Faculdade de Ciências da Universidade do Porto

Tese de Mestrado em Ecologia Aplicada

Study of the Marbled Newt (*Triturus* (*m.*) *marmoratus* and *T.* (m.) *pygmaeus*) hybrid zone by genetic markers

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Resumo

O tritão-marmorado, *Triturus marmoratus*, distribui-se aproximadamente desde Paris no centro de França até Gibraltar no Sul de Espanha. As duas subespécies reconhecidas, *T. (m.) marmoratus* e *T. (m.) pygmaeus*, são morfológica e geneticamente distintas. A sua distribuição é essencialmente parapátrica com uma zona de contacto que se estende ao longo do Sistema Central espanhol, chegando ao Tejo no centro de Portugal e prolongando-se para Norte ao longo da costa portuguesa até Aveiro. Quarenta e seis populações em cinco transectos que atravessam a zona de contacto, um dos quais em Espanha e quatro em Portugal, foram estudados para um marcador genético citoplasmático (o gene mitocondrial ND4) e quatro marcadores nucleares parcialmente diagnosticantes (as enzimas Pep A, Pep B, Pep D e Ldh2). Em áreas onde foi observada a ocorrência sintópica de *T. (m.) marmoratus* e *T. (m.) pygmaeus* também foi documentada a presença de híbridos F1 ou resultantes de retrocruzamentos e introgressão, mostrando que as duas formas não estão reprodutivamente isoladas. A modelação espacial, através de um Sistema de Informação Geográfica, de dados de distribuição geográfica e treze variáveis ecológicas seleccionadas criou um modelo com um bom ajuste preditivo. Esse modelo prevê a presença de um enclave de *T. (m.) marmoratus* na área de Caldas da Rainha no centro-oeste de Portugal, rodeado por *T. (m.) pygmaeus*. Uma análise detalhada nesta zona mostrou a presença de seis populações de *T. (m.) marmoratus* numa bolsa rodeada por 14 populações de *T. (m.) pygmaeus*, apoiando a validade do modelo. Os resultados obtidos parecem indicar que *T. (m.) pygmaeus* está a expandir a sua área de distribuição sobrepondo-se a *T. (m.) marmoratus*.

Abstract

The Marbled newt, *Triturus marmoratus*, has a distribution that goes from Paris in the centre of France to Gibraltar in the South of Spain. The two known subspecies, *T. (m.) marmoratus* and *T. (m.) pygmaeus*, are morphologic and genetically distinct. Their distribution is essentially parapatric with a contact zone running along the Spanish Sistema Central, reaching the Tejo in the centre of Portugal and prolonging itself to North along the Portuguese coast up to Aveiro. Forty six populations in five transects that cross the contact zone, one in Spain and the other in Portugal, were studied for a cytoplasmic marker (the mitochondrial gene ND4) and four partially diagnostic nuclear markers (enzymes Pep A, Pep B, Pep D and Ldh 2). In areas where the syntopic occurrence of *T. (m.) marmoratus* and *T. (m.) pygmaeus* was observed, F1 hybrids were documented, as well as ones resulting from backcrosses and introgression, showing that the two forms are not reproductively isolated. Spatial modelling, through a Geographical Information System, of distributional data and thirteen selected ecological variables created a model with good predictive power. This model predicted the existence of a *T. (m.) marmoratus* enclave in the western part of central Portugal, surrounded by *T. (m.) pygmaeus*. A detailed analysis in this area showed the presence of six *T. (m.) marmoratus* populations in a pocket surrounded by 14 *T. (m.) pygmaeus* populations, supporting the validity of the model. These results seem to indicate that *T. (m.) pygmaeus* is expanding its distribution range superseding *T. (m.) marmoratus*.

Résume

Le Triton marbré, *Triturus marmoratus*, a une distribution qui va de Paris au centre de France au Gibraltar dan le Sud de l'Espagne. Le deux sous-espèces connues, *T. (m.) marmoratus* et *T. (m.) pygmaeus*, sont morphologique et génétiquement distinct. Leur distribution est essentiellement parapatrique avec une zone de contacte qui va du Sistema Central espagnol, continues au longue du Tejo en le centre de Portugal et se prolonge au nord au long de la côte portugaise jusqu'à Aveiro. Quarante six populations dans cinq transects qui croisent la zone de contact, un en Espagne et les autres en Portugal, ont eté étudiés pour un marqueur cytoplasmique (le gène mitochondrial ND4) et quatre marqueurs partiellement diagnostiques (les enzymes Pep A, Pep B, Pep D et Ldh 2). Dans les secteurs où on a observé l'occurrence syntopique de *T. (m.) marmoratus* et de *T. (m.) pygmaeus*, hybrides F1 ont été documentés, aussi bien que ceux résultants des rétro-croisements et de l'introgression, prouvant que que les deux formes ne sont pas isolées reproductifment. Un model spatial, élaboré par un Système de Information Géographique, des données distributionnelles et de treize variables écologique sélectionné à créé un modèle avec une bonne puissance prédictive. Ce modèle a prevú l'existence d'une enclave de *T. (m.) marmoratus* dans la région occidentale du Portugal central, entouré par *T. (m.) pygmaeus*. Une analyse détaillée dans ce secteur a montré la présence de six populations de *T. (m.) marmoratus* dans une poche entourée par 14 populations de *T. (m.) pygmaeus*, support la validité du modèle. Ces résultats semblent indiquer que *T. (m.) pygmaeus* augmente sa gamme de distribution remplaçant *T. (m.) marmoratus*.

Introduction

1. Speciation and Hybrid zones

Speciation still remains a mystery to science, despite the enormous advancement that took place since Darwin. Many species concepts have been formulated by several authors, with the Biological Species Concept (MAYR, 1963) accepted by most authors. This model is, however, quite restrictive if one considers the total inexistence of reproduction as an indispensable condition for the elevation to a species status. Other concepts, such as the Phylogenetic Species Concept (Hennig, 1966), have emerged and try to be of more practical use (see AVISE, 2000; WU, 2001). Most of these concepts, however, still consider natural hybridization as a "step back" in the speciation process. Some authors defend greater flexibility of the species concept that allows for the incorporation of natural hybridization as a significant mechanism in evolution. Hybridization here is taken as 'crosses between individuals belonging to reproductively isolated species' (ARNOLD, 1997).

Hybrid zones show remarkable variation patterns and are, therefore, naturally appealing areas for many different kinds of studies. Recent studies on hybrid zones allowed for an enormous advancement in our understanding of evolutionary processes (HARRISON, 1993). The two main models developed on which hybrid zones theory is based on (the 'clinal' and 'mosaic' models) present complementary arguments in the domains of population genetics and landscape ecology, respectively. The clinal model of hybrid zone structure has been described by the result of the conflict between the opposing forces of migration and disruptive selection. While migration tends to broaden the zone, selection will have the inverse effect (Barton and Hewit, 1985). The mosaic model, on the other hand, is characterized by a patchy spatial structure. These occur when *taxa* that differ in habitat preferences interact in a patchy environment, hybridizing in the boundaries between patch types. These two models are linked by some spatial scale parameter (ARNTZEN, 1996). By changing the spatial scale in which one studies hybrid zones, it is possible to obtain results that support one or the other model. So, by analyzing mixed-model hybrid zones (where characteristics of both models are present) we will improve our understanding of the evolution of species. Perhaps all hybrid zones have components of both models, but it is rare to find a spatial scale amenable for investigation.

2. *Triturus marmoratus* as a model

2.1. Amphibians

Amphibians in general have a high dependence on suitable reproduction sites, their populations are highly structured and their dispersal capability is very limited making them suitable to serve as model species.

2.2. The genus *Triturus*

The genus *Triturus* contains 12 species of the common European newts - small amphibians with long tail and elongated bodies - that belong to the family Salamandridae - and share similar courtship behaviour (ARNTZEN & SPARREBOOM, 1987). The monophyly of this group was never put into question until recent phylogenetic studies showed that this genus might actually be paraphyletic (TITUS & LARSON, 1995; ZAJC & ARNTZEN, 1999). Newts are usually found in ponds or brooks, though in fact, most species spend most of the year on land, returning to the water to mate every year.

2.3. The marbled newt (*Triturus marmoratus*)

Morphology

The marbled newt (*Triturus marmoratus*) is a big-bodied newt, similar in built to the newts of the crested newt group (superspecies *Triturus cristatus*), measuring up to 16 cm, and having a heavy built (Figure 1). The dorsal colouration is greenish with dark spots of variable size and number giving them their name, marbled. The belly has a colour that varies from white to black, with beige and grey as intermediate colours. It may present white and black spots that vary in size and number (FERRAND DE ALMEIDA *et al.*, 2001).

Sexual dimorphism is notorious in breeding season, in similarity with other members of this genus, when a dorsal crest with dark colour and green or yellowish bands grows on the males. Out of the breeding season, males are easily recognized by a white stripe on the tail. Throughout the year, females as well as juveniles, have a yellow or orange line in the middle of the dorsum, going from the head down to the tail. (see Figure 1). Females deposit their eggs individually on aquatic plants leaves, folding the leave in order to cover the egg.

Figure 1 - Photograph of a *Triturus marmoratus marmoratus* female.

Distribution

The *Triturus marmoratus* distribution area goes from the Southwest of France to the South of the Iberian Peninsula (Figure 2A).

Two forms of *Triturus marmoratus* are described. The nominatypical form, *T. (marmoratus) marmoratus*, occupies the Peninsula approximately north of the Tejo River and the mountains of the Sistema Central in Spain and the South of France. The South form, *T. (m.) pygmaeus* (Wolterstorff, 1908) occupies the South of the Peninsula and the Atlantic coast of Portugal up to Aveiro (Figure 2B).

These two forms were fundamentally distinguished on the basis of their ventral and dorsal colouration, shape of the dorsal crest present in the mature adults, body size and several osteological characteristics (GARCÍA-PARÍS *et al.*, 1993).

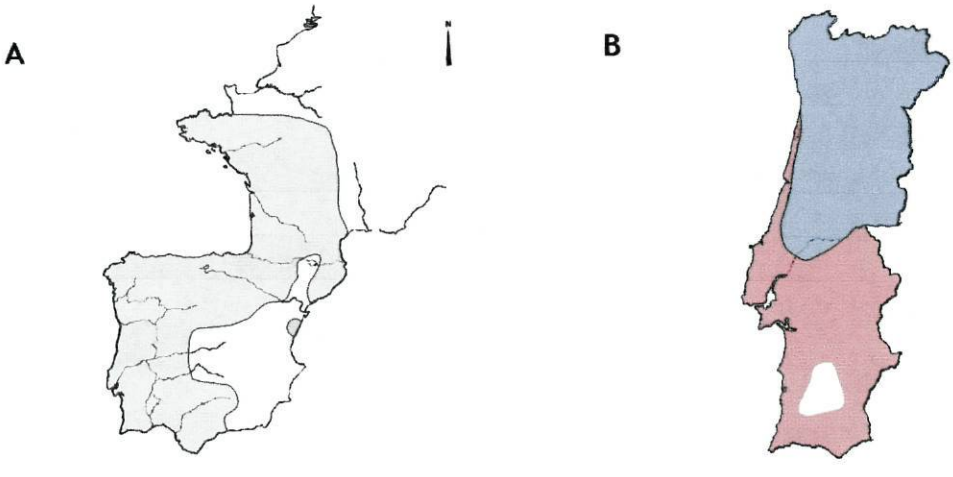

Figure 2 - **A**) Total distribution area of *Triturus marmoratus*; **B**) Distribution of the two described forms in Portugal: in blue, *T. (m.) marmoratus* and in red, *T. (m.) pygmaeus* (modified from FERRAND DE ALMEIDA *et al.*, 2001).

T. m. marmoratus / T. m. pygmaeus or *T. marmoratus / T. pygmaeus*?

The taxonomic status of these two forms is currently under debate, although recently, García-París and co-workers (1993, 2001) have (only apparently) solved this issue. On these papers, the authors claim that *T. m. pygmaeus* should be raised to the species status, based on the lack of evidence of natural hybridization along 300 km of a presumed contact zone in Spain and on the strict correspondence between morphological phenotypes and mitochondrial DNA haplotypes.

In Spain, the two forms are separated by the mountains of the Sistema Central, which prevents contact between the species, being *T. (m.) marmoratus* normally restricted to the Northern slopes and *T. (m.) pygmaeus* to the Southern slopes, except in the Sierra de Guadarrana and the Sierra de Gata. However, even on these areas, so far, no ponds were found were the two forms co-existed. The minimum distance between ponds described by the authors (6 km) exceeds the dispersal capabilities by generation of these amphibians.

Towards the west, i.e. in Portugal, the contact zone is of a different nature, without any obvious geographical barrier to the dispersal of individuals. Besides that, the density of populations is relatively high towards the Atlantic coast and diminishes gradually in the direction of the centre of the Peninsula. The contribution of this part of the contact zone can be determinant in the maintenance of gene flow between the two forms. This facts alone make that, before establishing the taxonomic status of these two forms, the occurrence (or not) of hybridization and introgression in Portugal should be verified, since there are conditions for the actual contact of individuals here.

Analysis of genetic variability, along with morphology data and the definition of ecological requirements of these two forms will allow confirming or rejecting the conclusions of our Spanish colleagues (GARCÍA-PARÍS *et al.*, 1993, 2001).

3. Genetic markers

Systematics and evolutionary biology have benefited a great deal with the development, over the last few decades, of molecular genetics techniques. Starch gel electrophoresis, introduced in the 60's, supplied an easy access tool, due to its low costs and to the great number of nuclear markers that can be studied. However, the phylogenetic relation between alleles is not evident (Avise, 2000). On the other hand, standardization of automatic sequencing of PCR-amplified DNA has largely increased the use of this technique in the domain of evolutionary biology and systematics. The two types of markers differ in the manner in which they are transmitted to the offspring. While the nuclear markers are diploid, mitochondrial DNA is haploid and maternally inherited. The comparison between nuclear vs. mitochondrial DNA will hopefully give us some insight on phenomenon like introgression and hybrid zone dynamics. Many studies of hybrid zones, including in amphibians and inclusively in the genus *Triturus*, have indicated the existence of asymmetric introgression (e.g. Arntzen & Wallis, 1991, 1999) or DNA capture (e.g. Babik et al, in prep; García-París *et al.*, 2003). This indicates that by only looking at one type of marker we might not understand completely the interactions that occur in hybrid zones.

The objectives of this work are to study the structure of the hybrid zone between *Triturus (marmoratus) marmoratus* and *T. (m.) pygmaeus*, to understand what factors are influencing this structure and whether some geographic structures (like the Tejo river) are barriers to gene flow between the taxa. Molecular markers will be used to compare different areas of the hybrid zone.

Methods

1. Sampling sites

Sampling sites were selected in a way that, in its whole, they form transects across the contact zone. Distribution of sample sites is biased towards this zone, that is, the density of sampled populations is greater in the centre of Portugal than in the North or the South (see Figure 3).

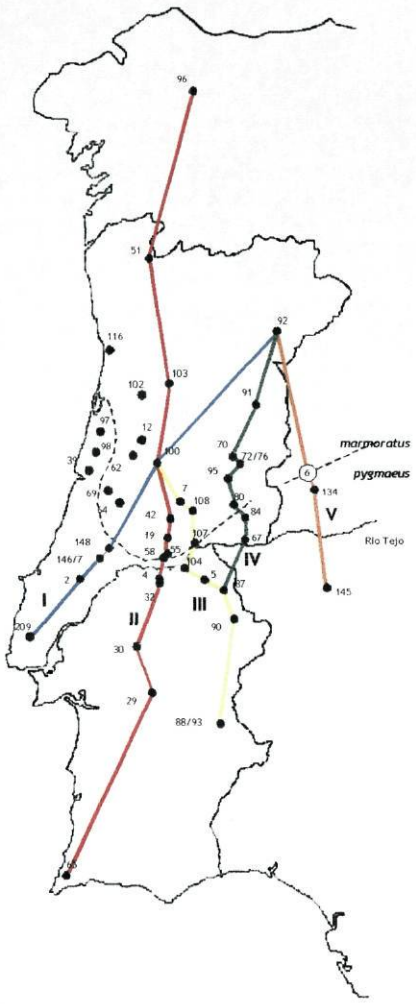

Figure 3 - Sample sites for *Triturus marmoratus*. Numbers indicate the localities sampled (see Appendix I); straight lines represent the transects; roman numerals represent the number of the transect; dashed line represents the centre of the contact zone; on transect V, a circle with the number six represents six localities marked with an asterisk on Appendix I.

Some populations had been previously sampled (ARNTZEN & FROUFE, unpublished) serving their results as a reference to this study.

Sampling was also performed on the coastal area, and particularly intensively between Leiria and Rio Maior, for in this area, characteristics of a clinal and a mosaic hybrid zones are supposed to be present. Namely, a *T. (m.) marmoratus* individual has been sited in an area south of a known population of *T. (m.) pygmaeus* (ARNTZEN, personal communication). Thirty two sites were visited in an area of approximately 1600 km^2 (see Figure 4).

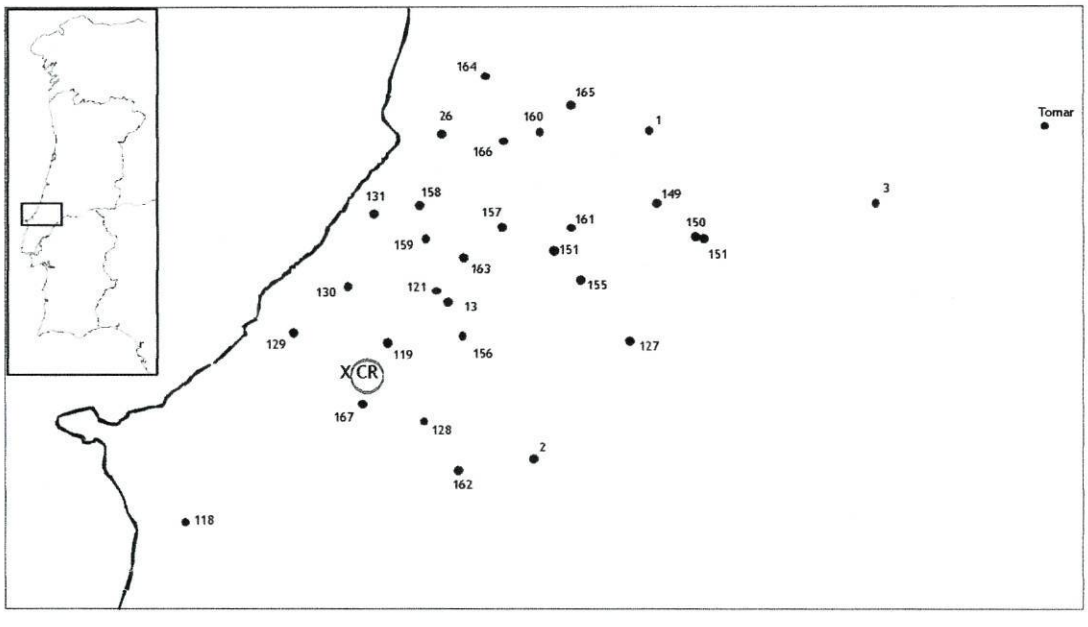

Figure 4 - Sample sites in the Caldas da Rainha area. Tomar population was included because it is the nearest known population of *T. (m.) marmoratus* (ARNTZEN, J.W. personal communication). Sampling area corresponds to the small rectangle on the upper left corner map.

On each visited site relevant geographical, biological and ecological information was registered. This information consisted on geographical coordinates, presence of other species and the ecological description of the ponds and surrounding landscape (see Appendix II). From this, we wished to retrieve information about any association between the distribution of newt taxa and ecological variables.

2. Capture methods

Adult animals and larvae were collected with the aid of dip-nets. In the area of greater sampling density, eggs were collected by scanning marginal vegetation, in a number no smaller than twenty, when possible. Even on these sites, when they were found, samples from adults were preferred over eggs. One of the nuclear markers (Ldh 2) could not be scored from eggs. Egg sampling allows for speedy sample collection from a great number of sites without significant harm to population survivorship. One female newt lays about 300 eggs per breeding season, resulting in a maximum of two breeding adults in the next generation. There is also a chromosomal length polymorphism that causes death to half the embryos of *T. marmoratus* and also the ones from a close species, *T. cristatus* (MACGREGOR *et al.*, 1990).

3. Sample conservation

Nine morphological and nine colouration characters were measured in every adult collected allowing the identification of each animal as being *marmoratus*-like or *pygmaeus*-like (ARNTZEN, personal communication - see Appendix III). After these measurements, the tip of the tail was clipped, promptly placed in *eppendorf* tubes, filled with a buffer solution (100 mM Tris, 1 mM EDTA, 0,05 mM NADP, pH corrected to 7,0 with HCl) and immersed on liquid nitrogen. Clipping allows for non-lethal sampling, and does not have grave consequences on the survival of the animal, because of the natural ability to regenerate the clipped tail (see Figure 5). After collecting the samples, animals were returned to the water. Larvae and eggs were collected whole and equally stored in liquid nitrogen. Samples were preserved in -80° C till the day they were analysed.

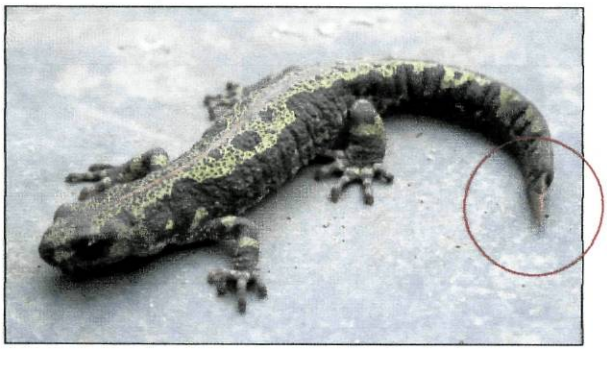

Figure 5 - *Triturus marmoratus* showing tail regeneration after a recent clipping.

4. Molecular and biochemical analysis

4.1. Allozymes

Sample preparation

A small portion of the samples was homogenised in a buffer (100 mM Tris, 1 mM EDTA, 0,05 mM NADP, pH corrected to 7,0 with HCl) and then submitted to ultrasounds in order to disrupt the cellular membrane and thus releasing the proteins into the buffer. These steps were performed on ice (0° to 4° C) to avoid protein degradation. Samples were then centrifuged for 15 minutes at 13 000 rpm (-4° C). Supernatant was decanted from each tube and submitted to a treatment with 2 mg of ditiotreitol (DTT) for an hour at 37° C (Ferrand, 1995).

Separation techniques

After a previous study (FROUFE *et al.*, unpublished) by protein electrophoresis (40 *loci*), four *loci*, peptidase A (*Pep A*), peptidase B (*Pep B*), peptidase D (*Pep D*) e lactate dehydrogenase 2 (*Ldh-2*), were selected on the basis of the amount of information that could be obtained from them (diagnostic power) and its practical usage (e.g. non-lethal sampling - some *loci* are not expressed on muscle tissue). High diagnostic power was also observed for the acid phosphatase *locus* (*Acp-1*). However, a technical problem in the consistent reading of the gels, forced the abandonment of this *locus*.

In order to study these diagnostic protein markers, conventional starch gel electrophoresis was performed on the three peptidases and isoelectric focusing on lactate dehydrogenase. Isoelectric focusing was preferred over starch gel electrophoresis for this *locus* because the capability to distinguish between alleles increased significantly by using this technique.

Conventional starch gel electrophoresis

For the separation of the peptidases the electrophoretic system developed by POVEY *et al.* (1972) and modified by FERRAND (1995) was used (Tris 0.1 M and $NaH_2PO_4.H_2O$ 0.1 M pH=7.6). The voltage used was 6.5 v/cm for 18 hours. Samples were applied to a starch gel (15% p/v) through small filter paper rectangles (6x8mm).

The gel was prepared by dissolving the starch in a buffer (Tris 0.01 M and $NaH_2PO_4.H_2O$ 0.01 M) for 15 to 20 minutes at 95° C.

Isoelectric focusing

As for *Ldh-2*, separation was accomplished in non-denaturing polyacrilamide gels. Gel ingredients are described in Table 1 following Pinho *et al.* (2003). Parameters used for separation are described in Table 2.

Table 1. Composition of the isoelectric focusing gel.

Ingredient	Quantity
Sucrose	2 g
Ready solution*	1.25 mL
Ampholines 5-8 (0,4 g/mL)	300 µL
Ampholines 6-8 (0,4 g/mL)	150 µL
Ampholines 7-9 (0,4 g/mL)	150 µL
TEMED (99% v/V)	10 µL
PSA (10 %)	70 µL
Distilled water	Up to 10 mL end volume

* Solution composed by Acrylamide (40%) and Bisacrylamide (3%)

Table 2. Electric parameters used in isoelectric focusing.

	Voltage (V)	Current (mA)	Power (W)	Time
Pre-focusing	1 500	25	1	30'
	1 500	25	2	15'
	1 500	25	3	15'
Focusing	1 500	25	4	1 h
	2 000	25	5	1 h
	2 500	25	6	1 h

Electrolytes solutions applied were L-aspartic acid 0.04 M (anode) and NaOH 1 M (cathode).

Detection techniques

For the detection of the different protein *loci*, several specific staining methods were used following Harris & Hopkinson (1976). Enzymatic activity was detected mixing the staining solution with previously heated agarose 1% (p/v). Then this mix was poured over the isoelectric focusing gels or over a slice of the starch gel. The gel was maintained at 37° C, till visualization of the bands was possible. Composition of the staining solutions is described on Tables 3 to 6.

Table 3. **Composition of the staining solution used to detect *PEP-A*.**

Ingredients	Quantity
Leucyl-Alanine	20 mg
MTT	14 mg
Snake Venom	4 mg
PMS	Traces
Buffer: Tris - HCl (Tris 6 g and HCl 37% 2,48 mL / 200 mL H_2O)	3 mL

Table 4. **Composition of the staining solution used to detect *PEP-B*.**

Ingredients	Quantity
Leucyl-glycyl-glycine	20 mg
Snake Venom	10 mg
Peroxidase	5 mg
3-amino-9-ethylcarbazole (in 1 mL Methanol)	10 mg
Buffer: Phosphate (KH_2PO_4 2.669 g/L and $Na_2HPO_4.2H_2O$ 14.327 g/L)	3 mL

Table 5. **Composition of the staining solution used to detect *PEP-D*.**

Ingredients	Quantity
Phenylalanine -Proline	20 mg
Snake Venom	10 mg
Peroxidase	5 mg
3-amino-9-ethylcarbazole (in 1 mL Methanol)	10 mg
Buffer: Phosphate (KH_2PO_4 2,669 g/L e $Na_2HPO_4.2H_2O$ 14,327 g/L)	3,5 mL

Table 6. **Composition of the staining solution used to detect *LDH-2*.**

Ingredients	Quantity
Calcium lactate	100 mg
NAD	7 mg
MTT	7 mg
Meldola	1 drop
Buffer: Tris - HCl (Tris 6 g and HCl 37% 2,48 mL / 200 mL H_2O)	3 mL

4.2. Mitochondrial DNA (mtDNA)

DNA extraction

Total DNA extraction was made according to the protocol originally described by Sambrook *et al.* (1989), with some modifications (see Appendix IV).

Amplification

Amplification of an mtDNA fragment through the PCR ("Polymerase chain reaction") technique was performed on *T3 Thermocycler* (Biometra), *My Cycler* (Biorad) e *PTC - 100* (MJ Research, Inc.) thermocyclers.

The amplified fragment was the terminal end of the mitochondrial gene that codes the subunit 4 of the NADH dehydrogenase (ND4). This fragment has a length of 905 base pairs (bp's) (see Figure 6). This mitochondrial genome region includes the end portion of the ND4 gene (751 bp's), the tRNA's for histamine, serine and leucyne.

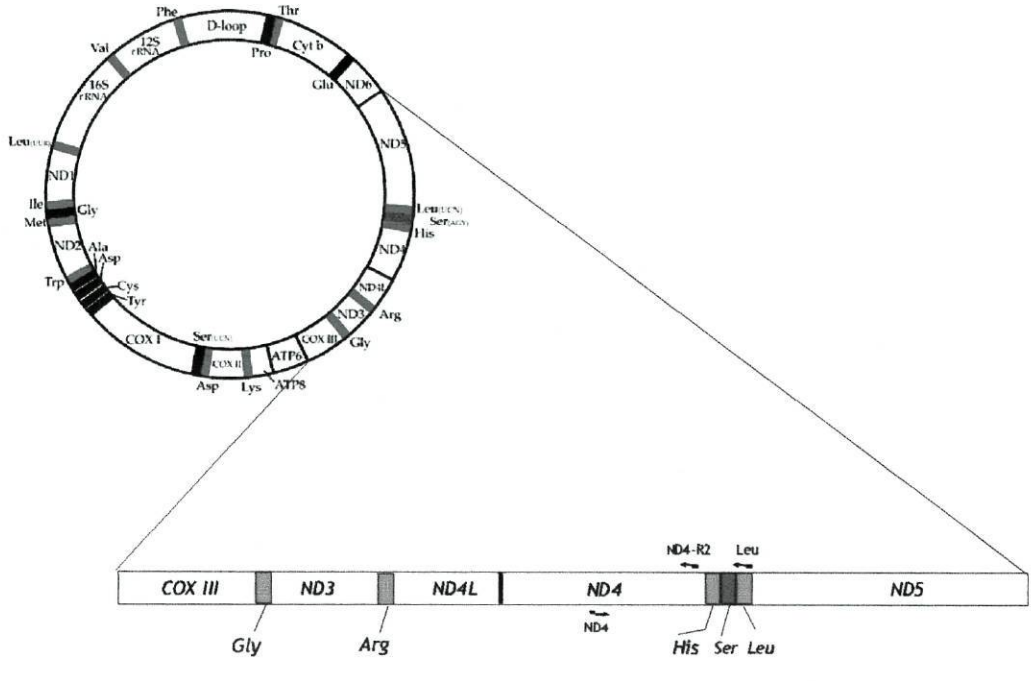

Figure 6 - Schematic representation of the studied fragment, its placement on the mtDNA molecule and indication of the relative position of the primers used for amplification.

The primers used to amplify the fragment are described in Table 7. Some initial results presented some ambiguities that lead us to the assumption that we were not obtaining pure mitochondrial sequences every time. Ambiguities in sequences can appear in nuclear DNA due to its diploid nature, but is only possible in mtDNA if there are different copies present, either by heteroplasmy or by the existence of mitochondrial-like copies in the nuclear genome. The analysis of these 'strange' sequences showed that there were no stop codons in the middle of the coding sequence. This would be a clear indicative that we were not in the presence of a mitochondrial sequence. However, these sequences possessed a higher g-content on the third codon position (ca. 15% against 10% in other sequences), characteristic of nuclear pseudogenes.

An RFLP-based analysis was developed, in order to recognize if more than one copy was present in the PCR products. By comparing restriction profiles between 'normal' and 'strange' sequences, the enzyme Eco24 I (recognition sequence: 5' GpuGCpy'C 3') was selected because it has two recognition sites in the "strange" sequence and only one in the normal one what would, theoretically, enable us to distinguish between the two. However, this trial did not yield any informative result. Some other alternatives could have been used to determine whether we were facing more than one mtDNA-like sequence: for example, single strand conformation polymorphism (SSCP) or cloning and sequencing of PCR products. This later option was too time consuming to execute in useful time and departed from the goals of this work.

Instead, we chose to avoid the pseudogenes. Again, there were several options. The mtDNA could be isolated from the remaining DNA by specific purification methods; or by RT-PCR. In our case, a phylogenetic reconstruction showed that the 'strange' sequences formed a well separated monophyletic group, appearing in the base of the tree, and so we took a simpler solution, by designing a specific primer for our mtDNA sequence (primer ND4-R2, see table 7). From the total of 137 sequences obtained, about 10 had to be repeated with this new primer. All of them were from *T. (m.) pygmaeus* populations close to the contact zone. However, an undetermined number of *T. (m.) pygmaeus* samples failed to amplify any product with the original set of primers, and we do not know if this relates to the pseudogene or not. Unfortunately, there was no time to test if the new primer worked for these samples.

Table 7 - List of primers used in the amplification and sequencing of the marbled newt's mtDNA (*Triturus marmoratus*).

Primer	Sequence (5' - 3')	Reference
ND4	CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC	Arévalo *et al.*, 1994.
Leu	CAT TAC TTT TAC TTG GAT TTG CAC CA	Arévalo *et al.*, 1994.
ND4 - R2	CCC TGA AAT AAG AGA GGG TTT AA	Developed in this study

Table 8 - Reagents and program used in the thermocycler for the amplification of the ND4 fragment of the Marbled newt's mtDNA.

Reagents	Quantity (µL)	Step	Time	Temperature	Cycles
Taq Polymerase (*EcoTaq*) (5u/ µL)	0,2				
Buffer (10x)	2,5	Separation	3'	94° C	1
$MgCl_2$ (50 mM)	3,0	Separation	30''	94° C	
Primer ND4 (10 µM)	0,8	Annealing	30''	56° C	30
Primer Leu (10 µM)	0,8	Synthesis	45''	72° C	
dNTP's (40 µM)	1,0	Synthesis	4'	72° C	1
Sample	1,0				
H_2O	15,7				

In Table 8 composition of the reaction and the program used in the amplification of the ND4 fragments on *T. marmoratus* samples is described.

After the reaction, amplification products were subjected to an electrophoretic run in agarose gels (2% p/v). In this electrophoresis a molecular weight marker was used (*Marker 5*, Eurogentec) to verify the amplified DNA fragments size. After electrophoresis, gels were stained with an ethidium bromide solution (0.05%, v/v) and DNA bands were observed under ultraviolet light.

Sequencing

Previously to sequencing, a purification of the PCR products was done using the enzymatic method, with exonuclease and alkaline phosphatase (*USB Corp.* - see Appendix V). Sequencing was done commercially in MWG (Germany), Macrogen (Korea) e Stabvida (Portugal) using the primers ND4 and Leu (or, in some samples, primer ND4-R2). Electrophoresis of the sequenced products was made in an automated sequencer *ABI Prism* 310 (Perkin-Elmer - Applied Biosystems, Inc.). Detailed protocol of these procedures is described in Appendix VI.

5. Data analysis

5.1. Allozymes

Allelic frequencies

For each *locus*, allelic frequencies were calculated using the program *GENETIX* v. 4.04 (BELKHIR *et al.*, 1996). Nei's unbiased genetic distance (NEI, 1978) between pairs of populations was calculated using the program *Biosys-1* (SWOFFORD & SELANDER, 1989). A Neighbor-Joining tree was built using the programs included in the *PHYLIP* v.3.6 software package (FELSENSTEIN, 1989). To evaluate the confidence in the tree, bootstraps were calculated from 1000 replicates (FELSENSTEIN, 1985).

Hardy-Weinberg equilibrium

The program *GENEPOP* (v 3.4) (RAYMOND & ROUSSET, 1995) was used to perform a Hardy-Weinberg exact test, following the Markov chain method. Fisher's exact tests to detect excess or deficit of heterozygotes were also performed using this program (ROUSSET & RAYMOND, 1995).

Population differentiation

Wright's (1978) F-statistics were calculated according to Weir & Cockerham (1984) and tested for significance using the program *Fstat* (Goudet, 2001). Three parameters were calculated: F_{is}, F_{st} e F_{it}. F_{is} measures reductions in heterozygosity (comparing expected and observed heterozygosity) due to non-random mating inside a subpopulation. F_{st} measures loss of heterozygosity due to population subdivision (i.e. the magnitude of heterozygosity when compared to total expected in a subset of populations with average heterozygosity found in each of these populations). F_{it} measures heterozygosity loss due to both effects.

Diversity measures

Average expected heterozygosity and average number of alleles per *locus* were determined by the program *GENETIX* v. 4.02.

Spatial analysis of the Caldas da Rainha zone

After determining allelic frequencies in the populations of the Caldas da Rainha region (see Appendix VIII), the form to which each population belonged was determined, assuming that the two forms are mutually exclusive. Following this determination, the program *PASSAGE* v.1.1 (ROSENBERG, 2001) was used to determine the area corresponding to each form by Voronoi polygons. Voronoi polygons create a cell around each point representing a population. The cell represents the area that is closest to that point than to any other point. Adjacent polygons belonging to the same form were merged into a single polygon.

5.2. mtDNA

Sequences were checked manually using the program Bioedit v. 5.0.9 (HALL, 1999), to correct any reading errors that the sequencer software eventually made. The obtained sequences were automatically aligned using the program Clustal W (THOMPSON et al., 1994), along with a mtDNA sequence of another Salamander, Luschan's salamander *Mertensiella luschani* (access number NC_002756) drawn from *GenBank* (http://www.ncbi.nlm.nih.gov/Genbank/index.html), serving this sequence as an outgroup to *T. marmoratus* sequences. Manual adjustments were made to maximize sequence correspondence.

The clustering method used in the phylogenetic analysis was the *Neighbor-joining* (NJ; SAITOU & NEI, 1987) constructed on the basis of a distance matrix between OTU's, calculated by the Tamura-Nei method (1993). To evaluate confidence on the tree, a bootstrap test was made with a 1000 replicates (FELSENSTEIN, 1985). These calculations were made in the program MEGA v. 2.1 (KUMAR *et al.*, 2001). *T. marmoratus* haplotype genealogy network was constructed with *Network* v.4.0.0.0 (BANDELT *et al.*, 1999).

Results

1. Allozymes

Of the 79 populations studied, 27 are *marmoratus*-like, 37 *pygmaeus*-like and 15 appear to be of mixed origin based on their morphological characters. One thousand three hundred and ten (1310) individuals were genetically analysed.

1.1. Polymorphism detection

In Figures 7 through 10, schematic representations of the polymorphisms detected for the four studied *loci* are represented.

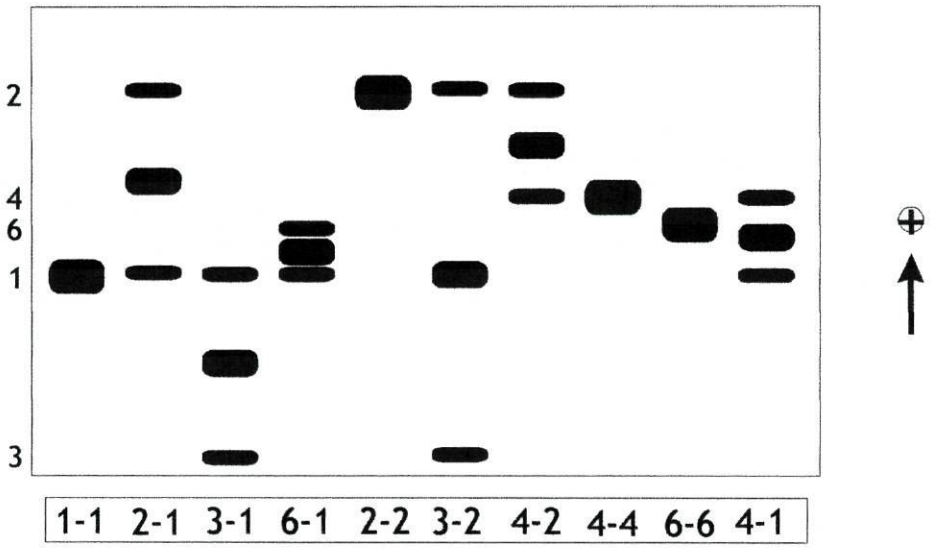

Figure 7 - Schematic representation of the polymorphism detected for the *locus* PEP-A. Horizontal legend refers to genotype, while vertical legend refers to alleles.

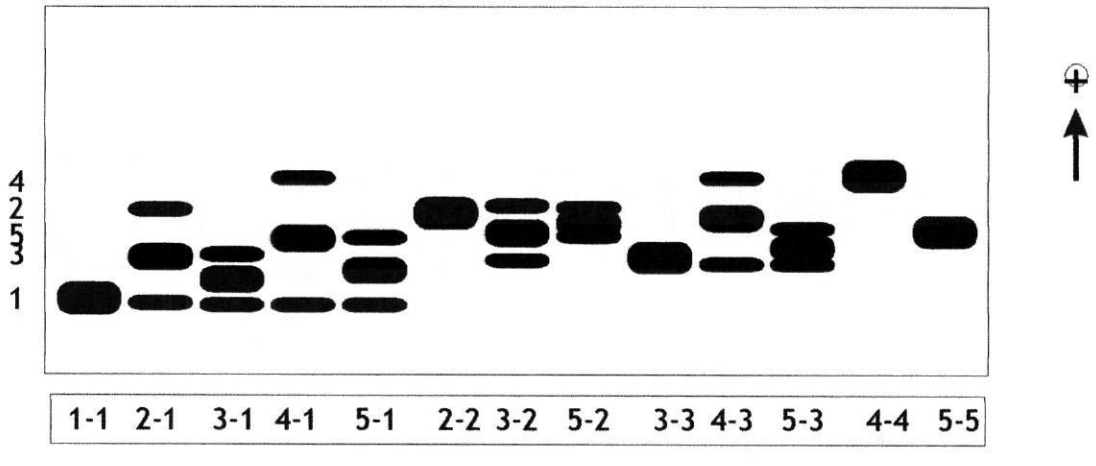

Figure 8 - Schematic representation of the polymorphism detected for the *locus* PEP-B. Horizontal legend refers to genotype, while vertical legend refers to alleles.

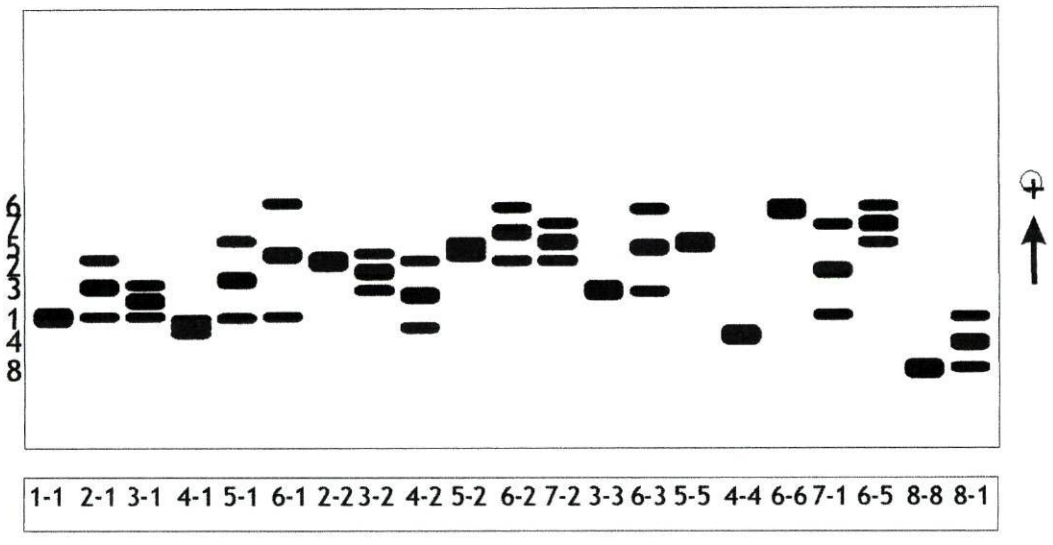

Figure 9 - Schematic representation of the polymorphism detected for the *locus* PEP-D. Horizontal legend refers to genotype, while vertical legend refers to alleles.

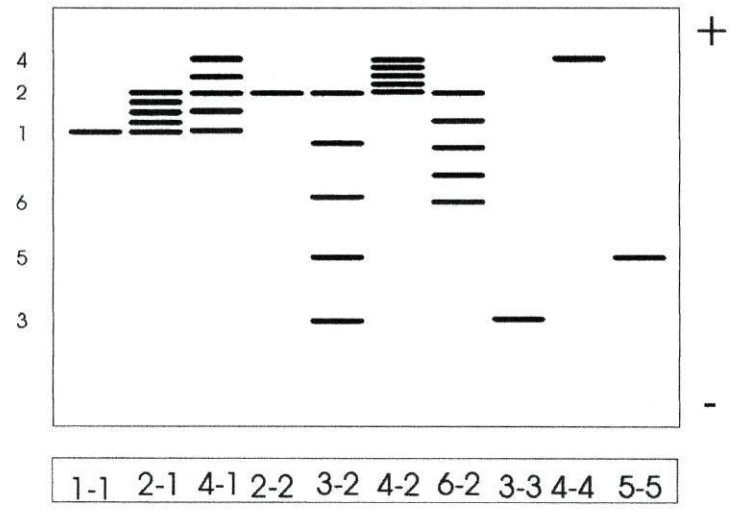

Figure 10 - Schematic representation of the polymorphism detected for the *locus* LDH-2. Horizontal legend refers to genotype, while vertical legend refers to alleles.

1.2. Population analysis *locus* by *locus*

The allelic frequencies found for each population and *loci are* given in Appendix VIII. Figure 11 represents allelic frequencies along the five transects for the four protein markers used. In Appendix IX results for the analysis on deviations to Hardy-Weinberg expectations is provided.

PEP-A

This marker is the most diagnostic of the protein markers used. In the contact zone, there is a sharp transition of the frequency of the two most common alleles, PEP-A*2 (the most common in *T. (m.) marmoratus* populations) and PEP-A*1 (most common in *T. (m.) pygmaeus* populations. A rare allele, PEP-A*4, is exclusive to the hybrid zone. Other rare alleles, PEP-A*3, PEP-A*5 e PEP-A*6, occur in some populations. PEP-A*5 did not occur on any other population after being discovered on Vila do Bispo - Sagres and the new allele, PEP-A*6, is private to Velada.

Significant deviations from Hardy-Weinberg expectation were found in 6 out of 79 populations (see Appendix IX).

PEP-B

In the reference transect (transect II), alleles PEP-B*1 and PEP-B *2 are the most frequent in *T. (m.) pygmaeus* populations, whereas PEP-B*3 is more frequent in *T. (m.) marmoratus* populations, decreasing its frequency gradually towards the South. However, with the results from the remaining populations it was realized that these alleles have a much wider geographical range.

A rare allele, PEP-B*4, appears in low frequency in four populations. The allele, PEP-B*5, appears only in Mora, Zebreira and Velada.

Significant deviations to Hardy-Weinberg expectations were found in thirteen out of 79 populations (see Appendix IX).

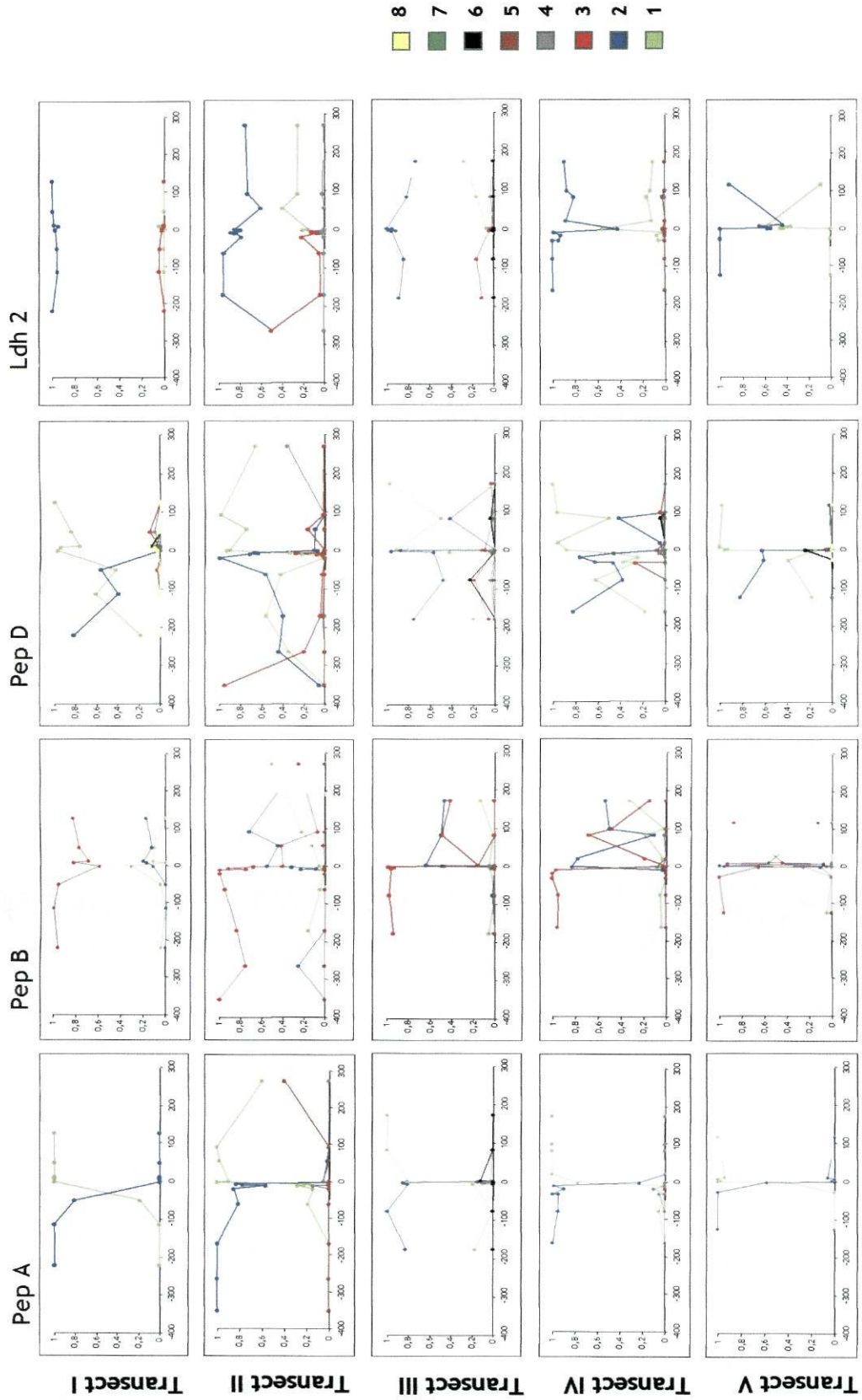

Figure 11 - Allelic frequencies over the five transects (N-S) for the four protein markers. Numbers under graphs correspond to sample sites as indicated in Appendix I. Allele numbers are indicated on the right.

PEP-D

In *T. (m.) marmoratus* populations, PEP-D*2 e PEP-D*3 are the most frequent, while PEP-D*1 is more frequent in *T. (m.) pygmaeus* populations. These alleles are not, however, exclusive to one or the other form, and in fact, occurred in high frequencies in some cases, in populations of the other form. Allele PEP-D *1 occurred in high frequencies in populations well distanced from the contact zone (see Appendix VIII).

Several rare alleles were found. Despite that PEP-D*4 occurred in high frequencies in Vila do Bispo - Sagres, in the other populations it occurs only in low frequencies. The new alleles, PEP-D*5 e PEP-D*6, appear restricted to the coastal zone (however they appear in three populations of transect IV - see Appendix VIII). As for the also new alleles, PEP-D*7 e PEP-D*8, they were only found in a few populations. All the samples that possess PEP-A*6 (except one individual) e PEP-D*7 are larvae or eggs, that suggests that there might be a differential expression between adults and non-adults (results not shown).

In this marker, 19 populations out of 79 show significant deviation from Hardy-Weinberg expectations (see Appendix IX).

LDH-2

In this marker, LDH-2*2 is the most frequent over the whole distribution area. The second most frequent allele (LDH-2*1 e LDH-2*3) in each population is characteristic for each form. LDH-2*3 is present in *T. (m.) marmoratus* and LDH-2*1 in *T. (m.) pygmaeus*. In the contact zone, both alleles LDH-2*1 and LDH-2*3 appear in the same populations. Other than these alleles, three rare alleles occur: LDH-2*4 is present in six populations; LDH-2*5 private to Fonte da Pena da Couvinha and LDH-2*6 private to Pedra do Altar. Once again, significant deviations to Hardy-Weinberg expectations were found in four out of 79 populations (see Appendix IX).

A more powerful analysis revealed that most cases of Hardy-Weinberg disequilibrium were due to heterozygote deficit. However, some exceptions occurred. In Soure and Casais dos Morgados disequilibrium detected for PEP-D appears to be due to an excess of heterozygotes instead.

1.3. Global analysis

Genetic distances

The tables in Appendix X show a matrix of the genetic distances between populations of each of the five transects calculated according to Nei (1978). Diversity measures (heterozygosity and average number of alleles per locus) are summarized in Appendix XI. Figure 12 shows the result of the phylogeny reconstruction by the Neighbor Joining method.

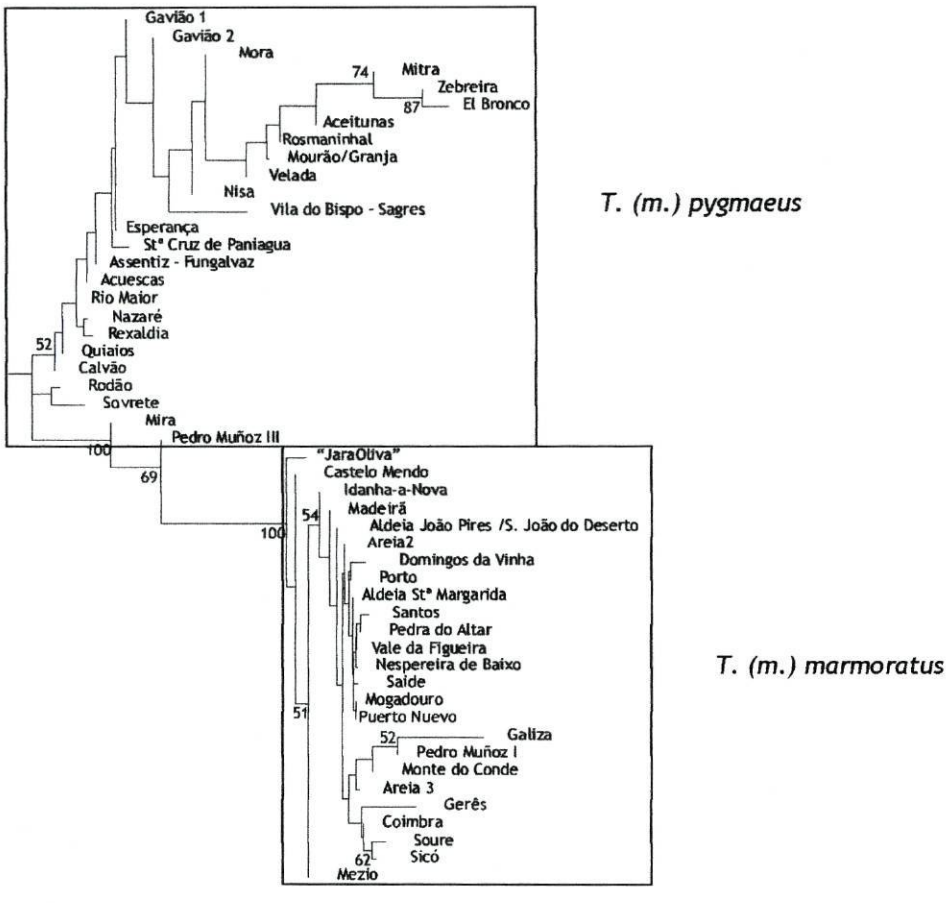

Figure 12 - Phylogenetic *Neighbor Joining* tree built with the allozyme data. The root of the tree was placed in the middle of the tree due to the impossibility to select an outgroup. Bootstrap values over 50 are shown next to the nodes.

The *Neighbor-Joining* tree clearly shows two groups with high bootstrap support corresponding to *T. (m.) marmoratus* and *T. (m.) pygmaeus*. Within each group, we found little support for any sub-structuring.

Population differentiation

Table 9 shows Wright's (1978) F-statistics values obtained for each marker in all populations studied. A sharp transition is observed between *T. (m.) marmoratus* and *T. (m.) pygmaeus* alleles (Fig. 11). There is also a pronounced population subdivision, as demonstrated by F-statistics. High values of these parameters are mostly due to one of the markers (Pep-A) whose F_{st} value (0.7773) is much higher than the global *loci* average (0.4876). If we take the F_{st} parameter for the two forms separately, we would see that its values are much lower (results not shown). These results are in agreement with the ones in the previous study that lead to choosing these markers (Arntzen *et al*, unpublished).

Table 9 - F_{is}, F_{it} and F_{st} values for all studied *loci* in all populations of *T. marmoratus*.

Locus	F_{is}	F_{it}	F_{st}
PEP-A	0,1434	0,8093	0,7773
PEP-B	0,2345	0,5118	0,3623
PEP-D	0,1752	0,5649	0,4725
LDH-2	0,0613	0,2688	0,2211
All	**0,1602**	**0,5697**	**0,4876**

All values are significant ($p<0.001$)

1.4 Detailed spatial analysis in the Caldas da Rainha area

Figure 13 - Distribution of *T. (m.) marmoratus* (striped) and *T. (m.) pygmaeus* (not striped) in the western part of Portugal, represented by Voronoi polygons. Note the presence of a *T.*

(m.) marmoratus enclave in the Caldas da Rainha Area (CR). VF - Valado dos Frades; PM - Porto de Mós.

The peptidases (A, B and D) were studied in the samples from the Caldas da Rainha area populations in a similar fashion as the populations in the five transects. It was not possible to detect Lactate dehydrogenase (LDH-2) from eggs. Allele frequencies are described in Appendix VIII. Hardy-Weinberg test results are shown in Appendix IX.

After assigning each population to a subspecies, results obtained from Voronoi polygons indicate the existence of a *T. (m.) marmoratus* enclave on the surrounding region to Caldas da Rainha and possibly another enclave further North in between Valado dos Frades and Porto de Mós (Figure 13).

2. Mitochondrial DNA

One hundred and thirty seven sequences were obtained from the amplification and sequencing of a fragment of the *ND4* mitochondrial gene. From these, 107 are *T. (m.) marmoratus* samples and the remaining *T. (m.) pygmaeus*. These sequences are aligned with positions 10811 and 11715 (904 base pairs) of the mitochondrial genome of the salamander, *Mertensiella luschani* (Zardoya & Meyer, 2001) and include the terminal end fragment of *ND4*, the Histamine tRNA, tRNA for Serine and for Leucyne. Thirty two haplotypes were found. Appendix VII presents a table with the variable positions of these sequences.

Appendix XII presents the nucleotide level divergence percentages between the defined haplotypes (see Appendix VII) used for the *Triturus marmoratus* phylogeny reconstruction. Average difference between *T. (m.) pygmaeus* and *T. (m.) marmoratus* samples is about 5%, while within each form differences are around 1%. However, some *T. (m.) marmoratus* haplotypes include samples that were identified as *T. (m.) pygmaeus* in the field and through protein markers, and *T. (m.) pygmaeus* haplotypes are only found in *T. (m.) marmoratus* samples (see Discussion). Average difference between each form and the outgroup used, *Mertensiella luschani*, is 30%, which is very close to the saturation limit of this marker.

Figure 14 presents the results of the phylogeny reconstitution through the Neighbor-Joining method. This tree demonstrates the existence of two groups well supported by high bootstrap values (>95) corresponding to *T. (m.) marmoratus* and *T.*

(m.) pygmaeus. Besides that, it also shows the existence of a well differentiated subgroup (bootstrap 98) within *T. (m.) marmoratus*. This subgroup seems to separate *T. (m.) marmoratus* populations into an Eastern and a Western group. Haplotypes H5, H10 and H14 are also found in samples classified by morphology and allozymes as *T. (m.) pygmaeus* (see Table 18). Haplotypes 22 and 24, despite grouping with *T. (m.) pygmaeus*, were found in *T. (m.) marmoratus* samples from Areia 2 and Areia 3 in the centre of transect II only.

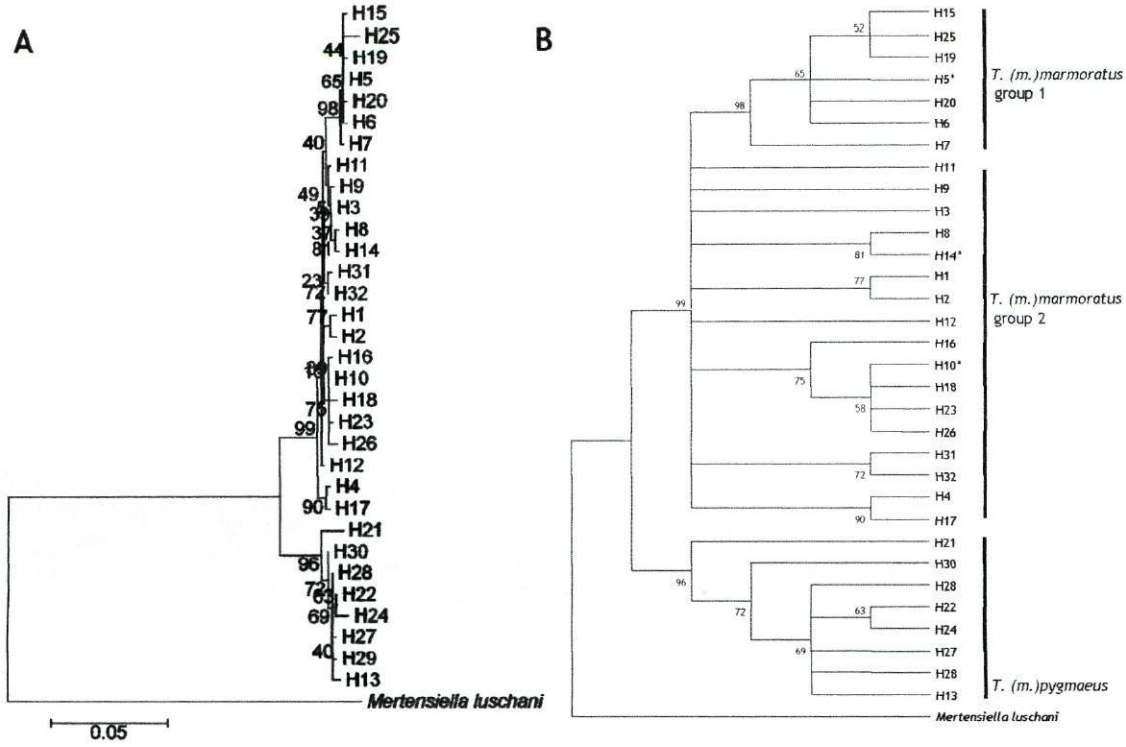

Figure 14 - **A**: NJ phylogenetic tree built from mtDNA sequences. Numbers adjacent to the nodes represent bootstrap values. **B**: Same tree, only condensed to bootstrap values lower than 50%. Clades supported by a bootstrap lower than 50 were turned into a polytomy. * - represents haplotypes that were found in samples from both forms.

Figure 15 represents the geographical distribution of the haplotypes corresponding to the three groups defined by the phylogeny reconstruction.

Figure 16 presents the haplotypic tree built with 137 samples grouped in 34 580 bp (the portion of ND4) haplotypes (Appendix VII). This haplotype network presents two distinct groups separated by 17 nucleotidic differences. *T. (m.) marmoratus* haplotypes (down and right) look more frequent, however, 3 times more

sequences of *T. (m.) marmoratus* samples were made than from *T. (m.) pygmaeus* samples, due to the sequence reading problem referred.

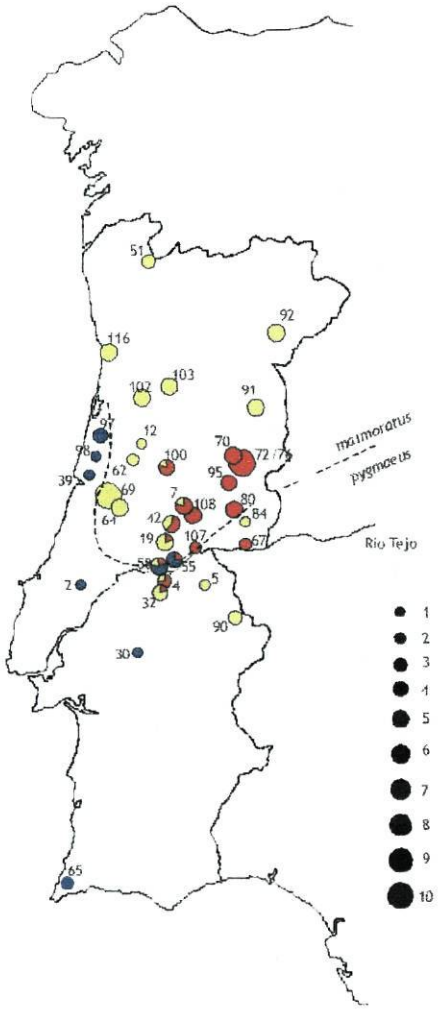

Figure 15 - Geographical distribution and frequency of the three major haplotype groups in the Marbled newt. Circle diameter indicates sample size. Blue - *T. (m.) pygmaeus*; Red - *T. (m.) marmoratus* group 1; Yellow - *T. (m.) marmoratus* group 2. Numbers on map indicate localities (see Appendix I).

Haplotype H5 is the most frequent with 34.3% and there are 7 haplotypes differing from this one by a single position. Haplotype 10 is the second most frequent with 13.9% and there are also 7 haplotypes differing from it in one or two positions. These two haplotypes have 5 nucleotide differences between them. In *T. (m.) pygmaeus*, the most common haplotype is H28, present in 4 samples, and all other *T. (m.) pygmaeus* haplotypes have only one or two differences from it, except haplotype H21, private to Vila do Bispo - Sagres, that has 5 differences.

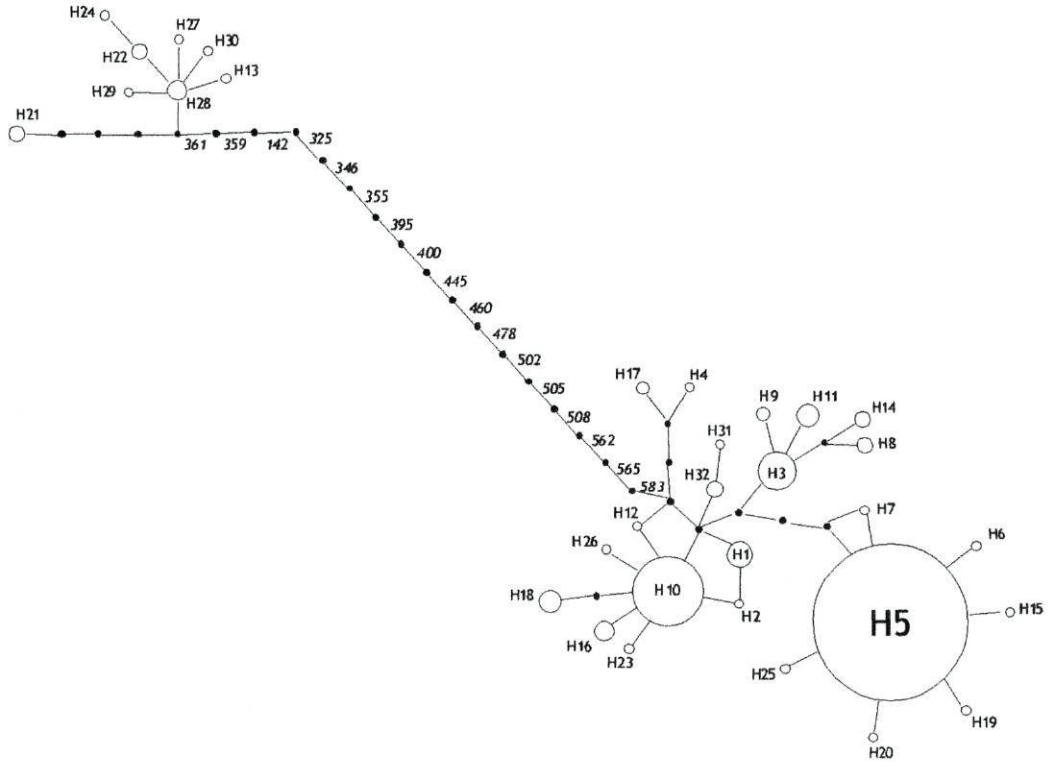

Figure 16 - Haplotype network representing genealogy of 34 haplotypes of a 583 bp fragment of *Triturus marmoratus* mtDNA. White circles represent haplotypes found where as black circles represent missing haplotypes. The size of the white circles is proportional to the number of individuals presenting that haplotype. Numbers in italic represent the position responsible for that mutation.

Discussion

1. Allozyme markers

In a previous study by E. Froufe, A. Geraldes and J.W. Arntzen (unpublished), thirteen populations forming a N-S transect across Portugal were studied (Galiza, Gerês, Mezio, Madeirã, Santos, Domingos da Vinha, Areia 2, Areia 3, Gavião 1, Gavião 2, Mora, Mitra and Vila do Bispo). In that study, five alleles were detected in PEP-A, four in PEP-B, four in PEP-D and four in LDH-2. In the present study, the existence of a new allele in PEP-A (PEP-A*6), another in PEP-B (PEP-B*5), four new alleles in PEP-D (PEP-D*5, PEP-D *6, PEP-D *7 e PEP-D *8), and two in LDH-2 (LDH-2*5 e LDH-2*6) was detected. The set of allozyme markers showed good diagnostic power, even though one of them (Pep-B) turned out to be not as diagnostic as previously thought.

The good diagnostic power of the allozyme markers allowed, in the zone of higher sampling density (the Caldas da Rainha region), that each population could be assigned to one of the forms on the basis of just this markers, and assuming, as it was said, that both forms are mutually exclusive (results not shown).

Distribution of the two forms in the Caldas da Rainha area seems to be associated with exogenous factors, that is, the hybrid zone was created due to the existence of (at least) one 'spot' of land with favourable conditions to the permanence of *T. (m.) marmoratus*, surrounded with habitat more favourable to *T. (m.) pygmaeus*. This *T. (m.) marmoratus* enclave can continue to exist if hybrids, having lower viability in any of the two habitat types, are continuously produced and immediately subjected to negative selection. The exogenous factors that appear to influence the most this distribution are relief (*T. (m.) marmoratus* being more predominant in steeper relief) and, although indirectly, fruit growing (specially pears), perhaps associated with different soil quality.

Deviations from Hardy-Weinberg equilibrium in all *loci* are, in most cases and as already referred, to a deficit in heterozygotes (Appendix IV). Two hypotheses were raised for the occurrence of heterozygote deficit. One related to technical problems that would lead to misreading gels. The other hypothesis raised was the existence of heterozygote inviability. Looking at Appendix IX where results from

Hardy-Weinberg tests are shown, we can see that most significant disequilibrium results are close to the contact zone. This can mean that there is a barrier to gene flow, probably resulting from a reduction of viability of the hybrids. However, if this hypothesis is correct, it becomes difficult to explain the occurrence of disequilibrium outside of the contact zone, namely in the cases of Gerês, Castelo Mendo or Vila do Bispo - Sagres.

Existence of alleles in a hybrid zone that do not occur in parental populations was first described by Woodruff (1989) that coined the term hybrizymes to refer to this phenomenon. The mechanism that seems to explain the occurrence of these alleles involves recombination of the genome of the parental forms, which would lead to a change in the electrophoretic mobility of the resulting allozymes (Schilthuizen *et al.*, 2001). The allele PEP-A *4 found in this study exists only on the contact zone. If this allele is in fact a hybrizyme, it would have resulted from the recombination between alleles PEP-A*1 and PEP-A*2. However, allele PEP-A*4 is present along the entire contact zone (see Appendix VII). This would imply that multiple events of recombination would result in the same change in electrophoretic mobility or alternatively, that this allele would have arisen from one recombination event and was able to expand its distribution area only along the contact zone, but could not penetrate the parental populations.

2. Mitochondrial DNA

One of the main problems that arise frequently in mtDNA studies is amplification of pseudogenes or nuclear copies of mtDNA. The possible existence of pseudogenes is frequently indicated by diverse syntoms: 'ghost' bands in a PCR; additional bands in a restriction profile; sequence ambiguities; stop codons in the middle of coding sequences; frameshift mutations and unexpected phylogenetic placement in a tree (BENSASSON *et al.*, 2001). In some of the sequences made (not included in the analysis) ambiguities were found and its phylogenetic placement was unexpected (all sequences that presented ambiguities were placed in a separate and basal group in a phylogenetic tree). Some of these sequences were repeated using the primer ND4-R2, and the 'symptoms' were no longer observed. According to a recent review paper, no pseudogene is reported for amphibians (BENSASSON *et al.*, 2001). *Triturus marmoratus* possesses a very large genome (about 10 time greater

than man (LIZANA *et al.*, 2000; VINOGRADOV, 1998; HORNER & MACGREGOR, 1983; CAPRIGLIONE *et al.*, 1987; DE SMET, 1981)) which indicates that a large amount of DNA in these newts is non coding, and relatively big amount of mitochondrial genome copies can occur inserted in the nuclear genome. The results, however, do not allow for the precise determination of the nature and origin of the ambiguities in the referred sequences.

In the mtDNA result, very frequent *T. (m.) marmoratus* haplotypes, H5 and H10, are distributed by several populations in the east and west of the centre of Portugal, respectively (See Appendix XIII). The presence of these haplotypes in an extensive area can be explained by the occurrence of successive bottlenecks followed by rapid expansion of populations possessing these haplotypes. In *T. (m.) pygmaeus*, haplotype H28 occurs along the coastal area between Calvão and Rio Maior which indicates recent expansion of *T. (m.) pygmaeus* into this narrow region of the Portuguese coast coming from the South. However, this result for itself is not conclusive due to the small sample number sequenced.

Haplotypes H5 e H10, referred in the previous paragraph, are the central haplotypes of the two *T. (m.) marmoratus* groups indicated by the phylogenetic tree. Both haplotypes are present in many populations in Central Portugal. The two groups of haplotypes seem to be geographically enclosed. Group 1 (see Figure 15) appears restricted to the East-Central Portugal. The true limits of these two groups would only be determined by sequencing samples from all the distribution area. Even though the protein markers used were not able to distinguish these groups, it is possible that a larger set of markers can.

3. Hybrid zone dynamics

The hybrid zone structure found appears to indicate that it is not static, but in movement, with *T. (m.) marmoratus* losing range to *T. (m.) pygmaeus*. Several lines of evidence point to this conclusion. Results from allozymes in the Caldas da Rainha area show the existence of a set of *T. (m.) marmoratus* populations surrounded by *T. (m.) pygmaeus*. A possible scenario for this phenomenon is the previous existence of a larger distribution of *T. (m.) marmoratus*. Later, *T. (m.) pygmaeus* increased its distribution range north and 'pushed' *T. (m.) marmoratus* out. This one remained only in a small population nucleus close to Caldas da Rainha

in more favourable habitat isolated from the remaining *T. (m.) marmoratus* populations. *T. (m.) pygmaeus* was able to extend its range North along the coastal dunes up to Aveiro, as the allozyme and mitochondrial markers show (see Figure 16). In another hybrid zone involving *T. (m.) marmoratus*, this time with *T. cristatus*, *T. (m.) marmoratus* also appears to be losing territory to this other close species (ARNTZEN & WALLIS, 1991).

Figure 16 - Esquematic representation of th hypothesis on the movement of the contact zone between *T. (m.) marmoratus* and *T. (m.) pygmaeus* in the Caldas da Rainha area. See text for details. CR - Caldas da Rainha; represents *T. (m.) marmoratus* populations; represents *T. (m.) pygmaeus* populations. Arrows show the probable path of *T. (m.) pygmaeus* expansion.

4. Introgression

With the markers used, evidence of introgression was found. In the allozymes we found alleles typical from both forms and hybrizymes. Besides that, nuclear and mitochondrial markers are not always in agreement, that is, some populations with *T. (m.) pygmaeus* haplotypes in mtDNA present *T. (m.) marmoratus* morphological phenotype, or vice-versa. In most cases, samples with *T. (m.) marmoratus* mtDNA but *T. (m.) pygmaeus* nuclear markers phenotype were found. Of this, several populations are an example (see Appendix XIII and Figure 14). On the other hand, only in Gavião 1 and 2 was the opposite result found. Several studies on hybrid zones have shown asymmetric introgression (*Gryllus firmus/pennsylvanicus*, ROSS & HARRISON, 2002; *Salamandra salamandra*, GARCÍA-PARÍS *et al.*, 2003) which is a consequence of a difference in the outcome of reciprocal matings. Probably, most

crossings occur between a *T. (m.) pygmaeus* male and a *T. (m.) marmoratus* female, leading to mixed origin offspring with, almost exclusively, *T. (m.) marmoratus* mtDNA.

Additionally, *T. (m.) marmoratus* mtDNA was also found in samples identified as *T. (m.) pygmaeus* both by their morphology and by allozyme markers. This introgression seems to occur mainly due to an expansion of the distribution range of *T. (m.) pygmaeus* from the South into regions up North. Persistence of *T. (m.) marmoratus* mtDNA can be explained by differential migration of males and females. Males from some salamander species are known to disperse further and more frequently than females (STAUB *et al.*, 1995). It has also been described that males and females do not arrive in synchrony at the breeding sites, with males arriving earlier than females (ARNTZEN, 2002). Breeding season also starts earlier for *T. (m.) pygmaeus* than for *T. (m.) marmoratus*. Consequently, in ponds where both forms occur, *T. (m.) marmoratus* males meet with *T. (m.) pygmaeus* females first than with females of their own kind, leading *T. (m.) marmoratus* females to have fewer breeding opportunities. It is expected that nuclear genes would cross the contact zone faster than mitochondrial genes due to the mode of transmission of the two kinds of genes (nuclear genes pass down four times more copies to the next generation than mitochondrial genes).

5. Variability in *T. (m.)* pygmaeus

A particular *T. (m.)* pygmaeus population (Vila do Bispo - Sagres) presents a highly divergent genetic profile whether in terms of allozyme markers or at the mitochondrial level. In the allozymes, it presents a private allele (PEP-A*5) and another allele (PEP-D*4) that, although it appears on other populations, it never shows high frequencies as in this population. In the mitochondrial marker, all samples present the same haplotype, which has at least five differences to all other *T. (m.) pygmaeus* haplotypes (see Figure 14). Such high divergence seems to indicate that this population belongs to a differentiated group from the other *T. (m.) pygmaeus*. However, the distance of the closest sampled populations (Mitra at about 175 km) does not allow to say that this is an isolated population that differentiated from other populations or if it belongs to a wider set of populations that differentiated but whose distribution area is no sufficiently known due to a defect in sampling.

6. Outcome of this work

This study comprises a great part of the contact zone between *Triturus (m.) marmoratus* and *T. (m.) pygmaeus* including a great extension of the zone in Portugal and part of Spain, and populations that are further away (from Gerês, Galiza and down to Vila do Bispo - Sagres). Population density in the centre of the zone allows the drawing of a highly detailed map of the limits of this hybrid zone and will allow for the construction of a powerful model of the distribution of these two forms. This model will allow for the prediction on future distribution of the species taking into consideration, for instance, climatic changes, like Teixeira and co-workers (2002) have done for the salamander *Chioglossa lusitanica*. This kind of prediction is certainly important for the definition of priority conservation areas as for the prediction of consequences (as well as for the definition) of conservation measures.

Concluding remarks

The number of allozyme markers used in this study is relatively low when compared to other studies of the same kind. However, the simultaneous use of nuclear and mitochondrial markers allows checking for congruence of the results. The distance found for the allozyme markers is not an artefact of the choice of markers, minimizing the potential overestimation due to the choice of *a priori* diagnostic markers.

Hybrid zone structure is maintained, according to the Tension Zone model (Barton & Hewitt, 1985; 1989), by selection against hybrids formed being counteracted by dispersal of individuals from parental populations into the contact zone and recombination of their genotype with the genotype of hybrids. The relative strength of these two factors will determine the width of the zone. So, if selection against hybrids is significantly greater than dispersal, the zone will be narrow, and vice-versa. The hybrid zone between *Triturus (m.) marmoratus* and *T. (m.) pygmaeus* is quite narrow, about 10 to 30 km. Introgression detected is limited to populations close to the centre of the hybrid zone; so are deviations to Hardy-Weinberg, which indicates some disadvantage of heterozygotes.

In future work, several aspects can be developed. In the Caldas da Rainha area, it will certainly be interesting to look for *T. (m.) marmoratus*-like DNA in populations with *T. (m.) pygmaeus* allozyme profile populations, specially in the 'corridor' separating the *T. (m.) marmoratus* enclave from other *T. (m.) marmoratus* populations. If *T. (m.) marmoratus* DNA is encountered in these populations, than isolation of this enclave occurred very recently, for it had not passed enough time to completely eliminate *T. (m.) marmoratus* DNA. Another possibility would be to repeat the detailed spatial analysis in other regions of the contact zone, especially in regions where the clinal aspect of the zone is more evident, as the ecological 'signal' that shapes the structure of the zone. The prediction is that this analysis will maintain the clinal aspect of the wider range sampling, and no enclaves will appear. Another case to study would be the structure of the contact zone in the North of Nazaré, namely the occurrence or not of other enclaves. Other enzymatic *loci* could also be explored to try to differentiate the two mitochondrial groups of *T. (m.) marmoratus*.

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Appendix

Appendix I - Sampling sites.

N	Locality	N	Locality
1	Porto de Mós	104	Velada
2	Rio Maior	107	Vila Velha de Ródão
3	Alqueidão	108	Vale de Figueira
4	Gavião 1	116	Porto
5	Nisa	118	S. Bartolomeu dos Galegos
7	Pedra do Altar	119	Salir de Matos
12	Saide	121	Fonte da Pena da Couvinha
13	Vimeiro	127	Mosteiro de Alcanene
19	Domingos da Vinha	128	Vidais
26	Valado dos Frades	129	Foz do Arelho
29	Mitra	130	Casais dos Morgados
30	Mora	131	Famalicão da Nazaré
32	Gavião 2	134	Aceitunas
39	Mira	135	Stª Cruz de Paniagua *
42	Santos	136	El Bronco *
51	Gerês	138	Pedro Muñoz II *
55	Areia 2	139	Pedro Muñoz III *
58	Areia 3	140	JaraOliva *
62	Coimbra	141	Puerto Nuevo *
64	Sicó	145	Acuescas
65	Vila do Bispo	146/7	Rexaldia I e II
67	Rosmaninhal	148	Assentiz - Fungalvaz
69	Soure	149	Chão
70	Monte do Conde	150	Covão da Fonte
72/76	Aldeia João Pires	151	Molianos
80	Idanha-a-Nova	153	Covas
84	Zebreira	155	Casais Monizes
87	Sovrete	156	Casal da Coita
88/93	Mourão / Granja	157	Carrascal
90	Esperança	158	Cela
91	Castelo Mendo	159	Genrinhas
92	Mogadouro	160	Juncal II
95	Aldeia Stª Margarida	161	Molianos II
96	Galiza	162	Stª Susana
97	Calvão	163	Ribeira da Maceira
98	Quiaios	164	Pataias Gare
100	Madeirã	165	Andam
102	Nespereira de Baixo	166	Cós
103	Mezio	167	Casal da Charneca
		209	Sintra

Appendix II - Form used on the field for the ecological characterization of the prospected sites.

Date	
Pond name	**Pond number**
Average slope of bank	<20, 21-40, 41-60, 61-80, >80
Pond depth (cm)	
Macrofauna	Sparse/moderate/abundant
Emergent vegetation	<1, 1-10, 11-20, 21-50, >50
Floating vegetation	<1, 1-10, 11-20, 21-50, >50
Marginal vegetation	<1, 1-10, 11-20, 21-50, >50
Submerged vegetation	<1, 1-10, 11-20, 21-50, >50
Fish	Present/absent
Waterfowl	Present/absent
Inflow and/or outflow	Present/absent
Pond permanency	Permanent/ impermanent
Pollution	None/slight/strong
Shade	<1, 1-20, 21-40, 41-60, >60
Pond size (m)	
Turbidity of water	Transparent/clear/moderate/opaque
Pond type	Field pond/lake/marsh/quarry/wheel/tank/other
Pond use	Undetermined/cattle/fowl/fish/multiple
Arable field	Present/absent
Ditch	Present/absent
Dry stone wall	Present/absent
Garden	Present/absent
Hedge	Present/absent
Marsh	Present/absent
Pasture	Present/absent
Pond	Present/absent
Relief	Flat/undulating/steep
Road	Present/absent
Scrub	Present/absent
Stream	Present/absent
Urbanisation	Present/absent
Woodland	Present/absent

Appendix II (cont.) - Form with information regarding the species of animals found in the prospected sites.

Species	Eggs	Larvae	Metamorphs	Juveniles	Adults	Sound

Apendix III - Morphological characters (A) and colouration (B) key used in the identification of the *T. marmoratus* individuals collected.

A

N°							
Population							
Sex							
SVL 1/2							
ILD							
FLL							
3FL							
HLL							
3TL							
HW							
HL							

SVL - Snout Vent Length; **ILD** - Inter Limb Distance; **FLL** - Fore Limb Length; **3FL** - Third Finger Length; **HLL** - Hind Limb Length; **3TL** - Third Toe Length; **HW** - Head Width; **HL** - Head Length.

B

N		population			
Belly´s color	white	white-grey	grey	grey-black	black
white spots	very few	few	medium	many	
	small	medium	big		
black spots	absent	very few	few	medium	many
	absent	small	medium	big	
links	left	right			
top-links	left	right			

	Belly colour	White spots size	White spots number	Black spots size	Black spots number	Links	Top-links
pygmaeus	white to grey	small	very few to few	absent or small	absent to few	>4	0 or 1
marmoratus	grey to black	big	medium to many	medium to big	few to many	<3	>1

Appendix IV - DNA extraction protocol

Ingredient

1 - Cell lysis buffer (0,5 M Tris, 0,1 M EDTA, 2% SDS, pH=8,8, autoclave)
2 - Protein precipitation solution (5 M ammonium acetate, pH=8,0, autoclave)
3 - DNA hydration solution (TE) (0,1 M Tris, 0,5 M EDTA, pH=8,0, autoclave)
4 - Proteinase K (25 mg/mL)
5 - Isopropanol (-20° C)
6 - Ethanol 70% (-20° C)

1. Put a small volume of tissue (10 - 20 mg) in 600 µL of cell lysis buffer.
2. Add 5µL of proteinase K and stir in the vortex.
3. Incubate at 55° C for at least 3 hours. Stir occasionally by inversion.
4. Add 300 µL of protein precipitation solution to the previous volume.
5. Mix and centrifuge 10 minutes at 14 000 rpm.
6. Place the supernatant in 1,5 mL *eppendorf* tubes.
7. Add 600 µL of Isopropanol (-20° C) and mix, inverting the tube several times.
8. Centrifuge for 10 minutes at 14 000 rpm.
9. Through away the supernatant.
10. Add 1 mL of ethanol 70% (-20° C) and vortex.
11. Centrifuge for 15 minutes at 14 000 rpm.
12. Through away the supernatant.
13. Let dry until ethanol evaporates.
14. Add 50 to 200 µL of TE.

Appendix V - PCR product purification by the enzymatic method.

Ingredients

PCR product

Exo-SAP (contains exonuclease e alkaline phosphatase)

1. Add 2 µL Exo-SAP for each 5 µL of PCR product.
2. Incubate at 37° C for 15 minutos.
3. Raise temperature to 80° C and incubate for 15 minutes. This is meant to inactivate the enzymes.

Appendix VI - Sequencing protocol

1. Sequencing

Ingredients	Amount
TRR or Big Dye	2 µL
PCR product	Up to 2,5 µL
Primer (10 µM)	0,27 µL
H_2O	until 10 µL

Sequencing program

Step	Temperature	Duration	N° of cycles
Initial denaturing	94° C	3 minutes	1
Denaturing	96° C	10 seconds	
Annealing	50° C	5 seconds	25
Extension	60° C	4 minutes	
	20° C	∞	

2. Precipitation of the sequencing product through *Sephadex* columns

1 Prepare a *Sephadex* G50 6,7% (p/v) solution.
2 Washout the columns with 550 mL of ultra pure water and centrifuge at 4 000 rpm for 4 minutes.
3 Stir the *Sephadex* solution.
4 Place a column in a collecting tube and pipette 750 µL of *Sephadex*.
5 Centrifuge at 4 000 rpm for 4 minutes.
6 Through the water and save the collecting tube.
7 Place the column in a 1,5 mL *eppendorf* tube.
8 Add 10 µL of the sequencing reaction.
9 Centrifuge at 4 000 rpm for 4 minutes.
10 Dry the *pellet* at 96° C for a few minutes with the tube cap open.

3. Ressuspension of the *pellet*

1 Add 14 µL of TSR (*Template Supression Reagent*) to the *pellet*.
2 Let the *pellet* hydrate for 10 minutes.
3 Mix in the vortex (≈ 40 seconds) and centrifuge briefly (≈ 10 seconds).
4 Denature the DNA at 94° C for 3 minutes.
5 Mix in the vortex (≈ 10 seconds) and centrifuge briefly (≈ 40 seconds).
6 Transfer the 14 µL to 0,5 mL ABI 310 tubes and cap.

Appendix VII - Variable positions in the sequences obtained from mitochondrial DNA in *Triturus marmoratus*.

	Nucleotide position																												
Haplotypes	96	106	109	112	119	120	122	126	127	133	138	142	190	199	238	269	284	307	325	331	346	355	359	361	365	370	373	376
H1	C	G	A	C	G	T	A	T	G	A	G	G	A	C	T	G	A	A	A	A	C	G	T	G	C	C	G	G
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H10	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H12	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H16	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.
H18	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A
H23	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H26	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H31	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H32	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
H17	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H5	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H6	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H7	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H15	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H19	.	A	.	T	.	.	.	G	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H25	.	A	.	T	.	.	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H20	.	A	C	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
H9	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
H11	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
H14	T	A	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.
H8	T	A	.	T	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
H13	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	A	C	A	.	.	A	.
H21	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	.	A	A	C	A	.	.	A	.
H22	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	G	A	A	C	A	.	.	.	.
H24	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	G	A	A	C	A	.	.	.	.
H27	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G	.	G	G	A	A	C	A	.	.	.	.
H28	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	G	A	A	C	A	.	.	.	.
H29	.	A	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	G	G	A	A	C	A	.	.	.	.
H30	.	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	A	A	C	A	.	.	.	.

Appendix VII (cont.) - Variable positions in the sequences obtained from mitochondrial DNA in *Triturus marmoratus*.

	Nucleotide position																									
Haplotypes	386	388	389	395	400	415	419	445	453	460	468	474	478	482	493	502	505	508	521	540	544	554	562	565	569	583
H1	G	A	T	C	G	C	G	C	T	G	A	C	T	G	C	C	A	G	A	T	G	A	C	A	C	A
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	T	.
H16	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H23	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H26	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H31	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H32	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.
H17	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	T	.
H5	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
H6	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	A	.
H7	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
H15	.	.	.	.	.	.	.	.	.	A	.	T	.	.	.	.	.	.	.	C	.	.	.	.	.	.
H19	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
H25	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
H20	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
H11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
H14	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H13	.	G	.	T	A	A	.	T	C	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T
H21	.	G	.	T	A	A	.	T	C	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T
H22	.	.	.	T	A	.	A	T	.	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T
H24	.	.	.	T	A	.	A	T	.	A	.	.	C	.	.	T	G	A	T	.	.	.	T	G	T	T
H27	.	.	.	T	A	.	.	T	.	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T
H28	.	.	.	T	A	.	.	T	.	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T
H29	.	.	.	T	A	.	.	T	.	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T
H30	.	.	.	T	A	.	.	T	.	A	.	.	C	.	.	T	G	A	.	.	.	.	T	G	T	T

Appendix VIII a - Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Transect I e V).

			Populations (Transect I)			Populations (Transect V)							
Locus		Allele	146	147	148	140	141	139	138	136	135	134	145
	N		*2*	*23*	*20*	*8*	*10*	*10*	*10*	*19*	*7*	*8*	*23*
PEPA		1	1	1	1	—	—	0,65	0,25	0,9737	1	0,9375	1
		2	—	—	—	1	1	0,35	0,75	0,0263	—	0,0625	—
		3	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—
	N		*2*	*21*	*20*	*18*	*19*	*19*	*10*	*21*	*7*	*8*	*24*
PEPB		1	—	0,2143	—	—	—	0,0263	—	—	—	—	—
		2	—	0,2619	0,175	—	0,0263	0,0789	0,15	1	0,0714	0,5625	0,125
		3	1	0,5238	0,825	1	0,9737	0,8947	0,85	—	0,9286	0,4375	0,875
		4	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—
	N		*2*	*23*	*20*	*16*	*15*	*20*	*10*	*21*	*7*	*8*	*25*
PEPD		1	1	0,6739	0,95	0,8438	0,1333	0,7	0,1	0,9286	1	1	0,98
		2	—	0,0652	—	0,1563	0,8667	0,275	0,5	0,0238	—	—	0,02
		3	—	0,0217	—	—	—	—	0,4	0,0476	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—
		6	—	0,1522	—	—	—	—	—	—	—	—	—
		7	—	—	—	—	—	—	—	—	—	—	—
		8	—	0,087	0,05	—	—	0,025	—	—	—	—	—
	N		*2*	*23*	*20*	*0*	*0*	*8*	*0*	*20*	*7*	*8*	*25*
LDH2		1	—	—	0,05	—	—	—	—	0,425	0,3571	0,5625	0,08
		2	1	1	0,95	—	—	1	—	0,575	0,6429	0,4375	0,92
		3	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—

Appendix VIII b - Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Transect II).

			Population												
Locus		**Allele**	96	51	103	100	42	19	58	55	4	32	30	29	65
	N		*10*	*25*	*28*	*24*	*7*	*14*	*27*	*12*	*20*	*10*	*34*	*23*	*10*
PEP A		**1**	—	—	—	0,1875	0,1429	0,2857	0,2222	0,1667	1	0,9	0,9853	1	0,6
		2	1	1	1	0,8125	0,8571	0,5714	0,7778	0,8333	—	0,05	0,0147	—	—
		3	—	—	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	0,1429	—	—	—	0,05	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	0,4
		6	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*10*	*10*	*28*	*24*	*7*	*14*	*24*	*12*	*22*	*10*	*37*	*29*	*10*
PEP B		**1**	—	—	0,1607	0,0417	—	—	0,0208	—	—	0,05	0,1216	0,2241	0,5
		2	—	0,25	—	—	—	—	0,2292	0,0833	0,3182	0,55	0,4459	0,7069	0,25
		3	1	0,75	0,8393	0,9583	1	1	0,75	0,9167	0,6818	0,4	0,4189	0,069	0,25
		4	—	—	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	0,0135	—	—
	N		*10*	*25*	*28*	*24*	*7*	*14*	*29*	*12*	*22*	*10*	*37*	*29*	*10*
PEP D		**1**		0,34	0,5536	0,4167	—	0,2143	0,069	0,3333	0,9318	0,9	0,7432	0,9828	0,65
		2	0,05	0,44	0,3929	0,5625	1	0,7143	0,6379	0,6667	0,0682	0,1	0,0946	—	—
		3	0,95	0,2	0,0357	0,0208	—	0,0714	0,2759	—	—	—	0,1622	0,0172	—
		4	—	—	0,0179	—	—	—	0,0172	—	—	—	—	—	0,35
		5	—	—	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—	—	—
		7	—	—	—	—	—	—	—	—	—	—	—	—	—
		8	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*0*	*25*	*28*	*24*	*7*	*14*	*30*	*12*	*22*	*10*	*38*	*38*	*10*
LDH 2		**1**	—	—	—	—	—	0,0357	0,0667	0,0417	0,2045	0,15	0,3947	0,25	0,25
		2	—	0,5	0,9643	0,9583	0,7857	0,8571	0,8833	0,8333	0,7955	0,85	0,6053	0,7237	0,75
		3	—	0,5	0,0357	0,0417	0,2143	0,0714	0,05	0,125	—	—	—	0,0132	—
		4	—	—	—	—	—	0,0357	—	—	—	—	—	0,0132	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix VIII c - Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Transect III).

		Population					
Locus	**Allele**	62	7	108	107	104	5/66
N		*24*	*36*	*31*	*34*	*21*	*40*
PEPA	1	—	0,0556	0,0645	0,8088	0,8095	1
	2	1	0,8472	0,8548	0,1471	0,0238	—
	3	—	—	—	—	0,0238	—
	4	—	0,0972	0,0806	0,0441	0,0238	—
	5	—	—	—	—	—	—
	6	—	—	—	—	0,119	—
N		*25*	*36*	*31*	*40*	*22*	*39*
PEPB	1	—	0,0417	—	—	0,0455	0,1282
	2	0,02	0,0278	0,0161	0,4875	0,6364	0,4615
	3	0,98	0,9306	0,9839	0,4625	0,1591	0,4103
	4	—	—	—	0,05	—	—
	5	—	—	—	—	0,1591	—
N		*24*	*36*	*31*	*40*	*22*	*39*
PEPD	1	0,0417	0,0417	0,0806	0,8	0,9091	0,8333
	2	0,4792	0,9583	0,871	0,0875	—	0,1538
	3	0,1875	—	—	0,1125	0,0909	0,0128
	4	0,0208	—	0,0484	—	—	—
	5	0,0417	—	—	—	—	—
	6	0,2292	—	—	—	—	—
	7	—	—	—	—	—	—
	8	—	—	—	—	—	—
N		*25*	*36*	*31*	*40*	*22*	*40*
LDH2	1	—	0,0278	—	0,05	0,0682	0,275
	2	0,84	0,9167	0,9839	0,95	0,9091	0,725
	3	0,16	0,0417	0,0161	—	0,0227	—
	4	—	—	—	—	—	—
	5	—	—	—	—	—	—
	6	—	0,0139	—	—	—	—

Appendix VIII d - Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Transecto IV).

			Population										
Locus		**Allele**	92	91	70	72/76	95	80	84	67	90	87	88/93
	N		*24*	*20*	*21*	*14*	*20*	*42*	*42*	*34*	*27*	*28*	*14*
PEPA		1	—	0,05	—	—	—	0,0119	0,7738	1	1	1	1
		2	1	0,95	0,9524	1	0,9	0,9881	0,2262	—	—	—	—
		3	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	0,0476	—	0,1	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—
	N		*25*	*21*	*18*	*18*	*21*	*35*	*32*	*34*	*27*	*38*	*14*
PEPB		1	0,04	0,0476	—	—	—	—	0,0625	0,0294	0,1852	0,0132	0,3214
		2	—	—	—	—	—	0,0286	0,8281	0,7794	0,1111	0,5	0,5357
		3	0,96	0,9524	1	1	1	0,9714	—	0,1912	0,6852	0,4868	0,1429
		4	—	—	—	—	—	—	0,0781	—	0,0185	—	—
		5	—	—	—	—	—	—	0,0313	—	—	—	—
	N		*25*	*21*	*13*	*12*	*21*	*38*	*41*	*35*	*27*	*40*	*14*
PEPD		1	0,18	0,619	0,2692	0,375	0,2381	0,4474	0,878	0,9571	0,963	0,5	1
		2	0,82	0,381	0,4615	0,625	0,7619	0,4605	0,0244	0,0429	—	0,4125	—
		3	—	—	0,2692	—	—	0,0395	0,0732	—	0,037	0,0125	—
		4	—	—	—	—	—	—	—	—	—	0,025	—
		5	—	—	—	—	—	0,0526	0,0244	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	0,0375	—
		7	—	—	—	—	—	—	—	—	—	0,0125	—
		8	—	—	—	—	—	—	—	—	—	—	—
	N		*25*	*21*	*20*	*18*	*21*	*39*	*44*	*42*	*27*	*40*	*14*
LDH2		1	—	—	—	0,0556	0,0714	0,0128	0,5568	0,119	0,1296	0,1625	0,1071
		2	1	1	1	0,944	0,9286	0,9872	0,4205	0,881	0,8704	0,8125	0,8929
		3	—	—	—	—	—	—	0,0227	—	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	0,025	—
		6	—	—	—	—	—	—	—	—	—	—	—

Appendix VIII e - Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Coastal area).

			Population							
Locus		Allele	116	97	39	98	102	12	69	64
	N		*18*	*26*	*7*	*20*	*21*	*10*	*35*	*26*
PEPA		1	0	1	0,8571	1	0,0238	—	0,1286	—
		2	0,8333	—	0,1429	—	0,8333	0,85	0,8429	1
		3	—	—	—	—	—	0,15	0,0143	—
		4	—	—	—	—	0,1429	—	0,0143	—
		5	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—
	N		*18*	*26*	*15*	*20*	*21*	*10*	*35*	*27*
PEPB		1	0,0556	—	0,0667	—	0,0238	—	0,0571	0,1111
		2	—	0,0192	—	0,025	0,0952	0,1	0,1143	0,0741
		3	0,9444	0,9808	0,9333	0,975	0,881	0,9	0,8286	0,7778
		4	—	—	—	—	—	—	—	0,037
		5	—	—	—	—	—	—	—	—
	N		*18*	*26*	*15*	*20*	*21*	*10*	*35*	*27*
PEPD		1	0,1944	0,8077	0,3333	0,925	0,1667	0,2	0,0429	0,0185
		2	0,75	—	0,3667	—	0,8333	0,8	0,4286	0,5926
		3	0,0556	0,0385	0,0333	—	—	—	—	—
		4	—	—	0,1	—	—	—	0,0714	—
		5	—	0,1538	0,0667	0,075	—	—	—	0,1111
		6	—	—	0,1	—	—	—	0,4571	0,2778
		7	—	—	—	—	—	—	—	—
	N		*18*	*26*	*15*	*20*	*21*	*10*	*35*	*27*
LDH2		1	—	—	—	—	—	0,1	—	—
		2	0,8889	0,9808	0,9333	1	0,881	0,9	0,8	0,7222
		3	0,1111	—	—	—	0,119	—	0,1714	0,2778
		4	—	—	—	—	—	—	—	—
		5	—	0,0192	0,0667	—	—	—	0,0286	—
		6	—	—	—	—	—	—	—	—

Appendix VIII f - Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Caldas da Rainha area). Population numbers are as indicated in Figure 4.

			Population														
Locus		**Allele**	1	3	13	118	119	121	127	128	129	130	131	149	150	151/7	152
	N		*13*	*12*	*10*	*6*	*22*	*10*	*10*	*11*	*10*	*10*	*0*	*10*	*10*	*10*	*10*
PEPA		1	1	—	0,4	1	0,0455	—	—	—	1	1	—	1	1	1	1
		2	—	—	0,6	—	0,9091	0,95	—	1	—	—	—	—	—	—	—
		3	—	—	—	—	0,0455	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	0,05	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*15*	*25*	*9*	*6*	*24*	*14*	*13*	*11*	*11*	*10*	*5*	*5*	*7*	*15*	*12*
PEPB		1	0,6333	0,22	0,0556	—	0,0417	0,0357	0,077	0,0909	0,2273	—	0,3	—	—	0,0333	0,0417
		2	—	0,24	0,0556	0,0833	0,0625	—	0,231	—	—	—	—	—	—	—	—
		3	0,3667	0,54	0,8889	0,9167	0,8958	0,9643	0,692	0,9091	0,7727	1	0,7	1	1	0,9667	0,9583
		4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*15*	*24*	*11*	*6*	*23*	*15*	*12*	*11*	*9*	*10*	*5*	*0*	*1*	*9*	*12*
PEPD		1	0,9667	0,6875	—	0,9167	—	0,0333	0,708	—	0,9444	0,5	0,9	—	1	0,9444	0,3333
		2	—	0,0208	1	0,0833	0,8261	0,8667	—	1	—	—	—	—	—	—	—
		3	—	0,0208	—	—	—	—	0,167	—	—	—	—	—	—	—	0,6667
		4	0,0333	—	—	—	—	—	—	—	0,0556	—	0,1	—	—	0,0556	—
		5	—	0,0208	—	—	—	0,0333	—	—	—	0,5	—	—	—	—	—
		6	—	—	—	—	0,1739	0,0667	—	—	—	—	—	—	—	—	—
		7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		8	—	0,25	—	—	—	—	0,125	—	—	—	—	—	—	—	—
	N		*5*	*20*	*0*	*6*	*23*	*3*	*6*	*0*	*0*	*0*	*0*	*0*	*1*	*2*	*1*
LDH2		1	—	0,025	—	—	—	—	—	—	—	—	—	—	—	—	—
		2	1	0,975	—	1	0,4348	0,6667	1	—	—	—	—	—	1	1	1
		3	—	—	—	—	0,5652	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	0,3333	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix VIII f (cont.)- Allelic frequencies and sample number (N) over four allozyme *loci* in populations of *T. marmoratus* (Caldas da Rainha area).

			Populações												
Locus		**Alelos**	153	155	156	158	159	160	161	162	163	164	165	166	167
	N		*7*	*10*	*10*	*2*	*10*	*9*	*10*	*3*	*2*	*10*	*10*	*10*	*10*
PEPA		1	1	1	—	1	1	0,4444	1	1	1	1	0,15	—	0,15
		2	—	—	1	—	—	0,5556	—	—	—	—	0,85	0,95	0,85
		3	—	—	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	—	—	—	—	—	—	0,05	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*8*	*10*	*11*	*2*	*9*	*16*	*0*	*3*	*1*	*4*	*14*	*14*	*21*
PEPB		1	0,0625	0,25	0,227	—	0,1667	0,0625	—	—	—	—	0,036	—	0,024
		2	0,0625	0,15	—	—	—	0,0938	—	0,167	—	—	—	—	0,024
		3	0,875	0,6	0,773	1	0,8333	0,8438	—	0,833	1	1	0,964	1	0,952
		4	—	—	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*7*	*10*	*20*	*2*	*8*	*16*	*2*	*3*	*1*	*11*	*15*	*14*	*20*
PEPD		1	1	1	—	1	0,8125	0,125	1	0,833	1	1	—	—	—
		2	—	—	1	—	0,0625	0,625	—	0,167	—	—	1	1	1
		3	—	—	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	0,125	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	0,0313	—	—	—	—	—	—	—
		6	—	—	—	—	—	0,0938	—	—	—	—	—	—	—
		7	—	—	—	—	—	0,125	—	—	—	—	—	—	—
		8	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		*8*	*10*	*0*	*0*	*0*	*14*	*0*	*3*	*0*	*0*	*1*	*0*	*0*
LDH2		1	—	—	—	—	—	—	—	—	—	—	—	—	—
		2	1	1	—	—	—	0,4643	—	0,833	—	—	0,5	—	—
		3	—	—	—	—	—	0,5357	—	0,167	—	—	0,5	—	—
		4	—	—	—	—	—	—	—	—	—	—	—	—	—
		5	—	—	—	—	—	—	—	—	—	—	—	—	—
		6	—	—	—	—	—	—	—	—	—	—	—	—	—

Appendix IX - Significance of heterozygosity deviation in relation to Hardy-Weinberg expectations in the transects.

	PEP A	PEP B	PEP D	LDH 2
TRANSECT I				
Rexaldia I	NA	NS	NS	NA
Rexaldia II	NA	NA	NA	NA
Assentiz - Fungalvaz	NA	NS	NS	NS
TRANSECT II				
Galiza				
Gerês	NA	0,0464	0,0059	NS
Mezio	NA	NS	NS	NS
Madeirã	NS	NS	NS	NS
Santos	NS	NA	NA	NS
Domingos da Vinha	NS	NA	0,0427	NS
Areia 3	NS	NS	NS	NS
Areia 2	0,0062	NS	NS	NS
Gavião 1	NA	NS	NS	NS
Gavião 2	NS	0,0150	NS	NS
Mora	NA	NS	NS	NS
Mitra	NA	NS	NA	NS
Vila do Bispo - Sagres	NS	NS	NS	0,0464
TRANSECT III				
Pedra de Altar	NS	NS	NS	NS
Vale da Figueira	NS	NA	NS	NA
Vila Velha de Ródão	0,0023	0,0075	0,0033	NS
Velada	NS	0,0026	0,0017	NS
Nisa	NA	0,0000	NS	NS
TRANSECT IV				
Mogadouro	NA	NS	NS	NA
Castelo Mendo	0,0256	0,0244	NS	NA
Monte do Conde	0,0244	NA	NS	NA
João Pires / S. João do Deserto	NA	NA	NS	NS
Aldeia Stª Margarida	NS	NA	NS	NS
Idanha-a-Nova	NA	NS	NS	NA
Zebreira	NS	0,0000	0,0005	0,0050
Rosmaninhal	NA	0,0001	NS	NS
Esperança	NA	NS	0,0189	NS
Sovrete	NA	NS	NS	NS
Mourão / Granja	NA	NS	NA	NS
TRANSECT V				
"JaraOliva"	NA	NA	NS	NA
Puerto Nuevo	NA	NA	NS	NA
Pedro Muñoz III	NS	NS	0,0002	NA
Pedro Muñoz I	0,0464	NS	0,173	NA
El Bronco	NA	NA	NS	NS
Stª Cruz de Paniagua	NA	NA	NA	NS
Aceitunas	NA	0,0490	NA	NS
Acuescas	NA	0,0209	NA	NS

NS - Not significant (p>0.05); NA - Not Applicable (monomorphic).

Appendix IX (cont.) - Significance of heterozygosity deviation in relation to Hardy-Weinberg expectations in the coastal and Caldas da Rainha area.

	PEP A	PEP B	PEP D	LDH 2
COASTAL AREA				
Porto	NS	NS	NS	NS
Calvão	NA	NA	0,0024	NA
Mira	NS	NS	NS	0,0345
Quiaios	NA	NA	NS	NA
Nespereira de Baixo	NS	0,0094	NS	NS
Saide	NS	NS	NS	NS
Coimbra	NA	NA	NS	NS
Soure	NS	NS	0,0114	0,0064
Sicó	NA	NS	0,0235	NS
CALDAS DA RAINHA AREA				
Valado dos Frades	NA	NS	0,0001	NA
Rio Maior	NA	0,0248	0,0023	NA
Porto de Mós	NA	NS	NA	NA
S. Bartolomeu dos Galegos	NA	NA	NA	NA
Salir de Matos	0,0471	NS	NS	NS
Fonte da Pena da Couvinha	NA	NA	NS	NS
Vimeiro	NS	NS	NA	NA
Alqueidão	NA	NS	0,0019	NA
Mosteiro de Alcanene	NA	NS	0,0167	NA
Vidais	NA	NS	NA	NA
Foz de Arelho	NA	NS	NA	NA
Casais dos Morgados	NA	NA	0,0069	NA
Famalicão da Nazaré	NA	NS	NA	NA
Chão	NA	NA	NA	NA
Covão da Fonte	NA	NA	NA	NA
Carrascal	NA	NA	NA	NA
Molianos	NA	NA	0,0437	NA
Casal da Coita	NA	0,0376	NA	NA
Cela	NA	NA	NA	NA
Genrinhas	NA	NS	NS	NA
Molianos I	NA	NA	NA	NA
Ribeira da Macieira	NA	NA	NA	NA
Pataias Gare	NA	NA	NA	NA
Andam	NS	NA	NA	NA
Cós	NA	NA	NA	NA
Casal da Charneca	NS	NS	NA	NA
Covas	NA	NS	NA	NA
Casais Monizes	NA	NS	NA	NA
Juncal II	NS	NS	0,0000	NS
Stª Susana	NA	NA	NA	NA

NS - Not significant (p>0,05); NA - Not applicable.

Appendix X

Genetic distances between populations in transect I.

Transect I	Population	*91*	*4*	*146*	*147*	*148*	*2*
91	Castelo Mendo						
4	Madeirã	0,011					
146	Rexaldia II	0,431	0,394				
147	Rexaldia I	0,330	0,319	0,055			
148	Assentiz - Fungalvaz	0,364	0,352	0,029	0,006		
2	Rio Maior	0,364	0,343	0,018	0,009	0,002	

Genetic distances between populations in transect II.

Transect II	Population	*96*	*51*	*103*	*100*	*42*	*19*	*58*	*55*	*4*	*32*	*30*	*29*	*65*
96	Galiza													
51	Gerês	0,224												
103	Mezio	0,198	0,096											
100	Madeirã	0,209	0,109	0,018										
42	Santos	0,298	0,117	0,126	0,058									
19	Domingos da Vinha	0,245	0,156	0,089	0,017	0,026								
58	Areia 3	0,147	0,107	0,087	0,043	0,043	0,035							
55	Areia 2	0,231	0,070	0,029	0,000	0,021	0,008	0,024						
4	Gavião 1	0,853	0,720	0,499	0,407	0,755	0,414	0,537	0,464					
32	Gavião 2	0,926	0,723	0,519	0,452	0,828	0,480	0,534	0,502	0,012				
30	Mora	0,948	0,825	0,661	0,560	0,923	0,550	0,597	0,603	0,029	0,020			
29	Mitra	1,462	1,078	0,845	0,806	1,422	0,882	0,871	0,872	0,096	0,024	0,048		
65	Vila do Bispo - Sagres	1,018	0,949	0,587	0,578	1,023	0,638	0,692	0,666	0,158	0,125	0,141	0,139	

Appendix X (cont.)

Genetic distances between populations in transect III.

Transect III	Population	*62*	*100*	*7*	*108*	*107*	*104*	*5/66*	*87*	*90*
62	Coimbra									
100	Madeirã	0,049								
7	Pedra do Altar	0,055	0,047							
108	Vale da Figueira	0,042	0,030	0,001						
107	Vila Velha de Ródão	0,633	0,345	0,623	0,571					
104	Velada	1,042	0,603	1,010	0,940	0,028				
5/66	Nisa	0,922	0,508	0,832	0,788	0,031	0,046			
87	Sovrete	0,686	0,396	0,555	0,535	0,052	0,102	0,036		
90	Esperança	0,758	0,396	0,752	0,688	0,050	0,107	0,040	0,100	

Genetic distances between populations in transect IV.

Transect IV	Population	*92*	*91*	*70*	*72/76*	*95*	*80*	*84*	*67*	*90*	*87*	*88/93*
92	Mogadouro											
91	Castelo Mendo	0,053										
70	Monte do Conde	0,025	0,026									
72/76	Aldeia João Pires / S. João do Deserto	0,007	0,014	0,011								
95	Aldeia Stª Margarida	0,001	0,044	0,021	0,003							
80	Idanha-a-Nova	0,024	0,004	0,011	0,000	0,019						
84	Zebreira	1,310	0,871	1,166	1,059	1,224	0,976					
67	Rosmaninhal	1,005	0,660	0,891	0,849	0,956	0,761	0,087				
90	Esperanca	0,690	0,405	0,569	0,566	0,631	0,503	0,286	0,112			
87	Sovrete	0,581	0,490	0,574	0,542	0,549	0,523	0,222	0,079	0,100		
88/93	Mourão / Granja	1,022	0,643	0,902	0,864	0,981	0,765	0,120	0,017	0,074	0,104	

Appendix X (cont. 2)

Genetic distances between populations in transect V.

Transect V	Population	*92*	*141*	*140*	*139*	*137*	*136*	*135*	*134*	*145*
92	Mogadouro									
141	Puerto Nuevo	0,000								
140	'JaraOliva'	0,126	0,143							
139	Pedro Muñoz III	0,231	0,129	0,246						
137	Pedro Muñoz I	0,048	0,143	0,049	0,153					
136	El Bronco	1,480	0,928	1,512	0,458	0,993				
135	Stª Cruz de Paniagua	0,711	0,383	0,742	0,095	0,524	0,290			
134	Aceitunas	1,067	0,600	1,103	0,257	0,778	0,054	0,073		
145	Acuescas	0,613	0,331	0,640	0,051	0,427	0,292	0,015	0,122	

Appendix XI - Diversity measures for the studied populations.

Population	Expected heterozigosity	Average number of alleles/ *locus*
Transect I		
Rexaldia II	0,2804	2,5000
Rexaldia I	0,0000	1,0000
Assentiz - Fungalvaz	0,1197	1,7500
Rio Maior	0,1597	2,0000
Transect II		
Galiza	0,0238	1,2500
Gerês	0,3814	2,2500
Mezio	0,2191	2,2500
Madeirã	0,2435	2,2500
Santos	0,1454	1,5000
Domingos da Vinha	0,3170	2,7500
Areia 3	0,3637	3,0000
Areia 2	0,2908	2,2500
Gavião 1	0,2216	1,7500
Gavião 2	0,2887	2,5000
Mora	0,3825	2,7500
Mitra	0,2206	2,5000
Vila do Bispo - Sagres	0,4838	2,2500
Transect III		
Pedra do Altar	0,1595	3,0000
Vale de Figueira	0,1323	2,5000
Vila Velha de Rodão	0,3257	2,7500
Velada	0,3012	3,5000
Nisa	0,3207	2,2500
Transect IV		
Mogadouro	0,0930	1,5000
Castelo Mendo	0,1643	1,7500
Monte do Conde	0,1832	1,7500
João Pires / S. João do Deserto	0,1569	1,7500
Aldeia Stª Margarida	0,1689	1,7500
Idanha-a-Nova	0,1794	2,7500
Zebreira	0,3471	3,2500
Rosmaninhal	0,1617	2,0000
Esperança	0,1951	2,2500
Sovrete	0,3508	3,2500
Mourão / Granja	0,1952	1,7500
Transect V		
'JaraOliva'	0,0659	1,2500
Puerto Nuevo	0,0706	1,5000
Pedro Muñoz III	0,2703	2,2500
Pedro Muñoz I	0,3025	2,0000
El Bronco	0,1687	2,0000
Stª Cruz de Paniagua	0,1480	1,5000
Aceitunas	0,2754	1,7500
Acuescas	0,1013	1,7500

Appendix XI (cont.)

Population	Expected heterozigosity	Average number of alleles/ *locus*
Coastal area		
Porto	0,2442	2,2500
Calvão	0,0995	2,0000
Mira	0,3057	3,0000
Quiaios	0,0469	1,5000
Nespereira de Baixo	0,2466	2,5000
Saide	0,2338	2,0000
Coimbra	0,2467	2,7500
Soure	0,3750	3,5000
Sicó	0,3340	2,7500
Caldas da Rainha area		
Valado dos Frades - Nazaré	0,2361	2,0000
Porto de Mós	0,1322	1,5000
S. Bartolomeu dos Galegos	0,0764	1,5000
Salir de Matos	0,2850	2,5000
Fonte da Pena da Couvinha	0,2126	2,5000
Vimeiro	0,1709	1,7500
Alqueidão	0,2787	2,7500
Mosteiro de Alcanene	0,2291	2,0000
Vidais	0,0413	1,2500
Foz do Arelho	0,1140	1,5000
Casais dos Morgados	0,1250	1,2500
Famalicão da Nazaré	0,1500	1,5000
Chão	0,0000	1,0000
Covão da Fonte	0,0000	1,0000
Carrascal - Évora de Alcobaça	0,0423	1,5000
Molianos	0,1311	1,5000
Casal da Coita	0,0878	1,2500
Cela	0,0000	1,0000
Genrinhas	0,1495	1,7500
Molianos II	0,0000	1,0000
Ribeira da Maceira	0,0000	1,0000
Pataias Gare	0,0000	1,0000
Andam	0,2060	1,7500
Cós	0,0238	1,2500
Casal da Charneca	0,0867	1,7500
Covas	0,0566	1,5000
Casais Monizes	0,1387	1,5000
Juncal II	0,4588	3,0000
Stª Susana	0,2083	1,7500

Appendix XII - Percentage of difference between *T. marmoratus* sequences, calculated following Tamura & Nei (1993). Above the diagonal are the values of Tamura-Nei distance calculated based only on tranversions between sequences. Below diagonal are the Tamura-Nei distance values calculated based on transitions and transversions between haplotypes. Numbers between 1 and 32 indicate the haplotypes defined on Appendix V. *Ml - Mertensiela luschani.*

H	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	27	28	29	30	31	32	*Ml*
1		0,000	0,002	0,002	0,002	0,003	0,003	0,002	0,002	0,002	0,002	0,002	0,007	0,002	0,002	0,002	0,002	0,002	0,003	0,003	0,007	0,002	0,010	0,003	0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,118
2	0,005		0,002	0,002	0,002	0,003	0,003	0,002	0,002	0,002	0,002	0,002	0,007	0,002	0,002	0,002	0,002	0,002	0,003	0,003	0,007	0,002	0,010	0,003	0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,118
3	0,009	0,010		0,000	0,000	0,002	0,002	0,000	0,000	0,000	0,000	0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
4	0,012	0,014	0,010		0,000	0,002	0,002	0,000	0,000	0,000	0,000	0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
5	0,014	0,016	0,009	0,016		0,002	0,002	0,000	0,000	0,000	0,000	0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
6	0,016	0,017	0,010	0,016	0,002		0,003	0,002	0,002	0,002	0,002	0,002	0,007	0,002	0,002	0,002	0,002	0,002	0,003	0,003	0,007	0,002	0,010	0,003	0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,118
7	0,014	0,016	0,009	0,016	0,002	0,003		0,002	0,002	0,002	0,002	0,002	0,007	0,002	0,002	0,002	0,002	0,002	0,003	0,003	0,007	0,002	0,010	0,003	0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,118
8	0,012	0,014	0,003	0,014	0,012	0,014	0,012		0,000	0,000	0,000	0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
9	0,010	0,012	0,002	0,012	0,010	0,012	0,010	0,005		0,000	0,000	0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
10	0,009	0,007	0,007	0,010	0,012	0,014	0,012	0,010	0,009		0,000	0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
11	0,010	0,009	0,002	0,012	0,010	0,012	0,010	0,005	0,003	0,005		0,000	0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
12	0,009	0,007	0,007	0,007	0,012	0,012	0,012	0,010	0,009	0,003	0,005		0,005	0,000	0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
33	0,043	0,045	0,041	0,041	0,043	0,043	0,043	0,045	0,039	0,041	0,043	0,037		0,005	0,005	0,005	0,005	0,005	0,007	0,007	0,003	0,005	0,007	0,007	0,005	0,003	0,003	0,003	0,003	0,007	0,005	0,007	0,122
14	0,012	0,014	0,003	0,014	0,012	0,014	0,012	0,003	0,005	0,010	0,005	0,010	0,045		0,000	0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
15	0,016	0,018	0,010	0,018	0,002	0,003	0,003	0,014	0,012	0,014	0,012	0,014	0,045	0,014		0,000	0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
16	0,010	0,009	0,009	0,012	0,014	0,016	0,014	0,012	0,010	0,002	0,007	0,005	0,039	0,012	0,016		0,000	0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
17	0,012	0,014	0,010	0,003	0,016	0,016	0,016	0,014	0,012	0,010	0,012	0,007	0,041	0,014	0,018	0,012		0,000	0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
18	0,012	0,010	0,010	0,014	0,016	0,018	0,016	0,014	0,012	0,003	0,009	0,007	0,045	0,014	0,018	0,005	0,014		0,002	0,002	0,005	0,000	0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
19	0,016	0,017	0,010	0,018	0,002	0,003	0,003	0,014	0,012	0,014	0,012	0,014	0,045	0,014	0,003	0,016	0,018	0,018		0,003	0,007	0,002	0,010	0,003	0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,118
20	0,016	0,017	0,010	0,018	0,002	0,003	0,003	0,014	0,012	0,014	0,012	0,014	0,045	0,014	0,003	0,016	0,018	0,018	0,003		0,007	0,002	0,010	0,003	0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,114
21	0,049	0,050	0,047	0,047	0,049	0,049	0,049	0,051	0,045	0,047	0,049	0,043	0,012	0,051	0,051	0,045	0,047	0,051	0,050	0,050		0,005	0,003	0,007	0,005	0,000	0,000	0,000	0,000	0,007	0,005	0,003	0,122
22	0,010	0,009	0,009	0,012	0,014	0,016	0,014	0,012	0,010	0,002	0,007	0,005	0,043	0,012	0,016	0,003	0,012	0,005	0,016	0,016	0,049		0,009	0,002	0,000	0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
23	0,054	0,056	0,052	0,052	0,054	0,054	0,054	0,056	0,050	0,052	0,054	0,048	0,017	0,056	0,056	0,050	0,052	0,056	0,056	0,056	0,005	0,054		0,010	0,009	0,003	0,003	0,003	0,003	0,010	0,009	0,007	0,127
24	0,021	0,023	0,016	0,023	0,007	0,009	0,009	0,019	0,018	0,019	0,018	0,019	0,050	0,019	0,009	0,021	0,023	0,023	0,009	0,009	0,056	0,021	0,058		0,002	0,007	0,007	0,007	0,007	0,003	0,002	0,010	0,118
25	0,012	0,010	0,010	0,014	0,016	0,018	0,016	0,014	0,012	0,003	0,009	0,007	0,045	0,014	0,018	0,005	0,014	0,007	0,018	0,018	0,051	0,005	0,056	0,023		0,005	0,005	0,005	0,005	0,002	0,000	0,009	0,116
26	0,049	0,050	0,047	0,047	0,049	0,049	0,049	0,051	0,045	0,047	0,049	0,043	0,012	0,051	0,051	0,045	0,047	0,051	0,050	0,050	0,003	0,049	0,009	0,056	0,051		0,000	0,000	0,000	0,007	0,005	0,003	0,122
27	0,047	0,049	0,045	0,045	0,047	0,047	0,047	0,049	0,043	0,045	0,047	0,041	0,010	0,049	0,049	0,043	0,045	0,049	0,049	0,049	0,002	0,047	0,007	0,054	0,049	0,002		0,000	0,000	0,007	0,005	0,003	0,122
28	0,049	0,050	0,047	0,047	0,049	0,049	0,049	0,051	0,045	0,047	0,049	0,043	0,012	0,051	0,051	0,045	0,047	0,051	0,050	0,050	0,003	0,049	0,009	0,056	0,051	0,003	0,002		0,000	0,007	0,005	0,003	0,122
29	0,045	0,047	0,043	0,043	0,045	0,045	0,045	0,047	0,041	0,043	0,045	0,039	0,012	0,047	0,047	0,041	0,043	0,047	0,047	0,047	0,003	0,045	0,009	0,052	0,047	0,003	0,002	0,003		0,007	0,005	0,003	0,122
30	0,009	0,010	0,007	0,010	0,012	0,014	0,012	0,010	0,009	0,007	0,009	0,007	0,041	0,010	0,014	0,009	0,010	0,010	0,014	0,014	0,047	0,009	0,052	0,019	0,010	0,047	0,045	0,047	0,043		0,002	0,010	0,114
31	0,007	0,009	0,005	0,009	0,010	0,012	0,010	0,009	0,007	0,005	0,007	0,005	0,039	0,009	0,012	0,007	0,009	0,009	0,012	0,012	0,045	0,007	0,050	0,018	0,009	0,045	0,043	0,045	0,041	0,002		0,009	0,116
32	0,050	0,052	0,048	0,048	0,050	0,050	0,050	0,052	0,047	0,048	0,050	0,045	0,014	0,052	0,052	0,047	0,048	0,052	0,052	0,052	0,005	0,050	0,010	0,058	0,052	0,005	0,003	0,005	0,005	0,048	0,047		0,127
35	0,296	0,293	0,294	0,291	0,296	0,299	0,296	0,294	0,291	0,291	0,294	0,291	0,307	0,294	0,299	0,294	0,291	0,294	0,299	0,297	0,298	0,294	0,306	0,307	0,296	0,304	0,301	0,304	0,298	0,291	0,291	0,306	

Appendix XIII - Number of sequences (N) and identification code of the samples corresponding to the haplotypes defined in Appendix VI.

	Haplotypes present									
TRANSECT II										
Galiza										
Gerês	H1	H1	H2							
Mezio	H8	H8	H8	H9	H9					
Madeirã	H5	H5	H5	H5	H10					
Santos	H5	H5	H5	H10	H10					
Domingos da Vinha	H5	H10	H10	H10	H10					
Areia 3	H5	H22	H22	H23	H24					
Areia 2	H5	H5	H5	H22						
Gavião 1	H5	H5	H25	H26						
Gavião 2	H5	H10	H10	H10	H10					
Mora	H13									
Vila do Bispo - Sagres	H21	H21	H21							
TRANSECT III										
Pedra de Altar	H5	H5	H5	H10	H15					
Vale da Figueira	H5	H5	H5	H5	H5					
Vila Velha de Ródão	H5	H5								
Nisa	H31									
TRANSECT IV										
Mogadouro	H3	H3	H3	H3	H3					
Castelo Mendo	H3	H3	H3	H3	H3					
Monte do Conde	H5	H5	H5	H6	H7					
João Pires / S. João do Deserto	H5	H5	H5	H5	H5	H5	H5	H5	H5	H5
Aldeia Stª Margarida	H5	H5	H5	H5						
Idanha-a-Nova	H5	H5	H5	H19	H20					
Zebreira	H14									
Rosmaninhal	H5	H5								
Esperança	H32	H32	H32							
COASTAL AREA										
Porto	H1	H1	H1	H1	H4					
Calvão	H28	H28	H28	H29	H30					
Mira	H27									
Quiaios	H28									
Nespereira de Baixo	H11	H11	H11	H11	H11					
Saide	H12									
Coimbra	H10	H10	H14	H14						
Soure	H10	H10	H10	H10	H16	H16	H16	H17	H17	H18
Sicó	H10	H18	H18	H18	H18					
CALDAS DA RAINHA AREA										
Rio Maior	H28									

Note: Numbers in italic correspond to samples with *pygmaeus*-like morphological phenotype.