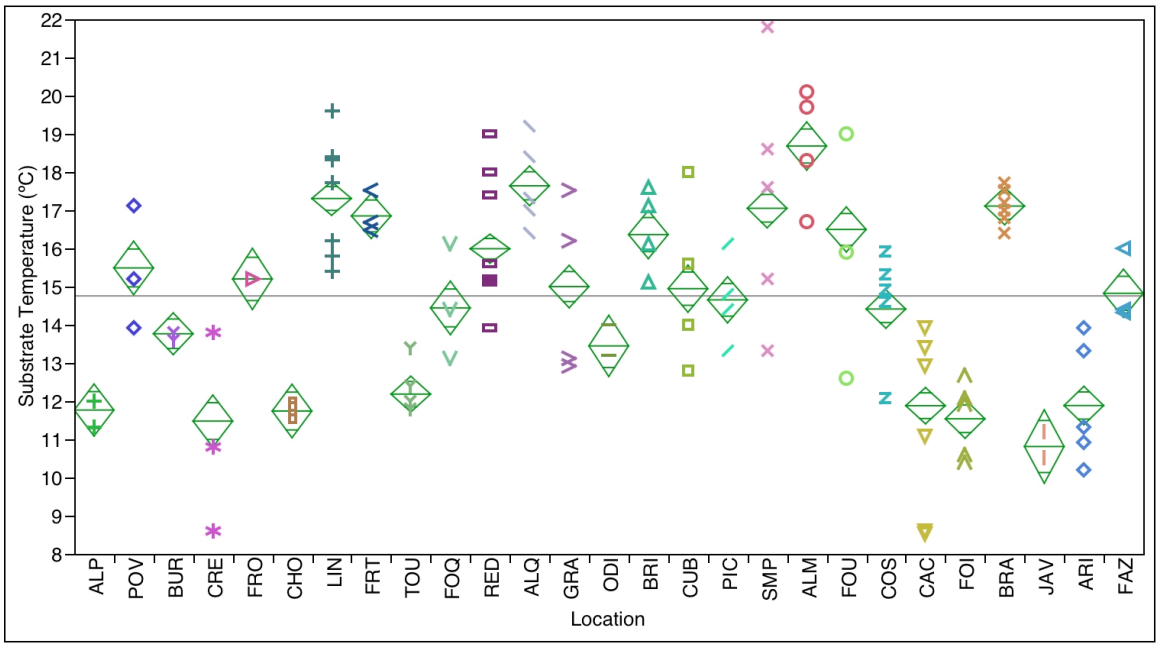


Figure 3.20. Temperature distribution in *Hyla meridionalis* sampled populations in Portugal.
Boxplots of male (top), air (middle) and substrate (bottom) temperatures measured for each recorded male of *H. meridionalis* in each population. The middle line of the diamond represents the group mean, and the top and bottom extremes represent the corresponding 95% confidence interval. The horizontal line in the graphic represents the global mean of all pooled samples. Populations are organised left to right according to their location along a north-south axis. For an explanation of population acronyms, see Materials and Methods, Table 2.1.

Temperatures of males and substrate are significantly positively correlated (Table 3.15). In the remaining analysis only male temperature will be used, as there are no missing values in the data set. Air temperature is also significantly correlated with both male and substrate temperatures, but with lower correlation values.

Table 3.15. Temperature correlation matrix for *Hyla arborea* sampled populations.
Pairwise correlation values for air, substrate and male temperature measurements.

	Air Temperature	Substrate temperature	Male Temperature
Air temperature (n=126)	–		
Substrate Temperature (n=120)	0.6245*	–	
Male Temperature (n=126)	0.6608*	0.8785*	–

Note: * $p<0.01$; *n* refers to the number of individuals

As there were significant differences among populations for both temperature and body size, and as some call parameters are typically affected by both temperature and/or body size,

first the relationship of call variables to male body and to temperature were assessed, by running a multivariate regression analysis model. For all populations as a single group, the effect of temperature and mass in each call variable was tested. All regression models revealed a highly significant correlation between male temperature and the dependent variable: call duration, intercall duration, number of pulses per call and mass all had an effect on call duration and on fundamental and dominant frequencies.

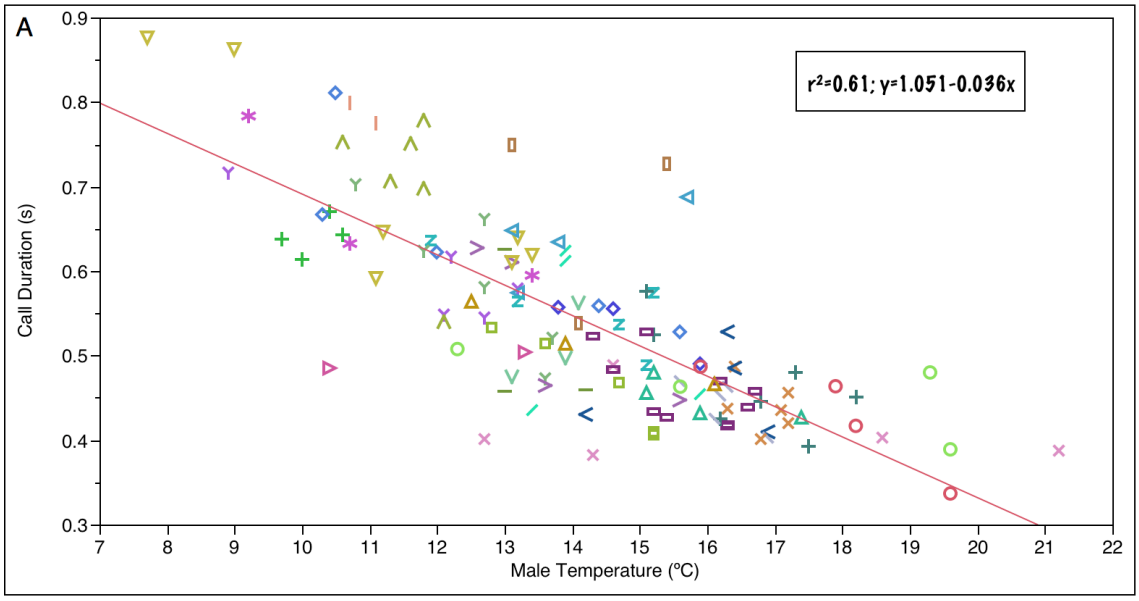
Table 3.16. Correlation matrix of call properties and male temperature and mass.

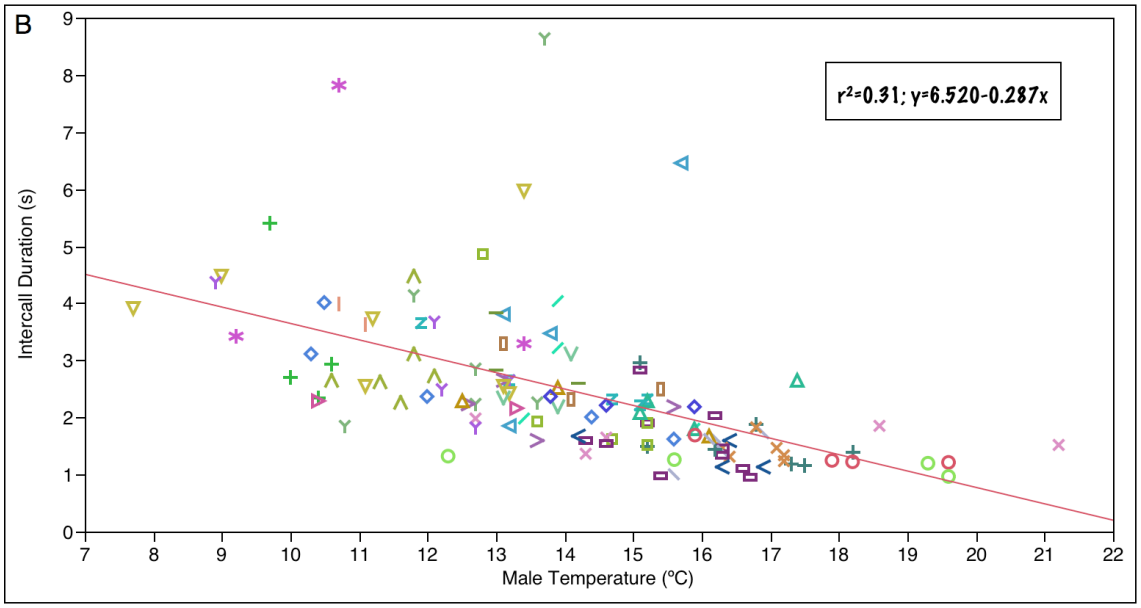
Correlation coefficients of call properties with male temperature and male mass in *Hyla meridionalis* (n=126).

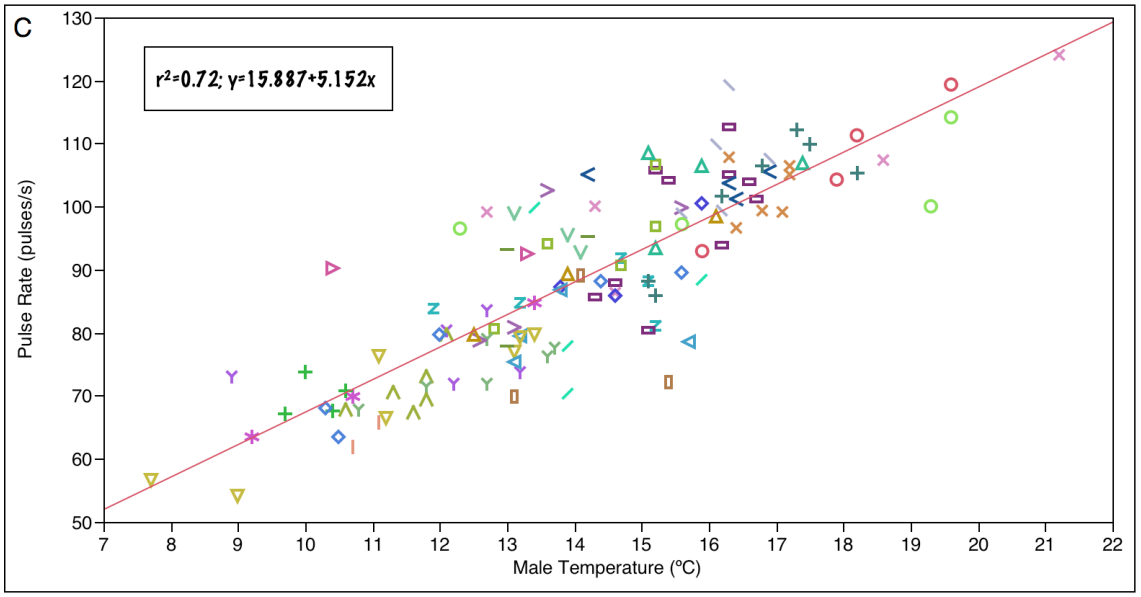
Call parameter	Correlation coefficient (r_s)	
	Male temperature	Male mass
Call Duration	-0.7804**	0.4439**
Pulse Rate	0.8497**	-0.4396**

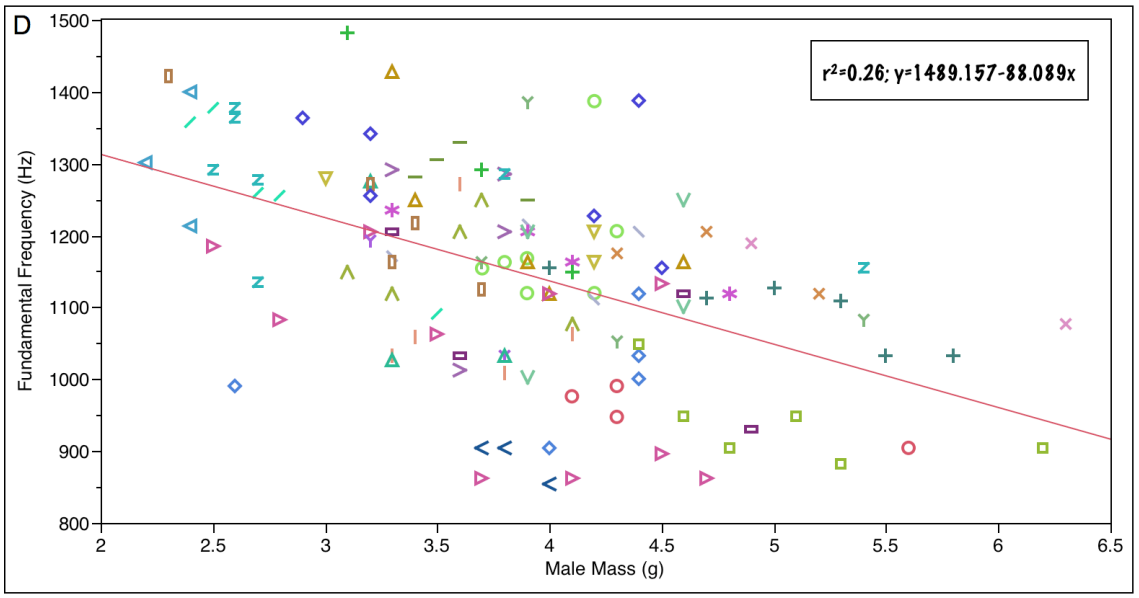
Note: **p<0.01

For call properties affected by both male temperature and male mass, correlation coefficients were used to select which factor had a stronger effect (higher correlation values) (Table 3.16). For those call properties, linear regressions of male temperature on temporal properties, and of male mass on spectral properties, were performed (Fig. 3.21) using the temperature and mass-independent residuals for subsequent analysis. The final decision on the independent variable was based on both correlation values and literature information, i.e. spectral variables are more dependent on body size than on temperature (e.g. Friedl & Klump 2002).




B
r²=0.31; y=6.520-0.287x
Intercall Duration (s)
Male Temperature (°C)


C
r²=0.72; y=15.887+5.152x
Pulse Rate (pulses/s)
Male Temperature (°C)


D
r²=0.26; y=1489.157-88.089x
Fundamental Frequency (Hz)
Male Mass (g)

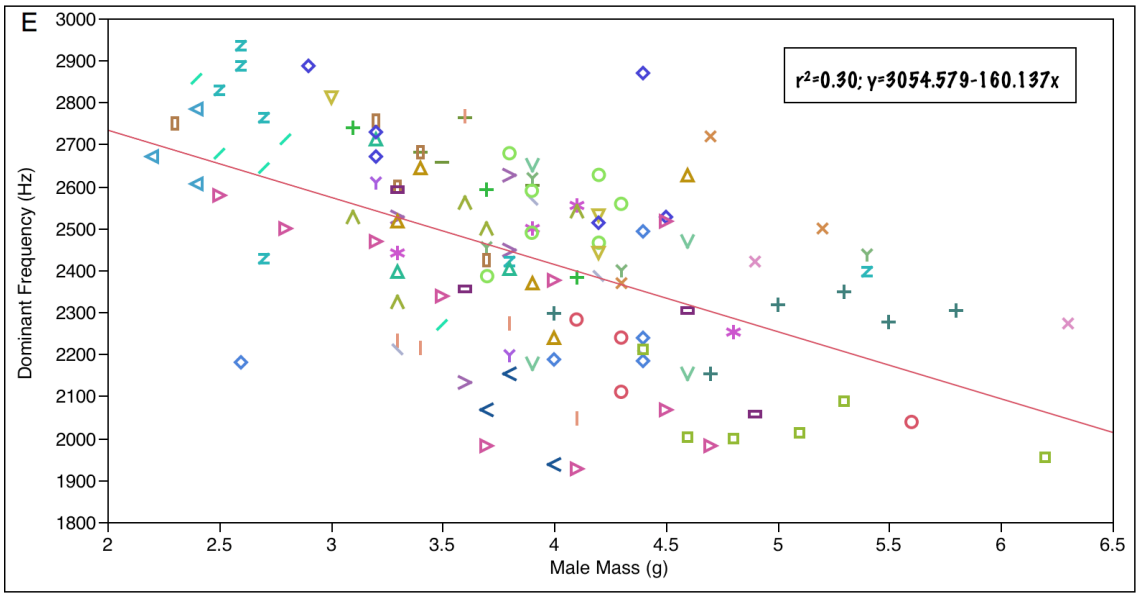


Figure 3.21. Relationships between call parameters and male temperature or male mass in *Hyla meridionalis*.

Linear regressions (with regression equations and coefficients of determinations) of male mass on fundamental (D) and dominant (E) frequencies, and of male temperature on call duration (A), intercall duration (B) and number of pulses per call (C). Coefficients of determination (r^2) and linear-regression equations are indicated in each graph, for each of the variable pairs.

For all call parameters affected by male temperature or body size, residuals will be used in subsequent analyses (ANOVA, DFA and PCA). In such cases, either the term 'adjusted' or 'residual' will follow the call-parameter name.

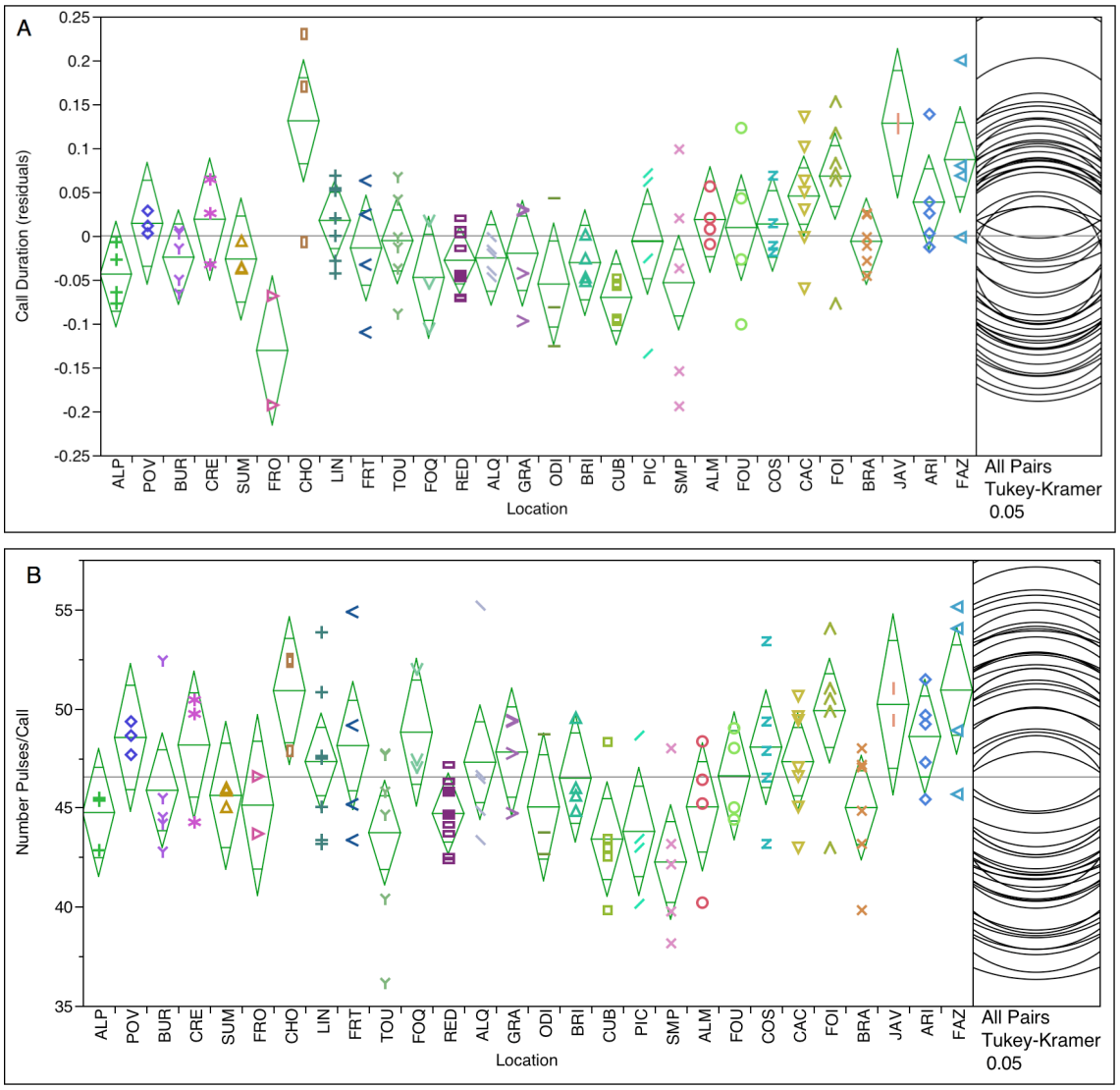
Call variation among groups

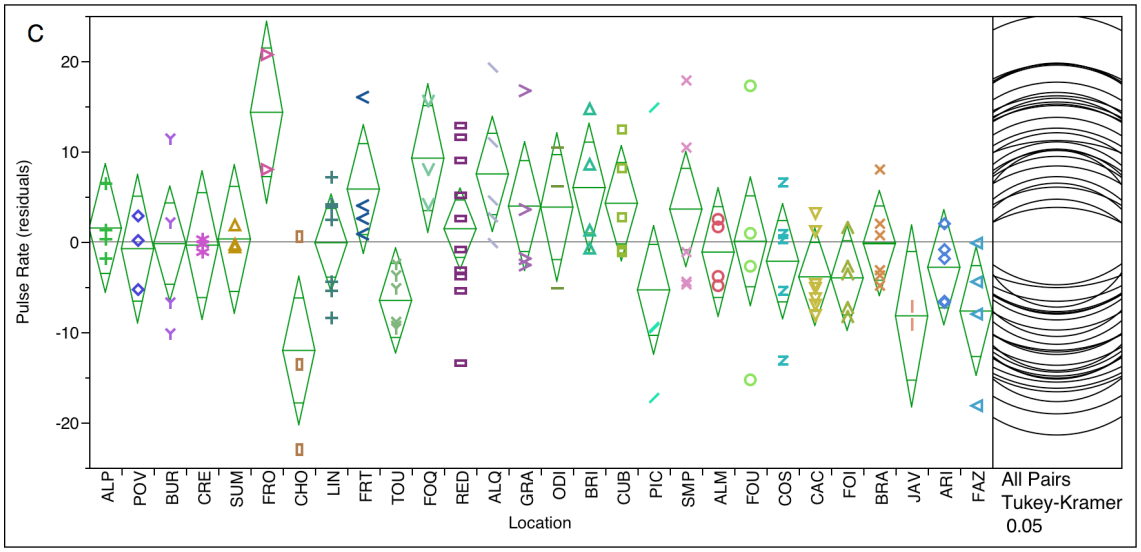
ANOVA

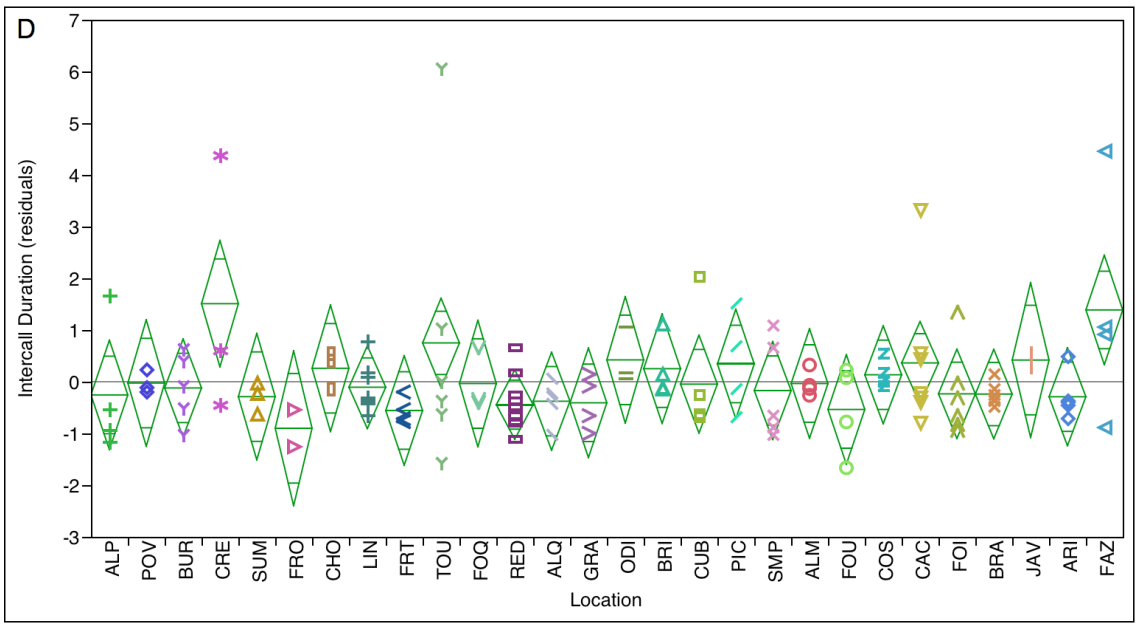
One-way ANOVA demonstrated a significant difference in the number of pulses per call (not adjusted) (n=126; $F_{27,98}$=2.2016; p=0.0026), call duration (adjusted) (n=126; $F_{27,98}$=2.9307; p<0.0001), pulse rate (adjusted) (n=126; $F_{27,98}$=2.1358; p=0.0037), fundamental frequency (adjusted) (n=126; $F_{27,98}$=5.1414; p<0.0001), dominant frequency (adjusted) (n=126; $F_{27,98}$=3.7719; p<0.0001), and difference between fundamental and dominant frequencies amplitudes (n=126, $F_{27,98}$=5.3824, p<0.0001), based on population group. No significant difference was found in adjusted intercall duration (n=131; $F_{30,100}$=1.0297; p=0.4395) between populations (see Fig. 3.22D for all the population comparisons).

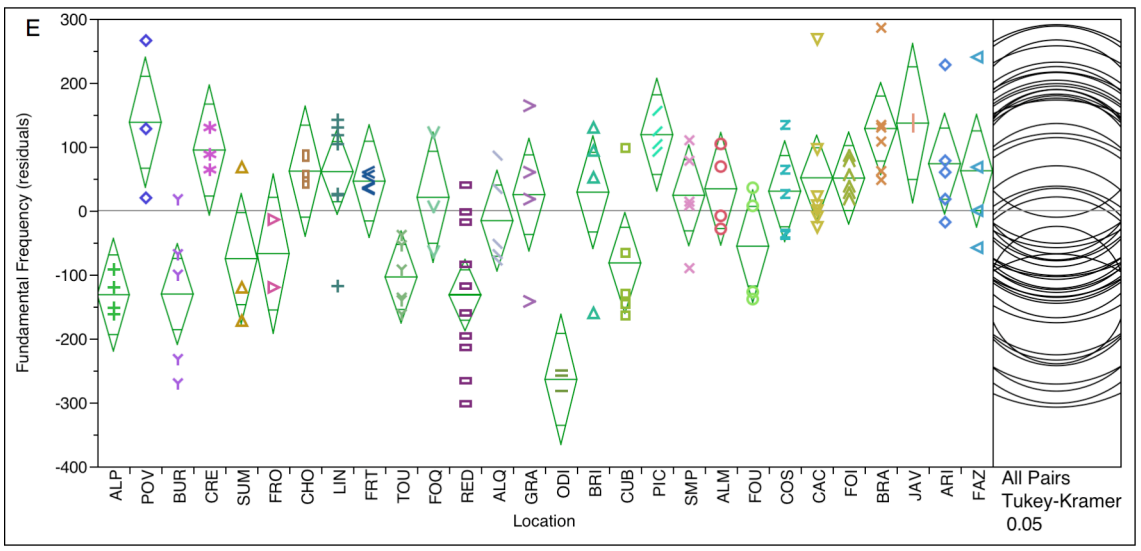
Tukey's Honestly Significant Difference (HSD) *post hoc* tests confirmed the significant differences among populations. For number of pulse calls (Fig. 3.22B), Tukey-Kramer HSD revealed SMP to be significantly different from FAZ and FOI. The Tukey-Kramer HSD on the difference between fundamental and dominant frequencies amplitudes (Fig. 3.22G) revealed

significant differences between POV and JAV and all the other populations. The T-K HSD procedure revealed that call durations (residuals, Fig. 3.22A) from RED, SMP, CUB and FRO were significantly different from all the other populations. For pulse rate (Fig. 3.22C), the T-K HSD procedure revealed that CHO was significantly different from FRO, but found no other significant differences among the other populations. For fundamental frequency (Fig. 3.22E), T-K HSD multiple-comparison test showed significant differences between ODI and almost all populations except FOU, FRO, SUM, CUB, TOU, BUR, RED and ALP; also between BRA, TOU, BUR, RED, ODI and ALP and the other populations. A T-K HSD applied to dominant frequency (Fig. 3.22F) detected significant differences between BUR, CUB, RED, TOU, ODI and all the others.




C
Pulse Rate (residuals)
20
10
0
-10
-20
ALP POV BUR CRE SUM FRO CHO LIN FRT TOU FOQ RED ALQ GRA ODI BRI CUB PIC SMP ALM FOU COS CAC FOI BRA JAV ARI FAZ
Location
All Pairs
Tukey-Kramer
0.05


D
Intercall Duration (residuals)
7
6
5
4
3
2
1
0
-1
-2
-3
ALP POV BUR CRE SUM FRO CHO LIN FRT TOU FOQ RED ALQ GRA ODI BRI CUB PIC SMP ALM FOU COS CAC FOI BRA JAV ARI FAZ
Location


E
Fundamental Frequency (residuals)
300
200
100
0
-100
-200
-300
-400
ALP POV BUR CRE SUM FRO CHO LIN FRT TOU FOQ RED ALQ GRA ODI BRI CUB PIC SMP ALM FOU COS CAC FOI BRA JAV ARI FAZ
Location
All Pairs
Tukey-Kramer
0.05

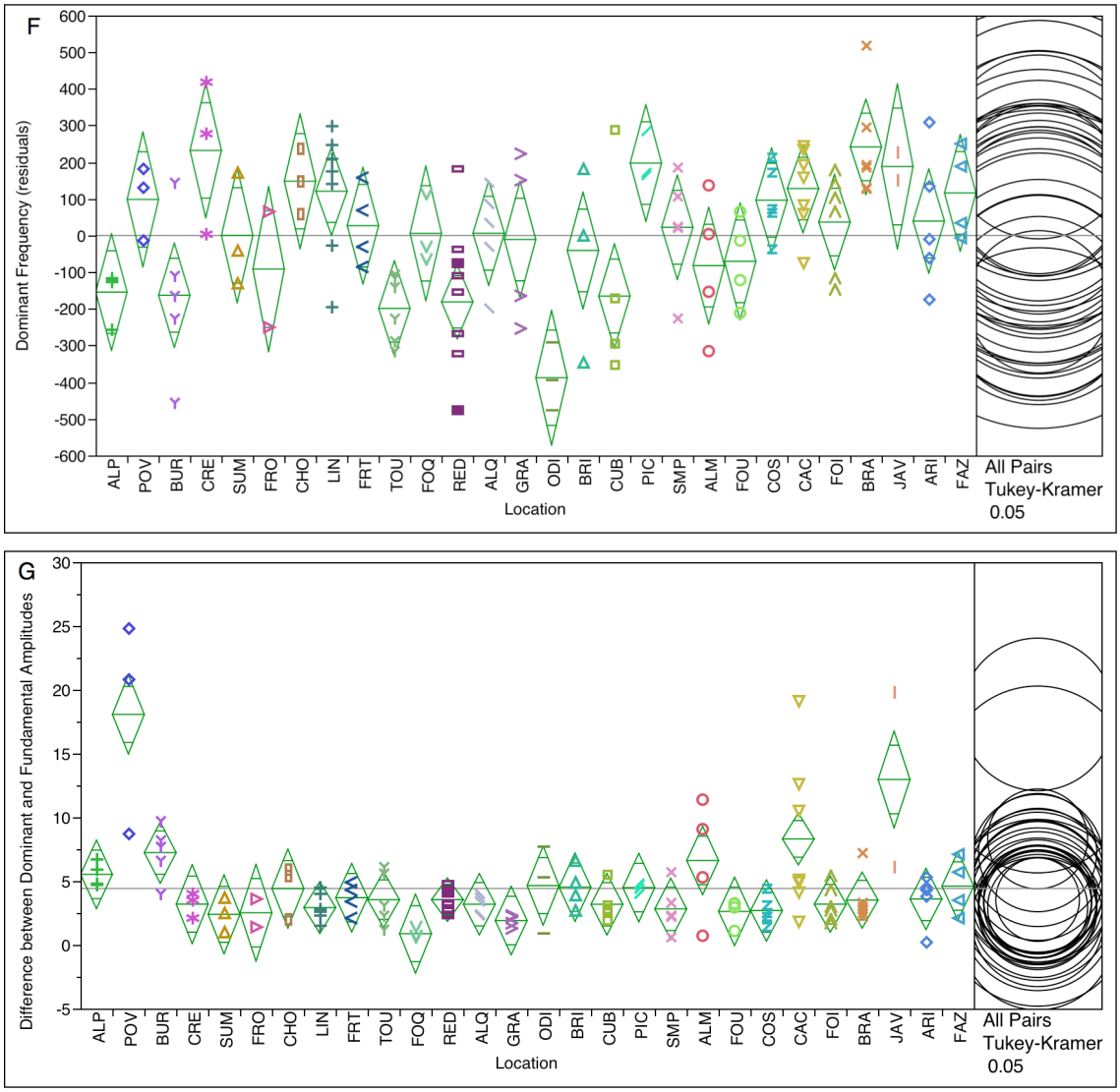


Figure 3.22. Call parameters distribution in *Hyla meridionalis* sampled populations in Portugal.
Boxplots of the temperature and mass-independent residuals of the acoustic variables analysed for *H. meridionalis* for each population. Representation of one-way analysis (green diamonds) and Tukey-Kramer multiple comparisons outputs (circles of the right windows, when suitable). The middle line of the diamond represents the group mean, and the top and bottom extremes represent the corresponding 95% confidence interval. The horizontal line in the graph represents the global mean of all pooled samples. Populations are organised left to right according to their location along a north-south axis.

Discriminant Function Analysis

Call properties pooled for each population were subjected to a discriminant analysis. Discriminant analysis, retaining all seven variables, was highly significant (Wilks' Lambda=0.0270, approximate $F_{189,637}$=2.3642, p<0.0001), even though the overlap among populations is obvious (see Fig. 3.23), and the percentage of individuals correctly assigned to their populations of origin was only 40.48% when all samples were pooled together. Only two populations (JAV and FOU) had no correctly assigned individual.

The first canonical function explained 41.74% of total variation (canonical correlation=0.835), and was positively correlated with call duration (adjusted) (CDadj standardised coefficient=0.716) and with the difference between dominant and fundamental frequencies amplitudes (dDAFA standardised coefficient=0.641). The second canonical function explained an additional 22.88% of the differences among populations (canonical correlation=0.747), and was negatively correlated with fundamental frequency (adjusted) (FFadj standardised coefficient=-0.469) and positively correlated with the difference between dominant and fundamental frequencies amplitudes (dDAFA standardised coefficient=0.742) (Table 3.17; Fig. 3.23).

Table 3.17. DFA on call parameters among sampled populations of *Hyla meridionalis*.
Standardised coefficients; percentage of variance explained by the individual functions and canonical correlations of the discriminant functions extracted. Analyses performed for the individuals of *H. meridionalis* sampled for advertisement calls.

Call parameters	Discriminant Functions	
	1	2
Call duration (CD)	-0.716	0.144
Intercall duration (ICD)	0.187	-0.108
Nr of pulses per call (PC)	-0.083	-0.292
Pulse rate (PR)	0.486	-0.181
Fundamental frequency (FF)	0.582	-0.469
Dominant frequency (DF)	0.174	-0.217
Difference of amplitude between dominant and fundamental frequencies (ADF)	0.640	0.742
Cumulative Variance Explained (%)	41.74	64.62
Canonical Correlations	0.835	0.747

From the DFA plot (Fig. 3.23) no populations or group of populations could be set apart. The confidence ellipses overlapped each other substantially.

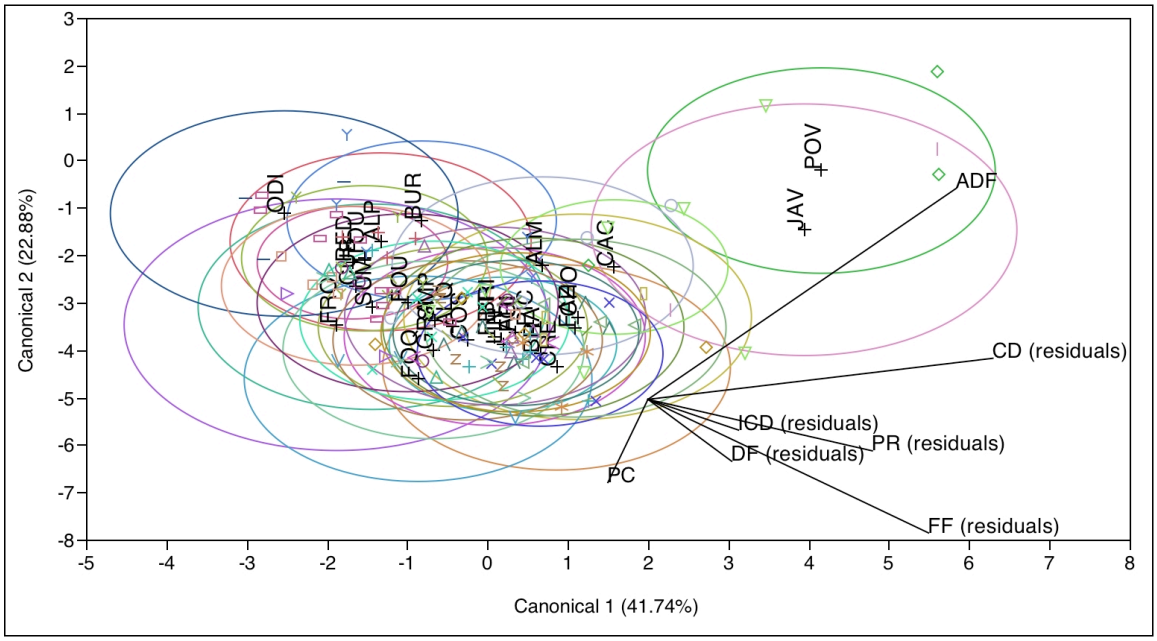


Figure 3.23. Canonical coefficients of the first two canonical functions extracted for *Hyla meridionalis* advertisement call parameters.

Two-dimensional space generated by discriminant function analysis of advertisement call of *H. meridionalis* (n=126) populations in Portugal. Canonical factor 1 accounts for 41.74% of the variation (canonical correlation=0.835), while canonical factor 2 explains 22.87% more of the variation (canonical correlation=0.747). The confidence ellipses are centred on the centroid of each group.

Principal Component Analysis

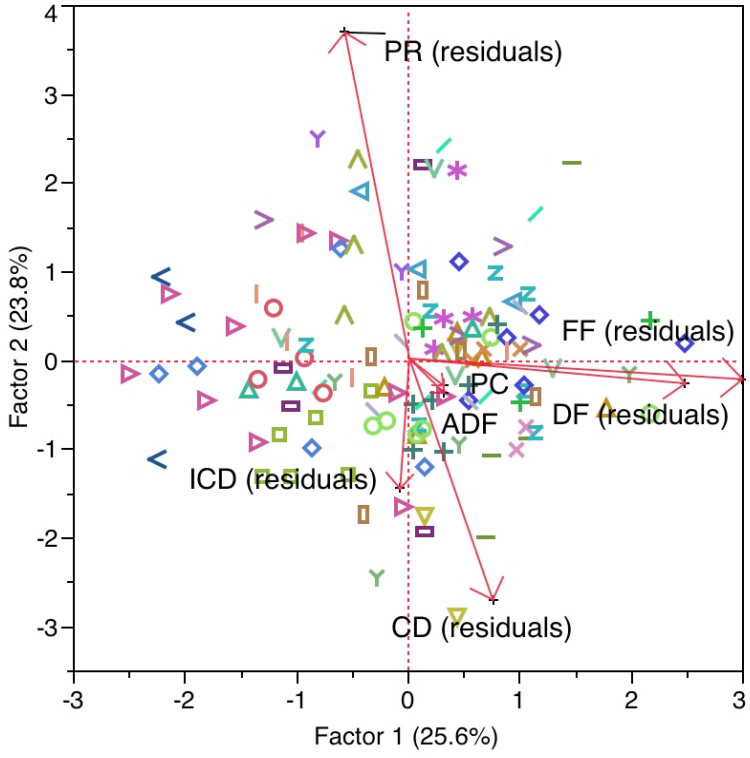
As for *Hyla arborea*, a Principal Component Analysis was conducted to examine if sampled populations of *H. meridionalis* could be grouped by their specific call parameters. Some call variables are significantly intercorrelated (see Table 3.16: Call Variable correlation), and, thus, the seven call variables were subjected to a PCA, followed by a maximum likelihood varimax (orthogonal) rotation. The first three components displayed eigenvalues greater than 1.0, together accounting for 68.30% of the total variance, emphasising the intercorrelations between the measured call properties. Corresponding factor loadings are presented in Table 3.18.

Principal component 1 reflected the spectral variables, as it was loaded most strongly by fundamental and dominant frequencies (0.99 and 0.82, respectively). In contrast, PC2 reflected the temporal variables of the calls, such as call duration and pulse rate (-0.72 and 0.98, respectively), with the exception of intercall duration and number of pulses per call. PC3 reflected another temporal variable, namely number of pulses per call (0.99) (Fig. 3.24).

Table 3.18. Principal Component Analysis of the advertisement call parameters of *Hyla meridionalis*.

Factor loadings for the three principal components derived from PCA of advertisement call properties of *H. meridionalis*, using Maximum Likelihood Varimax Rotation. Factor loadings greater than 0.60 (in absolute value) are indicated in bold. Analyses were performed for the 126 males of *H. meridionalis* with advertisement calls recorded.

Call parameters	Factor Loadings		
	PC1	PC2	PC3
Call duration (CD)	0.249287	**-0.724448**	0.567116
Intercall duration (ID)	-0.027754	-0.390837	0.053862
Number of pulses per call (PC)	0.102012	-0.081284	**0.991457**
Pulse rate (PR)	-0.192585	**0.980389**	0.041861
Fundamental frequency (FF)	**0.996001**	-0.061970	0.0089305
Dominant frequency (DF)	**0.824541**	-0.074676	0.083455
Difference of amplitude between dominant frequency and fundamental frequency (ADF)	0.103064	-0.102574	0.038179
Eigenvalues	1.7929	1.6653	1.3228
Cumulative variance explained (%)	25.613	49.403	68.300



Figures 3.24. PCA scatterplot of the first two principal components with Varimax rotation for *Hyla meridionalis*.

Bivariate plot of Varimax-rotated components from seven call variables, and *H. meridionalis* populations in relation to the first two axes of multidimensional space. For variable-factor loadings, Table 3.18.

3. Species Interaction: Comparison of populations in Allopatry versus Sympatry of *Hyla meridionalis* and *Hyla arborea*

From the 55 sampled sites in Portugal, 27 were located in potentially sympatric geographic areas. Within the sympatric area, only in ten populations were the two species detected calling together. Tissue samples were collected from all those populations. (see tables 2.1, 2.2 and 2.3 for details).

During the various field seasons, I observed several conspecific amplexus from both species, but no heterospecific amplexus were seen. Despite the absence of observations of heterospecific amplexus in the field, when placed together in an aquarium, a male and a gravid female of different species would begin amplexus in a relative short time period (less than 30 minutes). In the laboratory, both hetero and homospecific amplexus usually lasted for at least 5-6 hours. Not all crosses resulted in fertilised eggs, though all the females released their eggs after the 'set-up mating period'. Only one tadpole fully metamorphosed, but died soon after, while all others did not survive to that metamorphic stage. The newly metamorphosed individual did not present any external anomalies.

The laboratory crosses undertaken to obtain hybrid samples (i.e. to allow for tests of reciprocal heterospecific crosses and from conspecific crosses) resulted in a higher rate of success for ♂*H. meridionalis* x ♀*H. arborea* crosses than for ♂*H. arborea* x ♀*H. meridionalis*. There are no available frequencies for success rate, as the crosses' only objective was to obtain hybrids and not to observe survival rates.

3.1. Hybrid Fingerprinting using Restriction Fragment Length Polymorphisms (RFLPs)

To test for the reliability of the RFLP assays in the detection of F1 hybrids in nature (both in adults and tadpoles), a total of eight F1 hybrids, obtained in laboratory, were typed for the five REs (Restriction Endonuclease) showing the expected hybrid pattern of bands (see Fig. 3.25 for an example of RAG1 and Tyr1 genes). No differences were observed between reciprocal crosses, i.e. inverse male and female species. In Fig. 3.25, the hybrid pattern is easily identified, as it combines the bands from both parental species; i.e. in RAG1 *H. arborea* presents three bands after RE digestion, *H. meridionalis* two bands, and the hybrid presents four bands (the first one with the lowest size is common to both parental species). Eight non-hybrid individuals were tested as well, and revealed the expected parental pattern after digestion.

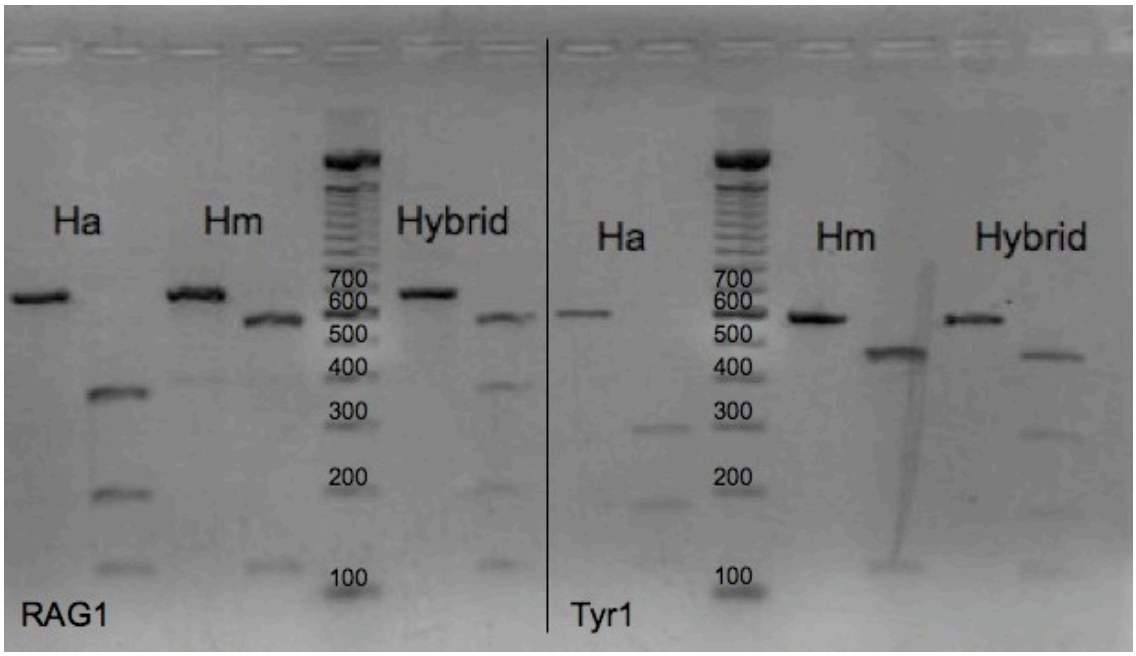


Figure 3.25. Restriction fragments obtained for RAG1 and Tyr1.

The fragments resulted from the digestion of the respective amplicons, and visualised on a 2% agarose gel stained with ethidium bromide. Each set of two consecutive columns corresponds to the PCR amplicon (1st column) and the restriction endonuclease fragments (2nd column), respectively. 'Ha' and 'Hm' stand for *Hyla arborea* and for *Hyla meridionalis*, respectively, and 'Hybrid' refers to the typed F1 laboratory hybrid. Ladder scale is in base pairs (bp).

301 individuals with typical morphotypes of *H. arborea* and *H. meridionalis* (of which 22 were adult females and 50 were tadpoles) from 19 populations (six allopatric, 13 sympatric of which 10 were strictly syntopic; see Table 3.19) were screened with the five RFLP assays.

None of the typed samples was identified as F1 hybrid, nor was any sample misidentified (i.e. samples that had previously been identified morphologically and/or acoustically as belonging to one species and, posteriorly, identified as a different species by RFLP typing). All the tadpoles tested originated from either one of two sampling sites (CRE or BUR, see Table 3.19), with the exception of the laboratory hybrid tadpoles. For practical reasons, and the difficulty of identifying tadpoles at the earlier stages of development to the species level, no tadpole was identified to species previous to RFLP assay. None of the screened samples from tadpoles revealed RFLP patterns of F1 hybrid, with each being identical to only one of the two *Hyla* species considered.

Table 3.19. Samples typed with RFLP of the two species of *Hyla*.

Location and number of samples per species is indicated. Tadpoles as mentioned before were not identified to species. Hybrids refer to individuals obtained in laboratory crosses. Rows in grey correspond to syntopic locations. *N/a* not applicable; '–' not captured in that location but in a potential sympatric geographic area.

Location	*H. meridionalis*	*H. arborea*	Tadpoles	Hybrids
ALM	1	*N/a*	–	*N/a*
ALP	12	4	–	*N/a*
BUR	31	24	26	*N/a*
ODE	5	–	–	*N/a*
COV	*N/a*	1	–	*N/a*
CRE	26	41	24	*N/a*
CUB	11	30	–	*N/a*
ENX	1	*N/a*	–	*N/a*
FRO	11	6	–	*N/a*
OBI	1	4	–	*N/a*
MEL	–	5	–	*N/a*
ODI	11	6	–	*N/a*
CHO	–	3	–	*N/a*
POV	5	4	–	*N/a*
SAG	–	4	–	*N/a*
STO	1	–	–	*N/a*
SMP	1	*N/a*	–	*N/a*
SOU	*N/a*	1	–	*N/a*
TRA	*N/a*	1	–	*N/a*
Laboratory	*N/a*	*N/a*	*N/a*	8
Total (n=309)	117	134	50	8

Note: For an explanation of population acronyms, see tables 2.1 and 2.2.

3.2. Advertisement Calls: Allopatry versus Sympatry

During the fieldwork, special attention was paid to any male producing calls with distinct audible characteristics (hybrid/intermediate), but none was ever recorded. Also, in all of the analysed audio-recordings, no male was identified as having sufficiently distinct call parameters to be considered as a potential 'hybrid', and so to be genetically tested for a better and decisive proof.

Within each species, allopatric populations call parameters were compared to sympatric call parameters, to evaluate the possible influence of the presence of individuals of the other *Hyla* species in the acoustic environment on the behaviour of males (i.e. 'behaviour' here refers to acoustic behaviour measured as the variation in advertisement call parameters). An independent-sample t-test was conducted comparing allopatric and sympatric population groups of both species.

In *H. arborea*, the allopatric populations group was formed by 30 males from seven populations, namely CAM, CAN, COV, FAL, CFO, SOU and TOI, and the sympatric group was formed by 28 males from seven populations, namely BUR, CHO, CRE, CUB, SAG, POV and SUM. There were no differences in call parameters of *H. arborea* between allopatric and sympatric locations except in call rate ($t\text{-test}_{56}$=-2.02715, p=0.0237, n=58), with the allopatric group having a higher mean rate than the sympatric group (see graphics in Appendix 6).

Table 3.20. Influence of Allopatry vs Sympatry in Advertisement Call Parameters.
Comparison of ANOVA results for call parameters between allopatry (A) and sympatry (S) groups of populations in *H. meridionalis* and *H. arborea.* Relationships presented in the table refer to mean values of each of the call parameters. Capital letters stand for higher means.

Call Parameters	***H. meridionalis***	***H. arborea***
Call duration	A>s*	A>s
Intercall duration	A~D	a<S
Number of pulses per call	A>s	A>s
Pulse rate	a<S	A>s
Fundamental frequency	A>s*	a<S
Dominant frequency	A>s*	a<S
Difference of amplitude between dominant and fundamental frequencies	a<S*	A>s
Calls per call group	*N/a*	A>s
Call group duration	*N/a*	A>s
Call rate	*N/a*	A>s*

Note: * p<0.05

In *H. meridionalis*, 95 males of 19 populations constituted the allopatric group (namely, ALM, ARI, BRA, BRI, CAC, COS, FAZ, FOI, FOQ, FRT, GRA, JAV, PIC, ALQ, RED, FOU, LIN, SMP, and TOU) and 31 males of 9 populations constituted the sympatric group (namely, ALP, BUR, CRE, CUB, FRO, ODI, CHO, POV and SUM). The t-tests helped to find significant differences for adjusted fundamental frequency ($t\text{-test}_{124}$=-3.25881 p=0.0007), adjusted dominant frequency ($t\text{-test}_{124}$=-2.24592, p=0.0132) and adjusted call duration ($t\text{-test}_{124}$=-1.91192, p=0.0291) – which showed higher mean values in the allopatric group than in the sympatric – and for the difference between fundamental and dominant frequencies amplitudes ($t\text{-test}_{124}$=2.245327, p=0.0265), which, conversely, showed higher mean values in the sympatric group. T-tests failed to find significant differences between allopatry and sympatry population groups for intercall duration, number of pulses per call and fundamental frequency amplitude (see graphics in Appendix 7).

Species apparently differ in behaviour for pulse rate, fundamental and dominant frequencies and the difference of amplitude between dominant frequency and fundamental frequency. This shows different behaviour tendencies in allopatry and sympatry (Fig. 3.20). For

example, *H. meridionalis* males had higher pulse rates in sympatry, whereas *H.arborea* males had higher pulse rates in allopatry. However, for call duration and number of pulses per call, both species have lower mean values in sympatry.

Chapter IV: Discussion

by Sara Maia

"What is a frog? At first, almost all persons will think, on meeting with this question, that they can answer it readily and easily. Second thoughts, however, will show to most that such is by no means the case"

Mivart, 1874

Discussion

Hylids have been a long-time favourite biological model of many scientists in several different disciplines, from natural history to physiology, acoustics, embryology, biogeography, etc. Although most of these studies focused on North and South American species, the *Hyla arborea* group (to which the two species of focus in this study belong) has also been the subject of many studies over the last few decades (see references in this thesis). However, to the best of my knowledge, few integrative studies of genetics and bioacoustics have been conducted, especially focusing on Iberian territories. This work is expected to provide an enhanced interpretation of the history of *Hyla arborea* and *Hyla meridionalis* within the Iberian Peninsula, using a combined populational approach of molecular genetic and acoustic data.

A correct delimitation of taxa is critical. Species is the basic taxonomic unit, and of extreme importance, as units of biological organisation analysed through ecology, macroevolution, biogeography, biodiversity and conservation, as well as unresolved species boundaries, can corrupt data interpretation and have a strong impact on subsequent conclusions (Sites & Marshall 2003, 2004). Both mitochondrial and nuclear genes should be used as complementary molecular markers for diagnostic identification of taxa (e.g. Garcia-París et al. 2003; Sequeira et al. 2005; Zangari et al. 2006), however, contradictory results from the two DNA sets are common among amphibians. For a long time, many of the currently accepted species of Euro-Asian Hylids were considered subspecies of just one species, *H. arborea*. The different species have recently been told apart, mostly differentiated by molecular genetic markers, but also by bioacoustic and morphological markers; nowadays, the *H. arborea* 'group' is composed of 16 well discriminated species (Faivovich et al. 1995; Smith et al. 2005; Wiens et al. 2005; Stöck et al. 2008).

Treefrogs, like many other amphibians, because of their particular biology – namely the high dependence on water bodies, many of which are of a temporary nature (this is so for many water bodies in the Iberian Peninsula), and the relatively low ability to migrate (low vagility) – have a populational structure organised as metapopulations – small population groups interconnected with a temporal-spacial dynamic. This is why most studies regarding amphibians focus on pond or other restricted water body breeding species, which are considered operatively as distinct 'populations'. Those breeding sites are thus considered discrete units in which a group of individuals comprises a relatively isolated entity (=population) (Sinsch 1990; Blaustein et al. 1994; Rowe et al. 2000; Smith & Green 2004). In this study, the sampling included both pond and lentic stream sites.

The lack of data, and the controversy around the historical origin, processes of colonisation, at large and small scales, and structure and genetic differentiation of the Iberian populations of the two species of Iberian *Hyla*, *H. arborea* and *H. meridionalis*, justify the type of approach to gaining understanding and knowledge used here. Both mitochondrial DNA sequence variation and acoustic properties of male advertisement calls were used to infer phylogeographic patterns of these two species by analysing the existence of mtDNA-defined clades and, simultaneously, acoustic-defined groups, and interpret their geographic correspondence (or not). The underlying rationale was to test whether genetic divergence was associated with acoustic divergence, assuming that calls are a relatively significant premating isolating mechanism between anuran species, and that acoustic parameters have a relatively strong genetic determinism, as they closely depend on the anatomical structures responsible for the production and reception of sound (contrary to what is observed in other vertebrates, in particular, birds and mammals.

1. The COI Mitochondrial Gene as a Molecular Marker

The COI gene is characterised by a lower evolution rate on average when compared to other mitochondrial genes, and by very conserved sequences within different taxa, especially among animals (Brown 1985). These facts make this mitochondrial marker a common, useful and reliable genetic marker used in numerous phylogenetic and phylogeographic studies. Sequences of COI gene fragments have been used successfully in different animal groups, such as arthropods (Trontelj et al. 2005; Chaves-Campos et al. 2011), birds (Lovette et al. 1999; Qu & Lei 2009; Stefka et al. 2011), reptiles (Macey et al. 1998; Douglas & Gower 2010), mammals (Tobe et al. 2010) and amphibians (including many with Hylids) (James & Moritz 2000; Burns et al. 2007; Recuero et al. 2007; Moen & Wiens 2009; Manzanilla et al. 2009; Nabil et al. 2010; Padhye et al. 2012). It is one of the chosen markers for the Barcoding Animal Life project (Hebert et al. 2003). All these studies suggested that COI could be a useful marker in assessing phylogenetic relationships within *Hyla* genus. Moreover, the recently published studies on *H. meridionalis* biogeography (Recuero et al. 2007) used the COI mitochondrial gene, as did Stöck et al. (2008) in their phylogeographic work on the western Mediterranean *Hyla arborea* species group. Using the same marker would allow for a direct comparison with previous results, as well as taking advantage of already sequenced individuals from the various regions of both species, determining geographic range, facilitating a broader analysis.

The biogeographic and phylogenetic histories of both *Hyla* species have been studied before, using both nuclear and mtDNA and allozymes (Busack 1986; Recuero et al. 2007; Stöck et al. 2008; Barth et al. 2011). Here, we focused not only on general patterns for each species' range distribution, reanalysing previous results, but – more importantly – we concentrated our

efforts on the Iberian Peninsula, and Portugal in particular, where there was a paucity of research until now. We greatly increased the sampled area of each species range within the Iberian region, especially within Portugal, and included many situations of allopatry and sympatry (or syntopy) of the two species. Furthermore, in the desire to know more about the interactions between the two *Hyla* species in sympatric situations, a comparison between allopatric and sympatric populations was conducted.

The mitochondrial DNA data obtained allowed us to 1) identify the main evolutionary lineages or clades within *Hyla arborea* and *H. meridionalis*; 2) estimate the phylogenetic relationship between central European *H. arborea* and Iberian *H. arborea*; 3) infer the phylogeographic and demographic processes that have shaped the genetic structure and current distribution of the species, namely contributing to the attemp of clarifying the controversial hypothesis of the *H. meridionalis* recent colonisation of the Iberian Peninsula.

1.1. *Hyla arborea*

Two highly divergent groups of *Hyla arborea* were detected in the data set (p-uncorrected distance of 0.125), namely a European group and an Iberian group, which formed well-supported clades in both ML and Bayesian phylogenetic trees (Fig. 3.2). These results suggest long-term divergence of the two geographic clades, and support previous studies that have proposed distinguishing the Iberian and European *H. arborea* into two independent taxa. Other authors have hypothesised that the Iberian *H. arborea* taxon may be a different subspecies, or even a distinct species, of the European *H. arborea* (i.e. Iberia not included) – *H. a. molleri* or *H. molleri*, respectively (Stöck et al. 2008; Barth et al. 2011). Stöck et al. (2008) compared the genetic diversity of *H. arborea* populations across Europe and found concordant patterns of genetic divergence at mitochondrial and nuclear DNA markers, suggesting the delimitation of an independent Iberian taxon. More recently, Barth et al. (2011) concluded, based on mitochondrial data from COI and 16S, that differentiation between *H. molleri* and *H. arborea* is constant among different populations (p-uncorrected distance around 14%). The p-distances obtained in the present study were similar to those found by Barth et al. (2011), i.e. around 12%, supporting the separation of the European and Iberian taxa and the theory of the resurrection of *Hyla arborea* var. *molleri* (Bedriagai 1890) previously described.

The European Clade. The European clade is subdivided into two subclades: one corresponding to individuals in continental Europe (including samples from Croatia, France, Germany, Switzerland, the Netherlands, Greece and Crete), and another identified as the Corsica group separated from the European *Hyla arborea*. Once considered as *H. arborea*, the endemic *H. sarda* of Corsica (also present in Sardinia) seems undoubtedly distinct. This had already been

reported by allozyme data (Nistri & Giacoma 2007) and bioacoustic analysis of the advertisement calls (e.g. Schneider 1974; Castellano et al. 2002), and more recently in the combined analysis of nuclear and mitochondrial markers by Stöck et al. (2008).

The strong genetic differentiation between the Iberian and the other two European groups of *Hyla* might be explained by the elevation of the Pyrenees acting as a geographical barrier to any potential gene flow and the isolation of Iberia from central Europe during the last glaciations. According to many authors, the *H. arborea* group originated from North American Hylids through two possible colonisation routes, one via Asia and the other directly from North America to Europe (see Introduction). Stöck et al. (2008) showed a closer relationship between the Iberian *Hyla arborea* and *H. orientalis* from Asia Minor and eastern Europe than between Iberian *Hyla arborea* and European *H. arborea* (Fig. 1.12). However, this relationship was evident only at the mitochondrial level and not at nuclear DNA (Stöck et al. 2008). Regardless of the ancestral lineage of the extant *H. arborea*, our results support the idea that the Iberian *Hyla arborea* is a relict of an ancestral group that has colonised the Iberian Peninsula and later became trapped in this region during the last glaciation, eventually differentiating into a distinct entity.

The Iberian Clade. Within the Iberian group, the COI network depicts two well-separated mitochondrial groups, identified as North and South (Fig. 3.3). All haplotypes recovered in our *H. arborea* sample cluster into two clades, suggesting that there are only these two divergent lineages within Iberian *H. arborea* (=*H. molleri*); however, this should not be taken for granted as more sampling could lead us to different conclusions. Previous works had not identified the two clades (Stöck et al. 2008; Barth et al. 2011), most probably due to the low number of Iberian samples used in their analyses. It is hard to suggest a divergence time for these two lineages as no molecular clock has been found so far that can be trusted.

The two groups exhibit somewhat geographically distinct distributions, with an overlapping region in northern Portugal (e.g. in SOU, TOI, CAM, COV, LCO; Fig. 2.2 and 3.4). The North group haplotypes were recovered from sample sites located north of the Mondego River and northwest of Spain, whereas the haplotypes belonging to the South group came from sample sites south of the Mondego River and the northeast Atlantic coast. Barth et al. (2011) also describe two geographically and slightly genetically differentiated groups – coastal Galicia and central Spain – which parallel our North and South groups. Though there is no clear geographic separation between the two groups, with corresponding geographic barriers, it seems that their distribution follows a pattern similar to others seen in Iberian/Portuguese amphibian species, where the Mondego River was identified as a barrier between clades, e.g. *Chioglossa lusitanica* (Sequeira et al. 2005, 2006) and *Rana iberica* (Teixeira et al. 2004). Haplotypes corresponding to the two different clades were found in the five populations

referred to above, located at the periphery of each lineage's geographic range (see also Fig. 3.3 in the results), which can probably be a sign of a secondary contact zone for the taxon.

The haplotype genealogy network showed star-like topologies for each of the two groups (Fig. 3.3), suggesting population expansion events (Slatkin & Hudson 1991) within the North and South groups following group divergence. The haplotype-sharing between populations suggests current gene flow or rapid population expansion, seen in other amphibian species. The star-like topologies conformed to these predictions based on population expansion models of neutrality tests, such as Tajima's *D*, Fu's F_S and R^2, and the mismatch distributions of the two Iberian groups (Slatkin & Hudson 1991). The mismatch distribution for the complete data set was clearly bimodal, one crest corresponding to the number of differences between the two groups and the other crest to the differences among individuals within groups.

By 'default', negative values of Tajima's *D* result from an excess of rare alleles (i.e. haplotypes) existing as a consequence of recent population expansion (Tajima 1989, 1993). However, negative values for *Fs* or *D*, as we found, can be explained by either sequences evolving under neutral-selection or natural-selection pressure. It is difficult to delineate between these two potential causes of deviation from expected selective neutrality. Also, the results mean that we have a case in which the mismatch distribution was bimodal, but Tajima's *D* test was not significant (though Fu's *Fs* and R^2 were). When histograms from mismatch distributions deviate from a unimodal shape, one may not always reject the null expectation (Schneider & Excoffier 1999). Mismatch distributions should be used to complement the neutrality tests as heuristic rather than statistical predictors of demographic history (Mahoney 2004).

Within the Iberian group, the mitochondrial genetic diversity found in *H. arborea* was fairly high, with a haplotype diversity value of 0.883; the South group showed higher diversity values relative to the North group (see Table 3.1), contrary to what was reported by Barth et al. (2011). The lower genetic diversity noted in the North group has a parallel with the patterns seen in Europe known as southern richness, and in Iberia (e.g. Sequeira et al. 2008; Gonçalves et al. 2009), which could be explained by a loss of diversity through northward colonisation, by a 'founder effect' event from individuals which originated from a southern refugium, or by events of population bottlenecking during species expansion. However, the proximity and partial overlapping of the two areas may not be significant enough to justify such differences in haplotype diversity, and the cause may instead be a sampling bias. In this case, increasing the number of sample sites, especially in the north of the Iberian Peninsula and in all Spanish territory, would probably help to answer this question. The high nucleotide diversity values observed in each clade, together with the generalised presence of only one or two mutational steps between most haplotypes of the star-like topology, indicate an increase in population size with an accumulation of genetic diversity, potentially due to an amelioration of climate

conditions post last-glaciation period. All other observed haplotypes derive from the most central haplotypes in both groups (i.e. North and South), and occur in much lower frequencies. The South dominant haplotype occurs only south of the Mondego River, in more than half of the populations south of the Tagus River (n=9 out of 13) and in three other populations north of the river. In the North group, the most dominant haplotype has a more scattered geographic distribution, apparently occurring only in limited areas of the distribution range.

Neigel & Avise (1993) argue that the older the haplotype the more widespread it tends to be, if under a restricted gene-flow model. The distribution of the mitochondrial groups follows the scenario proposed for other species of 'refugia within the refugia'. The North group appears to occupy what have been described as three different refugia: 1) along the Atlantic coast of the peninsula and likely the northern half of Portugal (*Chioglossa lusitanica* – Alexandrino et al. 2000, 2002; *Lissotriton boscai* – Martínez-Solano et al. 2006; *Podarcis bocagei* – Pinho et al. 2007); 2) in the western Cantabria mountains (*Salamandra salamandra* – García-Paris et al. 2003; *Lepus castroviejoi* – Pérez-Suárez et al. 1994); 3) in the northeastern part of Iberia (*Calotriton* – Carranza & Amat 2005; Pyreanean *Iberolacerta* – Carranza et al. 2004). The observed patterns suggest the survival of ancient groups in glacial refugia, and posterior colonisation of northern territories in both groups, as their geographic distributions seem to coincide with the idea of Iberia serving as a refugium during the last Pleistocene glaciations.

In other cases, like in *Chioglossa lusitanica* and *Rana iberica*, the Douro River acted as a major barrier during species' post-glacial expansion. This may have been the case here, too, as only a few populations from the north group occur south of the Douro basin. These populations may well be a result of post-glacial expansion to the south, and although there are not enough samples to test for genetic diversity depauperation as one would expect in such a case, the lower genetic-diversity level of the north group can give a rough indication of the expansion, which seems the most likely hypothesis.

Also, the contiguous and marginally overlapping ranges of the North and South groups, despite the low genetic differentiation between them (a p-uncorrected distance of 0.009), could be explained by an allopatric origin from two monophyletic groups (Avise 1989) or, alternatively, by a single, common origin with lineage sorting and extinction, which seems to be more probable in such a case. The little divergence between them suggests a recent separation.

1.2. *Hyla meridionalis*

Our results for *Hyla meridionalis* mitochondrial DNA biogeographic analysis reinforce the general patterns observed before by other authors. First, a low mitochondrial variation in the western groups (Tunisia and Algeria not included) when compared to other amphibians with a

similar geographic range (e.g. *Alytes* – Martínez-Solano et al. 2004; *Salamandra* – García-Paris et al. 2003; *Pleurodeles* – Carranza & Arnold 2004; *Discoglossus* – Zangari et al. 2006). Second, an increase in the sample size within Portugal and Spain, resulted in more but little-differentiated haplotypes and shared haplotypes on both sides of the Strait of Gibraltar (e.g. samples from Italy and Morocco shared two haplotypes with the northern Morocco group, H2 and H3). Recuero et al. (2007) described 21 COI haplotypes, and in the present study we have found 42 different haplotypes, half of which were newly identified haplotypes. Most of the new haplotypes were found in Iberia; 18 within the South Western group (SW), one in the N Morocco, NE Iberia and Canary Islands group (NM) and the other two in Algeria, which belongs to the Tunisia-Algeria group (TA) (Algeria was not represented in Recuero et al.'s (2007) work). Third, and contrary to Stöck et al.'s results (2008), the split of *H. meridionalis* into two clades was recovered, namely one clade (NM) from N Morocco, NE Iberia and the Canary Islands, and another (SW/WM) including W Morocco and SW Iberia. Moreover, the third and fourth clades obtained by Recuero et al. (2007) were also recovered, i.e. Central Morocco (CM) and Tunisia and Algeria (TA). Finally, the results seem to corroborate the hypothesis of a recent colonisation of the Iberia as well as an expansion of the species (very little genetic differentiation within Iberia).

The reduced mitochondrial variation observed in one of the sides of the Strait of Gibraltar has also been described in other cases (e.g. Busack 1986; Cosson et al. 2005). Additionally, the presence of the same or closely related haplotypes on both sides of the strait suggest a very recent isolation by the Strait of Gibraltar, or, as pointed out by Busack 1986, and more recently by Recuero et al. (2007), a permeability of the 'barriers' to *Hyla meridionalis* migration (natural or human). The first possibility is not very likely, since the last opening of the Strait of Gibraltar occurred about 5.3 Mya. Species like *H. meridionalis* would since then have been split in their distribution range, given the physical limitations on dispersal, thus restricting gene flow between the European and the African populations and originating vicariant, well-differentiated clades on both sides (Busack 1986; Castella et al. 2000; Sanmartím 2003; Gantenbein 2004), which is not the case.

The results seem to better support the second hypothesis, whereby a permeable barrier led to the presence of shared haplotypes on both sides of the strait. However, given the higher haplotype diversity within the Moroccan versus the Iberian groups, dispersal and gene flow seems to have occurred from the area of higher diversity, i.e. Morocco, to the area of lower diversity, i.e. the Iberian Peninsula. In addition, these results support a previous hypothesis: that a *H. meridionalis* African ancestor colonised European grounds (Recuero et al. 2007). The permeability of the strait is not unique to *H. meridionalis*, and has been shown for *Macroprotodon brevis* (Carranza et al. 2004) and *Crocidura russula* (Cosson et al. 2005).

Additionally, Carranza & Arnold (2004) found the opposite pattern in *P. waltl*: their analysis showed higher haplotype diversity in the European versus the Moroccan groups.

The mismatch distributions and related statistics pointed for a species in recent expansion. The low genetic variability may be indicative of this expansion, but according to modelling expansion scenarios of Sillero (2010), and despite the apparent exhistance of many potential areas where to expand, the analysis does not support the hypothesis of recent expansion events. This may be so either by climatic change reasons, being temperature related variables the ones with higher influence in the species distribution, according to Sillero (2010), or by the presence of *H. arborea* as a direct competitor. The second hypothesis seems less plausible as the two species are syntopic in many areas, and also the acoustic analysis developed in this study suggest an absence or very little interference from the presence of the other species.

Tunisia and Algeria Group (TA). The results confirm the existence of two main lineages within North Africa, i.e. Morocco (which includes samples from both NM and WM groups) and Tunisia-Algeria (TA), separated by a strong genetic divergence and into two geographic areas. Including samples from Algeria in the analysis confirmed the hypothesis proposed by Recuero et al. (2007) that the latter was part of the Tunisia lineage. New haplotypes were described, but they all grouped in the same phylogenetic clade with Tunisia samples. This pattern is also seen in other taxa, such as *Pleurodeles waltl/poireti/nebulosus*, *Discoglossus scovazzi/pictus* (Fromhage et al. 2004) and *Rana saharica* (Buckley et al. 1994, 1996 and Arano et al. 1998), with different explanations being given. For example, the differentiation between *P. waltl* (present in Iberia and Morocco) and *P. poireti/nebulosus* (present in Tunisia and Algeria) has been hypothesised as a consequence of the formation of the Strait of Gibraltar. The desiccation of the Mediterranean would have allowed the Iberian ancestor of *P. poireti/nebulosus* to reach northern Africa. Iberian *P. waltl* would have recently colonised Morocco (Carranza & Arnold 2003).

South Western (SW) and Western Moroccan (WM) Group. In this work the SW group – equivalent to the group with the same name in Recuero et al.'s (2007) work – is divided into several subgroups, but emphasis is given to the one with only Morocco samples, named here Western Moroccan (WM). In the Bayesian tree, also, no clear differentiation of subclades is observed. The splitting of WM is poorly supported, with a Bayesian posterior probability value of 0.63 (see Fig. 3.7), and in the haplotype genealogy (Fig. 3.8) WM appeared as one of the derived branches from the central SW haplotype (H7), forming itself into a simple, star-like structure. The relevance of this subgroup, despite the low pairwise p-uncorrected distance detected (0.3%) between it and the rest of the SW group, is the fact that it is geographically

restricted to the Moroccan area, sharing no haplotypes with Iberia. Moreover, its geographic isolation from the rest of the group supports the hypothesis of Recuero et al. (2007) that the south of the Iberia Peninsula would have been colonised by inviduals coming from southern to northern Morocco and from there to the Iberia. The population of northern Morocco would have then became extinct, but the two remaining southern Morocco and southern Iberia groups would have differentiated, leading to the SW and WM groups, as named in the present work.

The low diversification seen among the group samples, though not surprising, may have resulted either from a very recent colonisation of the south-western Iberian region or from a current gene flow within the Iberian populations going against a potential diversification. Corroborating these two hypotheses is the fact that the most abundant of all haplotypes (H7) is present in all but two sites in Spain, and all but eight sites in Portugal. Also, the population-demographic analysis suggests that the group (SW+WM) presents characteristics of a population or group of populations under demographic expansion according to observed unimodal mismatch distribution, the statistically significant negative values for Tajima's *D* and Fu's *Fs*, as well as highly significant R2 values. This can also explain the presence of many rare haplotypes.

The great difference observed in the number of haplotypes between Portugal and Spain (17 and six, respectively) suggest that colonisation was done initially through Spain and later Portugal, where the species suffers differentiation. This corroborates the hypothesis that individuals originating from Morocco would cross the Strait of Gibraltar, where the distance between the two continents is the shortest, and from there expand to the rest of the Iberian Peninsula. The differences could also be a result of the sampling method, as there are more sequences considered from Portugal (130 sequences) than from Spain (29 sequences).

Central Morocco Group (CM). Surprisingly, no individual sharing the unique CM haplotype, other than the two used by Recuero et al. (2007), was found (CM, H6 in our work and VI in Recuero et al. 2007). Even the sample obtained from a very close location (within a 10 km ray, see table 2.1 and map Fig. 2.1 and 3.9) did not match the haplotype nor the group (it belongs to the SW+WM group). More sampling should be done in the area to clarify this group in terms of its origin and relationship with the other Moroccan groups.

The unique haplotype H6 appears as a subclade within the same clade as NM (support 0.61/50, Bayesian posterior value and bootstrap value for ML, respectively, see Fig. 3.7), and in a separate branch in the haplotype genealogy (see Fig. 3.8). The mean p-uncorrected distance between CM and the other groups (exception made for TA) was around 1%, corroborating the values found by Recuero et al. (2007). The CM group, because of its close relationship within the phylogentic tree and its geographic location, as proposed by Recuero et al. (2007), seems to

have resulted from allopatric fragmentation between CM and the Moroccan populations of the NM group.

Northern Morocco, NE Iberia/ Southern France and Canary Islands Group (NM). Supporting Recuero et al's (2007) results the NM group was a well supported distinct clade from the SW group that also occupies both Europe and Africa continents. All the haplotypes found by Recuero et al. (2007) were recovered and a new haplotype was found – H42 – but only in Morocco. In northern Iberia, southern France and Italy only two haplotypes were found (H2 and H3), also present in Morocco and Tenerife. As proposed by Recuero et al. (2007), the low diversity seen in the European part of this group suggests a recent colonisation of the European locations from northern Morocco. This colonisation most probably resulted from human transport (passive or active), as it is unlikely that treefrogs could have travelled on natural rafts for such a long distance (over 100 km). For example, the presence of treefrogs in Tenerife is considered to be as introduced species (Pleguezuelos 2002). For the Canary Islands, a new haplotype was found – H3 – relating to the reports of Recuero et al. (2007). Contrary to what had been said (Recuero et al. had reported no genetic variation, with only one haplotype found), this is indicative of some genetic variation within the islands, which could be a sign of its introduction from different possible origins, Morocco but also Europe, where the haplotypes are also present.

The geographic distribution of NM and SW+WM remains intriguing. The absence of SW+WM haplotypes in northern Morocco could be explained by a local extintion of those haplotypes after the expansion event that would have taken place from southern to northern Morocco followed by recolonisation from a differentiated population, or by a differentiation *in loco* from the original WM haplotypes to NM haplotypes. The first hypothesis would support a natural colonisation of northern Iberia after the opening of the Strait of Gibraltar, whereas the second one suggests the influence of human translocation of specimens from Morocco to Iberia.

2. Microsatellites

Whereas mtDNA is commonly accepted to have less resolution power than microsatellites at showing geographical structure on a broader scale (Rowe et al. 2000), the use of a mitochondrial marker here proved to be fruitful in pointing out phylogeographical patterns in both studied species. The use of microsatellite markers (and other nDNA) together with mtDNA would have allowed a more detailed view of genetic structuring in the species, as well as a more fine genetic-structure image within smaller geographic scales. A better comprehension of gene-flow estimates and possible sex-biased and adult dispersal would also had been possible using

microsatellite markers. The use of multiple markers should not however be taken as granted for better results (Karl et al. 2012), but rather and above all be more critical of the obtained results.

Arens et al. (2000) and Berset-Brändli et al. (2008, 2008a) developed a set of microsatellite markers that have been used to clarify genetic patterns and sex-biased differentiation in fragmented populations of *Hyla arborea* in the Netherlands. In the present study the microsatellite loci specific for *H. arborea* were used for both Iberian *Hyla* species in an attempt to clarify patterns of gene flow and population structure. After experimenting with various PCR protocols – changing concentrations of the reagents – on the PCR reaction, and on the duration and temperature of the PCR thermal cycle protocol, the results obtained were not consistent and therefore did not allow for further analysis. Regarding this lack of data for *H. arborea*, perhaps further testing, with different PCR conditions, should be done. Also, the aforementioned microsatellite loci were designed for the European *H. arborea*, which, in this study, and in other recent studies (Stöck et al. 2008, 2011), has been shown to be very different from the Iberian *H. arborea*, which has consequently been proposed as a different species: *H. molleri*. Thus, the differences between the two taxa may be enough to prevent cross-amplification. The inconsistency seen on amplification may also be caused by the presence of null alleles, but it requires more investigation. The same can be said for the loci that failed to amplify for *H. meridionalis*, therefore leading to the conclusion that, at least for some loci, non-amplification may be artificial.

The loci that amplified and were scored for *Hyla meridionalis* showed high levels of homozigoty, but, as already mentioned, more scoring would be needed to be conclusive. Worth mentioning for *H. arborea/molleri*, though – and again, not conclusive yet – was that the previously described sex-biased tendency for WHA5-22A locus (Berset-Brändli et al. 2006), where all males analysed were heterozygous and females were homozygous, was not seen for the Iberian samples. This could be explained by the fact that the original study was on highly fragmented populations, but they also tested individuals from France obtaining the same pattern, which suggests that European *Hyla arborea* may be very different from the Iberian, as has been shown by the mtDNA and other nDNA markers. Interspecific variation of this locus was described by Klütsch (2006), sequencing 22 individuals from *H. arborea*, *H. meridionalis*, *H. sarda* and *H. savignyi* and observing size homoplasy; even though the alleles had the same size, sequences were different.

3. The Advertisement Calls as a Bioacoustic Marker

Bioacoustic data have long been used to study species relationship. In anurans in particular, advertisement calls of malesare considered species-specific and, thus, a very important primary, premating, isolating mechanism used with greater or lesser success to avoid heterospecific choices from females during reproductive seasons. Many studies have focused on geographic variation of advertisement-call properties. With a strong genetic component, anuran calls are, in general, thought to be innate, and individuals are less prone to learning or imitation than are those of other groups, such as birds and mammals (Crespo 1993).

The data obtained with this study allowed me to 1) identify the geographic structure of acoustic variation within *H. arborea* and *H. meridionalis* Portuguese populations; 2) analyse the effect of the presence of a similar species on call properties by comparing sympatric with allopatric sites (i.e. detect signals of character reinforcement or displacement); and 3) infer if there is a parallel between acoustic and mtDNA variation patterns, and therefore evaluate the pertinence of using this kind of complementary and integrative, double approach in these and other anurans.

3.1. *Hyla arborea*

Individual call variation **–** Call variation results showed similar patterns to those described in previous studies for other European treefrog populations (Castellano et al. 2002; Friedl & Klump 2002). Based on Gerhardt's (1991) criteria – to classify call properties into static or dynamic – the coefficients of variation indicated that spectral parameters were the least variable of the acoustic properties measured at the individual and population levels. In fact, most properties are highly stereotyped (static or intermediate properties, CVs <12%) within call groups (refer to tables 3.6 and 3.7), and only intercall duration and amplitude-related properties are highly variable (CVs> 12%). At the individual level, the spectral and temporal properties were stereotyped, with the exception of call-group duration and number of calls per group (CV> 12%). Low variability in spectral properties within males seems to be a general pattern among anurans (e.g. Gerhardt 1991; Castellano & Giacoma 1998; Bee et al. 2001; Friedl & Klump 2002). The low variability of spectral properties at the individual level, and its strong relationship with body size, suggest influence of the underlying mechanism of sound production. For instance, in anurans the laryngeal structures, arytenoids and vocal-cord mass are correlated with body size (Ryan 1988; McClelland et al. 1996), which may have an effect on the resonating frequencies of these structures.

Temporal properties within-individual variability, on the other hand, vary highly among different species of frogs and toads. Call duration was classified as a static property in green treefrogs – *Hyla cineria* (Gerhardt 1991), spring peepers – *Pseudacris crucifer* (Gerhardt 1991)

and European treefrogs – *Hyla arborea* (Gerhardt 1991; Friedl & Klump 2002; Castellano et al. 2002); it was classified as an intermediate property in green frogs – *Rana clamitans* (Bee et al. 2001), bullfrogs – *Rana catesbeiana* (Bee & Gerhardt 2001) and the Túngara frog – *Physalaemus petersi* (Boul 2003). Here it was classified as dynamic, as it is in gray treefrogs – *Hyla versicolor* (Gerhardt 1991) and green toads – *Bufo viridis* (Castellano & Giacoma 1998). Call group duration and number of calls per call group are dynamic properties, with coefficients of variation above 20% at the within-individual level analysed, for all except two sampling sites (i.e. COV and SOU). The same results were obtained in European treefrogs for a southern German population (Friedl & Klump 2002) and a north-Italian population (Castellano et al. 2002), and for *H. sarda and H. intermedia* from northern Italy (Castellano et al. 2002).

As suggested by Friedl & Klump (2002), categorising a certain within-individual call property as static or dynamic does not say much about how it varies between individuals, as static properties can differ significantly from one male to another, and from one population to another. Similarly, properties classified as dynamic may or may not differ between individuals and/or populations (e.g. Gerhardt 1991; Bee et al. 2001; Bee & Gerhardt 2001). In this study, as in Friedl & Klump (2002), spectral properties showed low variability within-individuals (CV <5%), but nevertheless were significantly different among populations.

Population call variation **–** The patterns of call variation found in this study are largely consistent with results from other European Hylids (e.g. Schneider 1974; Friedl & Klump 2002; Castellano et al. 2002). A significant proportion of the variation in spectral characters (fundamental and dominant frequencies) was associated with body-size variation, whereas temporal properties of the calls (but not to call groups) were mostly affected by male body temperature. There were significant differences in spectral properties and in amplitude (i.e. the difference in amplitude between fundamental and dominant frequencies) among populations, in accordance with the findings of Castellano et al. (2002) regarding six Sardinian populations of *H. sarda*. These results were evident after eliminating the effect of some morphological and environmental factors (e.g. body size and temperature) on some of the call properties, and after correcting for the linear effects of male temperature and size.

Unexpectedly, the males of CFO poulation, located in Serra da Estrela, in the Central Plateau System of Central Portugal (see Fig. 2.3), had extremely long call groups (see Fig. 3.16A) and a high number of calls per call group (Fig. 3.16B), when compared to the other sampling sites. One could argue that this specificity was an altitude effect because the site is at high elevation, approximately 1570 masl (meters above sea level), when compared to the other sites.. Bergmann's rule, originally developed for endotherms, indicates that body size is larger at higher latitudes, in order to decrease temperature loss in colder climates. Several studies have showed similar results for ectotherms, with body size increasing towards higher latitudes

conforming to a Bergmann's cline (Lindsey 1966; Van Voorhies 1996; Atkinson & Sibly 1997), but the opposite has also been found (Mousseau 1997; Ashton 2002; Ashton & Feldman 2003). Cvetkovic et al. (2009) studied populations of the common toad, *Bufo bufo*, in Europe, and found that mean body size decreased as latitude increased, but not with altitude, where they found no significant differences between low and high altitude zones. In this case, males from CFO did not differ significantly in body size from in the other studied sites (Fig. 3.13).

Vocalising is temperature-dependent, at least for some call properties (Prestwich 1994; Navas & Bevier 2001). Temperature could also directly or indirectly influence call properties (Navas 1993, 2003, 2008), but neither of the two call parameters – call-group duration and number of calls per call group – seemed to be affected by temperature or body size. Energy for calling activity is mainly obtained through aerobic behaviour (Taigen et al. 1985; Grafe & Thein 2001), and is consequently less dependent on temperature than, for example, metabolic anaerobic pathways (Bennett 1994). Optimal temperatures for anuran activity can be accurately predicted by environmental temperatures (Navas 2003), matching the expected elevational thermal cline. Although anurans normally call when environmental conditions are more favourable, thermal stress can probably be overcome by adopting different behaviour strategies, such as avoiding the lowest-temperature periods that can influence aerobic activities (see strategy described for *Hyla labialis*, Navas 1996). Also, high-elevation species, such as the Eastern Colombian Andes tropical frogs, have temperature-independent calling rates (Navas 1996) or reduced thermal dependency of vocal activity (Navas 2003), as evidenced by the high calling rates of poison-dart frogs, *Colestethus subpunctactus* (reaching one call per second, Navas & Bevier 2002).

However, CFO males' calling rates were not significantly different from the other populations, and, therefore, the results do not support the hypothesis of temperature-dependent high calling rates. Nevertheless, the effort of calling many consecutive calls is less energetically costly than producing shorter groups of calls. Frogs have muscles capable of very high rates of aerobic metabolism, usually preventing considerable oxygen debts; consequent recovery costs are negligible, despite the high energy cost of vocalisation (Wells & Taigen 1992; McLister 2001). Grafe & Thein (2001) measured the rate of oxygen consumption of *H. arborea* in respiratory chambers, and their results indicated that males have been subjected to a persistent selection for high call rates, along with a respiratory physiology that sustains high levels of aerobic metabolism. European treefrogs showed high rates of oxygen consumption while calling and the highest factorial aerobic scope in ectothermic vertebrates, suggesting a 'smart' relationship between effort and energy consumption (Grafe & Thein 2001). Thus, the high calling rates for this species does not represent an extra cost when compared to other taxa.

Spectral parameters are known to be less affected by temperature than temporal parameters. However, tuning of the receptors in the inner-ear organ, particularly to low

frequencies, are highly temperature-dependent (Narins 2001). Such differences in thermal sensitivity between sound producers (males) and sound receivers (females and males) can promote higher chances of mismatches between potential mates that might be avoided or reduced by varying some call traits, such as the length of the call bout. Work on female preferences and thermal adaptation experiments could clarify these questions. Also important would be to know if the CFO males would keep their long group of calls if subjected to other thermal regimes, or if the oxygen availability is instead the limiting factor.

Advertisement calls are an important premating, isolating mechanism in anurans; therefore, one would expect a selection towards call uniformity within any species. Thus, variation from population to population would not affect communication or, theoretically, mating success. Our results for call variation among populations fit this principle. Calls were generally uniform among the range of populations analysed, even though some call parameters revealed significant differences from site to site (see ANOVA, DFA and PCA results).

Most of the differences among these populations were explained by the frequency (fundamental and dominant) and call-group related parameters (duration, number of calls and call rate). If differences in environmental conditions among sites pose distinct selective pressures to the individuals (e.g. Morton 1975), and differences in call properties result from it, then comparing different sites would not tell us anything useful about population relationships, but more about relationships between population and environment, and how the second affects the former. Frequencies would represent differences in male morphology, given the relationship between body size and mass of vocal cords and vocal sac (Martin 1972; McLlelland 1996, 1998). However, the analysis used adjusted frequencies independent of male size. Hence, these differences should not reflect environmental selection pressures.

3.2. *Hyla meridionalis*

Individual call variation – Results of individual call variation for *H. meridionalis* in Portugal showed that most of the call parameters are highly stereotyped, based on Gerhardt's (1991) criteria (CV <5% static; 5% <CV <12% intermediate and CV> 12% dynamic properties), with CVs lower than 3.5% for all spectral and temporal parameters. Exceptions included intercall duration, classified as dynamic and amplitude related parameters (i.e. intermediate or dynamic). Intercall duration classification can, however, be misleading because it is highly dependent on social environments such as chorus size and researcher presence. In a high-density chorus, and in situations of strong acoustic competition, males of other species have often elevated their call rate (e.g. Wagner 1989; Gerhardt et al. 2000; Wong et al. 2004). I am unware of any other studies done on *H. meridionalis* that show how call activity can be affected by social environment. However, anecdotal evidence from this study supports the hypothesis that the

presence (or absence) of other males' calling influenced the rate of call repetition: usually, males in less-active chorus have lower call rates. Also we may say that my presence when audio recording caused some disruption in calling activity, sometimes reducing or stopping it: this effect was more evident in smaller groups of active males, or on nights with full moon or otherwise more luminous.

Population call variation – Variation in call parameters can be affected by morphological and environmental factors, such as body size and air temperature and male body temperature (Wells 1977). As expected from the results, spectral parameters were mostly affected by body size, whereas temporal parameters were affected by temperature. These results are in accordance with descriptions for other species of anurans, including other European Hylids (e.g. Castellano et al. 2002). To my knowledge there are no other works analysing the relationship between body size and call parameters in this species to which the results could be compared. After correcting for linear effects of body size and temperature on the call, the static character of the call parameters is still demonstrated when making comparisons between populations. Homogeneity of calls is expected due to calls' important function as primary isolating mechanisms; variations seen among populations were not strong enough to differentiate groups or to isolate single populations (see DFA and PCA results).

4. Species Interaction: Hybrids and Reproductive Character Displacement in Calls

From the comparative analysis of the genetic and bioacoustic population structures in Portugal we can conclude that there is no coincidence in the varition patterns. The observed mitochondrial clades had no correspondence in bioacoustic population groups. In *H. meridionalis*, no population structure was found at the mitochondrial DNA level, nor at the biacoustic level. Apparently, neither of the markers show great variation between populations. This might be explained by the (proposed) recent colonisation of the Iberian Peninsula: that there has not yet been enough time to induce changes. Also, the probable gene flow between populations, indicating the migration of individuals from one site to another, may also have contributed to the homogenisation of both genetic and bioacoustic pools. In *H. arborea*, two mitochondrial clades were identified within Portugal, but not at a bioacoustic level. European *Hyla* have also been described as not significantly bioacoustically differentiated, and the same can be observed within Portugal.

Laboratory-conducted crossing experiments between males and females of the two species were successful in producing tadpoles, indicating that no absolute reproductive pre-

mating decisive barrier or genomic incompatibility during the embryos and tadpole development were present. The purpose of these crossings was not to follow the embryo tadpole development, but to provide some insight into the possibility of hybrid generation in the wild. In their crossing experiments, Rosa & Oliveira (1991) reported that, hybrid mortality was not significantly different from the control group (i.e. embryo development from homospecific crosses) until the stage of food intake, when mortality, of the hybrids, resulted mainly due to poor development of the mouth. Also, the hybrids that completed metamorphosis presented high rates of malformations, particularly in the hind limbs (Rosa & Oliveira 1991). The only hybrid obtained in the present study that completed metamorphosis had no obvious malformations, but no minute observations were done.

Despite previous reports on the occurrence of hybridisation between *H. arborea* and *H. meridionalis* in the wild, we found no natural F1 hybrids in this study, suggesting that, at least in present times, heterospecific mating occurs in such a low percentage that the resulting offspring have low probability of being sampled during field work. Also, no heterospecific amplexus were observed during my study (2005-2010), whereas homospecific amplexus were seen with a relatively frequently, especially during mating peaks (i.e. nights when there was more acoustic activity and more individuals were seen). The absence of F1 hybrids from the test samples could be an indication of: 1) past hybridisation that no longer takes place (meaning that either there were no signals of introgression left, or we failed to identify them); 2) there are no hybrids yet – even though earlier studies have detected some in nature, they are likely to be very rare and also there might have been a recent introduction/colonisation of *H. meridionalis* in Iberia Peninsula; and/or 3) we may not have used a sufficiently appropriate technique to detect hybridisation.

It can be argued that the relative proportion of each species at syntopic sites may have an effect on mating choice. In theory, if males of species A are more abundant than males of species B, there is a potentially higher chance for females of species B to choose a mate from a different species of their own, i.e. from species A, consequently increasing the likelihood of producing hybrid offspring. However, female choice is not only based on relative abundance of males in the reproduction site, but also depends on other, more direct reproductive signals, such as acoustic (e.g. male-produced advertisement calls) and visual (e.g. males' inflating vocal sacs) cues, which tend to minimise mismatings in situations in which multiple species are present, and/or where there is a reduced number of males of the same species. Though no accurate quantitative data are available, different situations were registered during the sampling seasons, ranging from a disproportional bias towards one of the species to an almost one-to-one ratio in terms of active calling males in the syntopic sites. Where there was only one species present the chorus sizes (i.e. number of males calling) also varied a lot. The total number of females captured during the entire study was significantly lower compared to the number of males (only

20 females from *H. meridionalis* and 16 from *H. arborea*), but this may be due to a lower detectability of females compared to males, as females in these two species do not call.

Even though no specific analysis was done to test for differences between COI sequences of the two groups (i.e. allopatry vs. sympatry), individuals from syntopic sites seem not to be differentiated from those of allopatric sites. Syntopic and allopatric populations shared COI haplotypes in both species, sometimes related to their geographic distribution. One would expect a clear differentiation between specimens of syntopic and allopatric populations if there was introgression, meaning that RFLP assays might have failed to identify the presence of hybrids. This could be the case if F2 (or more) hybrids were to be sampled in nature, for example. However, it is not conclusive that historical hybridisation has occurred in the past; even if we have not observed clear signs of introgression, a more detailed study should be done.

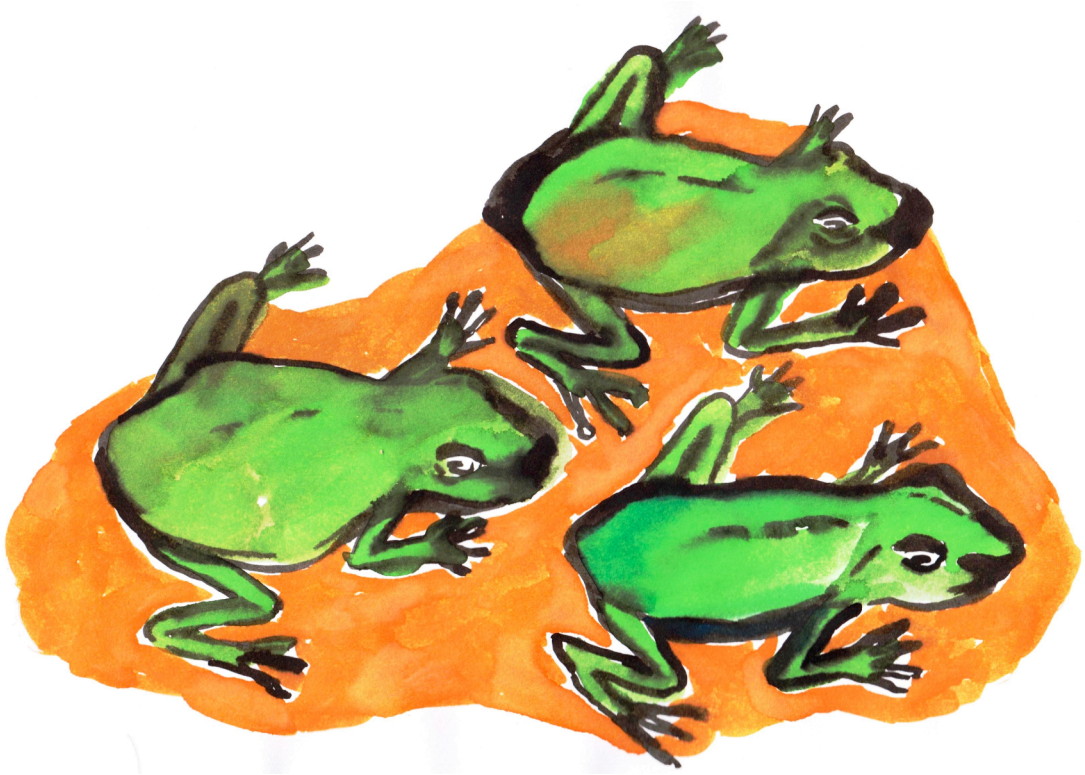
In what applies to the advertisement calls in sympatric conditions, especially involving relatively similar species (i.e. morphologically and bioacoustically similar), one can expect males to adopt one of two possible strategies: 1) emphasising the between-species differences by making their call parameters more extreme; 2) keeping call parameters constant, assuming females can recognise them even in the presence of other heterospecific calls. Incorrect female choice, leading to the selection of heterospecific mates, is well documented in anurans (Lamb & Avise 1986; Gerhardt & Huber 2002), and natural hybrids have been described for the two species of *Hyla* being studied. Thus, heterospecific matings are likely to occur. As mentioned before, however, no hybrids were found either by using a genetic approach or by using a bioacoustic approach.

The results suggest that *H. arborea* males use, at least more frequently, the second strategy. When comparing the allopatric group (i.e. composed of all populations where only *H. arborea* was present, n=7 populations) to the sympatric group (i.e. where both *Hyla* species were present, n=7 populations), there were no significant differences in call parameters – except for call rate – that had a higher mean value in an allopatric group. This result was not surprising, as no group was differentiated in the analysis of the whole set of populations and the call-rate differences may simply result from the inclusion of CFO (the exception found among the sampled populations) in the allopatric group. Therefore, data do not support the hypothesis of character-displacement in sympatry. All the populations in the sympatric group are in fact in strictly sympatric areas, i.e. syntopic populations, leading us to conclude that the presence of *H. meridionalis* seem not to be affecting the genetic and call evolution of *H. arborea*. Perhaps the calls are efficient in determining female mating preferences, and/or other behavioural strategies are adopted in mate recognition.

However, and contrary to observations in *H. arborea*, the results for *H. meridionalis* revealed significant differences in call properties between allopatry and sympatry situations. Both fundamental and dominant frequencies' mean values were significantly lower in the

sympatric populations than in the allopatric ones. Schlefer et al. (1986) and later Höbel & Gerhardt (2003) found the opposite pattern in *Hyla cinerea*, in which fundamental frequencies were lower in allopatric populations than in syntopic populations with *H. gratiosa*. Also, call duration was significantly different between the two groups, with higher mean values in allopatric populations. Schneider (1980, 1982) had tested for females' phonotaxis preferences in *H. meridionalis*, and reported that females preferred conspecific calls to heterospecific ones, and calls with high-temperature characteristics, i.e. shorter calls with fewer pulses. If this pattern applies to the populations studied here, then the results obtained suggest that *H. meridionalis* males in sympatry alter their calls to improve their mating success. The same has been shown in North American treefrogs: *Hyla chrysoscelis* males, when in sympatry with *H. versicolor* males, had significantly shorter calls (Ralin 1977). This behavioural change seen in sympatry conditions reflects the first strategy referred to above, whereby males exhibit extreme call properties in the presence of other species – especially related/similar species – so as to avoid mismatings via erroneous female choices.

Chapter V: Conclusions and Future Prospects



by Sara Maia

Then I saw three evil spirits that looked like frogs…

Revelation 16:13

Conclusions and Future Prospects

With the work presented in this thesis I intend to contribute to a better understanding of the evolutionary history of *Hyla meridionalis* and *Hyla arborea* in the Iberian Peninsula, and in particular in Portugal. A twofold approach was followed to study each species individually, and particularly their interactions within sympatric (and syntopic zones), using both molecular genetic and bioacoustic approaches. First, a solid scenario for the mitochondrial DNA of these two Hylid species was established, confirming the previously described lineages, and supporting the biogeographic scenarios for the origin of biological diversity within the Iberian clades. Second, corroborated the low levels of mitochondrial DNA diversity in *H. meridionalis* in both sides of the Strait of Gibraltar, with a lack or very weak differentiation of the populations within Iberia. Third, by identifying two significantly distinct clades between Central Europe and Iberian *H. arborea*, demonstrated that effectively these two groups should be distinct taxa, as it has been proposed. Fourth, the observed patterns of bioacoustic variation do not correlate to the genetic patterns, suggesting either a lag between evolution rates of genetic and bioacoustic traits or a phenotypic plasticity favouring environmental adaptation by individuals. Fifth, the absence of F1 hybrids within the sampled sympatric sites suggested a low level of hybridisation or an historical hybridisation no longer active, that could only be detected by using other molecular markers. Finally, it is shown that the genetic variability of the Iberian Hylids has been affected by Pleistocene climatic oscillations, and the observed patterns resemble those of other Iberian amphibians.

The present study allowed for a better comprehension of genetic and bioacoustic diversity patterns within Portugal. Nevertheless, many aspects of the evolutive history of both species remain untold. The use of independent *loci* (nuclear and mitochondrial) is necessary to study the history of populations and not only that of the chosen gene (Edwards & Beerli 2000). Moreover, a more detailed evaluation of gonads maturation (gonadal cycle) together with the observation of reproductive cycle phenology and an evaluation of the population effectives of both species in each situation (alopatry and sympatry) in as many sites as possible would have been helpful in clarifying some more aspects of the relationship between the two species within syntopic circunstances.

The combined analysis of population genetic and bioacoustic signal data revealed differences among the studied populations of both 'European' and 'Stripeless' treefrogs in Portugal. Genetic differences among populations did not correlate with geographic distance over the range of the study, but did form distinct clades within each species. However, a divergent pattern was revealed by the bioacoustic differences, which did not cluster into groups

congruent with the genetic groups, nor any other clustering patterns. The presence of males of other species calling at the same time, and in the same site, apparently had no effect on advertisement calls. Sympatric and allopatric populations did not show significant differences, thus seeming to exclude the hypothesis of reproductive character displacement by one or both species. This, together with the absence of F1 hybrids in the studied sites, suggests that – most probably – the rate of heterospecific mating is very low, and that advertisement calls are, indeed, effective premating barriers. Other possible premating barriers, such as a lag between species' sexual cycles, seem not to be prominent, as the species' reproductive season strongly overlaps. Further studies on female preferences would be required. Are sympatric females more prone to choosing and mating with males from their own species, when compared with allopatric females? More extensive hybridisation experiments would also help to better understand the post-zygotic mechanism of isolation. There have been reports of F1 hybrids in nature, but in this study no hybrids were found despite the great effort done during sampling to significantly cover the sympatric area of both species as well as capturing both adults and tadpoles (at pre- and post-metamorphic stages of development).

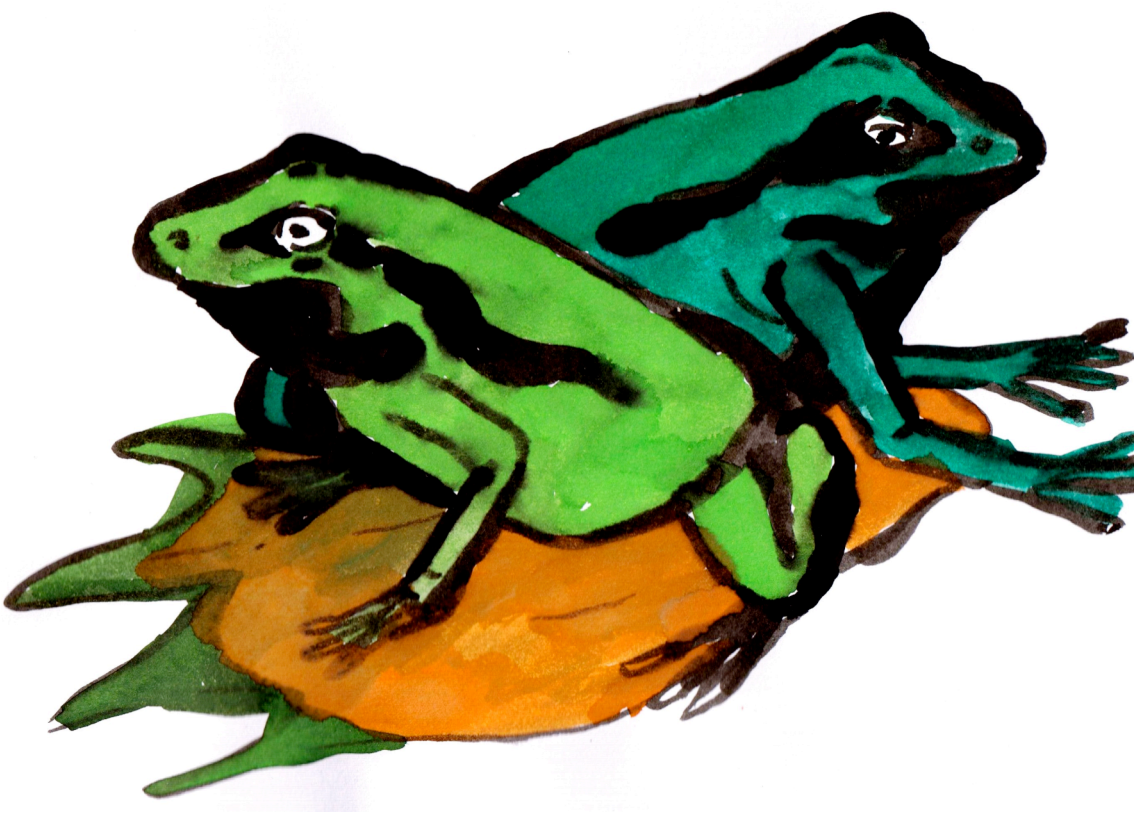
By sequencing a larger sample of individuals from a wider geographic range within Iberian than previous studies, we were able to corroborate the relationships between the various *H. meridionalis* clades described by Recuero et al. (2007). Also the partition of genetic variability across the Strait of Gibraltar, with higher haplotype diversity within Moroccan groups and shared haplotypes in both sides, add strength to the hypothesis of a colonisation of the Iberian grounds from Northern Morocco after the latest opening of the Strait (~5.3 Mya). Regarding the timing of these events as yet no accurate molecular clock has been found, by caution no information is given. Moreover, because nuclear markers were not used future work with respect to the testing of these hypotheses should use both mitochondrial and nuclear (namely, microsatellites) markers and investigate a proper accurate calibrated molecular clock specifically for the species.

The study of mtDNA variation of *H. arborea*, once again greatly increasing the sampled sites, in particular in Portugal, allowed the detection of an undescribed differentiation between populations. The two observed groups, herein named North and South, with a few sites sharing haplotypes of both clades in a overlapping unclear geographic region along a NW-SE axis, exhibited somewhat complementary geographic distributions, separated in part by the Mondego River. It would be of extreme importance to use here microsatellite markers for a characterisation of the population structure, and to clear the frontiers between the two groups.
The use of bioacoustic markers brought to light unknown information in call parameters variation within Portugal for the two studied species. Also showed that with high chance the advertisement calls may be acting as a relevant pre-mating barrier reducing the number of heterospecific crosses, but nevertheless experimenting with female choice preferences and male

acoustic behaviour in different situations (e.g. single or multispcies choruses, more or dense choruses, etc.) would permit a better illustration of the scenarios.

Regarding the double used approach, this work is clearly the beginning. More multi-approach and more diverse types of data will allow for a deeper understanding of each species and of their interspecific relationships. The analysis in more detail of the syntopic zones will allow for the description of species interaction and its consequences. For such a multidisciplinary framework making use of morphological, ecological and physiological information is crucial, and should be the next step in the nearest future.

Chapter VI. References



by Sara Maia

I have not attempted to discuss the question whether the soul of the frog possesses consciousness, because this appears to me to be a totally insoluble problem.

Thomas H. Huxley (1870), 'Has a frog a soul,
and of what Nature is that soul, supposing it to exist?'

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Appendix

by Sara Maia

Appendix 1. Variability in the lateral bands in *H. arborea* and *H. meridionalis* in sampled populations of Portugal.

H. arborea CFO

H. arborea CUB

H. arborea CUB

H. meridionalis LIN

H. meridionalis CHO

H. meridionalis BRA

H. meridionalis ALQ

H. meridionalis ARI

Appendix 2. Temperatures and Body Measurements of Audio-Recorded Populations of *Hyla arborea*.

Mean and Standard Deviation of temperatures measured at each site where males were audio-recorded, and body measurements of the recorded males.

LOC	Nr of Males	Male Temperature (ºC)		Air Temperature (ºC)		Substrate Temperature (ºC)		SVL (cm)		Mass (g)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
COV	3	18.83	1.46	13.87	0.76	22.00	0.00	3.80	0.20	4.20	0.44
FAL	5	16.28	2.08	10.20	0.00	18.80	1.12	3.50	0.20	3.58	0.48
CAM	3	15.73	1.90	16.00	1.39	15.60	1.04	3.57	0.12	4.37	0.75
TOI	2	15.45	1.91	10.00	0.00	15.40	1.98	3.45	0.35	4.00	1.84
CFO	3	13.07	0.12	11.00	0.00	13.40	0.00	3.97	0.25	4.53	0.75
SAG	4	14.10	0.48	10.78	0.21	*m.v.*	*m.v.*	3.63	0.29	4.40	0.50
SOU	11	20.09	2.48	17.31	2.60	20.75	2.82	3.51	0.25	3.09	0.78
BUR	3	10.70	1.30	9.93	3.35	14.40	0.35	3.47	0.06	3.77	0.38
POV	3	11.53	1.26	10.80	0.00	14.20	4.19	3.90	0.30	4.10	0.46
CRE	5	15.85	2.14	12.24	3.64	16.54	3.80	3.58	0.18	3.34	0.33
CAN	3	13.50	1.87	15.20	1.04	13.27	2.44	3.60	0.10	3.47	0.55
SUM	4	15.20	0.48	13.75	0.50	16.40	0.00	3.13	0.10	2.73	0.31
CHO	3	15.43	3.19	12.80	0.35	15.03	1.27	3.17	0.15	3.17	0.31
CUB	6	16.75	3.14	14.72	2.80	17.47	3.37	3.35	0.18	2.97	0.40

m.v., missing value

Appendix 3. Advertisement Call Parameters of *Hyla arborea* males recorded in Portugal.

Mean and standard deviations calculated for each call parameter of each recorded male in Portugal. In the table is indicated the location (LOC), the male code, alopatry and sympatry condition (ALO/SYMP) of each location, number of calls considered.

LOC	Male	ALO/SYMP	Nr of Calls	CGD (s)		CD (s)		ICD (s)		CCG		PC	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
COV	695	A	126	3.701	1.106	0.080	0.003	0.101	0.029	21.000	6.633	10.079	0.560
COV	696	A	115	3.653	0.537	0.077	0.004	0.084	0.060	23.000	3.391	10.661	0.736
COV	697	A	97	3.113	0.270	0.081	0.005	0.084	0.021	19.400	1.517	10.340	0.644
FAL	413	A	90	5.707	1.983	0.083	0.004	0.111	0.026	30.000	11.790	11.211	0.918
FAL	414	A	99	5.163	1.754	0.087	0.005	0.126	0.027	24.750	7.365	10.354	0.747
FAL	415	A	157	6.798	0.468	0.096	0.005	0.124	0.023	31.400	1.817	10.605	0.952
FAL	416	A	134	4.317	0.838	0.079	0.006	0.085	0.022	26.800	4.970	10.664	0.813
FAL	418	A	59	2.355	0.728	0.075	0.003	0.091	0.023	14.750	4.113	9.000	0.965
CAM	421	A	101	5.211	2.044	0.094	0.004	0.117	0.035	25.250	9.535	10.782	0.890
CAM	422	A	80	2.257	0.876	0.085	0.008	0.092	0.024	13.333	5.391	10.613	1.049
CAM	424	A	140	4.301	0.683	0.085	0.004	0.104	0.026	23.333	3.724	10.286	0.682
TOI	758	A	157	8.310	0.964	0.095	0.003	0.120	0.048	39.250	5.123	10.057	0.612
TOI	759	A	121	10.011	3.929	0.097	0.004	0.155	0.036	40.333	15.373	9.983	0.532
CFO	700	A	648	55.211	24.991	0.087	0.003	0.169	0.046	216.000	93.579	9.950	0.400
CFO	701	A	522	43.789	13.707	0.097	0.004	0.155	0.046	174.000	50.715	10.126	0.649
CFO	702	A	1146	45.028	8.213	0.096	0.008	0.167	0.061	382.000	199.015	10.416	0.803
SAG	748	S	94	4.063	1.460	0.090	0.005	0.133	0.033	18.800	6.611	10.872	0.930
SAG	749	S	47	3.535	1.518	0.085	0.007	0.236	0.058	11.750	5.852	9.213	1.197

LOC	Male	ALO/SYMP	Nr of Calls	CGD (s)		CD (s)		ICD (s)		CCG		PC	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SAG	750	S	63	4.349	0.677	0.083	0.010	0.131	0.041	21.000	1.732	9.667	1.576
SAG	751	S	76	4.912	0.788	0.082	0.008	0.117	0.015	25.333	4.041	9.013	1.281
SOU	108	A	103	2.473	0.409	0.064	0.003	0.085	0.026	17.167	2.927	9.650	0.801
SOU	109	A	87	1.844	0.516	0.066	0.003	0.066	0.018	14.500	4.930	11.977	0.849
SOU	660	A	117	2.986	0.471	0.072	0.003	0.085	0.026	19.500	3.834	11.368	0.772
SOU	661	A	117	2.905	0.239	0.072	0.003	0.081	0.025	19.500	1.871	11.248	0.890
SOU	662	A	159	3.594	0.081	0.072	0.003	0.066	0.018	26.500	0.837	10.893	0.690
SOU	665	A	163	5.412	1.395	0.082	0.004	0.122	0.035	27.167	5.456	10.742	0.828
SOU	667	A	116	4.322	0.993	0.087	0.005	0.103	0.062	23.200	4.712	11.681	0.881
SOU	668	A	198	3.364	1.019	0.083	0.003	0.129	0.042	16.500	6.172	9.222	0.597
SOU	89	A	100	2.834	0.416	0.059	0.003	0.087	0.020	20.000	3.082	11.160	0.861
SOU	90	A	129	2.169	0.247	0.056	0.002	0.084	0.046	16.125	2.031	9.876	0.829
SOU	91	A	151	2.902	0.683	0.063	0.002	0.096	0.011	18.875	4.291	10.278	0.776
BUR	190	S	77	5.987	1.629	0.110	0.007	0.128	0.029	25.667	7.638	10.974	0.743
BUR	200	S	57	4.861	1.573	0.101	0.005	0.164	0.068	19.000	6.083	10.368	0.698
BUR	222	S	67	4.190	1.463	0.101	0.005	0.159	0.058	16.750	5.500	10.866	0.851
POV	81	S	104	7.563	1.920	0.126	0.009	0.172	0.063	26.000	4.899	10.337	0.820
POV	82	S	108	7.989	1.268	0.129	0.009	0.174	0.049	27.000	2.449	10.602	0.947
POV	83	S	117	5.444	2.908	0.086	0.004	0.103	0.042	29.250	15.607	9.094	0.557
CRE	58	S	104	4.166	0.730	0.072	0.005	0.135	0.038	20.800	3.701	9.817	0.879
CRE	59	S	128	4.982	1.364	0.070	0.008	0.129	0.035	25.600	5.941	8.477	1.087
CRE	648	S	151	9.376	4.336	0.096	0.005	0.156	0.058	37.750	13.326	10.570	0.927

LOC	Male	ALO/SYMP	Nr of Calls	CGD (s)		CD (s)		ICD (s)		CCG		PC	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CRE	650	S	94	3.289	1.028	0.085	0.006	0.133	0.034	15.667	4.082	10.404	0.859
CRE	651	S	193	12.470	8.867	0.089	0.003	0.173	0.054	48.250	31.500	9.933	0.383
CAN	655	A	97	7.585	1.122	0.107	0.004	0.215	0.085	24.250	4.573	10.113	0.575
CAN	658	A	73	3.237	0.489	0.092	0.004	0.140	0.050	14.600	0.894	10.356	0.609
CAN	659	A	183	9.110	4.210	0.096	0.006	0.157	0.066	36.600	13.576	9.231	0.981
SUM	241	S	102	5.222	0.765	0.092	0.004	0.118	0.041	25.500	4.041	11.157	0.686
SUM	245	S	59	5.172	1.557	0.073	0.007	0.161	0.039	23.250	6.898	9.339	1.321
SUM	246	S	110	5.698	1.673	0.085	0.004	0.127	0.040	27.500	6.608	10.445	0.724
SUM	248	S	45	8.797	1.678	0.087	0.004	0.183	0.041	35.333	6.429	9.978	0.866
CHO	628	S	103	4.368	0.521	0.110	0.003	0.107	0.031	20.600	2.881	12.382	0.745
CHO	629	S	147	7.343	2.823	0.094	0.004	0.161	0.050	29.400	9.762	10.054	0.617
CHO	630	S	168	6.381	1.397	0.089	0.007	0.144	0.061	28.000	6.899	9.673	0.738
CUB	148	S	66	3.476	0.206	0.091	0.003	0.128	0.056	16.500	2.082	10.894	1.266
CUB	606	S	134	3.249	0.745	0.069	0.005	0.106	0.026	19.143	4.100	11.201	0.793
CUB	609	S	129	4.520	1.773	0.072	0.003	0.107	0.027	25.800	9.602	9.132	0.617
CUB	610	S	93	3.871	0.845	0.081	0.004	0.090	0.042	23.250	5.560	10.011	0.599
CUB	682	S	115	5.922	3.253	0.097	0.007	0.168	0.046	23.000	12.570	8.609	1.182
CUB	686	S	160	6.440	1.233	0.087	0.005	0.118	0.026	32.000	5.657	10.281	0.737

Advertisement Call Parameters acronyms: CGD, Call Group Duration; CD, Call Duration; ICD, Intercall Duration; CCG, Number of Calls per Call Group; PC, Number of Pulses per Call

Appendix 3 (cont.). Advertisement Call Parameters of *Hyla arborea* males recorded in Portugal.

Mean and standard deviations calculated for each call parameter of each recorded male in Portugal. In the table is indicated the location (LOC), the male code, alopatry and sympatry condition (ALO/SYMP) of each location, number of calls considered.

LOC	Male Code	ALO/SYMP	Nr of Calls	CR		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
COV	695	A	126	5.652	0.268	0.127	0.006	1013.448	64.619	2107.873	54.550	4.544	1.408
COV	696	A	115	6.320	0.585	0.139	0.007	936.603	82.247	2128.989	44.590	4.480	0.835
COV	697	A	97	6.240	0.291	0.128	0.007	925.270	43.117	2203.058	38.998	3.259	0.661
FAL	413	A	90	5.200	0.274	0.135	0.010	1091.024	28.850	2385.898	75.513	13.784	2.490
FAL	414	A	99	4.856	0.284	0.119	0.007	1111.465	45.065	2381.293	35.465	11.372	2.555
FAL	415	A	157	4.623	0.142	0.110	0.009	1118.630	61.078	2616.371	49.643	16.579	3.330
FAL	416	A	134	6.222	0.265	0.135	0.009	994.063	59.289	2311.460	65.704	15.511	3.926
FAL	418	A	59	6.309	0.194	0.121	0.012	990.517	26.517	2164.275	41.247	13.710	3.132
CAM	421	A	101	4.875	0.269	0.114	0.008	1022.933	31.316	2260.356	44.581	18.311	3.348
CAM	422	A	80	5.868	0.207	0.126	0.009	928.096	39.308	2055.353	46.969	8.528	2.565
CAM	424	A	140	5.428	0.132	0.121	0.007	1062.524	67.835	2135.186	39.012	19.346	4.087
TOI	758	A	157	4.718	0.173	0.106	0.006	921.960	34.120	1973.111	43.014	1.598	0.334
TOI	759	A	121	4.039	0.098	0.103	0.005	1263.862	22.050	2666.556	82.386	2.861	1.145
CFO	700	A	648	3.931	0.097	0.117	0.005	1066.571	97.766	2222.583	53.072	5.992	0.743
CFO	701	A	522	3.997	0.111	0.104	0.006	986.487	93.092	1985.366	33.573	4.159	0.624
CFO	702	A	1146	8.720	4.605	0.107	0.007	1009.878	44.889	2110.462	36.945	5.207	1.363
SAG	748	S	94	4.636	0.096	0.121	0.008	1166.926	29.689	2365.013	57.077	2.402	1.581
SAG	749	S	47	3.239	0.315	0.108	0.010	1176.543	59.647	2549.181	75.477	3.097	1.008
SAG	750	S	63	4.863	0.339	0.117	0.010	1090.343	42.226	2221.019	69.926	4.990	1.481

LOC	Male	ALO/SYMP	Nr of Calls	CR		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SAG	751	S	76	5.157	0.023	0.110	0.010	1102.730	37.254	2248.542	48.369	2.906	0.864
SOU	108	A	103	6.941	0.294	0.150	0.010	1001.392	36.043	2257.873	42.890	17.374	4.345
SOU	109	A	87	7.735	0.699	0.182	0.010	1114.789	69.327	2577.560	107.809	14.491	5.628
SOU	660	A	117	6.497	0.338	0.158	0.009	1179.367	80.739	2694.043	60.471	6.782	1.625
SOU	661	A	117	6.709	0.198	0.156	0.010	1230.532	142.087	2524.740	42.957	8.336	1.982
SOU	662	A	159	7.373	0.089	0.152	0.008	1293.082	124.568	2767.604	42.921	8.808	1.558
SOU	665	A	163	5.096	0.490	0.131	0.008	1151.953	50.726	2571.577	50.351	5.920	1.030
SOU	667	A	116	5.399	0.309	0.134	0.008	1234.831	60.839	2760.317	53.570	8.232	2.377
SOU	668	A	198	4.820	0.587	0.112	0.007	1264.592	48.888	2617.944	31.456	8.835	1.979
SOU	89	A	100	7.053	0.242	0.188	0.013	1068.922	50.245	2540.074	56.429	43.903	7.224
SOU	90	A	129	7.441	0.510	0.177	0.012	1184.831	84.296	2722.194	62.463	34.660	6.080
SOU	91	A	151	6.512	0.156	0.163	0.013	908.666	99.181	2145.054	37.770	52.165	8.161
BUR	190	S	77	4.269	0.110	0.100	0.007	1007.856	27.304	2250.096	37.057	7.326	1.220
BUR	200	S	57	3.912	0.046	0.103	0.007	1072.896	34.794	2212.267	40.399	11.385	2.432
BUR	222	S	67	4.040	0.524	0.108	0.007	1085.034	31.113	2269.040	66.298	7.527	1.354
POV	81	S	104	3.484	0.268	0.082	0.006	1217.888	28.421	2538.436	26.709	28.090	3.823
POV	82	S	108	3.406	0.225	0.082	0.006	1114.929	20.661	2515.018	28.179	26.582	3.093
POV	83	S	117	5.362	0.137	0.106	0.007	1119.716	24.640	2530.615	30.253	32.408	5.890
CRE	58	S	104	5.004	0.449	0.137	0.009	1245.212	42.047	2810.897	208.170	29.118	5.265
CRE	59	S	128	5.182	0.231	0.121	0.008	1180.644	32.233	2627.066	50.983	27.859	5.069
CRE	648	S	151	4.148	0.406	0.110	0.008	1216.991	37.021	2591.419	31.765	6.944	1.388
CRE	650	S	94	4.831	0.546	0.122	0.009	1180.194	102.493	2644.013	76.875	2.799	1.174

LOC	Male	ALO/SYMP	Nr of Calls	CR		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CRE	651	S	193	4.011	0.441	0.112	0.004	1103.664	66.015	2366.887	39.755	9.218	1.922
CAN	655	A	97	3.182	0.132	0.095	0.006	1301.774	31.415	2797.085	47.092	5.764	1.829
CAN	658	A	73	4.564	0.480	0.113	0.006	1295.541	30.263	2806.377	111.200	1.816	1.038
CAN	659	A	183	4.140	0.363	0.096	0.011	1271.058	91.176	2644.010	63.488	6.182	1.994
SUM	241	S	102	4.879	0.142	0.122	0.006	1088.070	28.997	2642.267	45.603	8.527	1.611
SUM	245	S	59	4.516	0.522	0.128	0.010	1054.773	46.897	2346.769	46.886	5.348	1.098
SUM	246	S	110	4.909	0.636	0.123	0.007	1046.920	29.037	2487.302	39.644	11.526	2.125
SUM	248	S	45	4.022	0.040	0.115	0.007	1322.622	44.627	2784.947	50.295	2.955	0.945
CHO	628	S	103	4.714	0.313	0.113	0.007	1199.607	39.734	2693.115	48.534	6.216	1.392
CHO	629	S	147	4.050	0.210	0.107	0.005	1326.861	49.728	2778.497	62.418	3.711	1.146
CHO	630	S	168	4.370	0.225	0.109	0.007	1278.663	51.845	2802.379	32.522	7.534	1.693
CUB	148	S	66	4.733	0.326	0.120	0.011	1166.055	42.953	2471.130	58.403	10.823	3.049
CUB	606	S	134	5.910	0.207	0.162	0.010	1187.542	97.158	2548.004	47.875	6.482	1.267
CUB	609	S	129	5.796	0.464	0.127	0.008	1251.267	37.022	2710.171	54.406	7.699	1.593
CUB	610	S	93	5.980	0.306	0.124	0.007	1241.995	72.201	2558.530	46.327	5.527	0.993
CUB	682	S	115	3.908	0.224	0.089	0.009	1298.371	38.811	2556.651	29.824	2.904	0.806
CUB	686	S	160	4.981	0.086	0.118	0.007	1062.139	48.998	2342.564	31.349	4.190	0.731

Advertisement Call Parameters acronyms: CR, Call Rate; PR, Pulse Rate; FF, Fundamental Frequency; DF, Dominant Frequency; ADF, difference of amplitude between dominant and fundamental frequencies.

Appendix 4 .Temperatures and Body Measurements of Audio-Recorded Populations of *Hyla meridionalis*.

Mean and Standard Deviation of temperatures measured at each site where males were audio-recorded, and body measurements of the recorded males.

LOC	Nr of Males	Male Temperature (ºC)		Air Temperature (ºC)		Substrate Temperature (ºC)		SVL (cm)		Mass (g)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ALM	4	17.90	1.32	17.45	0.09	18.68	1.34	3.72	0.11	3.92	0.44
ALP	4	10.17	0.40	8.37	3.02	11.77	0.34	3.82	0.19	4.48	0.52
ALQ	5	16.20	0.43	15.95	0.15	17.64	1.01	3.70	0.18	3.56	0.35
ARI	5	12.66	2.15	13.08	0.74	11.88	1.41	3.53	0.16	3.77	0.45
BRA	6	16.83	0.38	17.04	0.18	17.11	0.44	3.61	0.15	3.71	0.66
BRI	4	15.90	0.92	16.08	0.16	16.36	1.00	3.59	0.07	3.62	0.22
BUR	5	11.99	1.52	8.60	0.41	13.76	0.08	3.61	0.19	3.94	0.73
CAC	7	11.40	2.09	12.32	1.40	11.88	2.20	3.71	0.12	3.99	0.21
CHO	3	14.05	0.89	11.47	0.56	11.74	0.13	3.64	0.20	3.71	0.61
COS		13.87	1.35	14.52	0.38	14.41	1.40	3.53	0.08	3.21	0.47
CRE	3	11.42	1.73	13.33	2.82	11.48	2.09	3.98	0.24	4.66	0.35
CUB	5	14.20	1.00	12.51	1.60	14.95	1.97	3.49	0.11	3.65	0.31
FAZ	4	13.95	1.07	14.76	0.29	14.82	0.76	3.74	0.18	4.38	0.69
FOI	6	11.49	0.50	12.73	0.29	11.54	0.85	3.94	0.21	4.99	0.59
FOQ	3	13.69	0.46	13.35	0.49	14.44	1.24	3.10	0.09	2.34	0.09
FOU	4	16.53	3.00	15.17	0.18	16.50	2.66	3.65	0.15	4.05	0.67
FRO	2	11.96	1.50	11.60	1.35	15.20	0.00	3.58	0.21	3.48	0.31
FRT	4	15.95	1.06	15.35	0.09	16.85	0.39	3.85	0.18	4.03	0.55
GRA	4	13.75	1.18	12.65	0.55	15.00	2.01	3.73	0.04	4.24	0.36
JAV	2	10.88	0.21	12.20	0.00	10.81	0.37	4.02	0.26	5.52	0.74
LIN	7	16.62	1.09	15.93	0.35	17.31	1.47	3.38	0.49	3.17	1.00
ODI	3	13.37	0.58	9.69	0.48	13.45	0.38	4.00	0.00	3.87	0.13
PIC	4	14.36	1.07	14.41	0.20	14.65	1.13	3.68	0.16	3.60	0.18
POV	3	14.82	0.88	14.80	1.67	15.49	1.34	3.70	0.09	3.66	0.41
RED	10	15.67	0.83	13.48	0.41	15.99	1.56	3.57	0.23	3.75	0.71
SMP	5	16.06	3.21	14.55	0.40	17.05	3.03	3.28	0.19	2.78	0.38
SUM	3	14.18	1.57	13.71	0.47	*m.s.*	m.s.	3.70	0.00	3.45	0.27
TOU	6	12.56	1.07	10.67	0.32	12.18	0.57	3.97	0.15	5.02	0.55

m.s. missing value

Appendix 5. Advertisement Call Parameters of *Hyla meridionalis* males recorded in Portugal.

Mean and standard deviations calculated for each call parameter of each recorded male in Portugal. In the table is indicated the location (LOC), the male code, alopatry and sympatry condition (ALO/SYMP) of each location, number of calls considered.

LOC	Male Code	ALO/SYMP	Nr of Calls	CD (s)		PC		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ALP	138	S	3	0.49	0.02	45.20	0.84	92.94	2.84	1214.50	19.23	2566.76	23.61	5.30	0.62
ALP	139	S	0	0.46	0.02	48.33	1.63	104.29	3.10	1169.98	17.60	2210.77	22.26	0.72	0.94
ALP	140	S	3	0.34	0.01	40.20	0.84	119.36	0.80	1205.88	30.44	2196.40	43.10	11.39	1.07
ALP	157	S	6	0.42	0.01	46.40	1.52	111.30	2.07	1111.10	19.23	2385.92	38.51	9.07	1.37
ALP	161	S	6	0.61	0.01	45.33	1.15	73.79	1.10	976.17	24.83	2282.50	0.00	6.67	0.43
POV	94	S	6	0.67	0.04	45.33	3.06	67.61	1.13	904.40	0.00	2038.47	108.37	5.93	0.59
POV	95	S	5	0.64	0.02	42.83	0.75	67.15	1.04	990.50	0.00	2239.50	0.00	4.83	0.91
BUR	193	S	4	0.64	0.01	45.50	0.84	70.79	0.94	947.50	0.00	2110.30	0.00	4.67	0.57
BUR	194	S	5	0.47	0.01	46.43	0.53	98.91	0.70	1248.90	0.00	2497.90	0.00	3.71	0.11
BUR	195	S	5	0.46	0.01	55.17	1.33	119.18	3.64	1076.70	0.00	2540.93	38.51	2.41	0.19
BUR	197	S	8	0.45	0.03	44.83	1.94	99.27	5.59	1205.90	0.00	2562.45	23.61	2.34	0.13
BUR	198	S	6	0.40	0.01	43.33	1.21	107.57	1.17	1119.70	0.00	2325.60	0.00	3.43	0.91
CRE	191	S	0	0.42	0.02	46.67	1.63	109.90	2.35	1148.45	35.15	2526.57	22.21	4.05	0.30
CRE	646	S	4	0.81	0.04	51.43	1.99	63.43	2.26	1427.36	16.29	2516.33	22.98	5.26	0.30
CRE	652	S	7	0.67	0.03	45.40	1.52	68.08	1.53	1162.80	0.00	2627.10	0.00	4.49	0.69
CRE	653	S	0	0.62	0.03	49.63	1.77	79.75	1.00	1248.90	0.00	2643.23	22.25	4.26	0.27
CRE	654	S	7	0.56	0.03	49.20	1.10	88.16	2.22	1119.70	0.00	2239.46	74.56	0.22	0.79
SUM	242	S	6	0.53	0.02	47.25	1.67	89.50	1.97	1162.80	0.00	2368.70	0.00	3.79	0.22
SUM	243	S	5	0.46	0.01	48.00	0.89	105.19	1.34	1227.38	36.01	2512.23	22.21	3.05	0.42
SUM	249	S	6	0.44	0.01	47.17	0.98	107.93	1.65	1256.08	17.60	2727.52	52.11	2.55	0.44
FRO	528	S	7	0.49	0.02	47.00	2.45	96.60	1.53	1363.77	22.21	2885.40	0.00	3.27	0.55

LOC	Male Code	ALO/SYMP	Nr of Calls	CD (s)		PC		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
FRO	529	S	6	0.42	0.02	44.83	1.72	106.56	2.53	1342.27	17.55	2670.10	0.00	7.16	0.59
CHO	631	S	6	0.40	0.03	39.80	2.17	99.36	2.13	1386.72	19.27	2868.20	112.30	2.79	0.51
CHO	632	S	4	0.44	0.01	43.17	0.98	99.19	0.94	1155.62	17.60	2526.57	22.21	2.23	0.35
CHO	633	S	7	0.48	0.01	44.83	1.33	93.54	2.13	1284.82	32.44	2447.62	32.44	3.85	0.69
LIN	634	A	7	0.43	0.01	45.60	0.89	106.84	0.80	1205.90	0.00	2627.10	0.00	2.68	0.29
LIN	635	A	6	0.46	0.01	49.50	1.00	108.46	1.36	1012.05	24.88	2131.80	24.83	6.65	0.53
LIN	636	A	6	0.43	0.01	46.00	1.26	106.39	1.62	1292.00	0.00	2526.57	22.21	4.91	0.59
LIN	638	A	6	0.62	0.03	44.50	3.11	72.03	1.74	1001.28	21.55	2185.63	21.55	3.96	0.28
LIN	639	A	6	0.72	0.02	52.40	1.34	73.14	1.51	1033.60	0.00	2239.50	0.00	7.67	1.19
LIN	642	A	6	0.55	0.02	44.20	1.64	80.44	1.30	904.40	0.00	2187.78	19.27	6.57	0.63
LIN	644	A	6	0.58	0.01	42.75	0.71	73.80	1.03	1119.70	0.00	2492.51	15.24	8.22	0.26
FRT	611	A	6	0.54	0.01	45.50	1.05	83.52	0.60	990.50	0.00	2182.03	22.26	9.74	0.63
FRT	612	A	6	0.62	0.01	49.40	1.14	79.82	1.68	1119.70	0.00	2489.28	19.27	12.56	1.25
FRT	613	A	6	0.64	0.01	50.60	0.89	79.16	0.87	1154.18	36.06	2385.90	49.09	19.11	1.95
FRT	614	A	6	0.61	0.02	47.00	0.93	77.07	1.25	1168.19	15.24	2589.39	27.62	10.46	0.89
TOU	531	A	5	0.59	0.07	45.00	4.55	76.25	1.43	1119.70	0.00	2465.55	64.61	4.13	1.18
TOU	532	A	6	0.86	0.02	46.60	0.89	54.06	0.73	1386.72	19.27	2627.10	0.00	5.13	0.15
TOU	533	A	6	0.88	0.01	49.60	0.89	56.66	1.34	1205.90	0.00	2558.14	23.61	1.84	0.11
TOU	534	A	7	0.65	0.02	43.00	1.22	66.52	0.87	1162.80	30.48	2678.74	70.74	4.94	1.97
TOU	535	A	6	0.75	0.02	52.33	1.37	69.81	1.08	1162.80	0.00	2440.43	22.26	1.98	0.08
TOU	536	A	8	0.73	0.01	52.50	1.00	72.19	0.67	1205.90	0.00	2530.18	107.65	5.38	0.32
FOQ	673	A	5	0.54	0.02	47.86	1.57	89.06	0.75	1279.69	21.03	2811.61	21.03	5.89	0.19
FOQ	675	A	6	0.57	0.01	46.50	0.55	81.09	1.90	1162.80	0.00	2598.37	22.26	3.09	0.14
FOQ	677	A	6	0.49	0.02	43.17	1.33	88.23	1.52	1421.20	0.00	2749.07	87.92	1.37	0.09

LOC	Male Code	ALO/SYMP	Nr of Calls	CD (s)		PC		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
RED	537	A	7	0.64	0.03	53.38	3.07	83.81	1.43	1216.65	19.91	2680.88	19.95	2.18	0.14
RED	538	A	7	0.54	0.01	49.33	0.82	91.91	0.68	1270.45	23.61	2756.22	27.23	2.47	0.20
RED	539	A	7	0.56	0.03	47.86	1.21	84.81	2.63	1125.86	16.29	2424.01	21.03	4.36	0.68
RED	542	A	6	0.78	0.02	49.75	0.96	63.51	2.19	1119.70	0.00	2497.90	0.00	3.96	1.40
RED	543	A	6	0.60	0.03	50.43	3.10	84.69	2.48	1205.90	0.00	2719.34	16.25	2.11	1.16
RED	547	A	6	0.63	0.02	44.29	2.36	69.92	1.78	1175.11	21.03	2368.69	43.07	3.51	0.39
RED	674	A	6	0.47	0.03	42.50	1.91	90.70	1.19	1270.45	24.88	2766.98	21.55	5.48	0.59
RED	681	A	6	0.53	0.02	43.00	1.55	80.71	1.01	1062.33	22.26	2045.65	23.61	1.92	0.24
ALQ	616	A	7	0.51	0.01	48.33	1.21	94.08	1.39	1033.60	0.00	2232.32	17.60	2.60	0.19
ALQ	617	A	6	0.41	0.01	39.80	1.30	96.97	0.50	1007.74	23.61	2273.92	36.01	2.76	0.07
ALQ	618	A	6	0.41	0.02	43.40	2.51	106.68	1.24	1059.46	23.61	2213.64	23.61	3.15	0.27
ALQ	620	A	6	0.64	0.02	55.14	2.67	86.88	4.11	1052.07	23.04	2399.43	32.53	5.73	0.97
ALQ	622	A	6	0.57	0.01	45.67	0.52	79.48	2.20	1385.30	32.41	2619.90	32.41	7.14	0.33
GRA	688	A	7	0.65	0.02	48.88	1.73	75.37	1.28	1082.08	15.20	2438.65	51.17	2.11	0.37
GRA	689	A	6	0.69	0.01	54.00	1.83	78.66	2.44	1162.80	24.88	2454.80	35.19	3.48	0.64
GRA	691	A	7	0.54	0.01	43.00	0.82	79.74	0.37	1108.95	21.50	2347.15	24.88	2.97	0.11
GRA	694	A	7	0.78	0.02	54.00	1.26	69.33	1.72	1033.60	0.00	2275.35	32.40	4.35	1.06
ODI	210	S	3	0.70	0.02	51.00	1.79	73.08	0.69	1155.62	17.60	2296.87	22.26	2.72	0.14
ODI	212	S	6	0.75	0.03	51.00	2.10	67.85	2.50	1126.88	17.60	2318.42	17.60	1.69	0.80
ODI	213	S	4	0.71	0.02	49.83	0.41	70.52	1.12	1112.53	17.55	2153.30	0.00	2.00	0.10
BRI	623	A	6	0.75	0.04	50.50	1.73	67.38	2.35	1033.60	0.00	2304.05	24.88	5.43	3.07
BRI	625	A	5	0.50	0.03	47.40	2.51	95.55	1.52	1300.62	19.27	2670.12	30.44	1.36	0.44
BRI	626	A	4	0.56	0.02	52.00	1.26	92.65	2.73	1399.65	36.06	2784.93	58.83	0.71	0.05
BRI	627	A	6	0.47	0.01	47.00	1.10	98.98	1.33	1213.07	17.55	2605.50	101.00	0.58	0.22
CUB	122	S	4	0.51	0.00	49.00	0.00	96.52	0.33	1205.90	0.00	2591.18	42.38	2.94	0.45

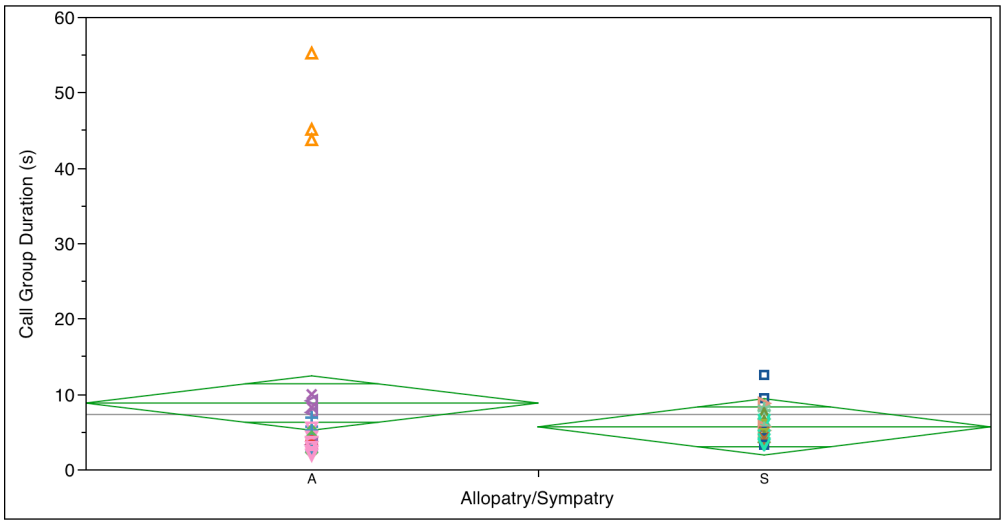
LOC	Male Code	ALO/SYMP	Nr of Calls	CD (s)		PC		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CUB	683	S	6	0.46	0.02	45.00	1.91	97.20	1.62	1033.60	0.00	2356.37	59.42	3.26	0.21
CUB	684	S	6	0.39	0.01	44.40	1.52	114.16	2.75	930.26	23.61	2058.60	77.06	1.10	0.57
CUB	685	S	5	0.48	0.01	48.00	1.10	100.05	2.64	1119.70	0.00	2304.07	45.18	3.21	0.23
CUB	687	S	5	0.50	0.01	46.57	1.27	92.43	1.03	1193.59	21.03	2608.61	65.10	1.45	0.18
PIC	333	A	7	0.48	0.02	43.67	1.86	90.12	0.61	1033.60	0.00	2196.40	0.00	3.59	0.08
PIC	334	A	5	0.49	0.01	49.17	1.17	101.27	1.31	1162.80	0.00	2555.28	52.16	3.44	0.42
PIC	338	A	7	0.41	0.02	43.33	2.80	105.49	2.62	1205.87	38.51	2497.87	60.91	4.93	0.62
PIC	340	A	4	0.53	0.01	54.83	1.17	103.80	1.53	1119.75	47.16	2253.82	52.15	4.34	0.58
SMP	548	A	6	0.43	0.01	45.17	1.17	105.05	1.82	1234.60	52.15	2440.47	205.05	2.09	0.49
SMP	549	A	6	0.63	0.03	49.43	1.72	78.91	1.77	1205.90	0.00	2651.67	22.98	2.25	0.13
SMP	550	A	8	0.61	0.02	49.33	1.51	80.83	0.85	1248.90	0.00	2469.17	44.51	1.30	0.06
SMP	551	A	6	0.45	0.01	44.71	1.60	99.82	1.51	1002.81	21.03	2177.93	33.91	2.35	0.32
SMP	552	A	6	0.47	0.01	47.71	0.49	102.65	2.29	1101.29	33.86	2153.31	24.85	1.74	0.13
ALM	301	A	5	0.77	0.04	51.00	2.16	65.87	1.72	1076.70	0.00	2271.75	21.50	19.84	0.38
ALM	302	A	6	0.80	0.03	49.40	0.55	61.83	1.91	1188.64	89.33	2420.34	153.47	6.09	0.87
ALM	305	A	5	0.45	0.02	47.57	2.23	106.54	2.28	1292.00	0.00	2830.06	40.95	1.50	0.37
ALM	306	A	5	0.58	0.02	50.83	0.98	88.21	2.90	1277.63	22.26	2763.43	152.67	2.64	0.38
FOU	567	A	6	0.39	0.01	43.17	0.98	109.87	1.56	1378.12	47.18	2885.43	38.51	2.79	0.18
FOU	568	A	7	0.43	0.01	43.33	1.51	101.76	3.37	1134.07	22.26	2426.07	22.26	4.53	0.31
FOU	569	A	5	0.52	0.02	45.00	1.26	85.80	1.01	1155.62	17.60	2397.35	35.15	3.98	0.24
FOU	570	A	6	0.48	0.01	53.83	1.33	112.20	2.47	1363.75	35.15	2935.70	57.25	2.71	0.13
COS	561	A	6	0.45	0.01	47.50	1.52	105.28	1.56	1284.82	42.38	2418.90	42.35	2.33	0.62
COS	564	A	8	0.63	0.02	48.67	1.53	77.79	0.15	904.40	0.00	2067.20	0.00	0.93	0.08
COS	565	A	6	0.46	0.01	42.67	1.03	93.30	0.86	854.13	17.55	1938.00	0.00	5.30	0.44

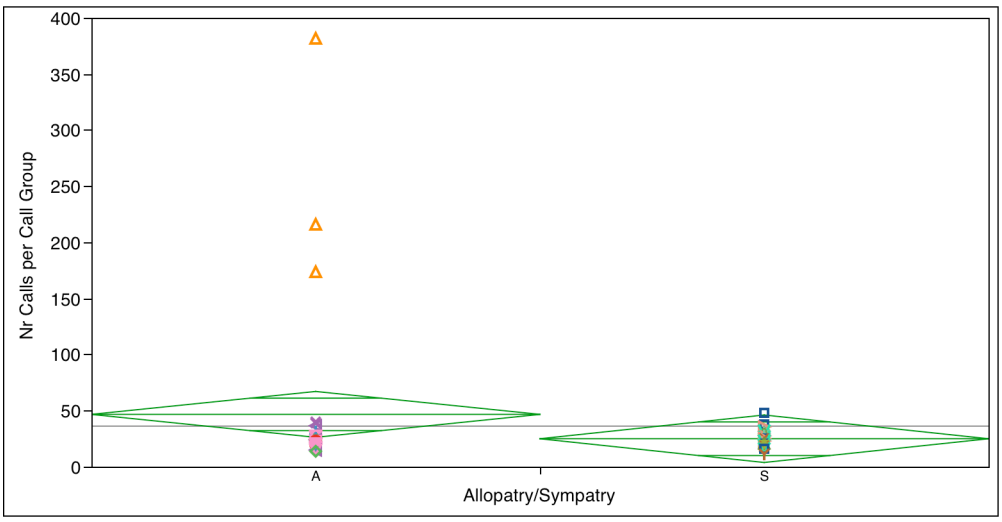
LOC	Male Code	ALO/SYMP	Nr of Calls	CD (s)		PC		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
COS	566	A	7	0.46	0.00	43.75	0.96	95.21	2.05	904.40	0.00	2153.30	0.00	7.72	0.78
CAC	312	A	5	0.44	0.02	43.43	1.27	99.79	2.23	1328.91	38.74	2762.36	16.29	4.37	0.26
CAC	313	A	5	0.62	0.02	48.60	2.61	77.95	2.18	1248.92	30.44	2601.24	38.55	4.72	0.47
CAC	315	A	8	0.45	0.01	40.14	1.07	88.45	3.19	1304.33	47.92	2657.86	81.38	4.84	0.80
CAC	560	A	4	0.61	0.01	43.00	1.41	70.25	1.32	1281.23	21.55	2680.88	21.55	4.04	0.35
CAC	573	A	5	0.49	0.02	49.33	2.73	100.60	2.33	1148.43	22.26	2383.03	22.21	8.67	2.93
CAC	574	A	5	0.56	0.02	47.67	2.50	85.86	1.53	1292.00	0.00	2591.18	17.60	24.77	0.91
CAC	575	A	5	0.56	0.01	48.60	0.89	87.16	1.48	1481.50	23.55	2739.00	23.55	20.76	1.13
FOI	584	A	4	0.44	0.02	45.71	2.81	103.96	3.52	861.30	0.00	1981.06	49.72	4.19	0.71
FOI	585	A	6	0.52	0.02	44.71	1.25	85.66	2.25	1132.01	21.03	2516.33	22.98	3.34	0.18
FOI	588	A	6	0.43	0.01	44.57	0.98	104.26	2.12	861.30	0.00	1925.69	21.03	4.29	0.48
FOI	589	A	6	0.47	0.01	44.14	1.21	93.96	2.36	1082.84	16.25	2497.87	35.15	4.27	1.28
FOI	590	A	6	0.53	0.03	42.33	1.75	80.29	1.51	1205.90	0.00	2469.17	22.26	2.32	0.19
FOI	591	A	4	0.48	0.02	42.50	1.64	87.99	2.81	1119.70	0.00	2375.83	42.32	3.21	0.18
BRA	596	A	6	0.42	0.01	43.67	1.21	105.02	1.63	897.22	17.60	2067.20	27.26	3.95	0.50
BRA	597	A	6	0.46	0.03	46.33	3.27	101.13	2.30	861.30	0.00	1981.10	0.00	4.84	0.22
BRA	598	A	6	0.42	0.01	47.17	0.75	112.68	1.85	1062.33	22.26	2339.97	22.26	2.67	0.19
BRA	600	A	6	0.43	0.01	45.83	1.47	105.74	1.89	1184.35	23.61	2576.82	32.44	2.41	0.18
BRA	603	A	6	0.40	0.01	43.17	1.47	107.36	2.29	1256.10	42.35	2713.18	27.23	3.29	0.19
JAV	44	A	4	0.39	0.02	48.00	1.41	124.00	3.00	1091.02	64.84	2268.17	44.47	2.23	0.16
JAV	45	A	5	0.40	0.01	39.75	1.28	99.24	2.42	1259.68	19.95	2643.23	22.25	5.71	0.60
ARI	557	A	7	0.49	0.01	42.17	0.98	86.35	2.08	1356.60	23.55	2856.73	22.21	2.30	0.15
ARI	577	A	5	0.38	0.03	38.17	0.98	99.99	4.38	1378.13	38.51	2677.30	32.41	0.59	0.25
ARI	579	A	5	0.56	0.02	45.00	1.79	79.73	2.20	1033.60	0.00	2404.53	17.55	3.65	0.16

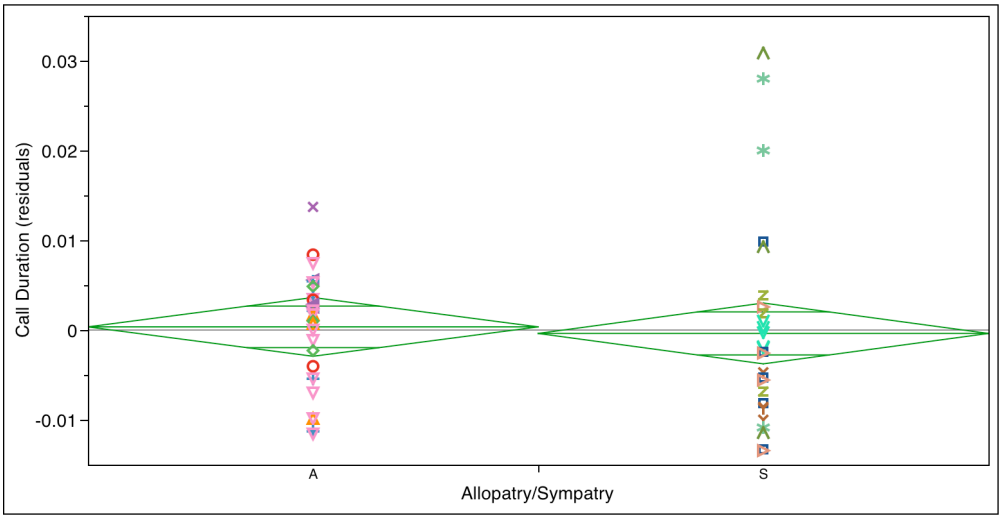
LOC	Male Code	ALO/SYMP	Nr of Calls	CD (s)		PC		PR		FF (Hz)		DF (Hz)		ADF	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
ARI	580	A	8	0.52	0.01	46.00	1.41	89.34	2.79	1274.76	23.61	2713.18	30.44	0.99	0.29
FAZ	553	A	7	0.47	0.02	45.83	1.72	98.50	1.92	1026.42	17.60	2397.40	52.15	2.55	0.28
FAZ	554	A	6	0.58	0.03	45.80	1.64	78.94	2.87	904.40	0.00	1955.24	23.61	3.05	0.47
FAZ	555	A	8	0.66	0.04	47.67	2.34	72.03	0.63	1047.97	22.26	2210.77	84.69	3.03	0.22
FAZ	556	A	7	0.47	0.02	36.17	1.94	76.36	1.57	882.85	23.61	2088.75	23.61	5.58	0.28

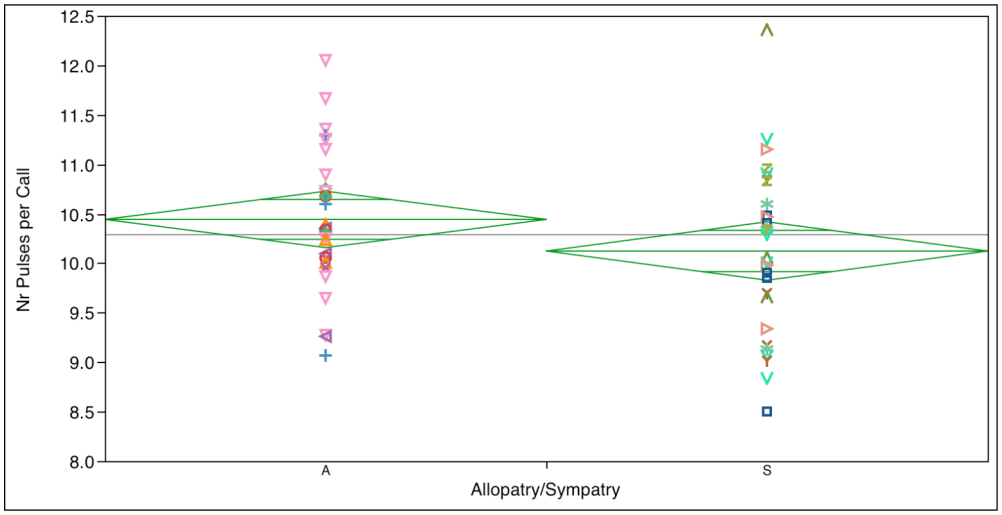
Advertisement Call Parameters acronyms: CD, Call Duration; PC, Number of Pulses per Call; PR, Pulse Rate; FF, Fundamental Frequency; DF, Dominant Frequency and ADF, difference of amplitude between dominant and fundamental frequencies.

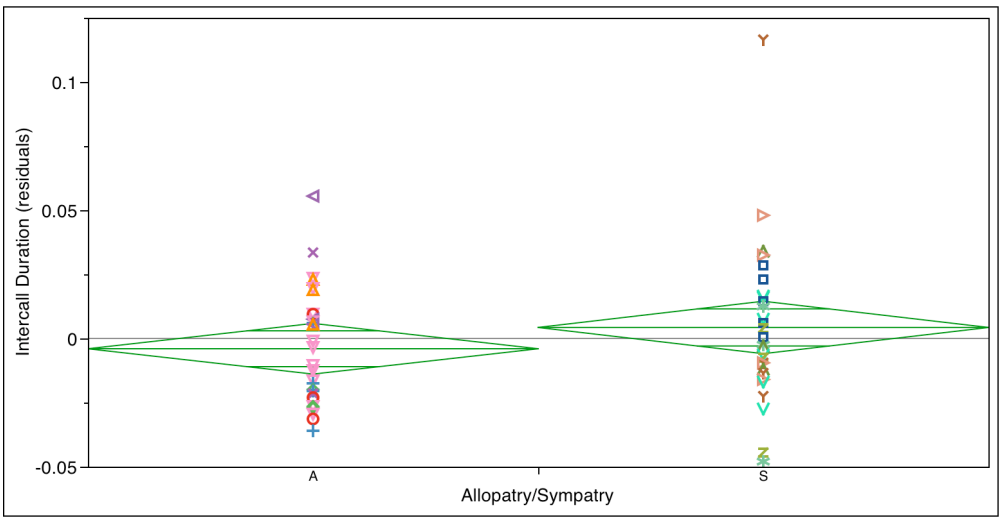
Appendix 6. Allopatry versus Sympatry Call Parameters distribution in *Hyla arborea* sampled populations in Portugal

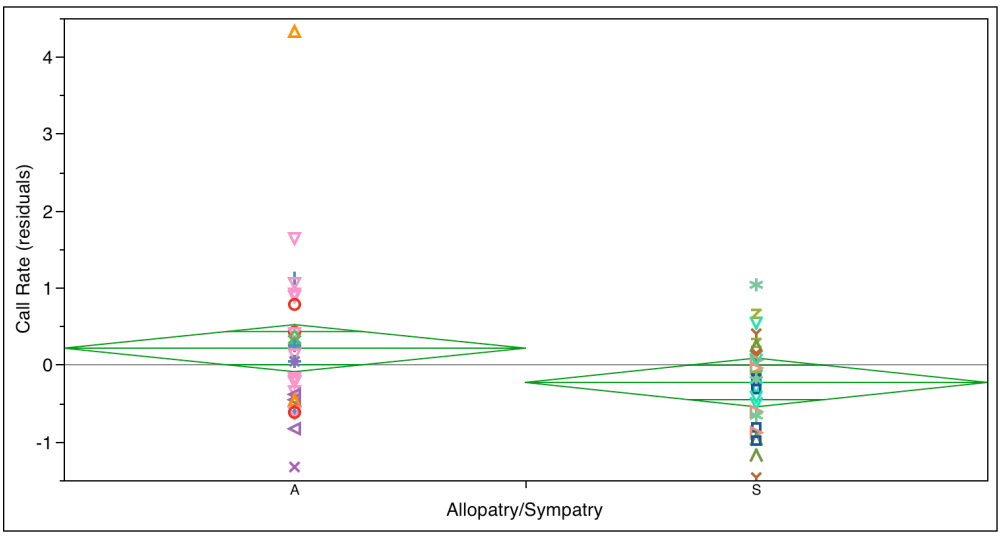


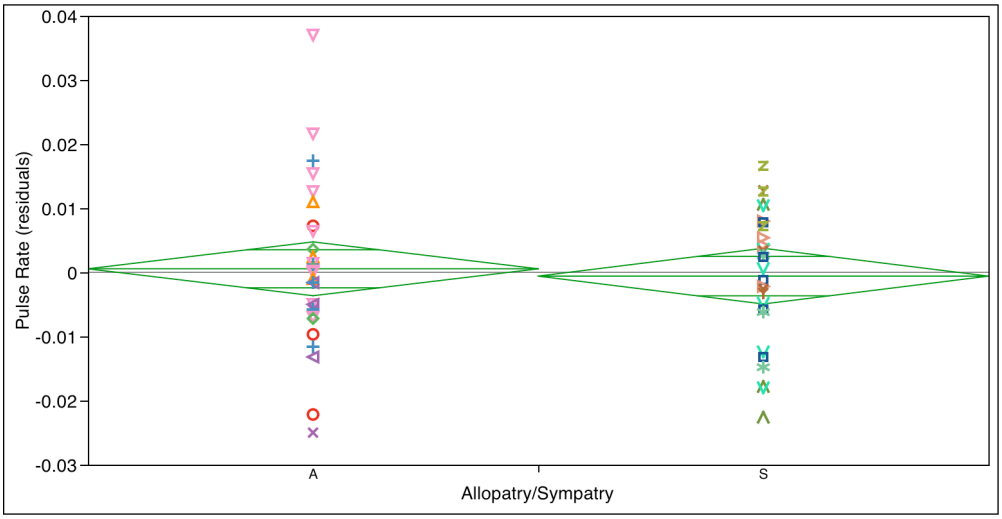


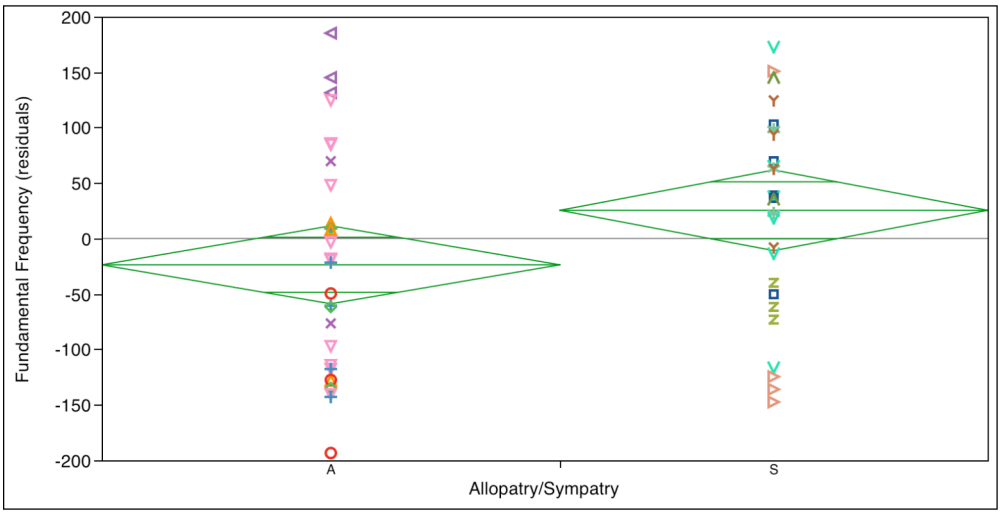


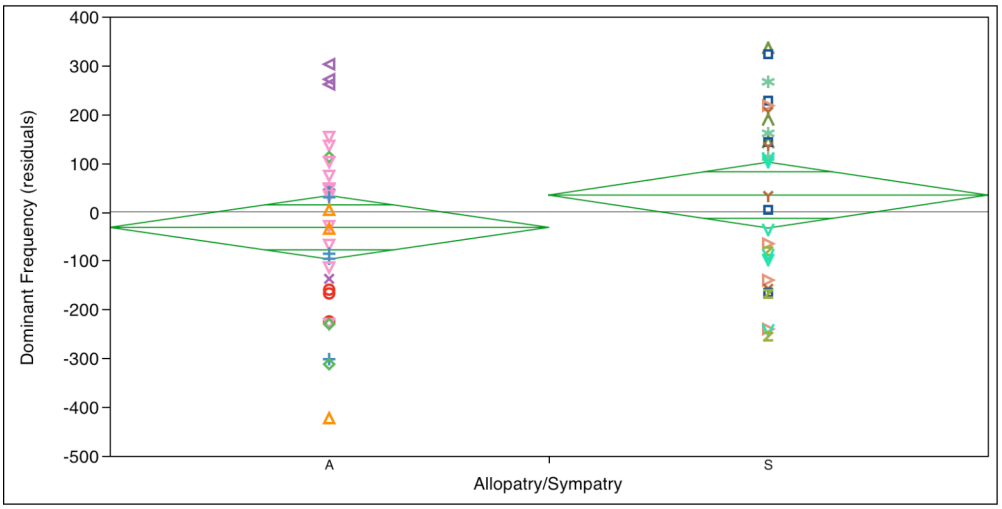

Nr Pulses per Call
12.5
12.0
11.5
11.0
10.5
10.0
9.5
9.0
8.5
8.0
A
S
Allopatry/Sympatry

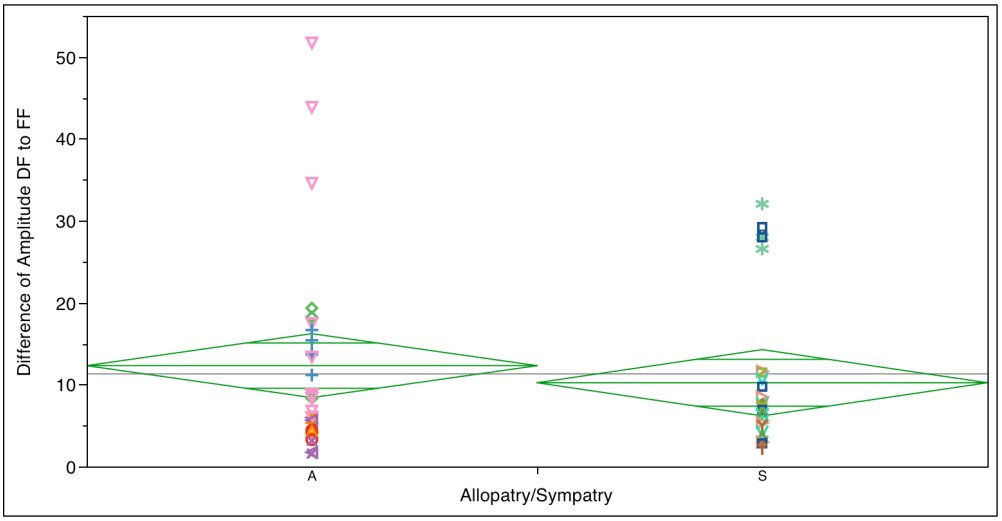

Intercall Duration (residuals)
0.1
0.05
0
-0.05
A
S
Allopatry/Sympatry


Call Rate (residuals)
4
3
2
1
0
-1
A
S
Allopatry/Sympatry

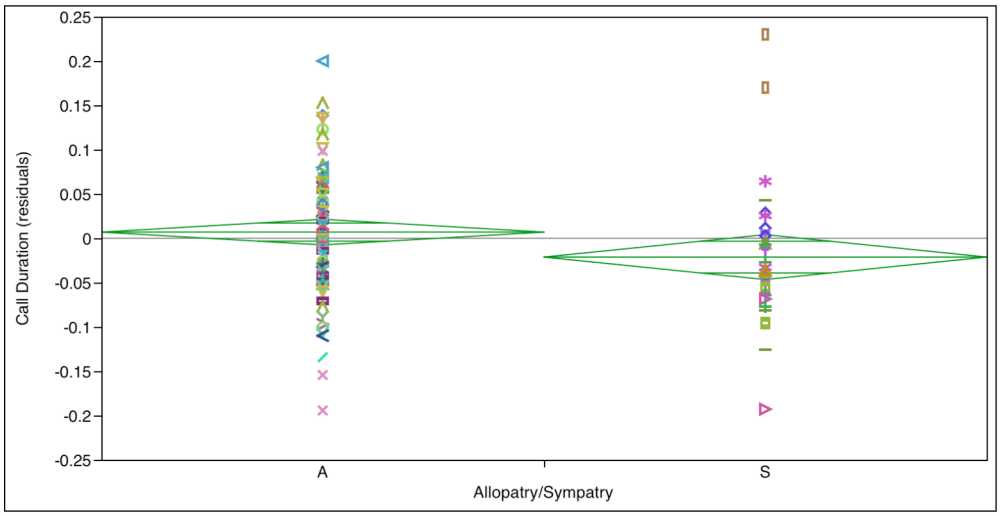

0.04
0.03
0.02
0.01
0
-0.01
-0.02
-0.03
Pulse Rate (residuals)
A
S
Allopatry/Sympatry

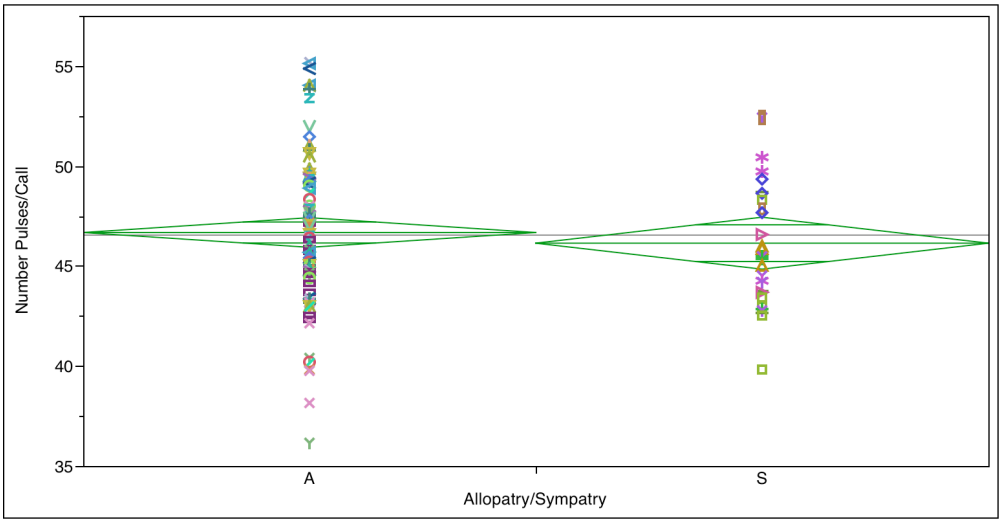

200
150
100
50
0
-50
-100
-150
-200
Fundamental Frequency (residuals)
A
S
Allopatry/Sympatry

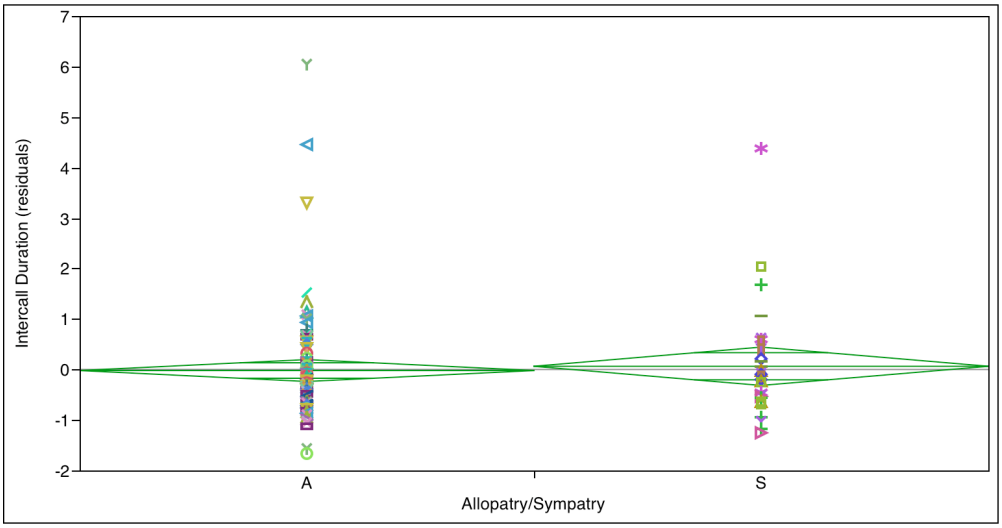

400
300
200
100
0
-100
-200
-300
-400
-500
Dominant Frequency (residuals)
A
S
Allopatry/Sympatry

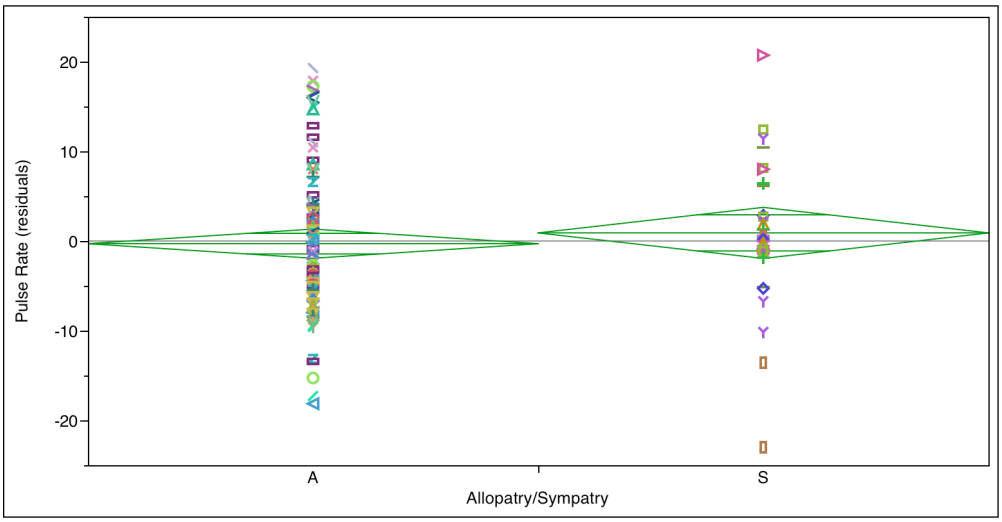

Difference of Amplitude DF to FF
50
40
30
20
10
0
A
S
Allopatry/Sympatry

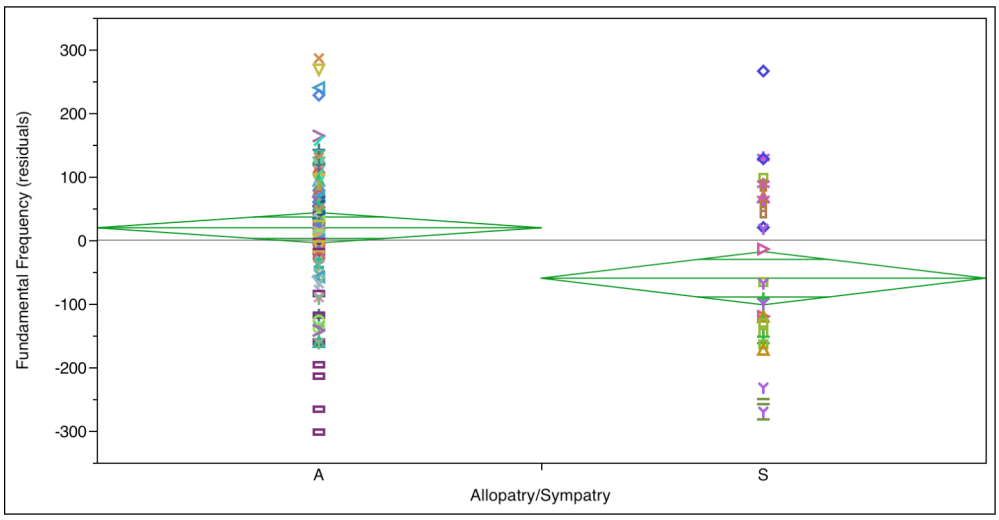
Appendix 7. Allopatry versus Sympatry call parameters distribution in *Hyla meridionalis* sampled populations in Portugal

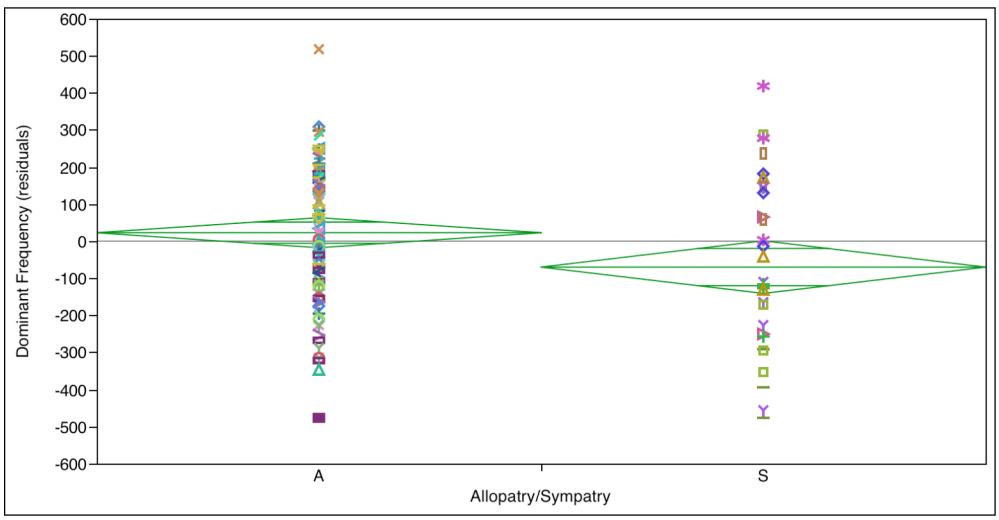


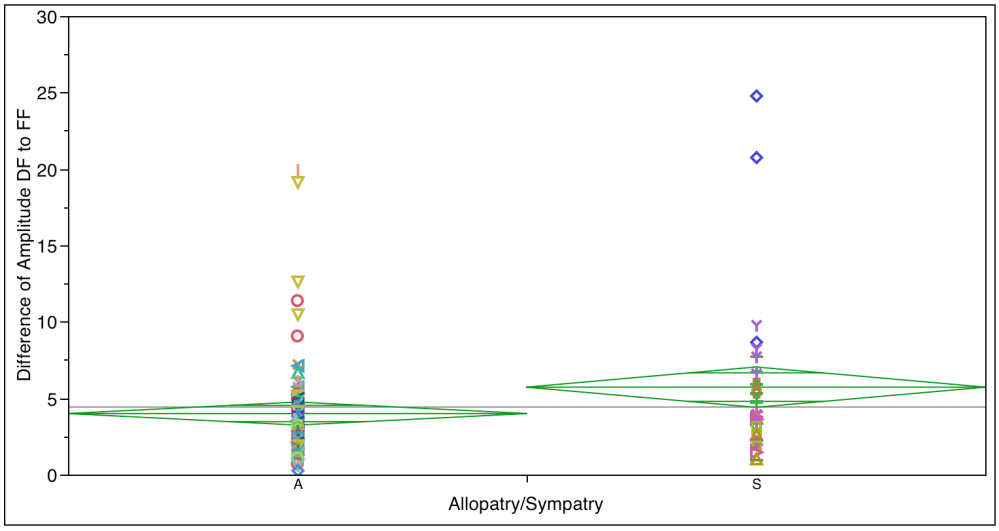





Pulse Rate (residuals)
20
10
0
-10
-20
A
S
Allopatry/Sympatry


Fundamental Frequency (residuals)
300
200
100
0
-100
-200
-300
A
S
Allopatry/Sympatry


Dominant Frequency (residuals)
600
500
400
300
200
100
0
-100
-200
-300
-400
-500
-600
A
S
Allopatry/Sympatry


Difference of Amplitude DF to FF
30
25
20
15
10
5
0
A
S
Allopatry/Sympatry

Agora é que está mesmo quase, quase…