

**Multidisciplinary approach to evaluate adequacy of protected areas in the Azores
for the preservation of current levels of Biodiversity.**

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Abstract:

Many of the endemic species that are found on islands have very restricted distributions and naturally small population sizes, being therefore highly susceptible to extinction. Arthropods are among the animal groups most severely affected by extinction on islands, and although they represent more than half of the Azorean terrestrial endemic species, they were not used to assess conservation priorities in the Azores. In this study we used a multidisciplinary approach, including field surveys, traditional taxonomy, molecular assays, and the speciose beetle genera *Tarphius* and *Trechus*, to evaluate the adequacy of protected areas in the Azores in biodiversity preservation. The data indicate that the area protected in the Azores is insufficient to guarantee the maintenance of species richness and genetic diversity of endemic *Tarphius* and *Trechus* beetles. Because all *Tarphius* and *Trechus* populations had exclusive haplotypes, and genetic variability within species is geographically structure, we argue that they should be treated as distinct conservation units and that the maximum number of populations should be protected in order to preserve current levels of biodiversity, and thus arrest the generalized trend of insular biodiversity decline.

1. Introduction

The volcanic archipelago of the Azores in the North Atlantic is the most remote of the Macaronesian Islands and a long period of evolution in isolation has led to the origin of many endemic species. However, the arrival of humans to the archipelago, more than 500 years ago, has shaped the original faunal and floral composition of these islands. The current biota composition is therefore the result of *in loco* evolution and anthropogenic activities, including the introduction of exotic species and the extinction of native species (Martins 1993, Silva & Smith 2004, Amorim 2005, Borges *et al.* 2006, Silva & Smith 2006, Borges *et al.* in press).

Many of the endemic species that are found on islands have very restricted distributions and naturally small population sizes, which make insular endemic species highly susceptible to extinction (Frankham *et al.* 2002, Gillespie & Roderick 2002, Whittaker & Fernández-Palacios 2007). Arthropods, particularly insects, are among the animal groups most severely affected by extinction on islands, and more than 3/4 of the insect species known to have gone extinct in the last 300 years vanished from islands (Gillespie & Roderick 2002).

Arthropods have been the focus of much of the biological research undertaken in the Azores and, in fact, represent more than half of the terrestrial endemic species found in the archipelago (Borges *et al.* 2005a). Nevertheless, species of endemic arthropods were not used to assess conservation priorities in the Azores, and therefore were not taken into consideration when deciding the areas that should be granted protection (*e.g.*, Nature Reserves, Natural Forest Reserves and NATURA 2000 sites).

In this study we used a multidisciplinary approach, including field surveys, traditional taxonomy and molecular assays, to evaluate how appropriate is the protected area in the Azores for the preservation of current levels of biodiversity. Beetles of the genera *Tarphius* (Coleoptera, Zopheridae) and *Trechus* (Coleoptera, Carabidae) which are among the most diverse insect genera found in the Azores (Borges *et al.* 2005a), and are therefore an important contribution to the total biodiversity of the archipelago, were chosen as the study organisms.

2. Methods

Field surveys

All islands Azorean islands (Fig.1), with the exception of Corvo, where no suitable habitat for the study beetle genera can be found, were sampled from 1999 to 2004.

Tarphius beetles were collected using three different methods: i) pitfall trapping, ii) visual search in specific habitats, and iii) leaf litter collection. Pitfall traps were set up along a 150 m long and 5 m wide transect, and a total of 30 small plastic pitfalls were used per transect. Transects were run in three main habitat types found in the Azores: **i)** native *Laurisilva* forest (dominated by *Juniperus brevifolia*, *Erica azorica*, *Ilex perado* ssp. *azorica* and/or *Laurus azorica*); **ii)** planted forests of *Cryptomeria japonica* (exotic), and **iii)** semi-natural grassland. Pitfall sampling took place over at least 14 consecutive days and a total of 90 transects were set up in 16 Natural Forest Reserves on Flores, Faial, Pico, São Jorge, Terceira, São Miguel and Santa Maria islands in the scope of project BALA - *Biodiversity of the Arthropods from the Laurisilva forest of the Azores* (see Borges *et al.* 2005b, 2006). Visual searches were conducted both during the day and at night. Loose bark, decaying wood and dead arborescents of the endemic plant *Euphorbia stygiana* (which are known to harbor *Tarphius*) were the main targets of the searching efforts. Leaf litter collection took place in different habitats and Berlese funnels (Berlese 1904) were used to extract small invertebrates from the plant and soil material collected.

Collection of epigeal forms of *Trechus* beetles was performed following the pitfall-transect methodology described above for *Tarphius*. Additionally, soil-dwelling arthropods were sampled by sifting soil and leaf litter (Gillerfors 1986), and by intensive visual searches in dark humid habitats. Pitfall traps and visual searches were used to sample cave populations of *Trechus*. Traps were set up along a 500 m transect extending from the cave entrance inwards. The sampling strategy included visiting a total of 49 lava tubes and volcanic pits, including all caves where *Trechus* have been recorded in the past. Traps were left in place for a minimum of seven days and up to a month. Intensive visual searches were also performed during pitfall traps set up and removal.

Pitfall traps were also set up in the Mesocavernous Shallow Stratum -MSS- (Borges 1993) on Terceira, Flores and São Jorge islands to investigate the presence of *Trechus* beetles.

Species affiliation was determined based on morphological characters and the number of individuals per species was recorded for all sampling localities. This information was uploaded into the ATLANTIS data base for the Azores (Borges 2005) and used to produce updated distribution maps of *Tarphius* and *Trechus* species in the Azores. The updated distributions per species per island were superimposed onto maps of protected areas in the Azores for each island where the species occurs to determine whether the distribution of the Azorean endemic species of *Tarphius* and *Trechus* is included in the archipelago's protected areas.

Molecular assays

One to eight individuals per species and per location were used for DNA extraction. Total genomic DNA was extracted from each individual beetle using the DNeasy™ Tissue Kit (Qiagen).

Mitochondrial DNA (mtDNA) has been shown to be a useful marker to reconstruct phylogenetic relationships at a variety of taxonomic levels in insects and other taxa (Crozier *et al.* 1989, Simon *et al.* 1994, Avise 1994, Juan *et al.* 1995, Lunt *et al.* 1996, Avise 2000). Namely, the mtDNA genes cytochrome oxidase I (COI) and cytochrome oxidase II (COII) are particularly valuable to infer phylogeographic patterns within beetle species (Emerson *et al.* 1999, Stauffer *et al.* 1999, Emerson *et al.* 2000, Moya *et al.* 2004) and were used in this study to characterize populations of Azorean endemic *Trechus* and *Tarphius* beetle populations. DNA amplification of the target genes (a fragment of about 1,500 bp, comprising part of the COI gene, the leucine tRNA gene and the complete COII gene) was performed by PCR and the purified PCR products were cycle-sequenced in both directions with the PCR amplification primers and internal primers.

Sequences were collapsed to haplotypes with the program COLLAPSE 1.2 (Posada 1999) and analyzed using Maximum Parsimony (MP) and Bayesian Inference (BI) methods. The program MODELTEST v. 3.07 (Posada & Crandall 1998) was used to select the model of evolution that best explains the nucleotide variation in the sequence data set, and the software PAUP* v. 4.0b10 (Swofford 2002) was used to generate MP trees, while the Bayesian analyses were performed using MRBAYES v 3.1 (Huelsenbeck & Ronquist 2001).

Analysis of molecular variance, AMOVA (Excoffier *et al.* 1992), was used to investigate genetic structure and the analyses were performed with the software

ARLEQUIN 2.000 (Schneider *et al.* 2000) assuming the model of sequence evolution selected by MODELTEST. The basic grouping unit in all AMOVA designs was defined as conspecific sequences that occur on a particular island. To investigate within island population structure of cave populations, *i.e.*, if there is population structure associated with cave of origin, we used the two most wide spread cave *Trechus* species in the Azores, *Trechus terceiranus* from Terceira island and *T. picoensis* from Pico island. Due to the small number of mtDNA sequences generated per species per cave, AMOVA was not appropriate to test population structure. Instead, we calculated pairwise genetic distances between haplotypes, assuming the model of molecular evolution (TrN+I+G) suggested by MODELTEST, and compare it to geographic distances. To test if genetic distance between haplotypes is correlated to geographic distance between the caves where they are found, *i.e.*, isolation by distance (Wright 1943), we compared genetic and geographic distances for individual haplotypes and mean pairwise genetic distances between groups of haplotypes from caves at different geographic distances. Pairwise genetic distance matrixes and pairwise geographic distance matrixes were compared using Mantel tests (Mantel 1967) in the program GENETIX v4.05 (Belkhir *et al.* 2004). The nonparametric statistical tests Kruskal-Wallis and Mann-Whitney implemented in the statistical package SYSTAT 9[®] (SPSS Inc., Evanston, IL, USA) were used to test the null hypothesis that the groups of haplotypes from caves at different geographic distances being compared had identical mean genetic distances.

3. Results

Field surveys

As part of project “BALA”, a total of 2,700 pitfall traps were set up in Natural Forest Reserves on Flores, Faial, Pico, São Jorge, Terceira, São Miguel and Santa Maria islands (see Borges *et al.* 2005b). Additionally, 1,660 pitfall traps were set up to collect live specimens: 240 pitfall traps set up on the surface (Flores, Terceira, Faial, Pico, Santa Maria and São Miguel islands), 40 in the MSS (Flores, Terceira and São Jorge), and 1,380 in the 49 lava tubes and volcanic pits visited (Faial, Pico, São Jorge, Terceira, Graciosa, São Miguel and Santa Maria islands).

With the exception of Graciosa and Faial islands, *Tarphius* specimens, assigned to eight species, were collected on all other surveyed islands. *Tarphius* were for the first time collected on São Jorge island (*T. azoricus*), the species *T. acuminatus* was for the first time collected on São Miguel island and the species *T. depressus* was for the first time collected on Pico and São Miguel islands. An updated map of *Tarphius* species distribution in the Azores is present in Figure 2.

No beetles of the genus *Trechus* were collected in the pitfall traps set up in the MSS, and the pitfalls set up on the surface were only successful in capturing *Trechus* on Terceira island (*T. terrabravensis* Borges, Serrano & Amorim; see Borges *et al.* 2004). In contrast, pitfall traps were very effective sampling cave fauna and *Trechus* were collected in 21 of the 49 lava tubes and volcanic pits surveyed. Specimens of *Trechus* beetles, representing eight species, were found on four of the eight islands surveyed, namely on Faial, Pico, Terceira and São Jorge islands. Several of the *Trechus* specimens collected belong to four species yet unknown to science: the surface-dwelling *T. terrabravensis*, and the cave-dwelling species *T. pereirai*, *T. oromii* (Borges *et al.* 2004) and *T. isabelae* (Borges *et al.* 2007). Moreover, several of the specimens collected represent first records, for example, *Trechus* were for the first time collected on Faial island (*T. oromii* Borges, Serrano & Amorim) and surface-dwelling *Trechus* were for the first time collected on Terceira island (*T. terrabravensis* Borges, Serrano & Amorim). An updated map of *Trechus* species distribution in the Azores is present in Figure 3.

When the updated distributions of *Tarphius* and *Trechus* beetle species endemic to the Azores were mapped onto a map of the archipelago with all the terrestrial protected areas marked, species distributions in many instances fall outside the protected areas. The lack of overlap between populations/species distribution ranges and protected areas is in some cases complete. For example, the only area on Terceira island where *Tarphius azoricus* are known to occur is not protected (Fig. 4), neither are the sites on Santa Maria islands where *Tarphius depressus* have been collected (Fig. 5). However, the more striking cases involve single island endemics, *i.e.*, species that are only known to exist on a particular island. All *Trechus* species found on Pico island, *T. jorgensis* from São Jorge island, *T. oromii* from Faial island and three of the *Tarphius* species found on Santa Maria island (*T. rufonodulosus*, *T. serranoi* and *T. pomboi*) are single island endemic species whose distribution range does not overlap with any protected areas (Fig. 6, 7, 8 and 9, respectively).

Molecular assays

Sixty eight sequences were generated for seven species of *Tarphius* beetles collected in the Azores, corresponding to 56 haplotypes, and 76 sequences were generated for eight Azorean endemic species of *Trechus* beetles, corresponding to 68 haplotype (Fig. 10 and 11). With the exception of two *Tarphius* individuals from different species collected on the same collecting site in São Miguel island that had the same sequence, haplotypes were only shared by conspecific individuals collected in the same location. The phylogenetic reconstructions show that for the species of *Tarphius* that occur on multiple islands (*T. tornvalli* + *T. azoricus*, and *T. depressus*), populations of the same species found on different islands are genetically divergent (Fig. 10). For the cave *Trechus* haplotypes in particular, although the lack of a clear geographic structure of haplotypes according to cave of origin, the phylogenetic tree shows that distribution of haplotypes is not completely random with regard to cave affiliation (Fig. 11), as some haplotypes from the same lava tubes group together.

The analyses of molecular variance (AMOVA) for both genera suggested that most of the mtDNA variation found can be attributed to variability among species, (Table I and II). For the genus *Tarphius*, an appreciable proportion of the total variation (40.62%, Table I) separates populations of the same species found on different islands, and for the genus *Trechus*, 25.80% of the total variation (Table II) is due to haplotypic differences among islands.

For the cave species *T. terceiranus* and *T. picoensis* the Mantel tests performed for matrices of pairwise genetic and geographic distances between conspecific individual haplotypes showed in both cases a significant correlation between genetic distances and the distances between the caves where the haplotypes were found ($Z=90,514.34$ and $43,311.03$ respectively, $P<0.05$). The nonparametric statistical tests Kruskal-Wallis and Mann-Whitney tests revealed for both species that the average genetic distances between individuals from the same cave and individuals from different caves, and between individuals from caves at different distances are not the same ($P<0.05$). The statistical tests indicate that there is an association between genetic and geographic distance *i.e.*, haplotypes from the same cave tend to be more closely related than haplotypes from different caves, which suggests a pattern of isolation by distance (Wright 1943, Slatkin 1993).

4. Discussion and Conclusions

It is now widely recognized that conservation of biodiversity requires urgent and effective measures (Frazee *et al.* 2003, Strange *et al.* 2006). The field surveys performed revealed that the range of many of the *Tarphius* and *Trechus* species endemic to the Azores is not encompassed by the current network of protected areas in the archipelago, which therefore raises conservation concerns.

The loss of genetic variability is one of the major concerns of any conservation/management program as it may jeopardize the ability of a population to adapt to a changing environment, and thus compromise its long-term evolutionary potential (Meffe & Carroll 1994, Reed & Frankham 2003). The molecular data revealed that all *Tarphius* and *Trechus* populations sampled in the Azores had exclusive haplotypes, and that genetic variability within species is geographically structure. Therefore, we suggest that those populations should be treated as distinct conservation units in order to avoid erosion of genetic diversity and to protect the processes that sustain it (Moritz 1994, Witting *et al.*, 1994, Moritz 1995, Vane-Wright 1996, Fraser & Bernatchez 2001).

Habitat destruction caused by clearing of native vegetation, which has occurred at a particularly fast pace in the last century (Martins 1993, Borges *et al.* 2006, Borges *et al.* in press), constitutes the most serious threat to *Tarphius* and *Trechus* beetles in the Azores. Fragmentation of the natural forest, *Laurisilva*, to which both genera are intimately associated, may have a severe and irreversible effect on the genetic diversity of those beetle species, particularly for the low vagile *Tarphius* beetles. *Trechus* beetles in the Azores are mainly found in lava tubes and therefore this type of habitat deserves special attention. Because subterranean ecosystems are directly dependent on processes occurring above ground (Wilkens *et al.* 2000, Culver 2001), and the capacity of lava tubes to support life is related to the quality of the habitat found directly above them (Howarth 1983), the replacement of native and semi-native forest by pastures throughout the Azores (Martins 1993, Borges 1996, Borges *et al.* 2006, Borges *et al.* in press) is of concern for the sustainability of the subterranean ecosystems. Some of the caves in the Azores where *Trechus* are found are in protected areas (*e.g.*, Montoso on São Jorge isl., Malha and Coelho on Terceira isl.) and a few have special protection status (Carvão and Natal on Terceira isl., and Torres on Pico isl.), but the majority of them are not under any kind of protection. This issue is of particular relevance because

five of the seven troglobitic species of *Trechus* endemic to the Azores are only found in non-protected caves. The most pressing examples include *T. jorgensis* and *T. oromii*, whose distribution is restricted to a single cave on São Jorge and Faial islands, respectively. Given the vulnerability of their habitat, most cave *Trechus* species that occur in the Azores are potentially endangered, and special conservation efforts should be allocated to preserve the integrity of lava tubes and volcanic pits in the archipelago. The presence of unique *Trechus* haplotypes in all caves sampled suggests that a conservation plan aimed at protecting the current levels of genetic diversity should include as many caves as possible on all of the islands where the beetles are found to prevent the loss of unique lineages.

Based on these results, a report was made to the Azorean Government suggesting the creation of new protected areas for *Tarphius* species, one in Fontinhas (Terceira island) to protect one population of *Tarphius azoricus* (see Fig. 4) and another one in Pico Alto (Santa Maria) to protect the unique known location for the species *Tarphius rufonodulosus*, *T. serranoi* and *T. pomboi* and also a population of *T. depressus*. This site on Santa Maria island is also a hotspot of diversity in the Azores (see Borges *et al.* 2005b). An official proposal to integrate those areas into IUCN categories has been produced, but unfortunately it may take a considerable amount of time before it is approved. Special management measures are therefore in need to be implemented as soon as possible. Concerning the cave habitats, the GESPEA group is currently working with the Azorean Government to create special conservation management measures for the most important Azorean caves.

In conclusion, field surveys and molecular data clearly indicate that the area protected in the Azores is insufficient to guarantee the maintenance of species richness and genetic diversity of *Tarphius* and *Trechus* beetles found in the archipelago. However, new opportunities are being created for the selection of new areas and implementation of management plans in this Atlantic archipelago.

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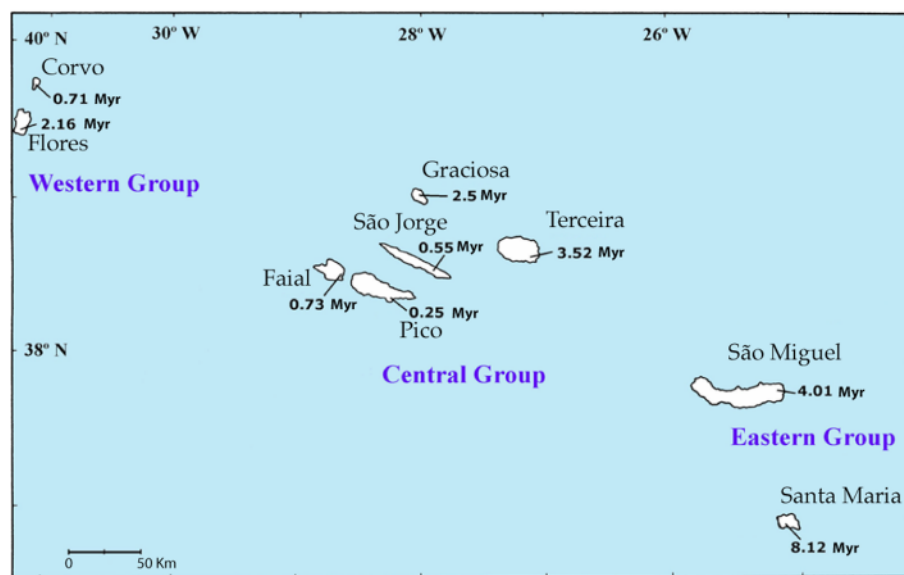


Figure 1. The archipelago of the Azores. Maximum geological age of each island in million year (Myr) as in França *et al.* 2003.

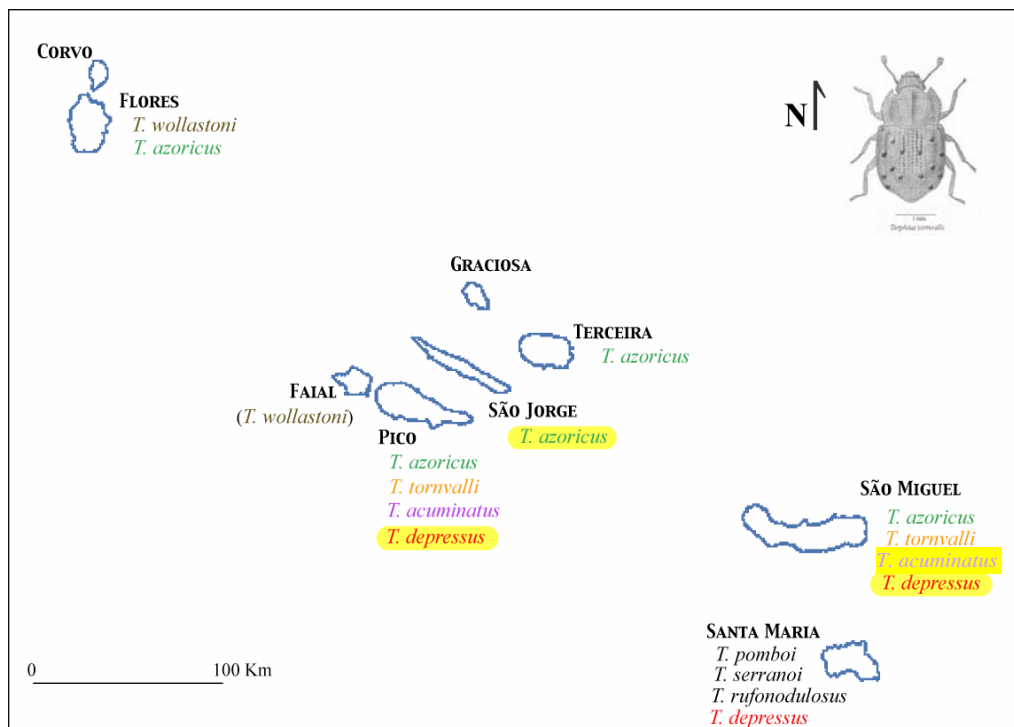


Figure 2. Updated distribution of the genus *Tarphius* in the Azores. Species that occur on multiple islands are color coded, yellow boxes indicate species that were collected for the first time on a particular island, and parentheses indicate unconfirmed occurrences. Typical morphology of the genus *Tarphius* is illustrated by a drawing of *T. tornvalli* (from Gillerfors, 1986).

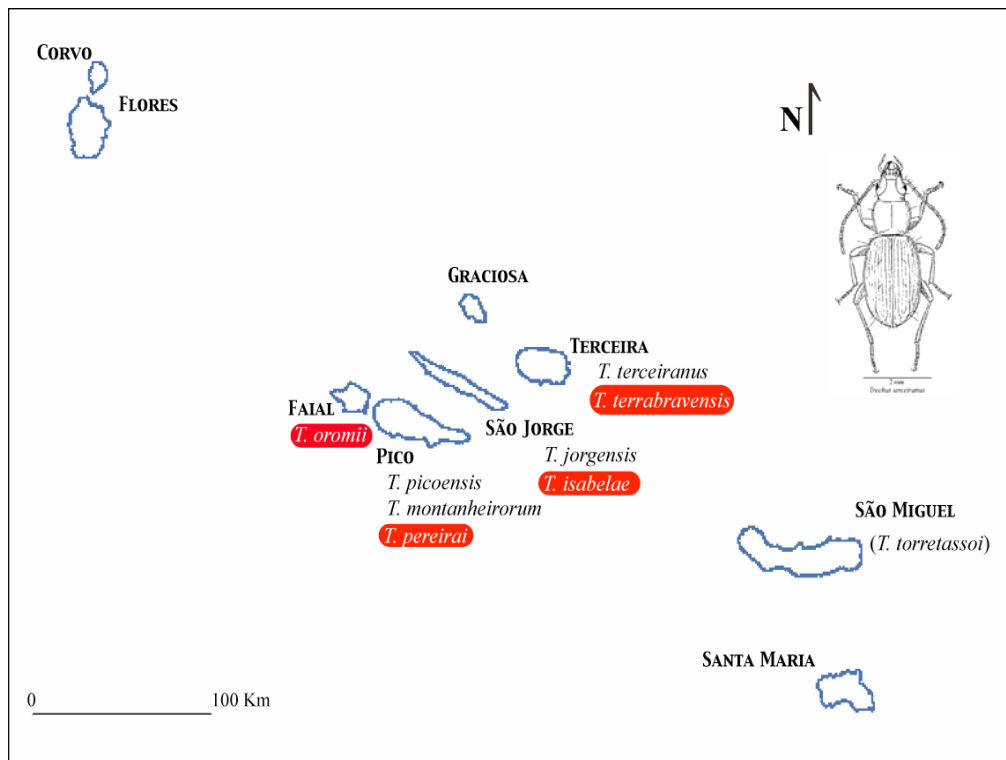


Figure 3. Updated distribution of the genus *Trechus* in the Azores. Red labels indicate species new to science, and parentheses indicate unconfirmed occurrences. Typical morphology of the genus *Trechus* is illustrated by a drawing of *T. terreiranus* (from Machado 1988)

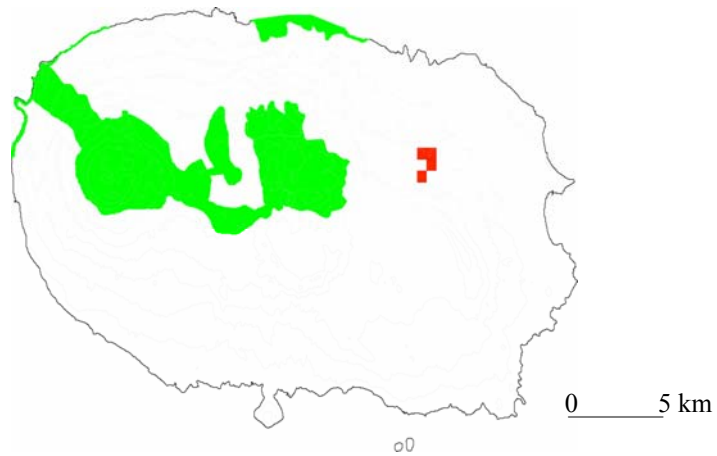


Figure 4. Distribution of *Tarphius azoricus* on Terceira island (red squares). Protected areas, including Nature Reserves, Natural Forest Reserves and NATURA 2000 sites (European Union network of nature conservation sites) are shaded green. ATLANTIS 2005.

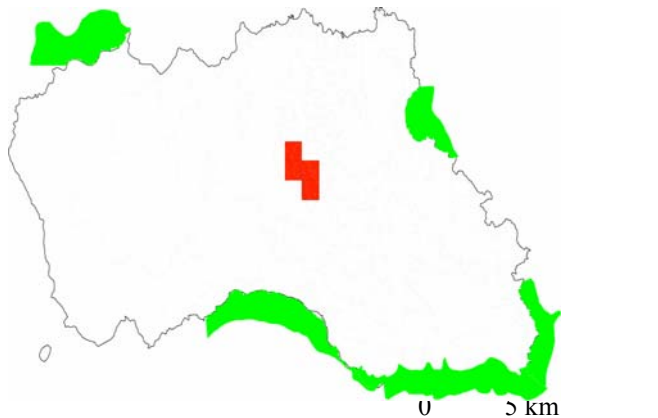


Figure 5. Distribution of *Tarphius depressus* on Santa Maria island (red squares). Protected areas, including Nature Reserves, Natural Forest Reserves and NATURA 2000 sites (European Union network of nature conservation sites) are shaded green. ATLANTIS 2005.

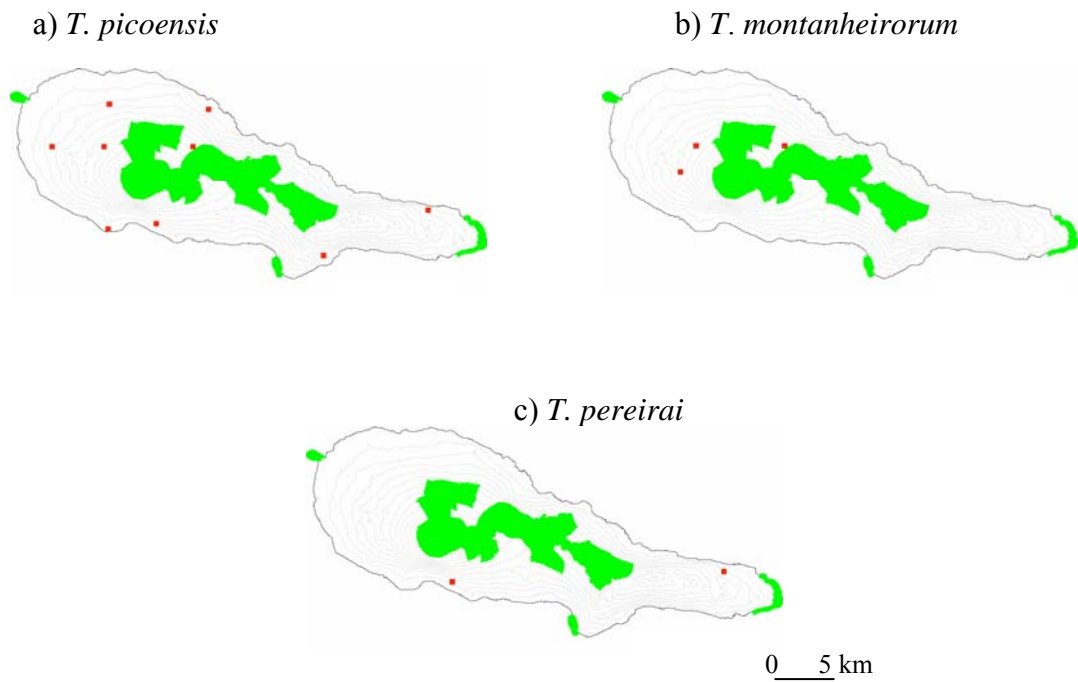


Figure 6. Distribution of *Trechus* species endemic to Pico island (red squares): a) *T. picoensis*, b) *T. montanheirorum*, and c) *T. pereirai*. Protected areas, including Nature Reserves, Natural Forest Reserves and NATURA 2000 sites (European Union network of nature conservation sites) are shaded green. ATLANTIS 2005.

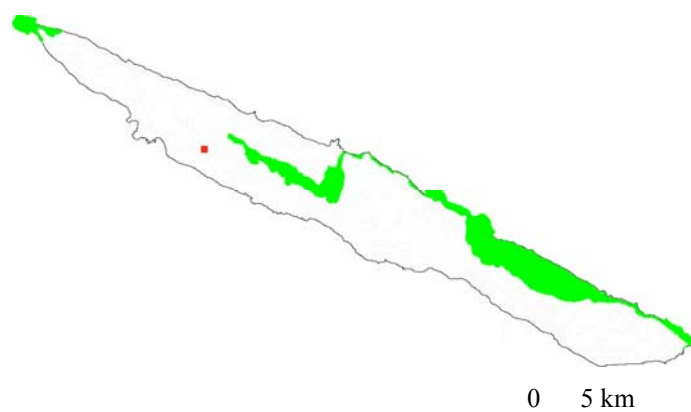


Figure 7. Distribution of *Trechus jorgensis* endemic to São Jorge island (red squares). Protected areas, including Nature Reserves, Natural Forest Reserves and NATURA 2000 sites (European Union network of nature conservation sites) are shaded green. ATLANTIS 2005.

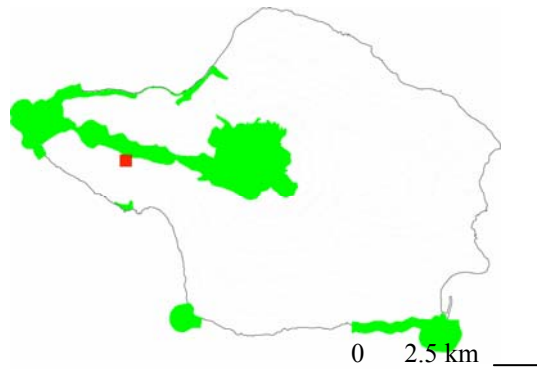


Figure 8. Distribution of *Trechus oromii* endemic to Faial island (red squares). Protected areas, including Nature Reserves, Natural Forest Reserves and NATURA 2000 sites (European Union network of nature conservation sites) are shaded green. ATLANTIS 2005.

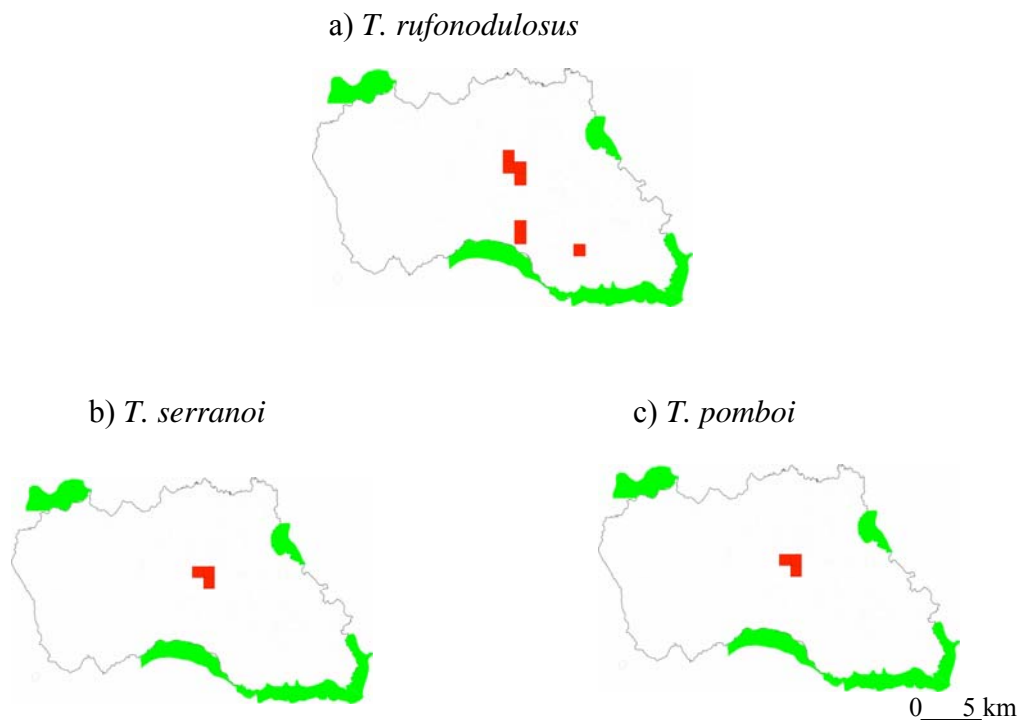


Figure 9. Distribution of a) *Tarphius rufonodulosus*, b) *T. serranoi* and c) *T. pomboi* on Santa Maria island (red squares). Protected areas, including Nature Reserves, Natural Forest Reserves and NATURA 2000 sites (European Union network of nature conservation sites) are shaded green. ATLANTIS 2005.

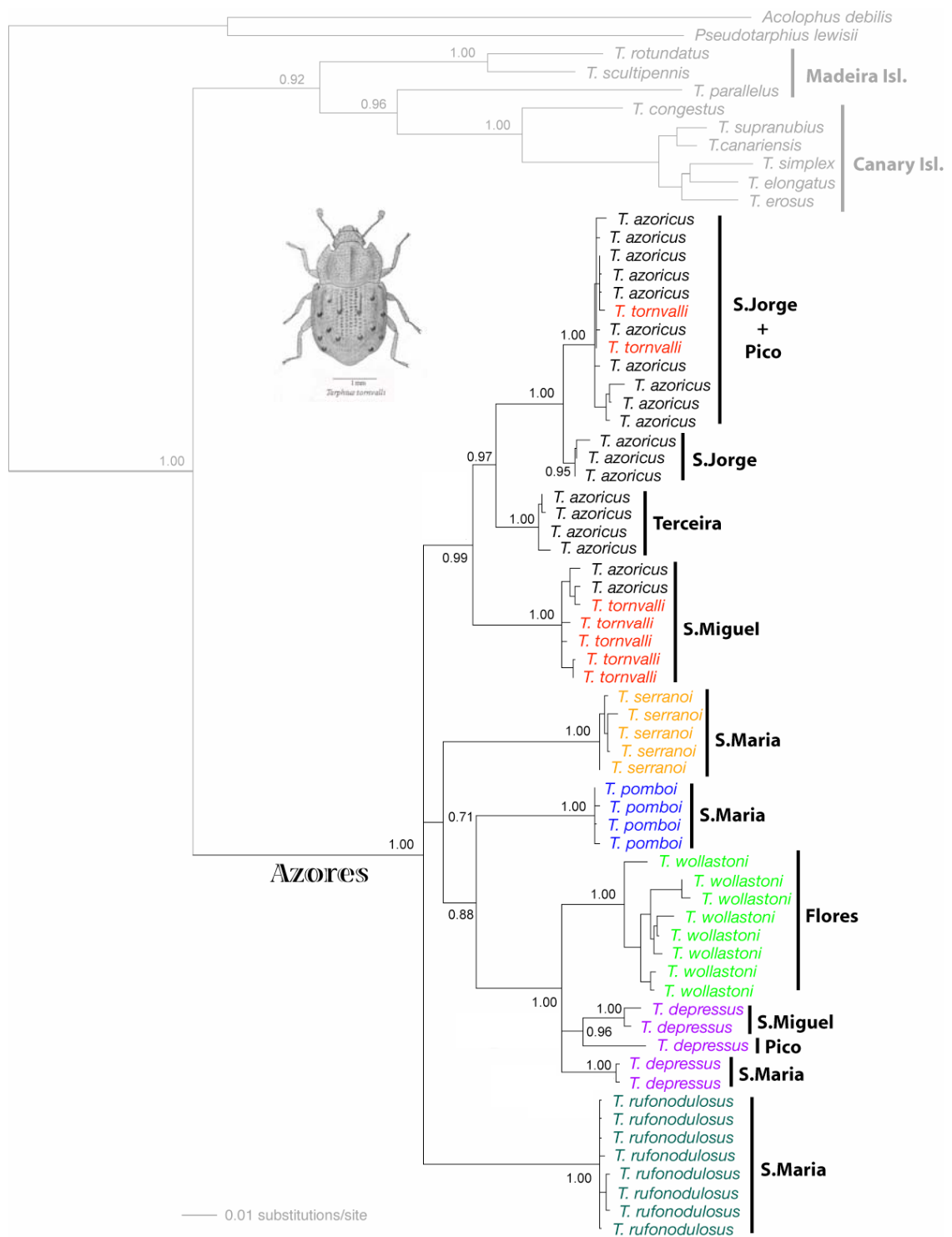


Figure 10. Mitochondrial DNA phylogenetic reconstructions of *Tarphius* species endemic to the Azores: Bayesian Inference tree. Numbers on nodes represent clade credibility for nodes with a posterior probability value ≥ 0.70 . Species are color coded.

Note – Clade credibility values were omitted on some of the terminal nodes for graphical clarity.

Table I. Hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) among *Tarphius* haplotypes.

Source of variation	d.f.	Variance	% of total variation	Fixation indices	P-value ¹
<u>With regional structure</u>					
(grouping by island)					
among islands	5	0*	0*(-24.53)	$\Phi_{CT} = 0^*$	0.99
among species within islands	5	63.93	118.64	$\Phi_{SC} = 0.953$	0
among individuals within species	57	3.18	5.89	$\Phi_{ST} = 0.941$	0
<u>No regional structure</u>					
(grouping by species)					
among species	5	34.72	54.39	$\Phi_{CT} = 0.544$	< 0.001
among populations within species	5	25.93	40.62	$\Phi_{SC} = 0.891$	0
among individuals within populations	57	3.18	4.99	$\Phi_{ST} = 0.950$	0

¹ – proportion of 10⁶ permutations that show fixation index values \geq than the observed value

* - negative values are presented as 0

d.f. – degrees of freedom

Table II. Hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) among *Trechus* haplotypes.

Source of variation	d.f.	Variance	% of total variation	Fixation indices	P-value ¹
among islands	4	9.03	25.80	$\Phi_{CT} = 0.258$	< 0.05
among species	5	20.75	59.26	$\Phi_{SC} = 0.799$	0
among individuals within species	58	5.23	14.94	$\Phi_{ST} = 0.851$	0

¹ – proportion of 10⁶ permutations that show fixation index values \geq than the observed value

* - negative values are presented as 0

d.f. – degrees of freedom

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