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Short Communication

The Lusitania Province as a center of diversification: The phylogeny of the genus *Microlipophrys* (Pisces: Blenniidae)

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ABSTRACT

The Lusitania Province has been considered a transition zone between the Atlantic northern cold waters and Tropical warm waters. Tropical species have expanded their ranges during warm periods and either retreated during cold periods or survived in local refuges. Successive waves of dispersion into this Province could have favored diversification through geographic isolation. Taxa that remained in this large Province may also have diversified *in loco*. We analyzed molecular markers of the genus *Microlipophrys* (family Blenniidae) that confirm the validity of this genus and of the seven recognized species. *Microlipophrys* and its sister clade apparently originated within Lusitania and dispersed into the tropics at a later stage.

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1. Introduction

The marine biogeographic province Lusitania, as defined by Briggs (1995), encompasses the warm temperate North-eastern Atlantic, extending northward from its tropical limit at the Cape Verde, Senegal, to the western entrance of the English Channel, where cold temperate conditions begin, and includes the Mediterranean and the archipelagos of the Azores, Madeira and Canaries. The province acted as a transition zone between tropical and cold temperate waters. Much of the fish fauna of the Lusitania Province is composed of fish of tropical origin that withstand lower temperatures and cold-adapted species that survive in warmer waters. In some cases, there is evidence supporting a history of substantial evolution and diversification in this Province, so that many genera and some subfamilies are endemic or almost endemic, e.g., taxa of Blenniidae, Trypterigiidae, Labridae and Gobiesocidae (e.g., Almada et al., 2008; Carreras-Carbonell et al., 2007; Hanel et al., 2002).

While tropical conditions prevailed in the area up to the Mid-Miocene Climatic Optimum (18–14 MYA), the area subsequently experienced a gradual cooling, with oscillations, that took momentum in the Pliocene and culminated in the Pleistocene glaciations (Briggs, 1995; Cronin, 2010). This cooling period that extended for several million years must have allowed the evolution of adaptations to the new temperate conditions and the formation of a true warm-temperate fauna. As the Mediterranean remained warmer, at least in some areas, than the surrounding Atlantic, progressive disjunction developed among the tropical and Mediterranean warm-waters (Thiede, 1978). This disjunction was both fostered by the intense upwelling in the northwest African coast and the more intense cooling of the North-eastern Atlantic, when compared with the Mediterranean, during glacial periods (Briggs, 1995).

Before the advent of phylogeography, the Pleistocene glaciations were already perceived by several authors as major disturbances that must have affected the fish fauna. Zander (1980) assumed that the Blennioidei, now present in the Mediterranean, would not tolerate the low glacial temperatures, and assumed that they survived in or near the tropics at the West African coast. Similarly, based on an analysis of the extant blenniid biodiversity, Almada et al. (2001) considered that the Lusitania Province contained several refugia little affected by the glaciations, namely in tropical West Africa and some warm pockets near Madeira. However, they postulated that conditions inside the Mediterranean would be sufficiently favorable to allow the persistence of fish of tropical origin that would become isolated from their West African relatives during glaciations. In this way, the Mediterranean would act as a refugium preserving tropical fish and as a secondary center of diversification, as fish spared from the glaciations would have

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had the opportunity to speciate and diverge ecologically. During interglacial periods, Mediterranean fish could migrate out into the Atlantic while new emigrants from the tropics could in some favorable climatic episodes, migrate into the Mediterranean. The operation of this two part system, with refugia in the tropics and in the Mediterranean, led to the prediction that many species-pairs should have a Mediterranean and a tropical member. Several phylogeographic studies supported these predictions, e.g., on the blennies *Parablennius sanguinolentus* and *Parablennius parvicornis* and the sister pair of damsel fishes *Chromis chromis* and *Chromis limbata*, in the Mediterranean and the tropics, respectively (Domingues et al., 2005, 2008).

The genus *Microlipophrys* was recently erected, based on mitochondrial DNA and morphology (Almada et al., 2005). These authors also showed that *Microlipophrys* is sister to a well-supported clade formed by *Lipophrys pholis*, *Lipophrys trigloides* and *Coryphoblennius galerita*. The genus *Microlipophrys* includes seven species: *Microlipophrys adriaticus* (Steindachner and Kolombatovic, 1883), *Microlipophrys bauchotae* Wirtz and Bath, 1982; *Microlipophrys caboverdensis* Wirtz and Bath, 1989; *Microlipophrys canevae* (Vinciguerra, 1880); *Microlipophrys dalmatinus* (Steindachner and Kolombatovic, 1883); *Microlipophrys nigriceps* (Vinciguerra, 1883); and *Microlipophrys velifer* (Norman, 1935). Four species occur in the Mediterranean, two of them extend their distributions as far as the Portuguese Atlantic coast. Three species occur only in the tropical Atlantic, namely in West Africa and the Cape Verde archipelago (see Table 1). These disjunct distributions render the genus especially interesting both phylogenetically and biogeographically.

A phylogeny of the genus will help to clarify if the genus originated in the tropics, having had a secondary diversification in the Mediterranean or, on the contrary, if it originated in the Lusitania Province and dispersed into the tropics at a later stage. We also address the suggestion that *M. caboverdensis* might have originated by hybridization of *M. bauchotae* and *M. velifer* (Wirtz and Bath, 1989). Wirtz and Bath (1989) speculated on this possibility because *M. caboverdensis* (endemic to the Cape Verde Islands) is intermediate between the two West-African species *M. velifer* and *M. bauchotae* in several character states.

The present study extends the analysis of Almada et al. (2005) by sampling two additional *Microlipophrys* species, *M. bauchotae* and *M. velifer*, and by including two additional nuclear markers (part of the Rhodopsin gene and the 1st intron of the S7 gene), to address the following issues: What are the phylogenetic relationships within *Microlipophrys* and in particular what is the relationship between the four Mediterranean and the three tropical specie?

2. Material and methods

2.1. Samples, DNA extraction, amplification and sequencing

We included all seven species of Microlipophrys, together with *L. pholis*, *L. trigloides* and *C. galerita*, and also included *P.* parvicornis and *P. sanguinolentus* as outgroups. New samples of *M.* nigriceps could not be obtained. We therefore used mitochondrial sequences already available for this species (Almada et al., 2005), but were unable to sequence nuclear markers.

Fish samples were collected by lab members or collaborators (see Acknowledgments) and voucher specimens deposited in the ISPA collection (ethanol preserved whole fish or tissue). Total DNA from remaining samples was extracted and PCR amplified using the REDExtract-N-Amp kit (Sigma–Aldrich, www.sigma.-com). We PCR amplified and sequenced two mitochondrial fragments (12S and 16S rDNAs) and two nuclear fragments (the first intron of the nuclear S7 ribosomal protein gene and part of the Rhodopsin gene) using the following pairs of primers: 12s rDNA – 12S For and 12SRev (Almada et al., 2005); 16s rDNA – 16SFor and 16SRev (Almada et al., 2005); first intron of the S7 – S7RPEX1F and S7RPEX2R (Chow and Hazama, 1998); and Rhodopsin – RhodF: CCG TCATGGGCGCCTA(CT)ATGTT(CT)(CT)T and RhodR: CAG-CACAGGGTGGTGATCAT(AG)CA(AG)TG. Amplification of the latter fragment was conducted as follows: 35 cycles of [94 °C (1 min), 60 °C (1 min) and 72 °C (1 min)]. PCR products were purified using microClean (MicroZone, www.microzone.co.uk), and sequenced in STABVIDA (http://www.stabvida.net/) using the same primers. A table with voucher name, collection location, and corresponding GenBank Accession Number, per gene fragment, is provided as Supplementary Material.

2.2. Phylogenetic analyses

Sequences were edited using BioEdit v. 7.0.1 (Hall, 1999) and aligned using ClustalW (Thompson et al., 1994). We applied Maximum Parsimony (MP), Minimum Evolution (ME), Maximum Likelihood (ML) and Bayesian Inference (BY) methods to each DNA marker separately, to a concatenated mitochondrial data set, and to all markers combined (except ME). For multi-loci data sets, partitioned models were implemented in ML and BY approaches.

MP and ME analysis were performed with PAUP*v4.0b10 (Swofford, 2003). For ME analyses the best-fit model of nucleotide substitution were selected with jModeltest 0.1.1 (Posada, 2008). The best models were chosen according to Akaike Information Criterion (AIC) were TIM2 + I + Γ, for the concatenated 12S and 16S fragments; TPM1uf + Γ for the Rhodopsin region; and TrN + Γ for

Table 1
Distribution and climate of *Microlipophrys* species and their close relatives.

	Distribution	Climate/Rage
Microlipophrys adriaticus	Mediterranean Sea, Sea of Marmara and the Black Sea	Warm Temperate
Microlipophrys bauchotae	Northeast Atlantic, Bay of Victoria, Cameroon and Bahia de Isabel, Fernando Poo	Tropical
Microlipophrys caboverdensis	Eastern Central Atlantic: endemic to Cape Verde	Tropical
Microlipophrys canevae	Mediterranean Sea and off southern Portugal	Warm Temperate
Microlipophrys dalmatinus	Mediterranean Sea and off southern Portugal	Warm Temperate
Microlipophrys nigriceps	Mediterranean Sea	Warm Temperate
Microlipophrys velifer	Eastern Atlantic: off west Africa from Senegal and Cape Verde to the Cunene River, Angola	Tropical
Lipophrys pholis	Mediterranean and North-eastern Atlantic: southwards from Norway to Morocco	Cold Temperate
Lipophrys trigloides	Mediterranean, Canary and Madeira Islands, and North-eastern Atlantic: from the coasts of France, the Iberian Peninsula, Morocco, and southwards to Senegal	Warm Temperate
Coryphoblennius galerita	Mediterranean, Canary and Madeira Islands, and North-eastern Atlantic: from the coasts of western England and the British Channel, France, Spain, Portugal, and southwards to Morocco	Warm Temperate
Blennius ocellaris	Mediterranean and Black Sea and Northeast Atlantic coast from Morocco to the English Channel	Temperate

the S7 region. Bootstrap analyses (1000 replicates) were used to assess the relative robustness of branches of the ME and the MP trees (Felsenstein, 1985). ML analyses was performed using RAxML v.7.0.3, with 1000 thorough bootstrap replicates (Miller et al., 2009; Stamatakis et al., 2008).

Bayesian analysis was performed using MCMC as implemented in Mr. Bayes 3.1 (Ronquist and Huelsenbeck, 2003), with two independent runs of four Metropolis-coupled chains of four million generations each, to estimate the posterior probability distribution. Topologies were sampled every 100 generations, and a majority-rule consensus tree was estimated after discarding the first 10^5 generations.

Age of most recent common ancestors were estimated using a linearized ME evolution tree, assuming a strict molecular using MEGA (Tamura et al., 2007). Molecular clocks for the mitochondrial and for the nuclear S7 were estimated from levels of mean net divergence between the blenny sister species *Hypsoblennius invemar* and *Hypsoblennius brevipinnis*, respectively 4.5% and 5.4% divergence for 12S and 16S (when 12S and 16S were analyzed as a concatenated alignment, we assumed the most conservative, slower 12S clock) and 8.3% divergence for S7, and assuming they were separated by the isthmus of Panama 2.8 MYA (Lessios, 2008). We also applied BEAST in order to implement a relaxed molecular clock (Drummond et al., 2006; Drummond and Rambaut, 2007), assuming a GTR + Γ substitution model with 6 gamma categories, a Yule process prior, the monophyly of the ingroup, *Microlipophrys* and the *Lipophrys* group, and running each analysis for 40 million generations, sampling every 1000 generations. Alignments and trees were deposited in Treebase (http://www.treebase.org, submission ID number: 10695). Available during submission for reviewers at http://purl.org/phylo/treebase/phylows/study/TB2:S10705?x-access-code=4c1d3a0360361625ebaae9dae3fce608&format=html.

3. Results

A total of 450, 490, 790, and 730 bp were aligned corresponding to 12S, 16S, Rhodopsin, and S7, respectively. Gene fragments included 121, 127, 52 and 179 parsimony informative sites, for the 12S, 16S, Rhodopsin, and S7, respectively. The two mitochondrial fragments combined yielded an 890 bp alignment. An alignment of all markers included 2373 bp and 24 specimens for which all 4 markers were sequences. Only *M. nigriceps* mitochondrial data was included in this alignment. The independent molecular information among the mitochondrial and the two nuclear regions were complementary and reinforced each other. Overall, the Bayesian approach inferred more resolved and better supported topologies. Among the three regions, the Rhodopsin fragment proved the most effective in singly resolving the relationship among species.

Overall, the most salient features that are consistent among analyses are:

1. The *Microlipophrys* and *L. pholis–L. trigloides–C. galerita* form well supported sister clades. Therefore, the validity of the genus *Microlipophrys* (Almada et al., 2005) is supported by molecular data, both mitochondrial and nuclear, when considering all species of this genus and the most closely related species. Assuming a molecular clock for the concatenated mitochondrial sequences of 0.008 subst./MY for the mitochondrial regions and 0.0148 subst./MY for S7 (Almada et al., 2009), suggests the divergence between *Lipophrys–Coryphoblennius* and *Microlipophrys* occurred *ca.* 2–11 MYA.
2. Results are consistent with *M. nigriceps–M. canevae* being sister species that comprise a basal clade within the genus *Microlipophrys*. This is evident in mitochondrial analyses, as we were unable to obtain nuclear data for *M. nigriceps*, but holds up in total evidence analyses, wherein only *M. nigriceps*'s mitochondrial

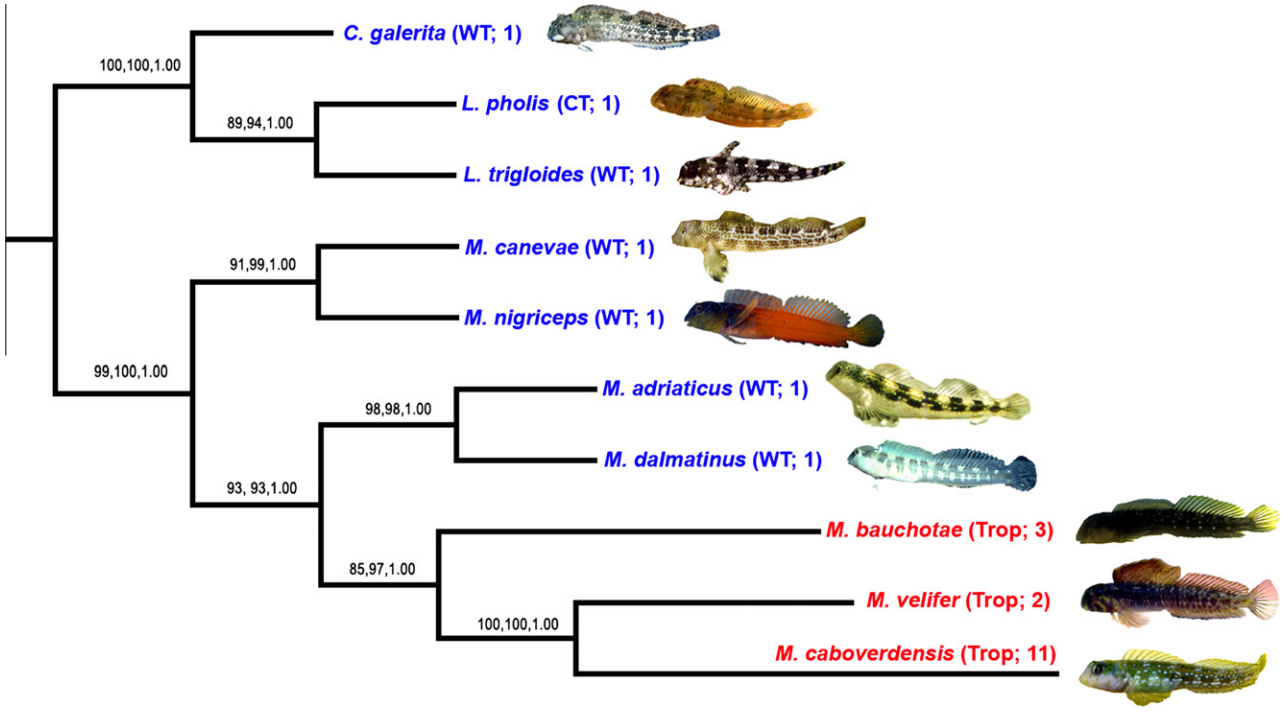

Fig. 1. Maximum Parsimony phylogram estimated from a full evidence approach, including the mitochondrial 12S and 16S regions and the S7 and Rhodopsin nuclear regions (only mitochondrial data was used for *M. nigriceps*). *Parablennius parvicornis* and *P. sanguinolentus* were used as outgroups and to root the tree. Numbers in parenthesis after species names indicate the number of specimens of each species included in this analysis. (The larger sample of *M. caboverdensis* may be responsible for the relative larger size of its branch length.) Support values near each node correspond to maximum parsimony bootstrap values (based on 1000 replicates), maximum-likelihood bootstrap values (based on 1000 replicates) and Bayesian posterior probabilities. Species habitat is indicated as CT (Cold Temperate), WT (Warm Temperate) and Trop (Tropical). Topologies based independently on the mitochondrial regions and each nuclear region, with corresponding support values, are available as Supplementary Material. Fish are not to scale.

sequence was used (Fig. 1). Analysis of the concatenated mitochondrial DNA revealed 88%, 99%, and 73% bootstrap support for the *M. nigriceps–M. canevae* clade in the MP, ME and ML analyses, respectively, and 0.95 posterior probability in the BY analysis. The remaining *Microlipophrys* form a well-supported clade in the full evidence analysis (Fig. 1). This result is also supported when considering the most phylogenetically informative mitochondrial fragment (16S) and when analyzing the concatenated mitochondrial DNA. Independent analyses of the two nuclear fragments (where *M. nigriceps* sequence is absent) were consistent with this result: the unconstrained topologies did not differ significantly from topologies constrained to form a clade containing all *Microlipophrys* except *M. canevae*. For instance, the unconstrained ME topology using Rhodopsin did not differ significantly from the constrained estimate (Shimodaira–Hasegawa test: $p > 0.1$). Molecular clock analyses estimated the *Microlipophrys* clade to be 2.1–11 MYA.

3. The full evidence approach and analyses of mitochondrial and Rhodopsin suggest a clade formed by the Mediterranean *M. adriaticus–M. dalmatinus*. This relationship was not revealed in the analyses of S7, although the supported topology was not inconsistent with the existence of this clade, for instance the unconstrained ME topology did not differ significantly from one constrained to have a *M. adriaticus–M. dalmatinus* clade (S–H test; $p > 0.1$).
4. All analyses of each DNA region suggest a well-support clade formed by *M. velifer* and *M. caboverdensis*. Support for this clade is very strong in the full evidence approach (Fig. 1). Moreover, support is also robust from the mitochondrial, S7 and Rhodopsin analyses: from 72–100% (for MP), 91–99% (for ME) and 69–100% (for ML), and from 0.7–1.0 posterior probability (for BY). Despite the substantial sampling of *M. caboverdensis* fish (12–19 specimens, depending on the gene marker), there is no evidence of close phylogenetic affinity between any mitochondrial or nuclear sequence of *M. caboverdensis* and *M. bauchotae*.
5. The full evidence approach supports the association of *M. bauchotae* with the species pair *M. velifer–M. caboverdensis*, forming a tropical *Microlipophrys* clade. Evidence from independent analyses among the markers varies, although the several *M. bauchotae* sequences always form a well-supported clade. For instance, all analyses of S7 sequences revealed strong support for the full evidence topology. Although, BY analysis of mitochondrial and Rhodopsin data revealed a closer proximity of *M. bauchotae* with the *M. adriaticus–M. dalmatinus* clade, analyses of these fragments with other methods (MP, ME and ML) were not inconsistent with a tropical *Microlipophrys* clade. For instance the ME estimate using Rhodopsin did not differ from the constrained estimate (S–H; $p > 0.1$). Molecular clock analyses estimated the tropical *Microlipophrys* clade to be 1.5–12 MYA.

4. Discussion

Our results, comprising all the *Microlipophrys* species and four genetic markers, including two nuclear markers, confirm the validity of the monophyly of *Microlipophrys*, as suggested by Almada et al. (2005), and of the seven currently recognized species. Based on molecular data, this genus and the *Lipophrys–Coryphoblennius* group form well-supported clades, estimated to have diverged at least 5 MYA, in the late Miocene. In addition, *Microlipophrys* is characterized by a smaller size (from 4 cm in *M. dalmatinus* to 7 cm in *M. canevae*), use of tightly fitting nest holes by males, the presence of colorful head markings in males associated with fast head moving displays in the breeding season (Abel, 1993), and the presence of 12 pectoral rays, in contrast with the 13 pectoral rays of *Lipophrys* (Almada et al., 2005; Bath, 1977; Wirtz and Bath, 1982; Wirtz and Bath, 1989). *Coryphoblennius*, the basal species of the *Lipophrys–Coryphoblennius* clade, has 12 pectoral rays (Bath, 1977), perhaps having retained the ancestral character state of both groups.

The extensive sampling of *M. caboverdensis* and its well supported relationship with *M. velifer* across all markers also suggests the rejection of the hypothesis of a hybrid origin of *M. caboverdensis*, from the presumed parentals *M. velifer* and *M. bauchotae* (Wirtz and Bath, 1989), although we cannot discard indication of such a hybrid origin in other loci.

Almada et al. (2001) suggested the African tropical coast, Madeira and warm Mediterranean pockets served as refugia during colder periods. Successive waves of recolonization from these areas into the warm temperate North-Atlantic and Mediterranean may have favored diversification. The group of species included in this study seems to indicate an alternate pattern.

The clade *L. pholis–L. trigloides–C. galerita* and *Blennius ocellaris* (Almada et al., 2005), include cold-tolerant species, reaching latitudes as high as the North Sea, in the case of *L. pholis*, or British Isles in the case of *C. galerita* (Table 1). Within *Microlipophrys*, the basal clade *M. nigriceps–M. canevae* is mostly endemic to the Mediterranean, as are *M. adriaticus* and *M. dalmatinus*. Among these Mediterranean *Microlipophrys*, *M. dalmatinus* and *M. canevae* extend their distribution into the Atlantic, along the coast of Portugal, but no further. The hypothesis that *Microlipophrys* originated and had its center of diversification at mid-latitudes, as early as the Miocene, is supported by their close relation with cold-tolerant species, the peak of diversity of *Microlipophrys* in the Mediterranean, and the fact that its basal clade is distributed in the Mediterranean.

Diversification may have taken place in the Mediterranean itself, promoted by changing conditions in this sea and successive stages of isolation of suitable pockets, as seems to be suggested by the near restriction of the northern *Microlipophrys* to the Mediterranean. The distribution of the *Lipophrys–Coryphoblennius* clade, extending along the Atlantic coast of Europe and northwestern Africa, and present in the Azores, Madeira and Canaries Islands, also suggests the hypothesis that these two sister clades evolved inside in the Lusitania Province and acquired varied adaptations to colder temperatures.

Thus, our results suggest that the tropical subclade, namely *M. bauchotae*, *M. caboverdensis* and *M. velifer*, has a single origin, by dispersal of a northern ancestor into tropical West Africa.

If correct, this finding is counter-intuitive, as we tend to assume that the tropics, with their high diversity, are exporters to higher latitudes (Jablonski et al., 2006). It also illustrates the role of the Mediterranean and the Lusitania Province as a center of diversification. Due to the imprecision of our molecular clock, we cannot ascertain if the southward migration of the ancestor of the tropical African *Microlipophrys* took place when tropical conditions extended to the North-eastern Atlantic, or later when Pliocene cooling was already ongoing. Even with this limitation, our estimates clearly reject a Pleistocene origin of this tropical clade (TMRCA of tropical clade > 2 MYA). The Plio-Pleistocene cooling would in any case, hinder the migrations between the Mediterranean and tropical West Africa, thus helping to maintain the disjunction in the distribution areas of the genus.

Other genera, with similar peaks of diversity and endemism in the Mediterranean, suggest the importance of subtropical/temperate regions as sites of diversification may be more general. The Mediterranean has nine endemic genera teleost reef fish, representing 11% of its total genera (Floeter et al., 2008). For instance, the genus *Tripterygion* (Blennioidei: Trypterigiidae) has four recognized species (Carreras-Carbonell et al., 2007), all of which occur in the Mediterranean. Only *Tripterygion delaisi* is not endemic to this

Sea, extending its distribution to the Atlantic, both northward to the English Channel and southward to Senegal. Thus when considering the distribution of the genus as a whole, its ancestor may have been subtropical or temperate, perhaps located in the Mediterranean, rather than in the tropics.

Hanel et al. (2002) identified a monophyletic Labrini tribe (familiy Labridae), whose members are at present largely presently distributed in the Lusitania Province, and provide a further example of this province as a center for diversification in several genera. The molecular study of Lepadogastrinae (family Gobiesocidae) also suggest this region played an important role in diversification of several genera (Almada et al., 2008).

In conclusion, we think our study and others suggest that biogeographic importance of the Lusitania Province should be reconsidered. In several cases it does not function as a mere transition region, but constitutes a region capable of fostering diversification in marine species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.12.008.

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