





First record of the exotic snail *Zonitoides arboreus* (Eupulmonata, Gastrodontiidae) in the Brazilian oceanic island of Trindade, South Atlantic

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Abstract: This study aims to refine the taxonomy of land snails previously identified as *Happia* sp. from the oceanic Trindade Island (SE Brazil). Based on a recent sampling of live specimens, we sequenced the mitochondrial COI barcode marker from two specimens. The so-called *Happia* sp. is actually the exotic *Zonitoides arboreus*, which is native from the Nearctic but widespread worldwide due to human introductions. The single COI haplotype from Trindade Island is identical to one from a specimen sampled from a Botanical Garden in The Netherlands, and more research is needed in order to elucidate possible introduction routes of *Z. arboreus*. This species feeds on fungi and plant material, requiring the existence of trees/wood debris and mild climatic conditions, and it is sometimes recorded as an intermediate host of some species of Cestoda and Nematoda. With the present record, the number of known native terrestrial gastropods from Trindade Island is reduced to six, whereas the number of introduced species rises to three, in addition to two cryptogenic species.

Keywords: DNA; Gastropoda; non-native species; orchid snail; *Zonitoides nitidus*.

Primeiro registro do caracol exótico *Zonitoides arboreus* (Eupulmonata, Gastrodontiidae) na ilha oceânica brasileira de Trindade, Atlântico Sul

Resumo: Este estudo visa refinar a taxonomia de caracóis terrestres previamente identificados como *Happia* sp. da oceânica Ilha de Trindade (SE Brasil). Baseado em recente amostragem de espécimes vivos, nós sequenciamos o marcador mitocondrial 'barcode' COI de dois espécimes. O morfotipo antes identificado como *Happia* sp. representa na verdade o exótico *Zonitoides arboreus*, que é nativo do Neártico, mas distribuído globalmente em razão de introduções antrópicas. O único haplótipo COI da Ilha de Trindade é idêntico ao de espécime obtido de um Jardim Botânico nos Países Baixos, e mais pesquisa é necessária a fim de elucidar possíveis rotas de introdução de *Z. arboreus*. Esta espécie se alimenta de fungos e plantas, necessitando da existência de árvores ou detritos vegetais e condições climáticas amenas, e é por vezes registrado como um hospedeiro intermediário de algumas espécies de Cestoda e Nematoda. Com este registro, o número conhecido de espécies nativas de gastrópodes terrestres da Ilha de Trindade cai para seis, enquanto o número de espécies introduzidas aumenta para três, além de duas espécies criptogênicas.

Palavras-chave: DNA; Gastropoda; espécie não-nativa; caracol de orquídea; *Zonitoides nitidus*.

Introduction

Zonitoides Lehmann, 1862 (Gastrodontiidae) is a genus of land snails currently comprising 17 extant species (MolluscaBase 2024), but mostly known by two widespread species: *Zonitoides arboreus* (Say, 1817) and *Zonitoides nitidus* (Müller, 1774). Whereas *Z. nitidus* is regarded as native from the Holarctic and mainly a temperate species, mostly found on wet/moist sites (Didier and Rondelaud 1989, Kuznik-Kowalska

2011, Capinha et al. 2014), *Z. arboreus* is a Nearctic species that can withstand drier and warmer conditions (Evangelista et al. 2013, Capinha et al. 2014). Both species have been introduced worldwide through traded plants on greenhouse cultivation, forestry plantations, botanical gardens or private nurseries (Barker 1999, Herbert 2010, Evangelista et al. 2013, Capinha et al. 2014). The exotic range of *Z. nitidus* is not well defined, but it possibly comprises countries in Asia and South America (Gude 1901, Haas 1959). On the other hand, *Z. arboreus* has

been introduced to Central and South America, Europe, Africa, Asia and Oceania (Evangelista et al. 2013, Hausdorf 2023), i.e., it is currently a global species with a long list of synonyms (MolluscaBase 2024). Despite the widespread range of these two species, there are very few genetic studies on *Zonitoides*, and by far most DNA sequences from the GenBank repository are from a single country (Canada). The feeding behavior of *Z. nitidus* comprises both the consumption of plant material and the seasonal predation on land or freshwater gastropods – and even small bivalves (Moens 1982, Didier and Rondelaud 1989, Barker and Efford 2004, Rondelaud et al. 2006). In contrast, *Z. arboreus* was only recorded feeding on fungi or plant material (e.g. Anonymous 1925, Bartsch and Quick 1926, Barker 1999, Barker and Efford 2004, Herbert 2010, Jaskula et al. 2019, Hirano et al. 2020).

The range of the exotic *Z. arboreus* in Brazil is much underestimated in the literature (e.g. Boffi 1979, Silva 2007, Agudo-Padrón 2011, Pilate et al. 2015); based on iNaturalist records with adequate photographs (Rosa et al. 2022), this species seems mainly concentrated in South/Southeast Brazil. Those records would benefit from further confirmation based on DNA evidence, as shells of *Z. arboreus* are similar to those of *Z. nitidus* (Evangelista et al. 2013). The non-figured records of *Z. nitidus* in Brazil, stemming from northern Minas Gerais, Teresópolis (Rio de Janeiro), Amparo (São Paulo) and Santa Catarina (Thiele 1927, Haas 1959), also require confirmation (see Discussion). The unprecise records of *Zonitoides* sp. from Ilha Grande (Rio de Janeiro) and Santa Maria (Rio Grande do Sul), respectively by Nunes and Santos (2012) and Silva et al. (2019), exemplify the need for a comprehensive revision of this genus in Brazil.

The curious record of *Happia* sp. (Scolodontidae) from Trindade Island (Salvador et al. 2014) caught our attention as a possible occurrence of *Zonitoides*. Trindade Is. lies about 1,140 km from the SE Brazilian coast and is the largest island of the Trindade and Martin Vaz archipelago, located in the eastern end of the Vitória-Trindade Seamount Chain. The terrestrial fauna and flora of Trindade Is. were severely impacted due to centuries of predation and herbivory by introduced mammals, especially goats. These were eradicated in 2005 (Silva and Alves 2011), though the house mice remain as the last invasive mammal on the island (Cunha et al. 2015, Alves and Silva 2016). That history led to the probable extinction of most native terrestrial gastropods, currently known mostly by empty shells, and to the introduction of exotic species (Salvador et al. 2013, 2014, Cunha

et al. 2015). One of these supposedly native species is the above-mentioned record of *Happia* sp., found for the first time in 2013, under the decomposing bark of a dead, introduced tree of *Araucaria angustifolia* in the Desejado Peak. At that moment, only empty shells were available for study, but recent sampling of live specimens enabled us to conduct a DNA barcode analysis to ascertain its identity.

Material and Methods

Since the 2013 fieldwork, two of the authors (NGS, MGR) were able to find live individuals of this species from Trindade Is. in different occasions. Only in the first instance, in 2013, the animals were found under the bark of a tree; in all other cases, they were found on or under rocks, and on the soil, being active during humid days and retracted into their shells on dry days. Around the rocks, the vegetation is predominantly composed of endemic ferns, grasses and sedges. The collection of samples for this study were carried out from September 22–24, 2023, at night, during a fog passage at the Desejado Peak (approximate coordinates and altitude for the sampling site: 20°30'40"S, 29°19'28"W, 580 m a.s.l.) (Figure 1).

In situ photographs of the snails were obtained using a Nikon D7100 with a Nikkor 85 mm macro lens. Four specimens were sampled and fixed in absolute ethanol, and stored at the malacological collection of Museu Nacional, Universidade Federal do Rio de Janeiro (Brazil), under registration number MNRJ 61775. Shells were photographed *ex situ* through a Leica DFC450 camera coupled to a Leica M205C stereomicroscope. A piece of the foot from two specimens was removed in order to enable genetic procedures.

DNA was extracted with a NucleoSpin Tissue kit (Macherey-Nagel), following the manufacturers' protocol, with the quality of the extraction measured through a NanoDrop 2000 spectrophotometer. The following reagents were used for the amplification of the COI (cytochrome c oxidase subunit 1) gene barcoding fragment: 1 U Platinum Taq DNA Polymerase (Invitrogen), in proprietary buffer (1x), 3.0 mM MgCl₂, 0.8 mg/mL BSA, 0.2 mM dNTP, 0.2 μM for each primer, in addition to 30–50 ng of DNA, totaling 25 μL per reaction. Primers used for the amplification of the COI marker were LCO1490-HCO2198 (Folmer et al. 1994). The thermocycling profile was: initial denaturation at 95°C (5 min); 36 cycles of denaturation at 95°C (45 s), annealing at 50°C (45 s), extension at 72°C (1 min 30 s), followed by a final extension

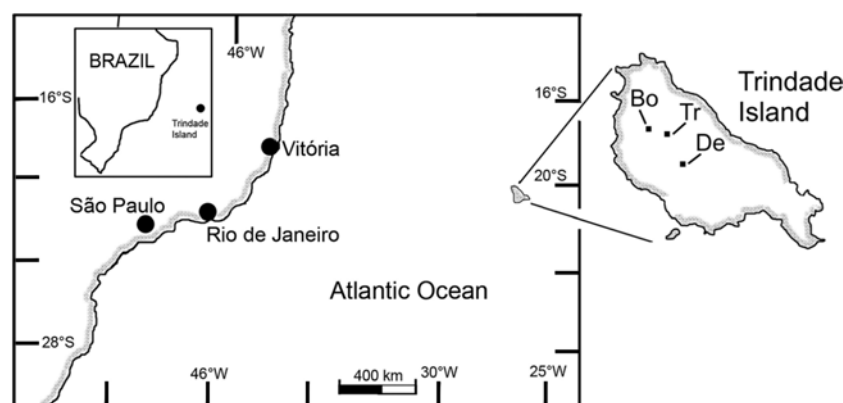


Figure 1. Map showing the location of Trindade Island (20°30'S, 29°20'W). Abbreviations: Bo, São Bonifácio Peak; De, Desejado Peak; Tr, Trindade Peak.

at 72°C (5 min). Results of PCRs were evaluated through an agarose gel electrophoresis. PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea) in both directions, using the same PCR primers. DNA sequences were aligned by the algorithm MUSCLE and merged in *contigs* through the software MEGA 7, and further submitted to GenBank (accession numbers PQ270250, PQ270251).

Outgroup selection for the phylogenetic tree comprised all COI sequences of *Zonitoides* stored in GenBank with more than 600 bp, in addition to the single available COI sequence of Gastrodontiidae (except *Zonitoides*) at GenBank [*Glyphyalinia indentata* (Say, 1822)] in order to root the tree; the final alignment had 608 bp. A Bayesian inference was conducted on MrBayes 3.2.7 (Ronquist et al. 2012) without *a priori* model, with one million generations applied to two independent runs with four chains each, trees sampled after every 100th cycle; the burn-in was defined to 25%. The software ASAP (Assemble Species by Automatic Partitioning; Puillandre et al. 2021) was used to evaluate the number of partitions (species) in the matrix of 75 COI sequences of *Zonitoides*, using all three available options (P-distance, Jukes-Cantor, Kimura). Genetic distances ('p', uncorrected) between the main partitions of *Zonitoides* were calculated on MEGA 7.

Results

Specimens of *Zonitoides* were exclusively found on the Desejado Peak (Figure 1), which is the most known mountain of the island and the first to be monitored during the process of natural regeneration, after the eradication of the feral goats (Silva and Alves 2011). Agreeing with previous descriptions on the morphology of *Zonitoides*, the sampled specimens have a glossy shell and dark head-foot (Figure 2). The Bayesian tree placed specimens from Trindade Is. within *Z.*

arboreus, in a clade herein referred as 'clade B' (posterior probability, PP, only 55%), which also contains sequences from Canada, The Netherlands and Malaysia (vs. only sequences from Canada in clade A, with PP = 97%) (Figure 3). All three models evaluated by the software ASAP provided two partitions (*Z. arboreus* and *Z. nitidus*) as the most proper scenario (lower scores) for the COI matrix of *Zonitoides*, whereas the second-best scenario comprised three partitions (*Z. nitidus* plus clades A and B of *Z. arboreus*) (Table 1). Genetic 'p' distances between *Z. arboreus* and *Z. nitidus* were 10.7–12.2%, and between clades A and B of *Z. arboreus* they were 2.8–4.9%; within *Z. nitidus*, the intraspecific variation was 0–1.0%, albeit greater for clades A (0–2.5%) and B (0–2.1%) of *Z. arboreus*.

Discussion

As expected, the species from Trindade Is. is *Zonitoides arboreus* and not *Z. nitidus*, owing to the apparent higher environmental tolerance of the former to (sub)tropical conditions than the strictly temperate *Z. nitidus* (Capinha et al. 2014). The terrestrial mollusc fauna of Trindade Is. is now comprised of six native species, two cryptogenic [*Gastrocopta barbadensis* (Pfeiffer, 1853) and *Pupisoma macneilli* (Clapp, 1918)], and three exotic species [*Z. arboreus*, *Bradybaena similaris* (Férussac, 1822), and *Vertigo* sp.] (Salvador et al. 2013, 2014, Cunha et al. 2015). The specimens of *Z. arboreus* were at first considered to be a native species of *Happia*, heavily influenced by the fact they were found atop one of the island's tallest peaks, which serve as refuge for one of the native snails (Salvador et al. 2014). Similarly to Trindade Is., the mountainous localities where *Z. arboreus* were sampled in Borneo (Malaysia) varied from 1,750–2,000 m a.s.l., always in human-disturbed sites (Capinha et al. 2014). The colder climate in moderate to high altitudes from tropical countries apparently facilitate the survivorship and reproduction of this Nearctic species (Capinha et al. 2014). Its colonization mainly requires the existence of trees or at least wood debris (Archer 1939, Caldwell 1996, Herbert 2010, Arruda 2014, Steury and Pearce 2014, Wackenheim et al. 2022).

Up to now, only *Z. nitidus* was confirmed as feeding on both plant and animal material, whereas *Z. arboreus* feeds only on fungi and plants (Barker and Efford 2004). Moreover, individuals of *Z. arboreus* tend to stick to human-disturbed sites (Capinha et al. 2014), although it may expand to adjacent undisturbed areas (Herbert 2010). Therefore, it is a species whose impacts on the native populations of snails are expected to be negligible; but of course, additional studies are welcome to confirm this and assess potential impacts to the flora and fauna. *Zonitoides arboreus* can serve as an intermediate host of cestode parasites of birds (Dick and Burt 1971) and of the nematodes *Angiostrongylus cantonensis* (Chen, 1935) (Sakamoto and Uga 2013, Walden et al. 2017) and *Parelaphostrongylus tenuis* Dougherty, 1945 (Pickles et al. 2013). However, the establishment of such parasites in Trindade Is. is unlikely, as most parasites tend to "miss the boat" when their host species colonize new regions, particularly oceanic islands (Paterson et al. 2003, MacLeod et al. 2010).

The localities from which there are DNA barcode sequences of *Z. arboreus* and *Z. nitidus* are just a little fraction of the species' total range. This severely limits genetic studies aimed to identify the exact native range of both species, to trace introduction routes, and also to define whether *Z. arboreus* is indeed a species complex (Capinha et al.

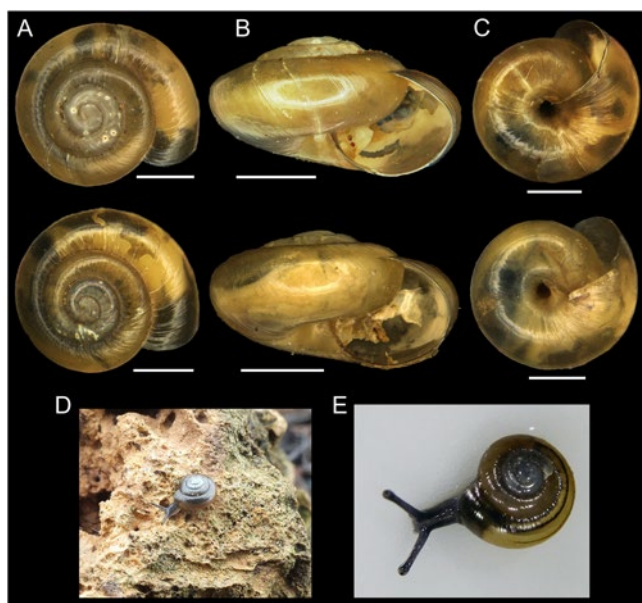


Figure 2. A-C. Shells of the two DNA-sequenced individuals of *Zonitoides arboreus* from Trindade Island, MNRJ 61775 (upper: GenBank code PQ270250; lower: GenBank code PQ270251); A, apical view; B, apertural view; C, abapical view. D-E. Live specimens of *Zonitoides arboreus* from Trindade Island: D, photographed *in situ*; E, photographed *ex situ*. Scale bars: 1 mm.

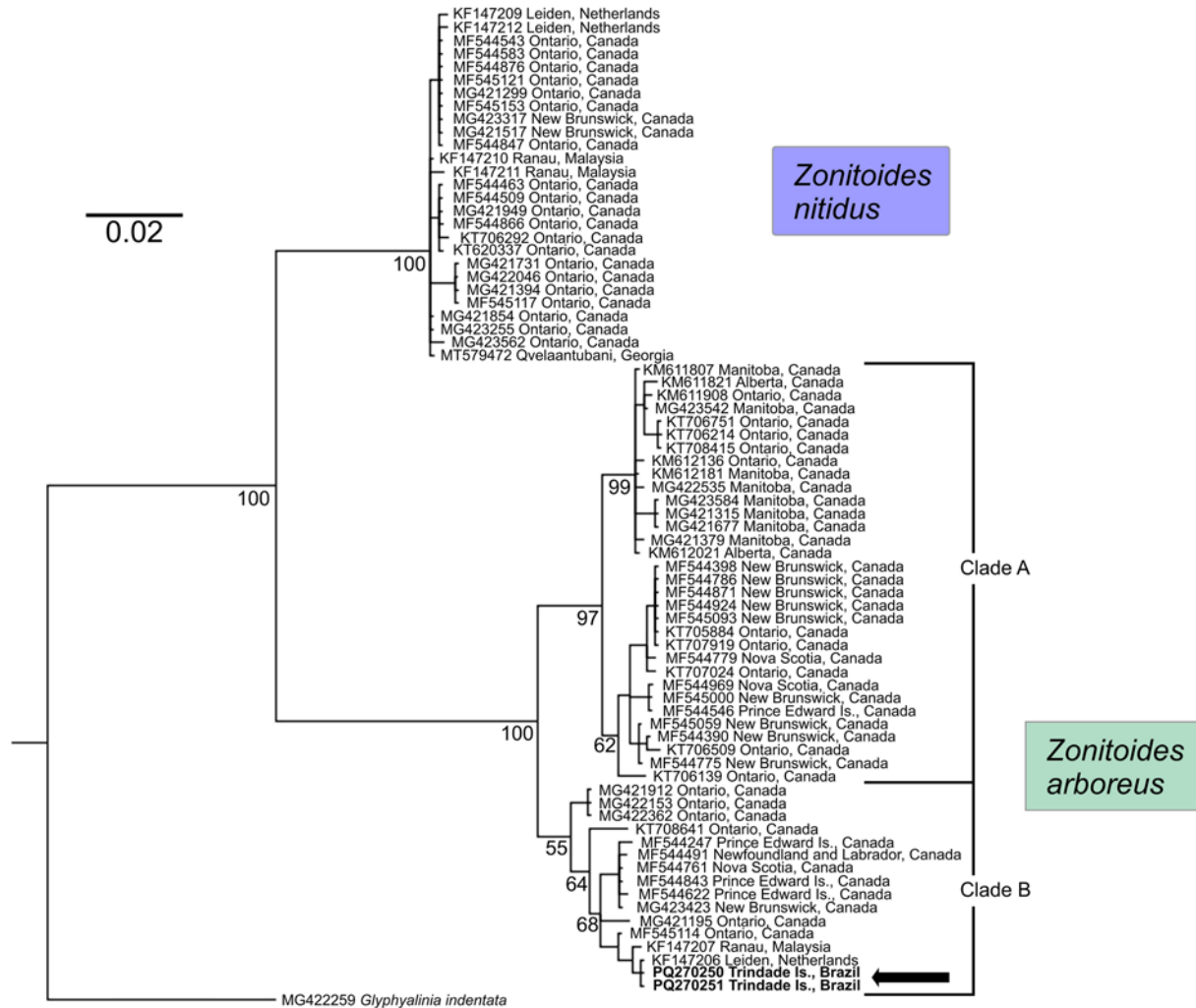


Figure 3. Bayesian tree obtained for the COI barcode marker, with posterior probability values (%) displayed below branches comprising most relevant clades. Sequences from Brazil are marked in bold and indicated by a dark arrow.

Table 1. Number of partitions, score values and threshold (genetic distances) evaluated by the software ASAP for 75 COI sequences of *Zonitoides* spp.

Model	P-distance		Jukes-Cantor		Kimura	
	1 st	2 nd	1 st	2 nd	1 st	2 nd
Best scenarios						
N ^o of partitions	2	3	2	3	2	3
Score	3.5	4.5	2.5	4.5	3.5	6.0
Threshold	6.74%	2.30%	7.19%	2.34%	7.36%	2.33%

2014). Clades A and B within *Z. arboreus* had respectively high and poor support values (Figure 3), and the species delimitation method (ASAP) suggested that *Z. arboreus* is a single species, though most DNA sequences are from Canada. The sequences from Trindade Is. were identical to a haplotype from the Botanical Garden of Leiden (The Netherlands), suggesting a high tolerance to temperature variations and a potential common route of introduction. According to Capinha et al. (2014), the clade which we termed as 'B' would be more tolerant to

warm conditions than clade A, although there is no real basis to infer that on the limited information currently available.

A global taxonomic revision of *Zonitoides* is essential, because most of the 17 extant and accepted species were described over a century ago (MolluscaBase 2024) and genetic data is scarce (except for the location-biased DNA sequences of *Z. arboreus* and *Z. nitidus*). In Brazil, the inclusion of new DNA sequences and a detailed examination in scientific collections could evaluate whether there were more than

one introduction event of *Z. arboreus*, and whether *Z. nitidus* is indeed present in cold refuges in Brazil, such as montane areas or in southern Brazil. Due to global climate changes, it is possible that *Z. nitidus* was present in SE/South Brazil many decades ago (Thiele 1927, Haas 1959), but perhaps not anymore owing to constant heat waves, deserving investigation if there were failed or successful introductions of *Z. nitidus* in Brazil, or merely incorrect taxonomic identifications.

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Author Contributions

Maurício R. Fernandes: morphological and genetic investigation; preparation of figures; writing – original draft.

Nilber G. da Silva: sampling license; sampling of material; *in situ* photographs; preparation of figures; writing – review and editing.

Márcia G. Rogério: sampling license; sampling of material; *in situ* photographs; preparation of figures; writing – review and editing.

Rodrigo B. Salvador: funding acquisition; preparation of figures; writing – original draft.

Conflicts of Interest

The authors declare that they have no conflict of interest related to the publication of this manuscript.

Ethics

The authors always adopted ethical procedures during the course of the study.

Data Availability

The two COI sequences of *Zonitoides arboreus* from Trindade Is. are available at the GenBank repository (<https://www.ncbi.nlm.nih.gov/genbank/>), under codes PQ270250-PQ270251.

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