

## Molecular systematics of the endemic Leptaxini (Gastropoda: Pulmonata) on the Azores islands

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### Abstract

The Azorean representatives of the Leptaxini (Pulmonata) are single island endemics, where a high-spired shell distinguishes the monotypic genus *Helixena* from two slightly different low-spired forms within *Leptaxis* (*azorica* and *caldeirarum* type). We studied the evolutionary history of putative taxa and the three shell-types using 12 allozyme loci and sequences of nuclear (ITS-1 and ITS-2) and mitochondrial DNA (COI and 16S rRNA). While little variation was found in both ITS genes, allozyme and mtDNA divergence was among the highest reported for pulmonate land snails. Generally, phylogeographic patterns are indicative of allopatric differentiation via the successive colonization of (younger) islands, while a major role for adaptive evolution is not supported. The *azorica* shell-type is monophyletic and has no common history with other sympatric shell-types on the same islands. The (ambiguous) position of *Helixena sanctaemariae* makes *Leptaxis* paraphyletic on the Azores and possibly also the *caldeirarum* shell-type. *Helixena* can therefore not be distinguished as a separate genus on the Azores. Following a lineage-based concept, representatives on all (ancient) islands should be considered distinct species.

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### 1. Introduction

Oceanic islands with well-documented geological histories provide excellent settings and time frameworks to evaluate the processes that lead to speciation and endemic radiations (Barton, 1998; Emerson, 2002; Gillespie and Roderick, 2002; Orr and Smith, 1998). This is nicely illustrated by several studies of endemic island radiations in terrestrial snails such as *Partula* on the Pacific Ocean Islands (Goodacre and Wade, 2001), *Cerion* on the Bahama Islands (Gould and Woodruff, 1990),

*Achatinella* in Hawaii (Thacker and Hadfield, 2000), *Albinaria* in Crete (Douris et al., 1998), and *Mandarina* in the Bonin Islands (Chiba, 1996). Yet, the relative importance of natural selection (adaptation) and common history in shaping phenotypic diversity of endemic snail radiations in islands remains unclear (Barton, 1998; Davison, 2002; Gittenberger, 1991; Orr and Smith, 1998). This is not only because of the confounding interplay between selection, drift, migration, and hybridization, but probably also the fact that most of these snail radiations involve very speciose taxa that show either a conspicuous phenotypic diversity or, conversely, a high degree of (convergent?) phenotypic similarity. However, understanding the evolutionary dynamics of the phenotypic diversity of endemic snail radiations would benefit from studies

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dealing with island radiations that are less speciose, but at the same time comprise both highly divergent and similar phenotypes which evolved over varying time intervals. A good example of such a radiation is the Azorean Leptaxini (Hygromiidae), a group of endemic terrestrial snails whose evolutionary history will be dealt with in the present paper.

The archipelago of the Azores [latitude: 36°55'–39°43'N (530 km); longitude: 25°00'–31°17'W (320 km)] comprises nine strongly isolated islands astride the Mid-atlantic Ridge. The islands are of volcanic origin and vary in age from 0.3 to 8 million years (My; Fig. 1). The recurrent volcanic activity in the archipelago may have promoted repeated extinction, fragmentation, (re)colonization, and allopatric speciation (e.g., Cook, 1996). Volcanic activity also changed the geomorphology of the islands and in particular established the current shape of the largest island (São Miguel) about 0.05 My ago by producing a land bridge between an older eastern island that originated 4 My ago and a younger western island that originated 0.55 My ago (Fig. 1) (Van Riel et al., 2003 and references therein).

The extant Azorean Leptaxini involves two genera (*Leptaxis* Lowe, 1852 and *Helixena* Backhuys, 1975) and seven nominal (sub)specific taxa. One of these, viz. *Leptaxis erubescens* (Lowe, 1831), was probably introduced by human from Madeira (Backhuys, 1975) and does not seem to have established itself on the Azores. Therefore, this species will not be considered here. The

other extant nominal *Leptaxis* taxa from the Azores are either regarded as distinct species or as subspecies of one single species (Backhuys, 1975; see Table 1 for names, authorships and abbreviations). Because the evidence supporting these alternative taxonomic interpretations is still indecisive, we will arbitrarily treat the different nominal taxa distinct species (Table 1). Most of these taxa are supposed to be restricted to single islands (Table 1), and the recent discovery of two unnamed *Leptaxis* sp. from the islands of Pico and Graciosa (Table 1) has further strengthened this assumption. Within Azorean *Leptaxis* two slightly different shell forms can be distinguished, viz., the *azorica* type (Fig. 3) with a more globose shell and an obtuse apex vs. the *caldeirarum* type (Fig. 3) with a more compressed shell and a pointed apex. In contrast, the high-spired shell of *Helixena sanctaemariae* (Fig. 3) is so different from that of *Leptaxis* sp. that the species was originally assigned to a different family (Enidae). Anatomically, however, *H. sanctaemariae* is nearly indistinguishable from *Leptaxis* OTUs. Therefore, Backhuys (1975) transferred the species to the Leptaxini and created the monotypic genus *Helixena* in recognition of the species' aberrant shell form (here referred to as the *Helixena* type). Interestingly, *H. sanctaemariae* lives sympatrically with *Leptaxis minor* (*azorica* type) on Santa Maria, the oldest island of the archipelago, while *L. caldeirarum* (*caldeirarum* type) lives sympatrically with *L. azorica* (*azorica* type) in the western (i.e., younger) part of São Miguel (Table 1).

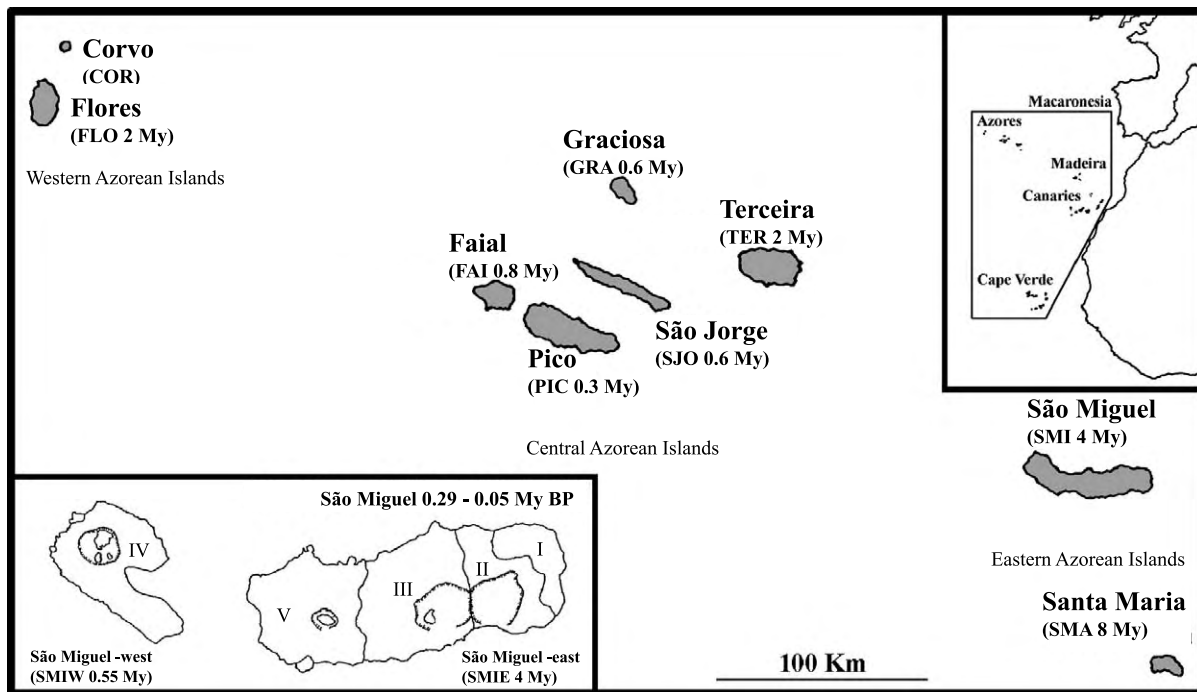


Fig. 1. Maps of the Azores Islands (abbreviation and geological age indicated below), Macaronesia (top inset) and São Miguel (geological settings between 0.29 and 0.05 My ago; ages of volcanologic regions: I, 4.2–0.95 My; II, 3.2–0.65 My; III, 0.8 My; IV, 0.55 My; V, 0.29 My) (Forjaz, 1998; Morton et al., 1998).

Table 1

Taxonomy (provisional names for OTUs and abbreviations in square brackets) and distribution (Island; see Fig. 1 for abbreviations) of the Azorean Leptaxini (*Helixena* and *Leptaxis*) following Backhuys (1975), number of specimens used for allozyme analysis ( $N_{\text{allo}}$ , number of populations in parentheses) and GenBank accession numbers for ITS-1, ITS-2, COI, and 16S sequences

(Sub)species/OTUs	Island	$N_{\text{allo}}$	ITS-1	ITS-2	COI	16S
<i>L. azorica</i> (Albers, 1852) [ <i>L. a.</i> “east”; Lae]	SMIE	183 (7)	AY747110	AY747120	AY747127	AY748297
[ <i>L. a.</i> “west”; Law]	SMIW	128 (5)	AY747111	AY747121	AY747128	AY748298
[ <i>L. a.</i> “Flores”; Laf]	FLO	34 (2)	AY747112	—	AY747129	AY748299
	COR <sup>a</sup>	—	—	—	—	—
<i>L. minor</i> (Backhuys, 1975) [Lm]	SMA	36 (2)	AY747113	AY747122	AY747130	AY748300
<i>L. caldeirarum</i> (Morelet and Drouët, 1857)[Lc]	SMIW	49 (2)	AY747114	—	AY747135	AY748305
<i>L. terceirana</i> (Morelet, 1860) [Lt]	TER	38 (1)	AY747115	—	AY747133	AY748303
<i>L. drouetiana</i> (Morelet, 1860) [Ld]	FAI	4 (1)	AY747117	AY747125	AY747131	AY748301
<i>Leptaxis</i> sp. <sup>a</sup> [ <i>L.</i> “Graciosa”; Lg]	GRA	7 (1)	AY747116	AY747124	AY747134	AY748304
<i>Leptaxis</i> sp. <sup>a</sup> [ <i>L.</i> “Pico”; Lp]	PIC	23 (1)	AY747118	AY747123	AY747132	AY748302
<i>L. vetusta</i> (Morelet and Drouët, 1857) <sup>b</sup>	SMA	—	—	—	—	—
<i>H. sanctaemariae</i> (Morelet and Drouët, 1857) [Hs]	SMA	309 (13)	AY747119	AY747126	AY747136	AY748306

<sup>a</sup> Martins, unpublished.

<sup>b</sup> Subfossil.

Against this background, we used allozyme and DNA sequence data from all known extant endemic Azorean Leptaxini to reconstruct the evolutionary history of this radiation. In this way, we aimed at four main goals: (1) assessing whether the *azorica* and *caldeirarum* shell forms reflect common history or evolved repeatedly as convergent adaptations, (2) inferring the evolutionary origin of the aberrant high-spined shell form of *H. sanctaemariae*, (3) estimating the rates of molecular change associated with the divergence of taxa and shell morphologies, and (4) providing a molecular framework to underpin the taxonomic interpretation of the Azorean Leptaxini.

## 2. Materials and methods

### 2.1. Sampling and sample preparation

Specimens ( $N = 811$ ; Table 1) were collected by hand and stored at  $-80^{\circ}\text{C}$ . All animals were dissected on ice

under a stereomicroscope. The digestive gland was removed, weighted, and homogenized in a 20%(w/v) aqueous sucrose solution ( $5\ \mu\text{l}/\text{mg}$  tissue). A piece of tissue from the foot muscle was kept at  $-80^{\circ}\text{C}$  for DNA extraction and the remaining soft body parts were stored in 70% ethanol (material deposited in the Royal Belgian Institute of Natural Sciences, IG 30325).

### 2.2. DNA sequences

DNA extractions were carried out following Winnepeenninckx et al. (1993). For each OTU, two nuclear (ribosomal internal transcribed spacer 1 and 2; ITS-1 and ITS-2) and two mitochondrial gene fragments (16S rRNA and cytochrome oxidase subunit 1; COI) were amplified (2–8 individuals) via the polymerase chain reaction (PCR) using universal primers (Table 2). For COI, a new set of primers was designed by modifying LCO1490 via molluscan COI sequences available in GenBank (Accession Nos. NC001816, NC001636, and

Table 2

Genes fragments and primers sequences (5′–3′) used for PCR and sequencing

Gene	Primer sequence	Name
ITS-1 <sup>a</sup>	TCCGTAGGTGAACCTGCGGAAGGAT	ITS1L
	TGCGTTCAAGATATCGATGTTCAA	58C
ITS-2 <sup>b</sup>	TATGCTTAAATTTAGGGGGT	ITS2B-28S
	TGTGAACTGCAGGACACATG	ITS2A-5.8S
16S rRNA <sup>c</sup>	CCGGTCTGAACTCAGATCACGT	16Sbr
	CGCCTGTTTAAACAAAAACAT	16Sar
COI <sup>d</sup>	GGTCAACAAATCATAAAGATATTGG	LCO1490
	TAAACTTCAGGGTGACCAAAAAATCA	HCO2198
COI <sup>e</sup>	TTTCNACTAACCATAARGATATTGG	LCO2
	CGTTCMAGTGTTATACCTGGAGA	HCO2
	CGCTCTAAYGATATACCAGGAGA	HCO3

<sup>a</sup> Hillis and Dixon (1991).

<sup>b</sup> Beebe and Saul (1995).

<sup>c</sup> Palumbi et al. (1991)

<sup>d</sup> Folmer et al. (1994).

<sup>e</sup> Van Riel et al. (this study).

Z71696; Benson et al., 2003) and by selection of specific reverse primers (HCO<sub>2</sub> and HCO<sub>3</sub>, respectively, for *L. azorica*/*L. minor* and the remaining OTUs), internal to LCO1490 and HCO2198 (see Table 2).

PCRs (25  $\mu$ l) were carried out with 0.2  $\mu$ M primer, using the standard protocol given with Promega PCR Core System I (Promega Benelux BV). The thermal cycling program consisted of an initial denaturation step (95 °C for 5 min) followed by 30 cycles of 95 °C (2 min), 45–55 °C annealing (1 min), and 72 °C (1 min) extension, ending with a final extension step of 5 min at 72 °C. Sequence reactions were carried out accordingly and analyzed with an ABI 373A automated sequencer (Applied Biosystems). Sequence data of outgroup taxa (families Helicidae and Hygromiidae) were obtained from GenBank and used for rooting trees. Different (combinations of) outgroups did not change the results. In the final analyses, *Arianta arbustorum* was used with ITS-1 (AF124053), ITS-2 (AY014136), and COI (AF296940.1), while *Phaeohelix phaeogramma* was selected for the analyses with 16S (AF098714.1). All sequences obtained for this study were submitted to GenBank (Table 1; Benson et al., 2003).

After alignment of the sequences with CLUSTAL X v1.83 (default options; Thompson et al., 1997), phylogenetic analyses were carried out with PAUP v4.0b10 (Swofford, 1998), using the criterion of maximum likelihood (ML; heuristic search via TBR, 100 randomized trees) and maximum parsimony (MP; exhaustive search) for optimal tree selection. Support for internal nodes was evaluated via 1000 bootstrap replicates (TBR). MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) was used for evaluating node support via posterior probabilities obtained via Bayesian analysis (BA) with Metropolis-coupled Markov chain Monte Carlo (mcmc) sampling (10<sup>6</sup> generations, sampled 1/100, discarding the first 10<sup>5</sup> generations = burnin value), using uninformative priors. The substitution model for the ML and BA was selected using likelihood ratio tests (LRT) implemented in Modeltest v3.06 (Posada and Crandall, 1998). Analyses using concatenated data were justified by failure to reject ( $\alpha = 0.05$ ) the null hypothesis of partition homogeneity using the incongruence length difference (ILD) test (10,000 replicates; invariable sites excluded) in PAUP v4.0b10 (Swofford, 1998) and the Swofford–Olsen–Waddell–Hillis test (SOWH test; Swofford et al., 1996), as described by Goldman et al. (2000), based on 500 parametric sequence simulations generated with the program Seq-Gen v1.2.7 (Rambaut and Grassly, 1997). Conflicting phylogenetic signals were also evaluated via spectral analyses using the program Spectrum v2.0 (Charleston and Page, 1999). The null hypothesis ( $H_0$ ) of constant evolutionary rates across lineages for a given phylogeny (likelihoods with/without molecular clock enforced via PAUP v4.0b10) was tested using the LRT calculator ( $\chi^2$ ; degrees of freedom = number of

OTUs—2) in Modeltest v3.06 (Posada and Crandall, 1998).

Local clocks were searched for via relative rate tests (RRT) as implemented in RRtree (Robinson-Rechavi and Huchon, 2000). Additionally, divergence times (mtDNA) and associated substitution rates were evaluated using the Bayesian relaxed molecular clock as implemented in the software package Multidistribute (Thorne and Kishino, 2002). Branch lengths and a variance–covariance matrix were determined (ESTBRANCHES) for each mtDNA fragment separately (F84 model + G = gamma distributed rate variation across sites) and analyzed simultaneously (MULTIDIVTIME) using the topology in Fig. 3. The mcmc algorithm was run (10<sup>6</sup> generations, sampled = 1/100, burnin = 10<sup>5</sup>) with prior values selected and evaluated following the recommendations by Thorne and Kishino (2002) (rttm = 6, rttmsd = 2, rtrate = 0.02, rtratesd = 0.02, bigtime = 100, nodata = 0, and commonbrown = 0, and default values for brownmean, brownsd, minab, newk, othk, and thek). The geological ages of the islands were used as maximum time limits ( $T_M$ ). Substitution rates were also evaluated via ML (F84 + G) with these dates as calibration points, under the assumption of a global molecular clock or with local deviations, as implemented in PAMLv3.14 (Yang, 1997).

### 2.3. Allozyme electrophoresis

Vertical polyacrylamide gel electrophoresis (PAGE) was used to screen variation in the following enzyme systems: non-specific esterases (EST; EC 3.1.1.-), aspartate aminotransferase (AAT; EC 2.6.1.1), glycerol-3-phosphate dehydrogenase (GPD; EC 1.1.1.8), glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), 3-hydroxybutyrate dehydrogenase (HBDH; EC 1.1.1.30), isocitrate dehydrogenase (IDH; EC 1.1.1.42), leucyl aminopeptidase (LAP; EC 3.4.11.1), peptidase (PEP; EC 3.4.13.18; substrate Leu-Ala), phosphoglucomutase (PGM; EC 5.4.2.2), and superoxide dismutase (SOD; EC 1.15.1.1). Staining recipes were modified after Harris and Hopkinson (1976). Genotypic data were used to calculate allele frequencies, mean number of alleles ( $A$ ), percentage unique alleles ( $U$ ), percentage polymorphic loci ( $P$ ), and Nei's (1978) unbiased genetic distances ( $D_{Nei}$ ) using the program BIOSYS-1 (Swofford and Selander, 1981).

## 3. Results

### 3.1. DNA sequence variation

Basic sequence characteristics are summarized for each gene fragment in Table 3. Both nuclear gene

Table 3

Sequence length (Bp), ML settings for base composition (% A/C/G/T), transition/transversion ratio ( $s/v$ ), substitution model (ML model) and likelihood of ML trees ( $-\ln L$ ), number of variable sites ( $S$ ), parsimony informative sites (PI) ( $S$  and PI with outgroup excluded in parentheses), number of MP trees (length in parentheses), consistency index (CI), retention index (RI), for each analyzed gene fragment (and COI + ITS-1 combined)

Gene	Base pairs	(A/C/G/T)	$s/v$	ML model	$-\ln L$	$S$	PI	MP	CI	RI
ITS-1	517–521	19/27/31/23	1.3	HKY <sup>b</sup>	1175.10	107 (16)	4 (0)	13 (108)	1.00	1.00
ITS-2	452 <sup>a</sup>	25/25/25/25	1.2	K2P <sup>c</sup>	1109.20	117 (18)	11 (11)	1 (122)	1.00	1.00
16S	370–374	37/13/17/33	—	GTR <sup>d</sup> + G	1404.87*	130 (77)	53 (43)	8 (210)	0.81	0.70
COI	378	28/14/16/42	—	GTR <sup>d</sup> + G	1583.28*	125 (112)	72 (67)	2 (247)	0.73	0.57
COI + ITS-1	895–899	25/25/25/25	—	GTR <sup>d</sup> + G + I	2948.03	—	82 (77)	1 (355)	0.81	0.64

H<sub>0</sub> = clock-like tree, rejected by LRT. See also text for abbreviations.

<sup>a</sup> Ambiguous region of 32 bp excluded.

<sup>b</sup> Hasegawa–Kishino–Yano model.

<sup>c</sup> Kimura 2-parameter model.

<sup>d</sup> General time reversible model with gamma distributed (G) rate variability across sites (shape parameter: G<sub>16S</sub> = 0.168; G<sub>COI</sub> = 0.174; G<sub>COI-ITS1</sub> = 0.579) and/or proportion invariable sites (I<sub>COI-ITS1</sub> = 0.443).

\*  $p < 0.01$ .

fragments showed little variation within the Azorean Leptaxini (Table 3). For convenience, we will here already refer to the two main clades that resulted from the phylogenetic analyses presented in 3.2 (clade A, *Lep-taxia azorica* and *L. minor*; clade B, remainder of OTUs; Fig. 3). For ITS-1, identical sequences were found for all OTUs of the *caldeirarum* shell-type and *H. sanctaemariae* (clade B in Fig. 3). Sequence divergence (ML) ranged from 0.2 to 0.9% within *L. azorica* (mean: 0.5%), 0.2 to 0.7% between *L. azorica* and *L. minor* (mean: 0.5%), and from 2 to 2.6% between clades A and B (mean: 2.3%). Additionally, one indel was shared between *L. minor* and the OTUs of clade B (5 bp, one of which differs between *L. minor* and clade B), while the latter were characterized by two indels of 1 and 3 bp.

No ITS-2 were obtained for *L. azorica* “Flores,” *L. caldeirarum* and *L. terceirarum*. Yet, in concordance with ITS-1, identical sequences were found for *L. “Graciosa,” L. drouetiana,* and *L. “Pico,”* but with ITS-2, these OTUs differed slightly from *H. sanctaemariae* (0.7%). Within the *azorica* shell-type, sequence divergence ranged from 0.2 (between *L. azorica* “east” and *L. azorica* “west”) to 1.3% (between *L. azorica* and *L. minor*). Sequence divergence between OTUs from clade A and B ranged between 2.9 and 3.4% (mean: 3.1%).

The mtDNA fragments showed much more variation between most OTUs. The COI fragment was most variable and characterized by a strong bias towards synonymous substitutions at third codon positions, which were saturated for transitions (data not shown). The substitution pattern within *L. azorica* differed conspicuously from all other OTUs as evidenced by a lower A + T content (mean: 77% vs. 88% in the remainder

of OTUs) and higher transition/transversion ratio ( $s/v$ ) at third codon positions (mean: 1.6 vs. 1.3). After translation (codon Table 5; Benson et al., 2003), only three amino acid changes were involved (one in *L. azorica* “east,” one in *L. caldeirarum*, and one in *L. “Pico”* with five additional amino acid changes in the outgroup) for a total of 112 variable nucleotide positions within the Azorean Leptaxini (14 at 1st, one at 2nd, and the remainder at 3rd codon positions). Sequence divergence (ML) within clade A ranged between 26.3 and 38.1% (mean: 30.9%), the minimum and maximum values found between *L. azorica* and *L. minor* (mean: 32.3%). Similar values (range: 23.3–55.8%) were found between clades A and B (mean: 38.4%). In contrast, sequence divergence between the OTUs of the central Azorean Islands only ranged between 1.7 and 5.0% (mean: 3.5%) and were most similar to COI in *H. sanctaemariae* (range: 9.0–11.9%; mean: 10.7%) and *L. caldeirarum* (range: 15.4–17.5%; mean: 16.3%; mean between *H. sanctaemariae* and *L. caldeirarum*: 19.1%).

Lower levels of variation were associated with the 16S fragment. In agreement with ITS and COI, the smallest distances (range: 1.4–3.5%) were found between the central Azorean OTUs (mean 2.2%). They formed a closely related group with *H. sanctaemariae* and *L. caldeirarum* (clade B), from which they were differentiated by 1.4–9.0% (mean: 4.3%; mean between *H. sanctaemariae* and *L. caldeirarum*: 5.3%). In contrast with the results from ITS and COI, *L. minor* was slightly less differentiated from clade B (range: 11.7–17.3%; mean: 15.4%) than from *L. azorica* (clade A; range: 13.8–19.7%; mean: 16.5%). Similar values were found within *L. azorica* (range: 10.5–15.1%; mean: 13.3%) while

divergence with all other OTUs, excluding *L. minor*, ranged between 20.8 and 33.5% (mean: 25.7%).

### 3.2. Phylogenetic analyses

The number of parsimony informative sites (PI), (retention) consistency index [(R)CI], number and length of the MP trees (identical or similar topologies), substitution model, and likelihood ( $-\ln L$ ) of the ML tree are summarized for each gene fragment in Table 3. The ML trees obtained with COI and 16S are shown in Fig. 2.

Using MP and ML analyses, both nuclear DNA fragments (ITS-1 and ITS-2) only showed meaningful bootstrap values ( $\geq 70\%$ ) supporting the monophyly of the OTUs with the *azorica* shell-type (clade A; 85–96%), while ITS-2 also distinguished *Leptaxis minor* from *L. azorica* (85–93%) and *H. sanctaemariae* at the basis of clade B (96–99%).

The mtDNA produced a higher resolution. Strong support for the monophyly of clade A and *L. azorica* was only found with COI in the BA (92 and 93%, respectively), while MP and ML only weakly supported clade A (57 and 69%, respectively) and *L. azorica* (67 and 61%, respectively). Based on 16S, a basal position of

*L. minor* in clade B was strongly supported in the MP (92%) and BA (71%) analyses, while the monophyly of *L. azorica* was only weakly supported (68 and 57%, respectively). Further disparity between the mtDNA genes involved the positions of *H. sanctaemariae* and *L. caldeirarum*. A basal position in clade B for *L. caldeirarum* and *H. sanctaemariae* as the sister taxon of the central Azorean OTUs (i.e., the paraphyly of the *caldeirarum* shell-type) was well supported by 16S with all phylogenetic inference methods (88–99%). With COI, the position of *H. sanctaemariae* is only resolved in the MP analysis (82%). Monophyly for the central Azorean OTUs was supported in both mtDNA data sets in the MP and BA analyses (75–99%), while only weakly supported in the ML analyses ( $<50\%$  with COI and 68% with 16S). Terminal nodes were generally only supported with COI, i.e., the clusters *L. drouetiana*/*L. "Pico"* (85–98%), *L. terceirana*/*L. "Graciosa"* (MP: 87%, BA: 69%) and *L. azorica* "east"/*L. azorica* "west" (73–99%).

Similar bootstrap support values were found by assuming a simpler model in the ML analyses (Jukes-Cantor + G) or by using different weights for the codon positions in COI in the MP analyses. Inconsistent patterns and/or lack of bootstrap support resulting from

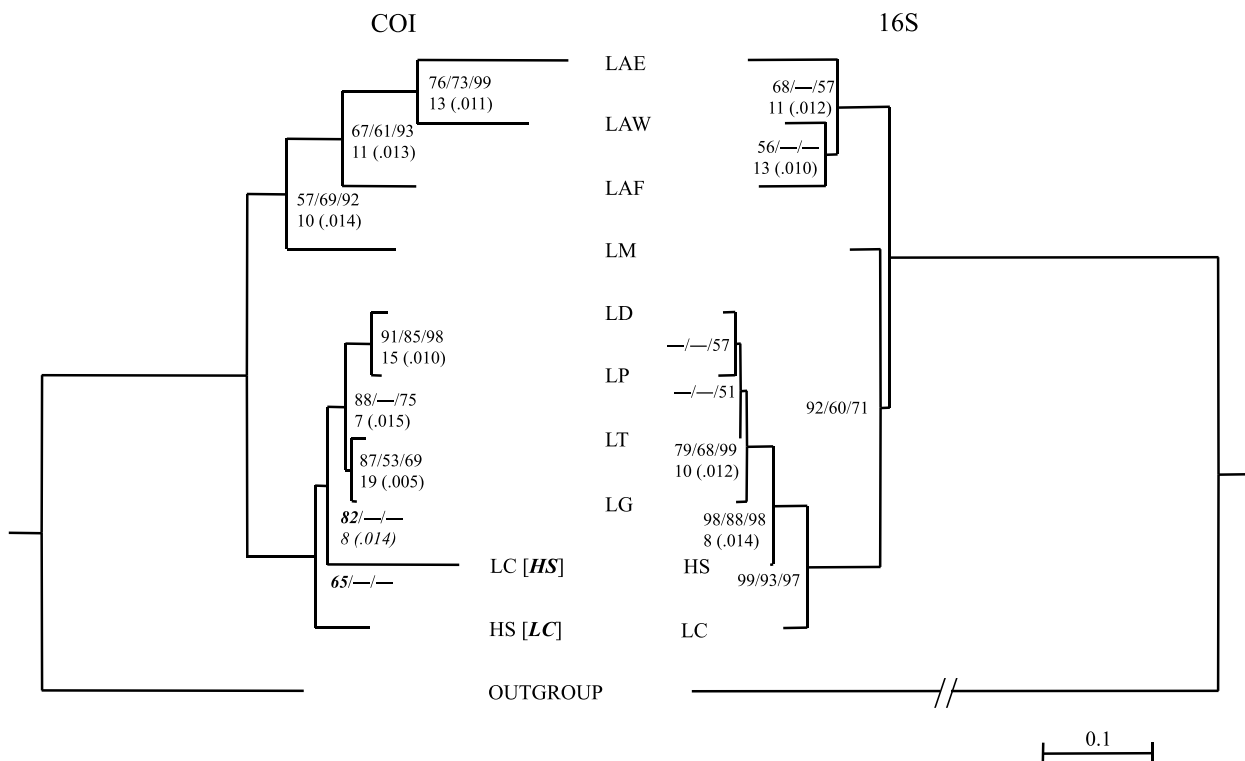


Fig. 2. Maximum likelihood optimal topologies obtained with COI (left) and 16S (right) for all OTUs of the Azorean Leptaxini (see Table 1 for abbreviations). Bootstrap support values (1000 replicates; MP, ML) and Bayesian posterior probabilities (BA; 10,000 trees sampled) are given for nodes supported  $>50\%$  (MP/ML/BA; support  $<50\%$  indicated by a horizontal line) with rank order and support from the spectral analysis given below (for support values  $>0.001$ ). The MP trees for COI support the positions of LC and HS depicted in the 16S topology (square brackets and bold italics). Outgroups: *Arianta arbustorum* (COI) and *Phaeohelix phaeogramma* (16S; terminal branch scaled to  $\pm 1/5$ ). Substitution models,  $-\ln L$ , CI, RI, PI, length, and number of MP trees are given in Table 3.

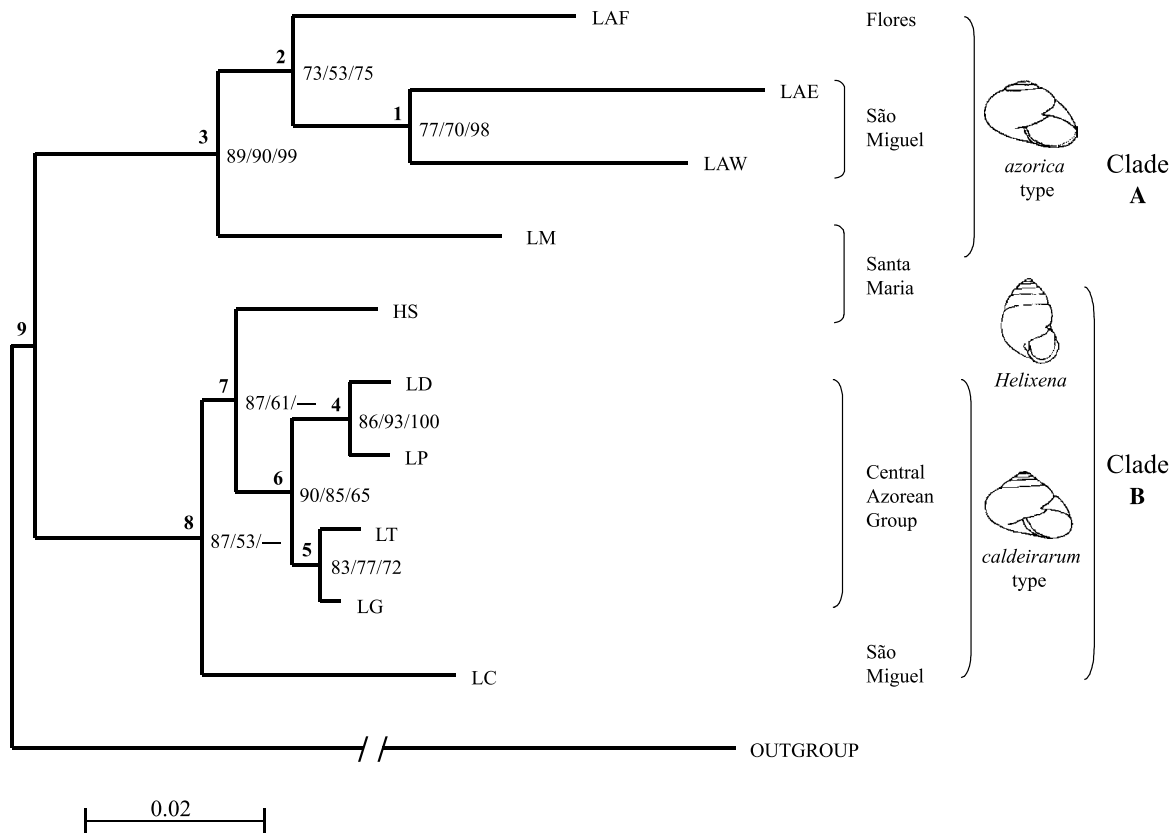


Fig. 3. Tree topology obtained via ML analysis of a concatenated data set with COI and ITS-1, for all OTUs of the Azorean Leptaxini (see Table 1 for abbreviations; Fig. 1). Outgroup branch scaled to 1/5. Substitution models are given in Table 3. Bootstrap (1000 replicates; MP, ML) support values and Bayesian posterior probabilities (10,000 trees sampled) are given for nodes supported >50% (MP/ML/BA; support <50% indicated by a horizontal line). Node numbers refer to the BA/ML analyses of divergence times and substitution rates (Tables 4 and 5).

the ML/BA analyses as a result of model mis-specification. This was indicated by the large differences in model parameter values (rate matrix,  $s/v$  and gamma-shape parameters using GTR + G and F84 + G; Swofford et al., 2001) when estimated for *L. a. azorica* and the other OTUs separately.

Although most bipartitions had many incompatible splits in the spectral analyses, some nodes lacking meaningful support in the MP, ML or BA analyses (node 2, 6, and 7 in Fig. 3; see next paragraph) were generally the best supported bipartitions (number of compatible minus incompatible splits  $\geq 0.01$ ; support values and rank numbers are indicated in Fig. 2).

Although the ILLD test justified ( $p = 0.18$ ) the joint analysis of ITS-1, COI, and 16S (but see Yoder et al., 2001), the difference between the ML topology based on COI (null tree) or 16S (alternative tree) was significant according to the SOWH test (difference in  $-\ln L = 6.99969 > 4.1721$ , the 95% point of the ranked list of differences, calculated from the simulated data sets). As the topologies obtained from COI were not contradicted by ITS-1, these two gene fragments were used in a concatenated data set. This generally yielded the same topologies as from the analyses with COI sepa-

rately (Fig. 2), but the monophyly of clade A (node 3; 89–99%) is clearly stronger supported (see Fig. 3).

### 3.3. Substitution rates and divergence times

According to the LRTs, the likelihoods of the mtDNA ML topologies were significantly lower ( $\alpha = 0.05$ ) under the assumption of a molecular clock. This was further confirmed for COI by the pairwise RRTs, which showed significant differences for comparisons between *L. azorica* “east” and *L. minor*, between the former and all OTUs in clade B and between *L. azorica* “Flores” and *L. “Graciosa”/L. terceirana*. The Bayesian estimates of divergence times obtained via the joint analysis of ITS-1 and COI, based on the topology in Fig. 3, are summarized in Table 4. The separate analysis of 16S (with node 8 at the basis of clade B) resulted in comparable age estimates for most nodes in clade B. The substitution rates, associated with these Bayesian time estimates, together with the ML values (local clock for *L. azorica* vs. the other OTUs), are given in Table 5.

In summary, the branch rates associated with the Bayesian time estimates via  $T_{M1}$  imply that the COI

Table 4

Divergence times (My;  $T_{D1}$  and  $T_{D2}$ ; standard deviation in parentheses) for node numbers given in Fig. 3, resulting from the simultaneous analysis of COI and ITS-1, under the relaxed Bayesian molecular clock (Thorne and Kishino, 2002) and two sets of maximum time limits ( $T_{M1}$  –  $T_{M2}$ ; determined by the age of the islands, putatively involved with a colonization event)

Node	Islands $T_{M1}$	$T_{M1}$	$T_{D1}$	$T_{M2}$	$T_{D2}$
1	SMIE/SMIW	0.55	0.39 (0.12)	4	0.97 (0.47)
2	SMIW/FLO	2	0.89 (0.33)	4	1.43 (0.54)
3	SMA/SMIE	4	1.26 (0.44)	4	1.83 (0.63)
4	FAI/PIC	0.3	0.15 (0.07)	0.3	0.17 (0.07)
5	TER/GRA	0.6	0.20 (0.11)	0.6	0.23 (0.12)
6	TER/FAI	0.8	0.39 (0.16)	0.8	0.45 (0.16)
7	SMA/TER	2	0.77 (0.31)	2	0.97 (0.35)
8	SMIE/SMA	4	1.03 (0.40)	4	1.30 (0.46)
9	SMA/SMIE	4	1.81 (0.61)	4	2.38 (0.71)

See Fig. 1 for island abbreviations.

Table 5

Mean values for substitution rates (per site per My per lineage; standard deviation in parentheses) in *L. a. azorica* (A) and the other lineages (B) via Bayesian statistics (Thorne and Kishino, 2002) and maximum likelihood methods (Yang, 1997) from the joint analysis of COI and ITS-1 and the separate analyses of 16S

Gene	ML1	BA1	ML2	BA2
COI-A	0.130 (0.029)	0.084 (0.051)	0.051 (0.014)	0.061 (0.036)
COI-B	0.028 (0.001)	0.054 (0.027)	0.026 (0.004)	0.043 (0.020)
ITS-1-A	0.002 (0.001)	0.003 (0.002)	0.001 (0.000)	0.002 (0.002)
ITS-1-B	0.004 (0.001)	0.003 (0.002)	0.004 (0.001)	0.002 (0.002)
16S-A	0.056 (0.012)	0.043 (0.025)	0.044 (0.012)	0.028 (0.017)
16S-B	0.016 (0.003)	0.033 (0.020)	0.019 (0.003)	0.023 (0.014)

fragment evolved  $\pm 1.5$  to 2 and  $\pm 20$  to 30 times faster than the 16S and ITS-1 fragment, respectively. Compared to the other lineages, the average substitution rate in *L. azorica* was estimated  $\pm 1.4$  to 1.6 and  $\pm 1.2$  to 1.3 times higher for COI and 16S, respectively. When the time limit for the origin of the three lineages within *L. azorica* (Lae, Law, and Laf; see Table 1 and Fig. 3) was set to 4 My ( $T_{M2}$ ), thus assuming a long-term differentiation on eastern São Miguel, estimated substitution rates became  $\pm 0.7$  to 0.8 times lower and node ages  $\pm 1.5$  to 2.5 times older for *L. azorica*. In each of these scenarios, the mcmcmc algorithm converged to similar values when the maximum age of node 9 (ingroup root) was set to 8 My, thereby assuming a sympatric origin for the lineages that gave rise to *L. minor* and *H. sanctaemariae* (see Fig. 3). Substitution rates (per site/My) estimated via ML for COI with  $T_{M1}$  resulted in a considerably higher value for *L. azorica* ( $0.130 \pm 0.029$ ) compared with the Bayesian estimates ( $0.084 \pm 0.051$ ), while similar values were found for 16S in these lineages (ML:  $0.056 \pm 0.012$ ; BA:  $0.043 \pm 0.025$ ). Much lower values ( $\pm 50\%$ ) were obtained by ML for the substitution rate in all other lineages (COI:  $0.028 \pm 0.001$ ; 16S:  $0.016 \pm 0.003$ ) and similar values were found using  $T_{M2}$  instead of  $T_{M1}$ . Likewise, the values for 16S in *L. azorica* were not affected ( $0.044 \pm 0.012$ ), while the high rates for COI in these lineages dropped to a value similar to 16S

( $0.051 \pm 0.014$ ) when calibrated on  $T_{M2}$ . For ITS-1, no conspicuous differences were found between lineages and/or by varying time limits ( $T_{M1}$  and  $T_{M2}$ ) in the BA, but the ML rate estimates for *L. azorica* were  $\pm 50\%$  lower ( $0.002 \pm 0.001$ ) than in other lineages ( $0.004 \pm 0.001$ ).

### 3.4. Allozyme analyses

Twelve putative allozyme loci were scored unambiguously, yielding 79 alleles. There was no enzyme activity for PEP2 in *H. sanctaemariae* and one individual (out of four) of *L. drouetiana*. All individuals with this characteristic were considered homozygotic for a hypothetical allele. Values for P, A, and U are summarized for each OTU in Table 6. Only little variation was detected at SOD, PEP-1, and PEP-2 within OTUs. Within the OTUs of the central islands and *L. caldeirarum*, most loci were monomorphic. Except for the ambiguous position of *H. sanctaemariae*, included with the *azorica* or *caldeirarum* shell-type depending on which clustering method was used (UPGMA and NJ, respectively), the topologies inferred from  $D_{Nei}$  largely agreed with the DNA topologies (see Fig. 3). Mean values for  $D_{Nei}$  between populations within OTUs were relatively low (range: 0.039–0.077). Extremely large values for  $D_{Nei}$  were found between OTUs of the *azorica* and *caldeirarum* shell-type (mean

Table 6

Percentage polymorphic loci (*P*), mean number of alleles (*A*: SD, standard deviation), and percentage of unique alleles (*U*: *v*, mean frequency)

	LAE	LAW	LAF	LM	LC	LT	LG	LP	LD	HS
<i>P</i>	38.1	58.3	29.2	66.7	25	25	0	8.3	16.7	48.7
<i>A</i>	1.2	1.7	1.4	2.1	1.3	1.3	1.0	1.1	1.2	1.7
(SD)	(0.2)	(0.2)	(0.2)	(0.3)	(0.2)	(0.2)	(0.0)	(0.1)	(0.1)	(0.1)
<i>U</i>	0	7	5	30	6	0	0	8	14	16
( <i>v</i> )	(—)	(.018)	(1.00)	(.294)	(.159)	(—)	(—)	(.087)	(.625)	(.199)

For OTUs with multiple populations, values for *P* and *A* were averaged over all populations with *N* > 7. See Table 1 for abbreviations.

$D_{\text{Nei}} = 1.879 \pm 0.588$ ; range: 1.023–4.348). Although *H. sanctaemariae* was most differentiated from the *caldeirarum* shell-type (mean  $D_{\text{Nei}} = 1.555 \pm 0.247$ ; range: 1.164–2.154), the values for  $D_{\text{Nei}}$  overlapped largely with those found between *H. sanctaemariae* and the OTUs of the *azorica* shell-type, i.e., *L. azorica* (mean  $D_{\text{Nei}} = 1.282 \pm 0.161$ ; range: 0.903–1.640) and *L. minor* (mean  $D_{\text{Nei}} = 1.226 \pm 0.129$ ; range: 0.940–1.423). The OTUs of the *caldeirarum* shell-type were clustered separately, where *L. caldeirarum* was most strongly differentiated (mean  $D_{\text{Nei}} = 0.738 \pm 0.069$ ). The central Azorean OTUs form a more closely related group with genetic distances (mean  $D_{\text{Nei}} = 0.254 \pm 0.121$ ; range 0.088–0.426) comparable to those found between populations of different OTUs within *L. azorica* (mean  $D_{\text{Nei}} = 0.188 \pm 0.064$ ; range: 0.078–0.335) or between the latter and *L. minor* (mean  $D_{\text{Nei}} = 0.347$ ; range: 0.214–0.520). The smallest genetic distance between OTUs was found for *L. terceirana* and *L. “Graciosa”* ( $D_{\text{Nei}} = 0.088$ ). If rare alleles with frequencies <0.1 were not considered, unique alleles were restricted to *L. azorica* “Flores” (LAP; diagnostic locus, i.e., all alleles are specific for a particular OTU), *L. minor* (diagnostic loci EST-2 and AAT; HBDH), *L. caldeirarum* (EST-2), *L. drouetiana* (GPD, PEP-2), and *H. sanctaemariae* (AAT).

## 4. Discussion

### 4.1. Genetic differentiation

Differentiation between ITS sequences (0–3.4%) agree with levels of variation found between congeneric species (0–1.8%) or genera (0–10.2%) in a wide range of land snail taxa (Wade et al., 2001). In contrast, extremely high levels of mtDNA divergence are commonly associated with stylommatophoran taxa, with maximum values found for 16S (31%; between *Partula* sp.) and COI (13%; within *Euhadra peliomphala*) (see Davison, 2002).

The maximum values for sequence divergence (uncorrected, *p*-distances) of 13.5% (16S) and 17.7% (COI) between the two shell-types of *Leptaxis* (*azorica* and *caldeirarum* shell-type) can thus be considered among the highest reported so far in stylommatophora (corrected, ML distances: respectively, 33.4 and 55.8%).

Although some bias could result from model mis-specification, the corrected values are probably realistic, as indicated by the extreme values for the allozyme genetic distances between several *Leptaxis* OTUs with different shell-types (maximum  $D_{\text{Nei}} = 4.348$ ). Values of  $D_{\text{Nei}}$  for most comparisons (0.078–1.640) fall more within a wide range also reported for other land snail taxa such as, e.g., *Placostylus* sp. (0.600–1.460; Triggs and Sherley, 1993), *Samoana* sp. (0.030–1.099; Johnson et al., 1986), and *Cepaea nemoralis* (0.002–0.829; Guiller and Madec, 1993).

In relation to the recent origin of the Azores, substitution rates estimated for mtDNA in *L. azorica* are also extremely high, approximately between 10 and 25% per My for COI and 10% per My for 16S, compared to values between 5 and 10% per My for COI and 5% per My for 16S in the remainder of OTUs (see Tables 4 and 5).

Rate estimates  $\geq 10\%$  per My reported for land snails seem to be commonly associated with taxa on volcanic archipelagoes (e.g., Chiba, 1996; Hayashi and Chiba, 2000; Thacker and Hadfield, 2000). Isolation, extinction, and (re)colonization processes in relation to volcanic activity are generally considered important for the evolution on volcanic archipelagoes (e.g., Hermans et al., 2003; Malhotra and Thorpe, 2000; Pook et al., 2000). Recurrent volcanic activity resulting in local extinctions or isolation of (small) populations and the (re)colonization of suitable habitat is expected to strongly enhance the effects of genetic drift. Evidently, these effects are much stronger for ground-dwelling organisms with poor dispersal capabilities and that require very specific (micro-) ecological conditions, such as land snails (Beheregary et al., 2003; Holland and Hadfield, 2002; Ibrahim et al., 1996; Shimizu and Ueshima, 2000; Van Riel et al., 2003). Nevertheless, other factors such as, e.g., generation time, mutation rates (or differences in DNA repairing), and natural selection cannot be excluded (see, e.g., Barton, 1998; Clarke et al., 1998; Davison, 2002; Thomaz et al., 1996).

### 4.2. Evolutionary relationships and taxonomic implications

The confusing effects of short internal and/or long branches (rate heterogeneity), frequency bias and mis-

specified models have been reduced by implementing phylogenetic inference methods that perform differently under these circumstances and by using a variety of models with different parameters (Huelsenbeck et al., 2002; Mooers and Holmes, 2000; Swofford et al., 2001).

Although equivocally supported in the mtDNA phylogenies, most of the evidence points to the monophyly of *L. azorica*, clade A (*azorica* shell-type), the central Azorean OTUs and clade B (see Fig. 3). The monophyly of the *caldeirarum* shell-type is apparent from the allozyme analysis, but because of the ambiguous positions of *H. sanctaemariae* and *L. caldeirarum* in the mtDNA phylogenies, a paraphyletic origin cannot be excluded. As the shells of (land) snails are subject to many selective pressures (Goodfriend, 1986), it seems reasonable to assume that adaptive changes have contributed to the evolution of shell morphology in the Azorean Leptaxini. A major role for adaptive evolution (natural selection) as evidenced by parallel (convergent) changes in many other land snail radiations (e.g., Chiba, 1996; Douris et al., 1998; Goodacre and Wade, 2001; Teshima et al., 2003) is not supported by our data for the origin of the *caldeirarum* and *azorica* shell-type.

The monophyly of the *azorica* shell-type and probable monophyly of the *caldeirarum* shell-type, sharing no common history with sympatric shell-types on the two oldest islands (Santa Maria and São Miguel), is indicative of a historical explanation (Davison, 2002; Holland and Hadfield, 2002; Rundell et al., 2004). From a systematic point of view, according to the phylogenetic species concept (see, e.g., Mayden, 1997), all OTUs within the Azorean Leptaxini should be considered as distinct species, including the OTUs and putative subspecies of *L. azorica* (see also Van Riel et al., 2003). The proper naming and taxonomic description of these species will be provided in a forthcoming revision of the Azorean Leptaxinae. The distinction of *H. sanctaemariae* in a separate genus makes the genus *Leptaxis* paraphyletic on the Azores. However, except for the aberrant shell shape, the genetic differences between *H. sanctaemariae* and *Leptaxis* OTUs fall completely within the range of variation observed in the latter, which should thus be considered the only genus within the Azorean Leptaxini.

#### 4.3. Timing of colonizations, speciation, and geological history of the Azores

The successive colonization of new islands from source populations on older islands is a pattern commonly observed on volcanic archipelagoes (e.g., Clarke et al., 1998; Fleischer et al., 1998; Juan et al., 2000; but see Rundell et al., 2004). Although the estimated node ages do not reject other islands as primary source (Table 4), the radiation of the Azorean Leptaxini presumably started on the oldest island, Santa Maria (8 My old). From here, the largest number of Leptaxini species ( $N = 3$ ) has been de-

scribed, including the subfossil *Leptaxis vetusta* and two of the most basal extant species in the phylogeny, *L. minor* and *H. sanctaemariae* (Figs. 2 and 3). The colonization of the Azores probably proceeded via the second oldest island, São Miguel (estimated  $1.81 \pm 0.61$  My ago), further to the much younger islands of the central Azorean Islands (presumably via Terceira, estimated  $0.77 \pm 0.31$  My ago). Compared to the available time (4–8 My), the age estimates for the oldest nodes appear young (roughly 1–2 My). The oldest split in the phylogeny is for example still estimated much younger ( $2.38 \pm 0.71$  My) than eastern São Miguel (4 My) and Santa Maria (8 My old). This should however not be surprising given the fact that environmental conditions may not always have allowed (re)colonization by land snails, as a result of destructive volcanic activity. The most active, destructive periods of volcanism on the two oldest Azorean islands are dated about 4 My ago. The last eruptive episode in the genesis of Santa Maria occurred about 2 My ago, but massive volcanic activity in the two oldest, easternmost volcanic regions of São Miguel lasted until 0.65 My ago (Fig. 1; Forjaz, 1998; Morton et al., 1998). Although Flores emerged around 2 My ago, the island may have only become available for (re)colonization around 0.65 My ago, after the formation of the upper geological complex started. Also the formation of Terceira, the oldest central Azorean island (2 My), involved a complex series of explosive, volcanic eruptions, and destructive earthquakes that lasted until about 0.3 My ago (Morton et al., 1998; Nunes, 2000). This could explain the absence of *L. azorica* from the central Azorean islands while the western islands (Flores and Corvo) have been colonized successfully, apparently  $0.89 \pm 33$  My ago.

Allopatric differentiation clearly seems to be the main mechanism underlying the radiation of the Leptaxini on the Azores. Nevertheless, the exact sequence of events still remains unclear with several alternative scenarios which can be considered equally probable. Besides the subsequent colonization of new islands, isolation of populations on single islands may have occurred via, e.g., adaptation to different habitats (ecological isolation) or fragmentation due to habitat destruction by lava flows or deforestation (Beheregary et al., 2003; Cook, 1996; Holland and Hadfield, 2002; Shimizu and Ueshima, 2000; Van Riel et al., 2003). Possibly, in a (hypothetical) scenario of sympatric (ecological) speciation on Santa Maria, non-hierarchical evolutionary relationships, and/or rapid speciation could explain the ambiguous phylogenetic positions of the three most basal taxa (e.g., Orr and Smith, 1998). Additionally, phylogenetic analyses should also include the Leptaxini from the other Macaronesian archipelagoes to determine the exact position of the outgroup root and/or evaluate the possibility of multiple colonizations to the Azores (e.g., Carranza et al., 2000; Emerson, 2002; Francisco-Ortega et al., 1996).

In summary, with reference to the four main goals set forth in this study, we conclude that

- (1) The *azorica* shell-type forms a monophyletic radiation through successive colonizations of islands, while paraphyly of the *caldeirarum* shell-type cannot be excluded because of the ambiguous phylogenetic position of *H. sanctaemariae*.
- (2) *H. sanctaemariae* is closely related to the *caldeirarum* group. The origin of the (aberrant) high-spired shell does not predate differentiation within *Leptaxis* (paraphyletic) and represents the only autapomorphic shell-type, possibly related to adaptive evolution.
- (3) Taxic differentiation in Azorean Leptaxini is relatively recent ( $1.81 \pm 0.61$  My), associated with extremely high rates of molecular change and three shell-types that originated within a relatively short time span, early in the history of the Azorean Leptaxini ( $1.81 \pm 0.61$  to  $0.77 \pm 0.31$  My).
- (4) *H. sanctaemariae* should be included within the genus *Leptaxis* and representatives on all (ancient) islands should be considered as separate phylogenetic species.

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