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ORIGINAL ARTICLE

Phylogeography and molecular phylogeny of Macaronesian island *Tarphius* (Coleoptera: Zopheridae): why are there so few species in the Azores?

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ABSTRACT

Aim We used a phylogenetic framework to examine island colonization and predictions pertaining to differentiation within Macaronesian *Tarphius* (Insecta, Coleoptera, Zopheridae), and explain the paucity of endemics in the Azores compared with other Macaronesian archipelagos. Specifically, we test whether low diversity in the Azores could be due to recent colonization (phylogenetic lineage youth), cryptic speciation (distinct phylogenetic entities within species) or the young geological age of the archipelago.

Location Macaronesian archipelagos (Azores, Madeira and the Canary Islands), northern Portugal and Morocco.

Methods Phylogenetic analyses of mitochondrial and nuclear genes of *Tarphius* beetles of the Azores, other Macaronesian islands and neighbouring continental areas were used to investigate the origin of island biodiversity and to compare patterns of colonization and differentiation. A comparative nucleotide substitution rate test was used to select the appropriate substitution rate to infer clade divergence times.

Results Madeiran and Canarian *Tarphius* species were found to be more closely related to each other, while Azorean taxa grouped separately. Azorean taxa showed concordance between species and phylogenetic clades, except for species that occur on multiple islands, which segregated by island of origin. Divergence time estimates revealed that Azorean *Tarphius* are an old group and that the most recent intra-island speciation event on Santa Maria, the oldest island, occurred between 3.7 and 6.1 Ma.

Main conclusions Our phylogenetic approach provides new evidence to understand the impoverishment of Azorean endemics: (1) *Tarphius* have had a long evolutionary history within the Azores, which does not support the hypothesis of fewer radiation events due to recent colonization; (2) the current taxonomy of Azorean *Tarphius* does not reflect common ancestry and cryptic speciation is responsible for the underestimation of endemics; (3) intra-island differentiation in the Azores was found only in the oldest island, supporting the idea that young geological age of the archipelago limits the number of endemics; and (4) the lack of evidence for recent intra-island diversification in Santa Maria could also explain the paucity of Azorean endemics. Phylogenetic reconstructions of other species-rich taxa that occur on multiple Macaronesian archipelagos will reveal whether our conclusions are taxon specific, or of a more general nature.

Keywords

Azores diversity enigma, Coleoptera, cryptic diversification, COI, COII, elongation factor 1α, island biogeography, island endemics, Macaronesia, speciation.

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INTRODUCTION

The Macaronesian islands (Cape Verde, Canary Islands, Salvage Islands, Madeira and the Azores) host a diverse and distinct biota and are recognized as an important component of the Mediterranean biodiversity hotspot (Myers *et al.*, 2000). Among these archipelagos, the Azores (Fig. 1) is comparatively depauperate, with fewer single-island endemic species, particularly in comparison with Madeira and the Canary Islands (Borges *et al.*, 2008a, 2010a; Arechavaleta *et al.*, 2010), a phenomenon that has been referred to as the 'Azores diversity enigma' (Carine & Schaefer, 2010). Arthropods represent approximately 60% of the endemic species found in the Azores (Borges *et al.*, 2010b) and recent studies have focused on this group to investigate why the number of endemic species in the Azores is reduced compared with other Macaronesian archipelagos, testing for correlations between the number of endemics and archipelago/island isolation, area, geological age, habitat diversity and native habitat destruction (Borges & Brown, 1999; Borges & Hortal, 2009; Cardoso *et al.*, 2010; Triantis *et al.*, 2010a,b). However, although a phylogenetic framework has been incorporated in the analyses of the Azorean flora (e.g. Carine & Schaefer, 2010; Schaefer *et al.*, 2011), such an approach has not been adopted for arthropods. Phylogenetic studies of Azorean taxa and Macaronesian relatives are few and involve single taxa and/or limited sampling of islands and mainland specimens. Most rely on single markers or short DNA sequences, and do not explicitly address the question of why there are fewer endemics in the Azores compared with the other Macaronesian islands (Cesaroni *et al.*, 2002; Fujaco *et al.*, 2003; Montiel *et al.*, 2008).

The genus *Tarphius* Erichson, 1848 (Insecta, Coleoptera, Zopheridae) is one of the arthropod groups with the highest number of endemic species in the Azores, with eight endemic species described (Oromí *et al.*, 2010a) and an undescribed new species from São Miguel (P.A.V.B., pers. obs.). Six species are single-island endemics (*Tarphius wollastoni* Crotch, 1867, *Tarphius acuminatus* Gillerfors, 1986, *Tarphius pomboi* Borges, 1991, *Tarphius serranoi* Borges, 1991, *Tarphius rufonodulosus* Israelson, 1984 and the undescribed species from São Miguel), and the other three species (*Tarphius tornvalli* Gillerfors, 1985, *Tarphius depressus* Gillerfors, 1985 and *Tarphius azoricus* Gillerfors, 1991) inhabit two, three and six islands, respectively (Fig. 1). The genus *Tarphius* also occurs in Madeira, the Canary Islands, North Africa, the Iberian Peninsula, Italy, South America and the Himalayas (Franz, 1967; Dajoz, 1977; Ślipiński, 1985), but the majority of species (86%) are endemic to the Macaronesian islands. *Tarphius* are apterous, fungivorous beetles with cryptic life styles, typically associated with the native forest of the Macaronesian islands (Borges, 1992; Emerson & Oromí, 2005). As is the case for other beetle genera (e.g. *Drouetius*/*Laparocerus*, *Calacalles*/*Acalles*, *Calathus*, *Trechus*), there are fewer *Tarphius* species endemic to the Azores compared with neighbouring archipelagos: Azores 9, Madeira 23, and Canaries 31 (Borges *et al.*, 2008b; Oromí *et al.*, 2010a,b; Machado, 2012; P.A.V.B., pers. obs.). Therefore *Tarphius* represents a good system for investigating, within a phylogenetic framework, the causes of impoverished endemism within the Azores.

Previous studies on Macaronesian *Tarphius* using phylogenetic approaches and mitochondrial markers (Emerson & Oromí, 2005) focused on species of the Canary Islands, including only a few taxa from Madeira, a single species from the Azores and no mainland relatives. Here we expand on the genetic and geographic sampling of Emerson & Oromí (2005) by employing nuclear (elongation factor 1 alpha, EF1α) and mitochondrial (cytochrome c oxidase subunit I, COI; leucine specific transfer RNA, tRNA-Leu; cytochrome c oxidase subunit II, COII) sequences to infer phylogenetic relationships among *Tarphius* species from all Macaronesian islands and the

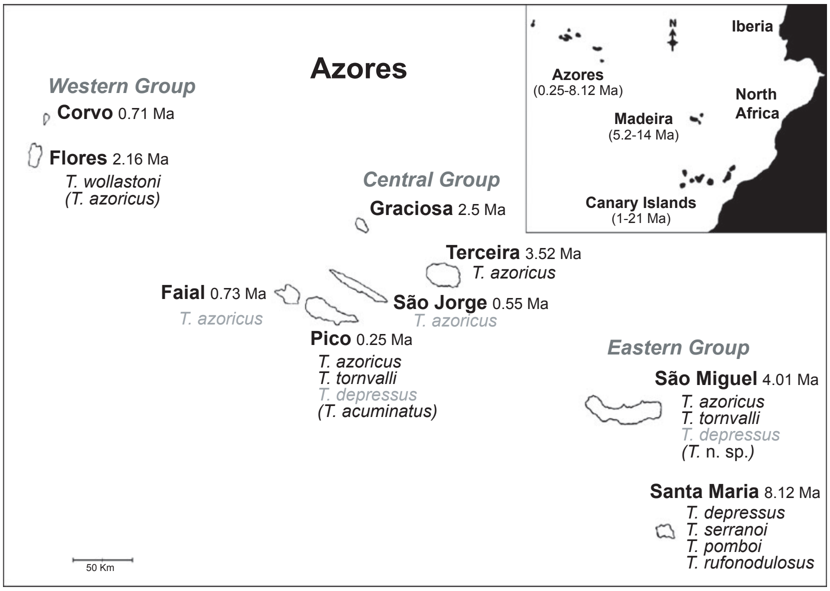

Figure 1 Location of the study. Island subaerial ages in million of years ago (Ma) and *Tarphius* species distributions are shown for the Azores. Taxa in grey are new records and taxa in brackets were not sampled. Inset: Macaronesian archipelagos with ages of emergent islands presented as a range.

closest continental areas of North Africa and Iberia. Special emphasis is given to the Azores, which comprise nine islands (Fig. 1) characterized by a rich and diverse volcanic history (Forjaz, 1998; Morton *et al.*, 1998; Nunes, 2000), with subaerial geological ages ranging from 0.25 to 8.12 Ma (França *et al.*, 2003). Although it has a total area of 2324 km^2, approximately half of the Azores (62%) is very recent in origin, less than 1 Ma (Triantis *et al.*, 2010b). The archipelago spans 615 km, and the closest land masses are Madeira and the Iberian Peninsula, at 840 km and 1584 km, respectively (Fig. 1).

Our sampling represents one of the most comprehensive phylogeographic and phylogenetic studies of a species-rich invertebrate genus in the Macaronesian islands that includes the Azores, allowing us to evaluate the Azorean diversity enigma (see Carine & Schaefer, 2010) by testing the following predictions: (1) due to their geographic proximity, Azorean lineages are more closely related to European species, and Madeiran and Canarian lineages are more closely related to African species; (2) Azorean taxa are monophyletic; (3) intra-island speciation has had a more important role in shaping patterns of genetic diversity of Canarian island *Tarphius* on older islands (Emerson & Oromí, 2005) and likewise we predict intra-island speciation to be the main mechanistic explanation for species diversity on the older Azorean islands (Santa Maria and São Miguel); (4) we predict that the oldest island of Santa Maria was the first to be colonized, and contains the most divergent lineages; (5) given the geographic distances among Azorean islands, low vagility of *Tarphius* and the presence of similar morphospecies on different islands, we predict that endemic taxa have been underestimated because of cryptic speciation; and (6) given the geographic isolation of the Azores, the relatively young age of much of its area and the intense volcanic activity in the archipelago, we predict Azorean lineages to be of recent origin, much younger than the maximum geological age of the archipelago (8.12 Ma).

MATERIALS AND METHODS

Sample collection

Collecting effort was focused on the Azorean and Madeiran archipelagos, mainland Portugal and North Africa (Morocco). *Tarphius* species were collected between 1999 and 2009 using a combination of pitfall trapping, manual searching in specific habitats, Berlese funnel extraction from leaf litter and canopy beating (Amorim, 2005). Specimens were stored individually in absolute ethanol or acetone and refrigerated prior to DNA extraction. Species identification was performed by P.A.V.B. and A. Serrano (CBA, FC, Universidade de Lisboa, Portugal), and voucher specimens were deposited in the Dalberto Teixeira Pombo Arthropod Collection (DCA, Universidade dos Açores, Portugal). Additional specimens from the Canary Islands and Madeira were collected during a previous study (Emerson & Oromí, 2005).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). A mitochondrial DNA (mtDNA) fragment of approximately 1500 bp, comprising part of the COI gene, the tRNA-Leu gene and the complete COII gene was amplified via the polymerase chain reaction (PCR) using a combination of primers described in the literature and their modifications (Simon *et al.*, 1994; Emerson *et al.*, 2000; Whiting, 2002). Amplification of approximately 950 bp of the nuclear EF1α gene was accomplished using a combination of primers designed specifically for *Tarphius* beetles. For details on DNA extraction and PCR amplification see Appendix S1 in the Supporting Information. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and cycle-sequenced in both directions with the PCR amplification primers and internal primers. Sequencing reactions were either performed using the ABI PRISM BigDye Terminator chemistry and separated by electrophoresis on an ABI PRISM DNA automated sequencer (Applied Biosystems, Foster City, CA, USA) or using the CEQ DTCS chemistry and run on a CEQ 2000 Genetic Analysis System (Beckman Coulter Inc., Brea, CA, USA).

Phylogenetic analyses

Sequence trace chromatograms were assembled, edited and aligned for both mtDNA and nuclear data sets with the software SEQUENCHER 4.8 (Gene Code Corporation, Ann Arbor, MI, USA). Sequence alignments were verified by eye and edited using MACCLADE 4.08 (Maddison & Maddison, 2005) and all sequences obtained for each partition were collapsed to unique sequences using DNACOLLAPSER (Villesen, 2007). The mtDNA sequences for all Canarian *Tarphius* species, some Madeiran species and the outgroup *Pseudotarphius lewisii* were obtained from GenBank (see Appendix S2).

The mtDNA and EF1α data matrices were examined for incongruence length differences (ILD) (Mickevich & Farris, 1981) using the partition homogeneity test (Farris *et al.*, 1995a,b) as implemented in PAUP* 4.0b10 (Swofford, 2002). Maximum likelihood (ML) and Bayesian inference (BI) methods were used to reconstruct phylogenetic relationships based on mtDNA and nuclear sequence data separately using *P. lewisii* as the outgroup and treating gaps and nuclear heterozygote positions as missing data. Mitochondrial sequences were analysed as a single fragment, partitioned according to gene, and partitioned according to codon position for the protein-coding fragments. The nuclear data were analysed as a single fragment, partitioned into exon and intron fragments and partitioned according to codon position for the exonic regions. The program JMODELTEST 0.1.1 (Posada, 2008) was used to select the model of evolution that best explains the nucleotide variation within each partition, excluding the outgroup and using the Akaike information criterion (AIC) (Posada & Buckley, 2004). BI analyses were performed with MRBAYES 3.1.2 (Ronquist & Huelsenbeck,

2003) and for each data set and partition scheme, two independent analyses starting from different random trees were run in parallel for five million generations, with four simultaneous Markov chains, and sampling every 100th generation. The average standard deviation of split frequencies was used as a convergence diagnostic, and the first 25% of samples were discarded as burn-in. Each analysis was repeated to test the robustness of the inferred phylogenetic relationships by comparing the topology and log-likelihood scores of the consensus trees obtained in each analysis. The software RAxML 7.2.8 (Stamatakis, 2006; Stamatakis *et al.*, 2008), run on the Cipres Portal 2.2 (Miller *et al.*, 2009), was used for ML phylogenetic inferences. Settings for ML searches were as follows: random seed value specified for the initial parsimony inference and for rapid bootstrapping, rapid bootstrapping conducted while searching for the best scoring ML tree (1000 iterations) and GTRGAMMA used both for the bootstrapping phase and the final tree (all other parameters using default values).

Comparative substitution rate and estimation of divergence time analyses

We combine intrinsic calibration points (island geological ages) with a molecular clock calibration derived from other studies using a Bayesian approach to infer divergence ages. Because accelerated rates of molecular evolution have been reported for groups that have radiated extensively on islands (Chiba, 1999), we performed a comparative rate analysis (Cicconardi *et al.*, 2010) to investigate whether *Tarphius* may be evolving at an anomalous rate compared with other Coleoptera and to choose an adequate rate for dating divergence events. Forty-four COII sequences representing the Coleopteran suborders Adephaga (20), Archostemata (1), Polyphaga (21) and Myxophaga (2) and two Diplura sequences (outgroups) were downloaded from GenBank (see Appendix S3) and added to an alignment of 49 *Tarphius* sequences representing all sampled species and clades. Coding sequences were aligned at the amino acid level (retro-aligned) using RevTrans 1.4 (Wernersson & Pedersen, 2003), with a final matrix of 675 bp. BEAUti 1.6.2 was used to generate the input file for the Bayesian Markov chain Monte Carlo (MCMC) analysis of molecular sequences implemented in beast 1.6.2 (Drummond & Rambaut, 2007). The analysis was run assuming an uncorrelated lognormal relaxed molecular clock (Drummond *et al.*, 2006) with the average number of substitutions per site per million years across the whole tree set to 1 (meanRate constrained under normal distribution, mean = 1, SD = 0.01), with individual branch rates unconstrained. Other priors included a Yule speciation process and enforced monophyly of the ingroup, each suborder, infraorder, superfamily, family and genus, to limit the tree search space. beast 1.6.2 runs were performed on the High Performance Computing Cluster supported by the Research Computing Service at University of East Anglia, UK. The analysis was run twice for 10 million generations, sampling every 100th generation, and Tracer 1.5 (Rambaut & Drummond, 2009a) was used to inspect for stationarity; both runs were combined in LogCombiner 1.6.2 (Rambaut & Drummond, 2009b) with burn-in set to 10%. A maximum clade credibility tree was obtained with TreeAnnotator 1.6.2 (Rambaut & Drummond, 2009c) and branch-specific rates and lengths were visualized with FigTree 1.3.1 (Rambaut, 2009). Rates for the groups of interest were calculated as the average of rates for all branches related to the specific group weighted by branch length.

Approximate divergence times within Macaronesian island *Tarphius* were estimated with beast 1.6.2 based on the mitochondrial protein-coding sequence data set (COI and COII, excluding the intervening tRNA-Leu) and using *Tarphius kiesenwetteri* (from northern Portugal) as the outgroup. Analyses were performed using an uncorrelated lognormal relaxed molecular clock (Drummond *et al.*, 2006) with unconstrained rates for individual branches and applying a mean substitution rate estimate derived from the comparative rate analysis of 2.1% pairwise divergence. Additional calibration was provided by subaerial ages of Macaronesian islands and eight nodes in the phylogeny were constrained under a uniform distribution to be no older than the age of a specific island represented within the clade, thus allowing for the possibility that colonization could have occurred at any time since island emergence until the present. Age constraints were applied to nodes with high support (posterior probability ≥ 95, bootstrap ≥ 90) and to Canary Island taxa (nodes not shown) that have been used for calibration in a previous study (nodes B, C, E and F in fig. 3 in Emerson & Oromí, 2005). Nodes II, III, VI and VII (Fig. 2) were constrained to be no older than the oldest island represented within the clade: Madeira plus Canary Islands (clade II) ≤ 14 Ma (Porto Santo); Azores (clade III) ≤ 8.12 Ma (Santa Maria); *T. azoricus* plus *T. tornvalli* (clade VI) ≤ 4.01 Ma (São Miguel); and *T. azoricus* plus *T. tornvalli* from the central group of Azorean islands (clade VII) ≤ 3.52 Ma (Terceira). Ten independent analyses, consisting of 10 million generations each and sampling every 1000th generation, were performed assuming a Yule speciation process tree prior and selecting the general nucleotide substitution model parameters (GTR + I + G) suggested by jModelTest 0.1.1 (Posada, 2008). Runs were checked for convergence to a stationary distribution and combined as described above. To estimate time range for intra-island diversification events in the Azores, Azorean taxa were limited to *Tarphius* lineages endemic to Santa Maria, the oldest and only island in the archipelago where there is evidence of intra-island speciation. Age estimation analyses were also performed considering codon positions to investigate the effect of different partition schemes on divergence time estimates.

RESULTS

Sample collection

All species of Azorean *Tarphius* were collected, except for *T. acuminatus* and one undescribed species from São Miguel.

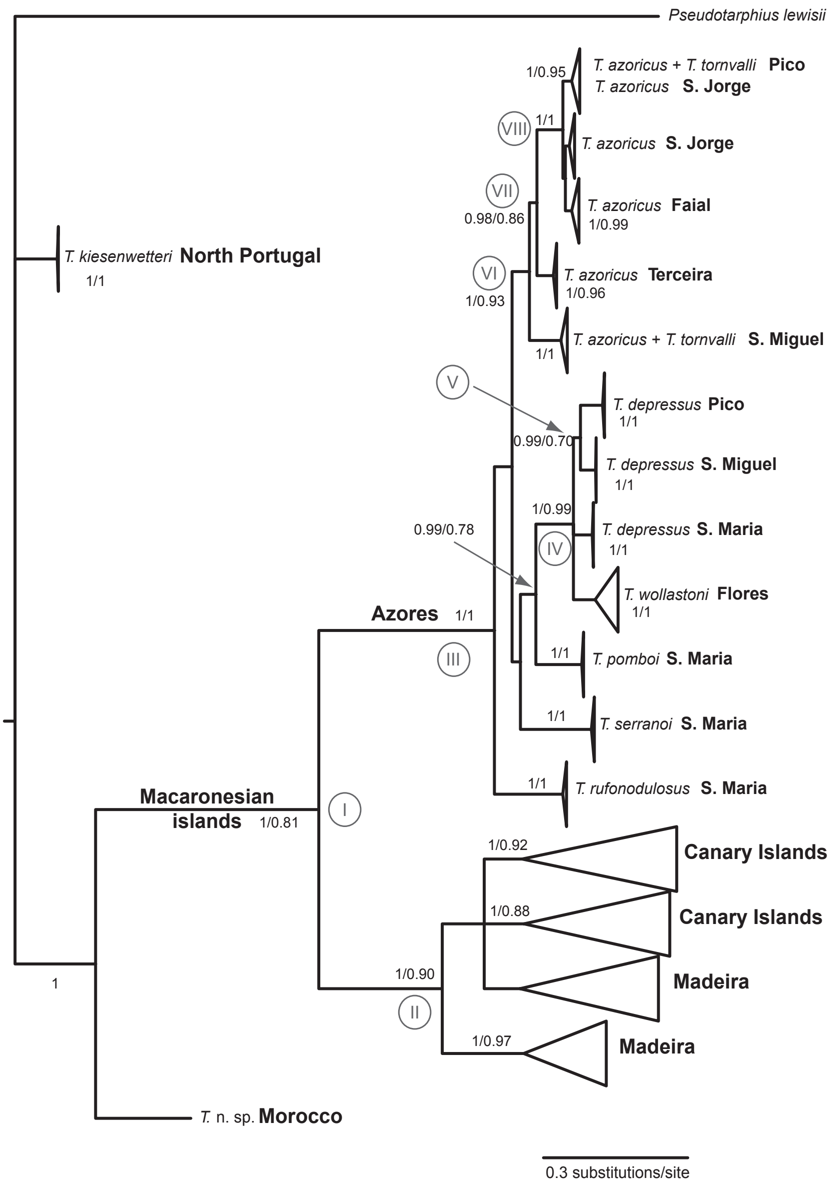

Figure 2 Bayesian inference tree for *Tarphius* beetles of the Macaronesian islands, northern Portugal and Morocco based on mitochondrial DNA (COI, tRNA-Leu and COII). Clade credibility values are shown for nodes with a posterior probability ≥ 0.95 and bootstrap support ≥ 0.70 in that order. Clades I–VII relate to divergent time estimates.

The genus *Tarphius* was collected for the first time on São Jorge and Faial islands (*T. azoricus* group), and the species *T. depressus* was collected for the first time on Pico and São Miguel islands. Azorean *Tarphius* species were typically found in the native humid evergreen broadleaf laurel forest (laurisilva), but were also collected on introduced plants, such as *Acacia* spp. and *Cryptomeria japonica*. Specimens of *Tarphius* were also collected in Madeira and Porto Santo islands, in the north of Portugal and a new species in the High Atlas of Morocco (species not yet described).

Phylogenetic analyses

Mitochondrial DNA and EF1α sequence data was obtained for 98 specimens of *Tarphius*: 68 from the Azores and 30 from Madeira, Porto Santo, northern Portugal and Morocco (unique mtDNA and EF1α sequences deposited in GenBank, accession numbers JQ689201–JQ689281 and JQ689282–JQ689387, respectively; see Appendix S2). Additional sequences of *Tarphius* of the Canary Islands and Madeira, and of the outgroup *P. lewisii* were retrieved from GenBank (see Appendix S2). This study includes the majority of Azorean *Tarphius* species (all except *T. acuminatus* and *Tarphius* n. sp. of São Miguel) and species from the closest and most likely sources of colonization to the archipelago: Madeira, 4 species; Canary Islands, 24 species; Iberia, 1 species; North Africa, 1 species) and North Africa, 1 species. The mtDNA alignment consisted of 1383 bp, comprising 642 bp of the COI gene, with a terminal 3 bp deletion in all continental *Tarphius* species, 64 bp of the tRNA-Leu gene, with a 1 bp deletion in the outgroup, and 677 bp of the COII gene. The EF1α alignment consisted of 920 bp, comprising two exonic regions totalling 709 bp intervened by an intron of 211 aligned nucleotide positions, ranging in sequence length from 163 to

204 bp. The final data sets consisted of 136 and 106 unique sequence for the mtDNA and EF1α markers, respectively. With the exception of *T. azoricus* and *T. tornvalli* of Pico and *T. azoricus* of São Jorge, sequences were only shared by conspecific individuals from the same island. The ILD test indicated significant incongruence between the mitochondrial and nuclear sequence matrixes (100 replicates, $P = 0.01$) and so data sets were analysed separately. For both data sets the nucleotide model of evolution that best fits the data, as suggested by the AIC in jModelTest, is the general time-reversible model assuming a proportion of invariable sites and a gamma distributed rate heterogeneity (GTR + I + G). This model was used for the parameterization of the ML and BI searches.

For both markers, the topologies of the ML and BI phylogenetic trees are similar, regardless of the partition scheme used, with minor terminal differences related to the placement of conspecific sequences. BI reconstructions from replicate runs show the same topology and very similar clade support and likelihood scores. Figures 2 & 3 represent the BI trees obtained for both gene regions with no partitioning of the sequence data. Both phylogenetic methodologies based on either of the molecular markers provided maximum support for the monophyly of *Tarphius* from the Azores, group taxa from Madeira and the Canary Islands together and place these as the closest relatives to the Azorean taxa. Other clades consistently recovered for the Azorean taxa include: *T. rufonodulosus* (Santa Maria), *T. serranoi* (Santa Maria), *T. pomboi* (Santa Maria), *T. depressus* (Santa Maria), *T. wollastoni* (Flores), *T. azoricus* (Terceira) and *T. azoricus* + *T. tornvalli* (São Miguel). Another common feature for both markers is that within the Azores, lineages from the oldest island Santa Maria (*T. rufonodulosus*, *T. serranoi* and *T. pomboi*) are sister taxa to lineages found on the younger Azorean islands (Figs 2 & 3). Specifically, in the mtDNA tree, for each major clade, lineages found on the oldest island within that particular clade show the deepest divergences and are sister taxa to the remaining lineages (Fig. 2). For example, in the *T. azoricus* + *T. tornvalli* clade, lineages from São Miguel (4.01 Ma) are sister taxa to the lineages found on the younger islands of Terceira (3.52 Ma), Faial (0.73 Ma), São Jorge (0.55 Ma) and Pico (0.25 Ma), and in the *T. azoricus* + *T. tornvalli* clade of the central group of islands (Fig. 1), lineages from the oldest island (Terceira) are sister taxa to lineages found on the younger islands of Faial, São Jorge and Pico (Fig. 2).

When compared with the EF1α tree (Fig. 3), the mtDNA tree (Fig. 2) provides finer resolution of the phylogenetic relationships within Azorean *Tarphius*, which is to be expected based on the faster rate of molecular evolution of the latter marker. In particular, the mtDNA tree groups most taxa according to morphospecies identity. The exceptions to this pattern involve species that occur on multiple islands, such as *T. azoricus*, *T. tornvalli* and *T. depressus*, where individuals group by island, rather than by morphospecies. The mtDNA tree also reveals that the species assemblages of Pico and São Miguel islands are not monophyletic, suggesting at least two colonization events to each of the islands by *T. azoricus* + *T. tornvalli* and *T. depressus* lineages.

Comparative substitution rate and estimation of divergence time analyses

The weighted average substitution rates and their standard deviations for the different Coleoptera lineages and for *Tarphius* are presented in Fig. 4. The *Tarphius*-specific rate in the comparative substitution rate analysis falls within the second quartile, revealing *Tarphius* to have been evolving at a 'typical' coleopteran rate. Therefore, a 2.1% pairwise divergence per million years, the median value of the range of substitution rates reported for Coleoptera (0.7–3.5%) (Gómez-Zurita *et al.*, 2000; Caccone & Sbordoni, 2001; Farrell, 2001; Contreras-Díaz *et al.*, 2007; Sota & Hayashi, 2007; Papadopoulou *et al.*, 2010), was used for the estimation of *Tarphius* divergence times.

The two partition schemes used to estimate divergence times (no partition and partition by codon position) produced concordant results. The estimated times to the most recent common ancestor (TMRCA) obtained for several nodes of interest on the mtDNA phylogeny (Fig. 2) are presented in Table 1. The mean time of divergence estimated for *Tarphius* of the Macaronesian islands (ingroup) was approximately 21 Ma, and 7 Ma for *Tarphius* endemic to the Azores. Within the Azores, colonization of the central group of islands (Fig. 1) by *T. azoricus* + *T. tornvalli* lineages (node VI) and *T. depressus* (node V) was estimated to have occurred approximately 3.7 and 2.9 Ma, respectively (Table 1), and species that colonized the western islands (Flores, Fig. 1) were estimated to have diverged around 3.3 Ma (Table 1, node IV). Estimates for divergence events within Santa Maria, the oldest Azorean island and the only one in the archipelago where there is evidence for intra-island speciation, reveal that: (1) the first divergence event (*T. rufonodulosus* from other lineages) occurred approximately 7.3 Ma [95% highest posterior density (HPD) interval: 6.27–8.12 Ma]; (2) the divergence of *T. serranoi* from other lineages occurred approximately 6.5 Ma (95% HPD interval: 5.28–7.63 Ma); and (3) the most recent divergence event in Santa Maria (*T. pomboi* and *T. depressus*) occurred approximately 4.9 Ma (95% HPD interval: 3.70–6.14 Ma).

DISCUSSION

Colonization of the Macaronesian islands and the Azores

Colonization of the Macaronesian islands is estimated to have occurred around 21 Ma, when divergence gave rise to a lineage that ultimately colonized the Azores approximately 7 Ma. Although Macaronesian islands that are now submerged may have been available for colonization possibly as early as 60 Ma (Fernández-Palacios *et al.*, 2011), the estimated time of

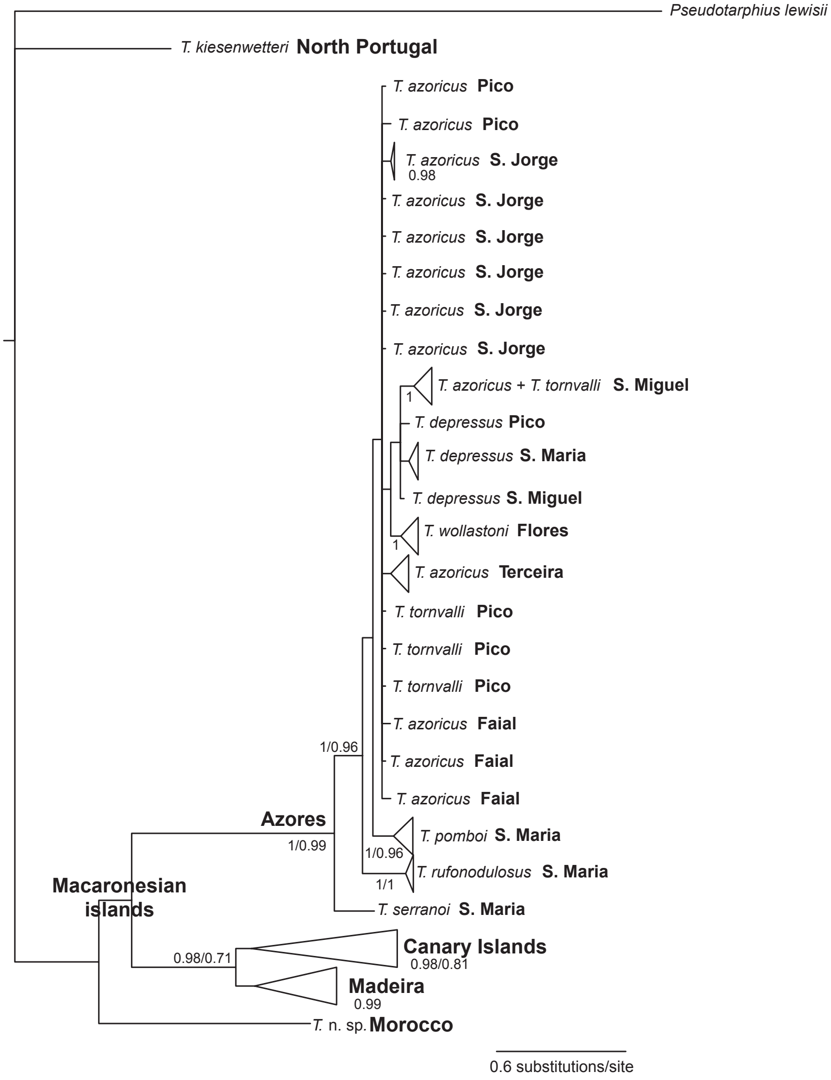

Figure 3 Bayesian inference tree for *Tarphius* beetles of the Macaronesian islands, northern Portugal and Morocco based on nuclear DNA (EF1α). Clade credibility values are shown for nodes with a posterior probability ≥ 0.95 and bootstrap support ≥ 0.70 in that order.

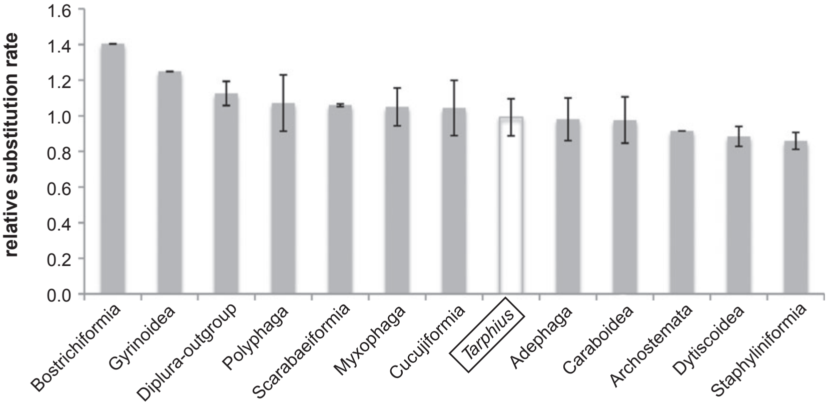

Figure 4 Weighted average relative nucleotide substitution rate for mitochondrial DNA (COII) of diverse Coleoptera groups and the genus *Tarphius*. Diplura was used as an outgroup. Black bars represent standard deviation.

colonization to the Macaronesian islands corresponds to the age of Fuerteventura (21 Ma, Canary Islands), the oldest currently emergent island among the focal archipelagos. Both phylogenetic trees reveal island *Tarphius* to be more closely related to each other than to continental taxa, suggesting a single colonization event. However, we cannot completely rule out the possibility that the record of multiple arrivals to the Macaronesian islands may have been erased by continental extinctions (Emerson, 2002). Present-day continental *Tarphius* species are potentially relicts of a more diverse and abundant

Table 1 Estimated times to the most recent common ancestor (TMRCA) for endemic *Tarphius* beetles of the Macaronesian islands based on mitochondrial protein-coding sequences (COI and COII). Eight age constraints were used as priors. Nodes B, C, E and F are from fig. 3 in Emerson & Oromí (2005). Mean value and the 95% highest posterior density (HPD) intervals are presented for tree nodes (see Fig. 2) in million of years ago (Ma). See text for details.

Tree node	Clade	Prior	Mean TMRCA (Ma)	95% HPD interval (Ma)
I	Macaronesian islands		20.63	15.94–25.78
II	Madeira + Canary Islands	≤ 14.00	13.52	12.65–14.00
III	Azores	≤ 8.12	7.42	6.38–8.12
IV	*T. wollastoni* + *T. depressus*		3.33	2.24–4.54
V	*T. depressus* (São Miguel + Pico)		2.90	1.81–4.11
VI	*T. azoricus* + *T. tornvalli*	≤ 4.01	3.65	3.10–4.01
VII	*T. azoricus* + *T. tornvalli* (Azores Central island group)	≤ 3.52	2.93	2.31–3.52
VIII	*T. azoricus* + *T. tornvalli* (Faial + São Jorge + Pico)		1.55	0.93–2.26
B	*T. setosus* (El Hierro + La Gomera)	≤ 1.00	0.86	0.64–1.00
C	*T. quadratus*	≤ 2.00	1.32	0.74–2.00
E	El Hierro + La Gomera	≤ 1.00	0.90	0.74–1.00
F	La Palma + La Gomera	≤ 2.00	1.11	0.50–1.85

fauna that inhabited laurel forests covering most of western Europe and North Africa during the Tertiary. Thus multiple colonizations of the Macaronesian islands may have occurred, but the signal for this may have been obliterated by the subsequent extinction of continental taxa following the disappearance of laurel forest. Likewise, limiting sampling of extant continental species may lead to an underestimation of the number of colonization events to the Macaronesian islands (Emerson, 2002).

Europe, Africa and other Macaronesian islands are potential sources of colonists to the Azores (Lindroth, 1960; Sundings, 1979; Borges & Serrano, 1993) but sequence data reveal that the closest relatives to the Azorean *Tarphius* are from Madeira and the Canary Islands. The inferred relationships may have been different if, as mentioned above, unsampled and extinct taxa were considered. However, the close relationship between Azorean and other island *Tarphius* species is also supported by morphological evidence, as *Tarphius* species of the Azores have been assigned to the subgenus *Atlantotarphius*, with representatives in the Canary Islands and Madeira, while *Tarphius* species from North Africa and Iberia have been placed in the subgenus *Tarphius* s.str. (Franz, 1967). This suggests that the Azores were colonized by stepping-stone dispersal via islands closer to the continental areas, and not directly from continental areas themselves. Within the Azores the trend is that lineages found on the oldest islands show the deepest divergences and are sister taxa to the remaining lineages (Figs 2 & 3), supporting the idea that, as new islands emerged, their colonization occurred from older islands within the archipelago, a pattern consistent with the progression rule (Funk & Wagner, 1995).

The Azores is the most isolated of the Macaronesian archipelagos, and land area has been limited until recently. The oldest island emerged 8.12 Ma, the second oldest island emerged only 4 million years later, and until about 1 Ma the total area of the Azores was only 62% of its current area (Triantis *et al.*, 2010b). The Azorean *Tarphius* species form a monophyletic clade, which argues for a single colonization event. Studies on other native Azorean taxa, including birds, butterflies, bats, mosses and other plants, also conclude that they result from single colonization events, supporting the hypothesis of low colonization frequency of the archipelago (Marshall & Baker, 1999; Cesaroni *et al.*, 2002; Salgueiro *et al.*, 2004; Werner *et al.*, 2009; Ferreira *et al.*, 2011). Isolation and small area may then operate as powerful filters limiting the number of propagules that arrive at the Azores, and ultimately the number of taxa endemic to the archipelago. Additionally, small population sizes and cataclysmic volcanic events on several of the Azorean islands (Serralheiro & Madeira, 1993; Nunes, 2000) may have led to extinctions of ancestral taxa (Sousa, 1985; Borges, 1992). However, our results clearly suggest ancient colonization, as the mean divergence time estimates for *Tarphius* endemic to the Azores is *c.* 7 Ma. Further, we find that Santa Maria, the oldest Azorean island (8.12 Ma), was the first to be colonized and that colonization occurred soon after its emergence. The genus *Tarphius* has had a long evolutionary history in the Azores and the failure of *Tarphius* to diversify more is not due to the recent arrival of ancestral stock. A similar result showing no support for the lineage age hypothesis for Azorean taxa (Wallace, 1881; Briggs, 1966) has also been reported for the plant genus *Pericallis* (Carine & Schaefer, 2010).

Diversification and extinction in the Azores

The Azores have had a history of active volcanism (Forjaz, 1998; Morton *et al.*, 1998; Nunes, 2000) which may have played an important role in shaping patterns of genetic diversity. Namely, habitat destruction, creation of barriers to dispersal and consequent population isolation due to volcanic activity may have promoted intra-island speciation (Carson *et al.*, 1990; Vandergast *et al.*, 2004; Emerson, 2008). Given that *Tarphius* beetles are flightless and some of the Azorean islands are characterized by relatively large inter-island distances, dispersal among islands is likely to be limited, and *in situ* speciation is expected to be the dominant mechanism for the origin of new species within islands. However, intra-island speciation in the Azores has only been inferred on Santa Maria, corresponding to the first diversification events within the archipelago. What might have promoted intra-island differentiation within Santa Maria is not clear. One potential explanation is vicariance followed by species range expansion and overlap, as *Tarphius* species on Santa Maria occur in sympatry. Santa Maria is the oldest Azorean island (8.12 Ma) and thus may be the only island where there has been sufficient time for intra-island speciation to have occurred. Therefore, the young geological age of most of the Azores and the consequent lack of time and opportunity for intra-island speciation could contribute to the small number of endemics in the archipelago. However, although in the Canary Islands intra-island speciation is responsible for many of the endemic *Tarphius* species found on all the older islands (Gran Canaria 14–16 Ma; La Gomera 12 Ma; Tenerife 11.6 Ma), divergence times are very recent, suggesting that island age may not be a key factor in intra-island speciation (Emerson & Oromí, 2005). This pattern of ancient lineages showing recent species diversification has been observed for other endemic invertebrates of the Canary Islands (Emerson & Oromí, 2005; Contreras-Díaz *et al.*, 2007). In contrast, there is no evidence for relatively recent speciation events on the oldest Azorean island of Santa Maria (8.12 Ma). Multiple species have originated within Santa Maria but the most recent speciation event was estimated to have happened between 3.6 and 6.1 Ma (split of *T. pomboi* and *T. depressus*). The last major eruptive phase in Santa Maria island dates from about 5.5 Ma (Serralheiro & Madeira, 1993) and may have contributed to these speciation patterns. Finally, Santa Maria is one of the islands most severely affected by human activities and only a small patch of 9 ha of very disturbed native forest persists (Gaspar *et al.*, 2008). Consequently, recent extinctions due to habitat destruction may have led to species loss on Santa Maria, but it seems improbable that such effects would have selectively targeted *Tarphius* taxa that are the products of recent speciation events. The absence of species that have originated within the last few million years on Santa Maria is therefore puzzling.

Despite the low vagility of *Tarphius*, it is clear that inter-island colonization within the Azores has played an important role in generating diversity and that it is associated with the most recent divergence events. The importance of inter-island colonization in shaping Azorean *Tarphius* diversity is supported by the presence of distinct but closely related clades of the same species on different islands (e.g. *T. azoricus* and *T. depressus*), and by the fact that island species assemblages are not monophyletic (e.g. Pico and São Miguel islands, Fig. 2). Inter-island colonization and isolation on different islands have also been acknowledged as important mechanisms responsible for the evolution of Azorean endemic land snails (Van Riel *et al.*, 2003, 2005; Jordaens *et al.*, 2009).

The paucity of species in the Azores could be exaggerated if the current number of *Tarphius* species described based on traditional taxonomy is an underestimate due to the presence of cryptic species. The phylogenetic relationships among taxa endemic to the Azores strongly support the taxonomic entities *T. serranoi*, *T. pomboi*, *T. wollastoni* and *T. rufonodulosus* (single-island endemics), as all specimens belonging to these species form monophyletic groups with high clade support (Figs 2 & 3). For species that occur on more than one island (*T. azoricus*, *T. tornvalli* and *T. depressus*), phylogenetic trees show that they mostly segregate by island of origin, and represent lineages that have been diverging in allopatry. If taxa accepted by traditional taxonomy and distinct phylogenetic lineages are combined, the total number of Azorean endemic *Tarphius* would be increased to 17: 14 taxa sampled plus three not included in this study (*T. azoricus* of Flores, *T. acuminatus* of Pico, and one undescribed species of São Miguel). This figure is much closer to the numbers of endemic *Tarphius* species reported for the other Macaronesian archipelagos, as Madeira and the Canary Islands are represented by 23 and 31 species, respectively.

At the time of their discovery, the Azores were almost entirely covered by native forest which was reduced to less than 3% of its original extent in less than 600 years (Gaspar *et al.*, 2008), the most dramatic destruction of native habitat recorded for the Macaronesian islands (Cardoso *et al.*, 2010; Triantis *et al.*, 2010a). Island *Tarphius* species are intimately associated with the native forest and the extensive deforestation in the Azores may have led to the extinction of some *Tarphius* lineages. Although the lack of fossil data does not allow us to test the hypothesis that more species of *Tarphius* may have existed in the Azores before human establishment, we cannot disregard the possibility of recent extinction also contributing to a low number of endemic species in the Azores.

CONCLUSIONS

The biota of the Azores is depauperate compared with that of other Macaronesian archipelagos. This lower biodiversity may result from a combination of young geological age, isolation, small area, small population sizes, lack of habitat diversity and the massive recent destruction of native forest. Our study provides a complementary approach to species presence/richness-based studies for exploring hypotheses concerning the limited diversity of endemic taxa in the Azores.

Based on phylogenetic reconstructions, *in situ* diversification within a monophyletic assemblage derived from a single colonization event appears to account for the origin of *Tarphius* species endemic to the Macaronesian islands. However, intra-island diversification, known to be an important mechanism for differentiation in older islands (Emerson & Oromí, 2005), amongst Azorean *Tarphius* endemics has only been demonstrated for species of Santa Maria, the oldest island in the archipelago. On the remaining Azorean islands, multiple species on the same island are the result of multiple colonization events. Therefore, the young geological age of the islands may be an impediment for intra-island speciation and thus limit the number of endemic taxa found in the Azores.

The molecular data also suggest that Azorean *Tarphius* are the result of a single colonization event. Colonization of the Azores is a rare event and likely to be the result of its isolation and small island sizes, which could also contribute to the smaller number of endemics compared with other Macaronesian archipelagos. Estimation of divergence times revealed that, as expected based on island age, the oldest divergence events in the Azores occurred in Santa Maria. However, speciation events on Santa Maria have not been detected at least since 3.6 Ma, which contrasts with the range of *in situ* diversification events extending to recent times within older islands of the Canaries (Emerson & Oromí, 2005). This intriguing phenomenon of an intra-island speciation hiatus on the oldest Azorean island when the island reached approximately middle age may also contribute to the smaller number of Azorean endemics. Molecular phylogenetic data also support the possibility that cryptic speciation accounts in part for the paucity of endemics in the Azores, and that the current taxonomy of Azorean *Tarphius* species should be revised.

Our data provide evidence that although most Azorean *Tarphius* species are the result of recent divergent events, *Tarphius* has had a long evolutionary history in the Azores, which is not consistent with the hypothesis that the lack of endemics is a consequence of the archipelago having been colonized only recently. The reconstruction of phylogenetic relationships for other species-rich taxa on multiple Macaronesian archipelagos, including the Azores, will facilitate testing whether our conclusions based on *Tarphius* are group specific or can be applied in general to explain the paucity of Azorean endemics.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Detailed DNA extraction and amplification methods.

Appendix S2 Sequences used in the phylogenetic reconstructions.

Appendix S3 Sequences used in the comparative substitution rate test.

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BIOSKETCH

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