DEVELOPMENT AND CHARACTERIZATION OF MICROSATTELITE LOCI FOR THE HARVESTED LIMPETS *PATELLA CANDEI* (D'ORBIGNY, 1839) AND *PATELLA ASPERA* (RÖDING, 1798) USING 454 SEQUENCING

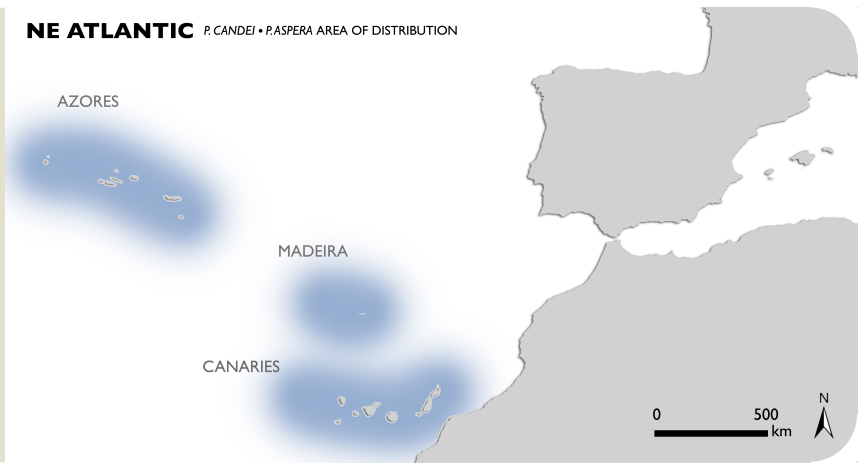
Rivas, M[1], **J Faria**[1,2], **P Ribeiro**[3], **P Presa**[4], **G Martins**[1,2], **AI Neto**[1,2]

(1) CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto.
(2) Center for Research in Natural Resources (CIRN), University of Azores.
(3) Center IMAR of University of the Azores.
(4) University of Vigo. Department of Biochemistry, Genetics and Immunology.

ABSTRACT Limpet harvesting in Azores (NE Atlantic) has been taking place probably since the islands were first colonized. These species are highly exploited and the stocks in most islands have declined steadily with catastrophic effects on coastal communities. They are a locally important resource but also ecologically important species and require prioritizing conservation strategies. Here we describe and develop species-specific microsatellite markers for the limpets *Patella candei* and *Patella aspera* using whole genome shotgun 454 sequencing. A total of 22 and 15 polymorphic microsatellite markers were described for *P. aspera* and *P. candei*, respectively. These novel genetic markers can be used to study the population genetic structure and evolutionary history of both patellid species and thus to contribute for stock conservation and management along their distributional area.

METHODS

- A total of 30 samples per species were collected in 3 different islands from Azores.
- Individuals were labelled and preserved in 96% ethanol.
- Genomic DNA was extracted from foot tissue using the E.Z.N.A. Mollusc DNA extraction kit following manufacturer's instructions.



PATELLA ASPERA (RÖDING, 1798)

Distribution: Azores, Madeira, Selvagens, the Canaries, and North of Africa.

Habitat: Rocky shores, lower intertidal and sublittoral zones

Description: The conical shell is light in colour and structurally strong, exhibiting thick and irregular margins. The underside of the muscular foot is yellowish.

Reproduction: *P. aspera* is a protandrous species. Organisms start off as male but many change to female as they age. This means that there are two types of males, permanent males and temporary (protandric) ones.

PATELLA CANDEI (d'ORBIGNY, 1839)

Distribution: Endemic to Macaronesia; archipelagos with different subspecies

Habitat: Rocky shores, intertidal and shallow sublittoral zones.

Description: The conical dark shell has regular margins, and is smother and thinner than *P. aspera*. The underside of the muscular body (foot) on which the limpet moves around is dark grey.

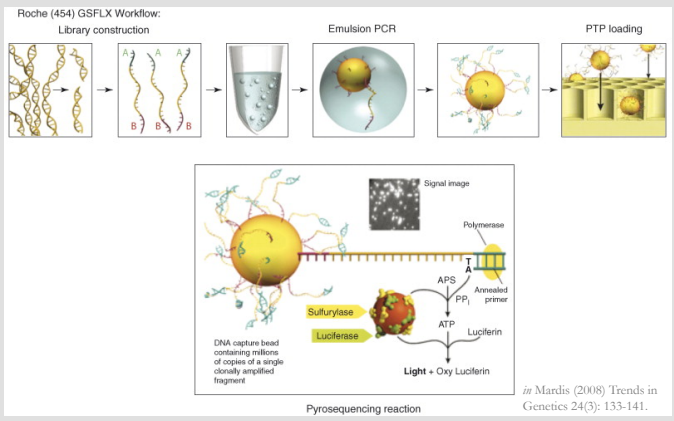
Reproduction: *P. candei* is a gonochoric species, meaning that individuals do not change sex throughout their lifes. Spawning occurs around the year but it is thought to peak during Autumn/Winter.

MICROSATELLITE DEVELOPMENT WORKFLOW

I. Microsatellite isolation

• DNA mix (>10 μg ml^{-1}) was sent to Genoscreen (France) for developing **microsatellite-enriched libraries**. Fragments from genomic DNA were enriched for SSR (single sequence repeats) content by using magnetic streptavidin beads and labelled TG, TC, AAC, AAG, AGG, ACG, ACAT and ACTC repeat oligonucleotides. High-throughput microsatellite isolation was made through **454 GS-FLX® Titanium pyrosequencing** of enriched libraries. QDD software was used to perform bioinformatic analyses.

		P. ASPERA		*P. CANDEI*
Sequences (raw datas)		28992		30683
Sequences containing microsatellites motifs		4754		1799
Bioinformatic validated pairs of primers		**309**		**107**
	Dinucleotides	88.7%		88.0%
	Trinucleotides	9.3%		10.1%
	Tetranucleotides	2.0%		1.9%

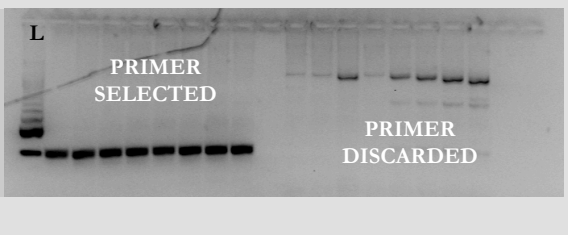


in Mardis (2008) Trends in Genetics 24(3): 133-141.

II. Primer selection

• **40 pairs of primers** per sp. with high and close Tm, compatible allelic ranges and no mispriming, were tested for amplification and annealing temperature. Only perfect motifs with simple repeats were selected (di-, tri- and tetra-nucleotides tandem repeats) and amplicon sizes varied from 90-315 bp (*P. aspera*) and 104-302 bp (*P. candei*).

PCR amounts	**Gradient PCR**	
~15 ng DNA template	95º - 15min	
1 × Qiagen™ Multiplex Kit	94º - 30sec	35×
0.4 µM of each primer	50-60º - 90sec	
ddH_2O	72º - 60sec	
Final volume = 10 µl	60 – 30min	



III. Marker validation

• Selected primers were fluorescently labelled with **NED, VIC, PET, or 6-FAM** (Applied Biosystems). A GTTT-sequence 'pig-tail' tag was added to the 5' end of all reverse primers. A total of eight individuals from 3 different populations (islands) were used for polymorphism detection. Marker amplification took place on a MyCycler™ thermal cycler (BioRad) using a touchdown PCR protocol with Ta adjusted for each primer pair. Amplified fragments were visualized using an ABI 3730 (Applied Biosystems) automated DNA sequencer with an internal size standard (GeneScan™ 500Liz®, Applied Biosystems) for accurate sizing. Genotypes were scored using GENEMAPPER™ v.4.2 (Applied Biosystems).

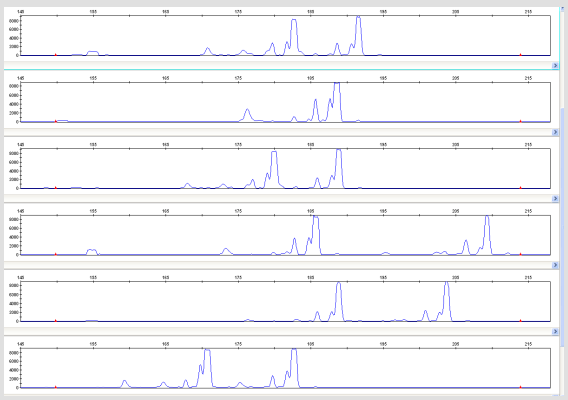
IV. Polymorphism

• Several markers had to be discarded either because they showed tri-allelic patterns, artifactual bands interfering with allele calling, and/or were monomorphic for the test-samples screened.

POLYMORPHIC MARKERS

P. aspera - **22 microsatellites** (2-9 alleles)

P. candei - **15 microsatellites** (2-10 alleles)



Ex. Locus ASP24 showing 8 alleles

• This study shows the usefulness of second-generation sequencing of microsatellite-enriched libraries to develop new microsatellites in non-model organisms.

• These novel microsatellite markers will be combined into the smallest number of multiplex reactions, reducing laboratory work and consumption of expensive reagents without compromising test utility.

• Conservation strategies in the NE Atlantic region should consider these novel markers to study the population structure of *P. aspera* and *P. candei* throughout their distribution area so that future efforts should be focused on **IDENTIFYING SCALES OF CONNECTIVITY AND PRESERVING STOCKS THAT HAVE BEEN SEVERELY DEPLETED**.

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T3.P5

ARCHAEOMALACOLOGICAL STUDY OF LA GARMA B CAVE (CANTABRIA, SPAIN)

M. T. Aparicio[1], E. Álvarez-Fernández[2], P. Arias[3], E. Gutiérrez[3], R. Ontañón[3]

[1]*Museo Nacional de Ciencias Naturales (CSIC). C. José Gutiérrez Abascal, 28003 Madrid (Spain)* teresa@mncn.csic.es

[2]*Dpto. de Prehistoria, Historia Antigua y Arqueología, Facultad de Geografía e Historia, Universidad de Salamanca (USAL). C. Cerrada de Serranos s/n. E-37002. Salamanca (Spain)* epanik@usal.es

[3]*Edificio interfacultativo de la Universidad de Cantabria, Av. de los Castros S/N, 39005 Santander (Spain)* ariasp@unican.es, egcuenca@gmail.com, ontanon_r@gobcantabria.es

La Garma B is one of the caves in La Garma Archaeological Complex (Omoño, Ribamontán al Monte, Cantabria). Two multiple burial phases were defined during the 1995-1999 archaeological excavation, one dated to *c.* 3000-2500 cal BC and corresponding to the early Copper Age (layer C), and another one dating to *c.* 2200-1600 cal BC and ascribable to the early Bronze Age (layer A). Most of the archaeological content of the cave corresponds to the latter, including human remains, mammal bones and molluscs, as well as pottery, lithic implements and some metal objects.

This contribution studies the archaeomalacological evidence (marine and terrestrial molluscs) in this burial cave from different points of view: taxonomy, taphonomy, technology, stratigraphy and spatial distribution. The marine species are dominated by limpets (*Patella intermedia, Patella ulyssiponensis* and *Patella vulgata*). Topshell *Osilinus lineatus* and the mussel *Mytilus* sp. are also present, but to a lesser extent. The marine molluscs were gathered on the coast, currently located about 5 km from the cave. A total of 12 terrestrial snails taxa have been found. *Cepaea nemoralis* is the more abundant and dominant species, and the only one that appears to have been gathered to be eaten. It shows a high proportion of apices among the MNI, which can be indicative of a poor state of conservation of the remains, perhaps derived from the humidity of the site. *Discus rotundatus* is the second more abundant land snail species. Its small size makes it unlikely that it was used as food. Yet its occurrence might be also indirectly derived from human activity. Snails of this species are frequently adhered to fallen leaves, so they might have been undeliberately introduced in the cave with some kind of vegetal matter. Finally, the relevance of malacological analysis in funerary archaeological contexts will be discussed.

T3.P6

ADAPTIVE RADIATION OF NERITOID GASTROPODS: TRANSITIONS BETWEEN MARINE AND FRESHWATER ENVIRONMENTS IN THE ONTOGENETIC CYCLE AND PAST EVOLUTIONARY HISTORY

Hiroaki Fukumori[1,2], Yasunori Kano[2]

[1]*Department of Natural Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8561, Japan* hirof@nenv.k.u-tokyo.ac.jp

[2]*Department of Marine Ecosystems Dynamics, Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan* kano@aori.u-tokyo.ac.jp

The transition from marine to freshwater habitats is one of the most significant steps in animal evolution. The Neritoidea are common and diverse members of the gastropod superorder Neritimorpha in rocky shores, seagrass beds, submarine caves and deep-sea hydrothermal vents and cold seeps, as well as in mangrove swamps, estuaries, rivers, streams and lakes. Interestingly, a previous phylogenetic reconstruction of this superfamily based on morphological characters suggested multiple habitat transitions from marine to freshwater, although the robustness of the tree topology was not tested and the history of their radiation has remained largely elusive.

Here we present a comprehensive, well-supported phylogeny of Neritoidea based on four gene sequences (5 kbp in total) from approximately 60 species representing most extant genera in Neritidae, Phenacolepadidae and Neritiliidae. Bayesian and likelihood reconstruction not only confirms the repeated invasions of freshwater by marine ancestors but also suggested multiple reinvasions of the sea from limnic habitats. A very plausible explanation for such frequent and unusual habitat shifts is the euryhaline nature of most limnic neritoids in their amphidromous life cycle: hatched larvae are swept downstream to the sea and metamorphosed juveniles settle at river mouths and then migrate upstream. Morphological and ontogenetic traits that would be adaptive in amphidromy have also been acquired in parallel. The most striking case concerns four independent losses of shell coiling and functional operculum to better facilitate upstream migration, resulting in the near-identical appearance of the polyphyletic *Septaria*. Our global investigation on the settlement size of neritoid planktotrophs revealed specific size ranges for individual clades that are useful in classifying fossils, although two amphidromous lineages were found to have independently acquired the smallest settler sizes, presumably to reduce probability of abortive dispersal through shorter metamorphic competence time while retaining ability of occasional long-distance trips with an extended delay period.

T3.P7

DEVELOPMENT AND CHARACTERIZATION OF MICROSATTELITE LOCI FOR THE HARVESTED LIMPETS *PATELLA CANDEI* (D'ORBIGNY, 1839) AND *PATELLA ASPERA* (RÖDING, 1798) USING 454 SEQUENCING

Manuel Rivas[1], **João Faria**[1,2], Pedro Ribeiro[3], Pablo Presa[4], Gustavo Martins[1,2], Ana I. Neto[1,2]

[1]*CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal* manulopezrivas@gmail.com

[2]*Center for Research in Natural Resources (CIRN), University of Azores, Apartado 1422, 9501-801 Ponta Delgada (Azores), Portugal* jfaria@uac.pt, gmartins@uac.pt, aneto@uac.pt

[3]*IMAR and Department of Oceanography and Fisheries (DOP), University of the Azores, 9901-862 Horta (Azores), Portugal* pribeiro@uac.pt

[4]*University of Vigo, Dep. Biochemistry, Genetics and Immunology, 36310 Vigo, Spain* pressa@uvigo.es

There is growing consensus that anthropogenic activities are impacting the structure and functioning of marine ecosystems and that these can have profound community-level effects, particularly when targeting keystone species. Limpet harvesting in Azores (NE Atlantic) has been taking place probably since the islands were first colonized. These species are highly exploited and the stocks in most islands have declined steadily with catastrophic effects on coastal communities. They are a locally important resource but also ecologically important species and require prioritizing conservation strategies. Such strategies should be supported by reliable data on the structure and dynamics of their populations, so that ecological hotspots are identified and protected. Characterizing the genetic diversity and structure of marine exploited populations is

thus of paramount importance to identify such units of conservation. Here we describe and develop species-specific microsatellite markers for the harvested limpets *Patella candei* and *Patella aspera* using whole genome shotgun 454 sequencing. A total of 309 bioinformatic-validated pairs of primers were obtained from *P. aspera* microsatellite-enriched library. The optimization of the amplification conditions of selected microsatellites (simplex and multiplex reactions) was performed in a gradient thermal cycler to optimize locus-specific amplification conditions and test their utility as genetic markers. Forty pairs of primers were tested, and about 28 revealed to be polymorphic. Using the same procedure, a total of 107 pairs of primers were validated for *P. candei* of which 15 turned out to be polymorphic. These novel genetic markers can be used to study the population genetic structure and evolutionary history of both patellid species e.g. levels of genetic variability within and between populations, and thus to contribute for stock conservation and management along their distributional area.

T3.P8
MOLECULAR SYSTEMATICS AND EVOLUTION OF THE HELICOIDEA (GASTROPODA, STYLOMMATOPHORA) OF THE WESTERN PALEARCTIC

Benjamín J. Gómez-Moliner[1], Oihana Razkin[1], Carlos Prieto[2], Alberto Martinez-Ortí[3], Benito Muñoz[4], José R. Arrébola[5], Mª José Madeira[1]

[1]*Dept. Zoología; Facultad de Farmacia, Universidad del País Vasco, Paseo de la Universidad nº 7, 01006- Vitoria, Spain* benjamín.gomez@ehu.es, oihana.razkin@ehu.es mariajose.madeira@ehu.es
[2]*Dept. Zoologia, Facultad de Ciencias y Tecnología, Universidad del País Vasco, Barrio Sarriena s/n 48940- Leioa, Spain* carlos.prieto@ehu.es
[3]*Museu Valencià d'Historia Natural, L'Hort de Feliu-Alginet, Apdo. 8460, 46018 Valencia, Spain and Departamento de Zoología. Facultad de Ciencias Biológicas, Universitat de València* alberto.martinez@uv.es
[4] *Dept. Biología Animal 1, Facultad Biología, Universidad Complutense de Madrid, Spain* titomu@bio.ucm.es
[5]*Dept. Fisiología y Zoología, Facultad de Biología, Universidad de Sevilla, c/ Profesor García González s/n, 41012-Sevilla, Spain* mastus@us.es

The Helicoidea, Rafinesque, 1815, is the most diverse group of the terrestrial molluscs of the Western Palaearctic, with its distribution center being located in the Mediterranean region where it locally can represent more than half of the molluscan biodiversity. It contains several families and genera of uncertain phylogenetic relationships.
The classification of the Helicoidea is mainly based on the morphology of shell and reproductive system, but there are still some controversies between the main classification systems. In addition the analysis of the possible ways of evolution within the Helicoidea requires other sources of information. In this work we are using the molecular techniques to progress in the resolution of the taxonomy, phylogeny, and evolution of this interesting group.
The main objectives of this work were to determine the phylogenetic relationships between the genera distributed throughout the Western Palearctic, as well as to clarify the phylogenetic relationships between some of the worldwide distributed families. We also studied the evolution of several organs used extensively to the classification of the Helicoidea.
We show the results of the analysis of 121 species belonging to 9 families (Sphincterochilidae, Elonidae, Helicodontidae, Trissexodontidae, Hygromiidae, Helicidae, Bradybaenidae and Humboldtianidae). One mitochondrial gene (16S rRNA) and one nuclear gene fragment (5.8S - ITS2 - 28S rRNA) were selected for this study in order to compare the new sequences with those obtained previously by other authors. The validity of the taxa considered in the main classification systems were contrasted with the phylogenetic tree obtained. Afterwards, we used the phylogenetic information to evaluate the evolution of different organs of the reproductive system, including the following: diverticulum of bursa copulatrix, dart sac with dart, accessory sac, mucous glands, as well as the relationship of the ocular retractor muscle and genital system.

T3.P9
PHYLOGENY OF PALAEOZOIC GASTROPODS INFERRED FROM THEIR ONTOGENY: HOW MANY HIGHER-LEVEL CLADES LIVED DURING PALAEOZOIC ERA?

Jiří Frýda

Faculty of Environmental Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Praha 6 – Suchdol, and Czech Geological Survey, P.O.B. 85, 118 21 Prague 1, Czech Republic bellerophon@seznam.cz

Gastropods are not only one of the most diverse groups of living animals occuring in marine, freshwater as well as terrestrial environments, but also have a rich fossil record extending back to the Cambrian. Because a third of all gastropod families are extinct, understanding of gastropod evolution and phylogeny necessarily involves study of both fossil and living species. Knowledge of the latter can be obtained from anatomical, morphologic, and molecular data, but for extinct forms virtually the only data source is the shell; typically it presents us with few characteristics. For both living and fossil gastropods, elucidation of ontogenic strategies is of prime importance in understanding the high-level phylogeny of this enormously diverse group. The analysis of Palaeozoic gastropods presented here relies heavily on ontogenies based on shell characteristics. It is argued that these results coordinate well with phylogenies of living gastropods inferred from the wider aggregate of anatomical, morphological, and molecular data. On the other hand, the analysis has highlighted problems with published phylogenies of living gastropods and, moreover, has produced evidence for the existence of several order-rank and long-lasting gastropod lineages forming an important part of Palaeozoic gastropod faunas but which failed to cross the Permian–Triassic boundary. Clearly, for understanding the phylogeny of the Gastropoda, it is imperative that the history of fossil gastropod clades be included.

T3.P10
MITOCHONDRIAL GENOMES OF EIGHT SPECIES IN VETIGASTROPODA (MOLLUSCA: GASTROPODA)

Hsin Lee[1], Wei-Jen Chen[1], Sarah Samadi[2], Chang-Feng Dai[1]

[1]*Institute of Oceanography, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei, 10617 Taiwan* leehsin.tw@gmail.com, wjchen.actinops@gmail.com, corallab@ntu.edu.tw
[2]*Department Systematique & Evolution, Museum National d'Histoire Naturelle 57 Rue Cuvier, 75231 Paris, France* Sarah@mnhn.fr

Mitochondrial DNA sequences have been commonly used in the studies of molecular phylogenetics and evolutionary biology. The gene order of mtDNA in animal kingdom

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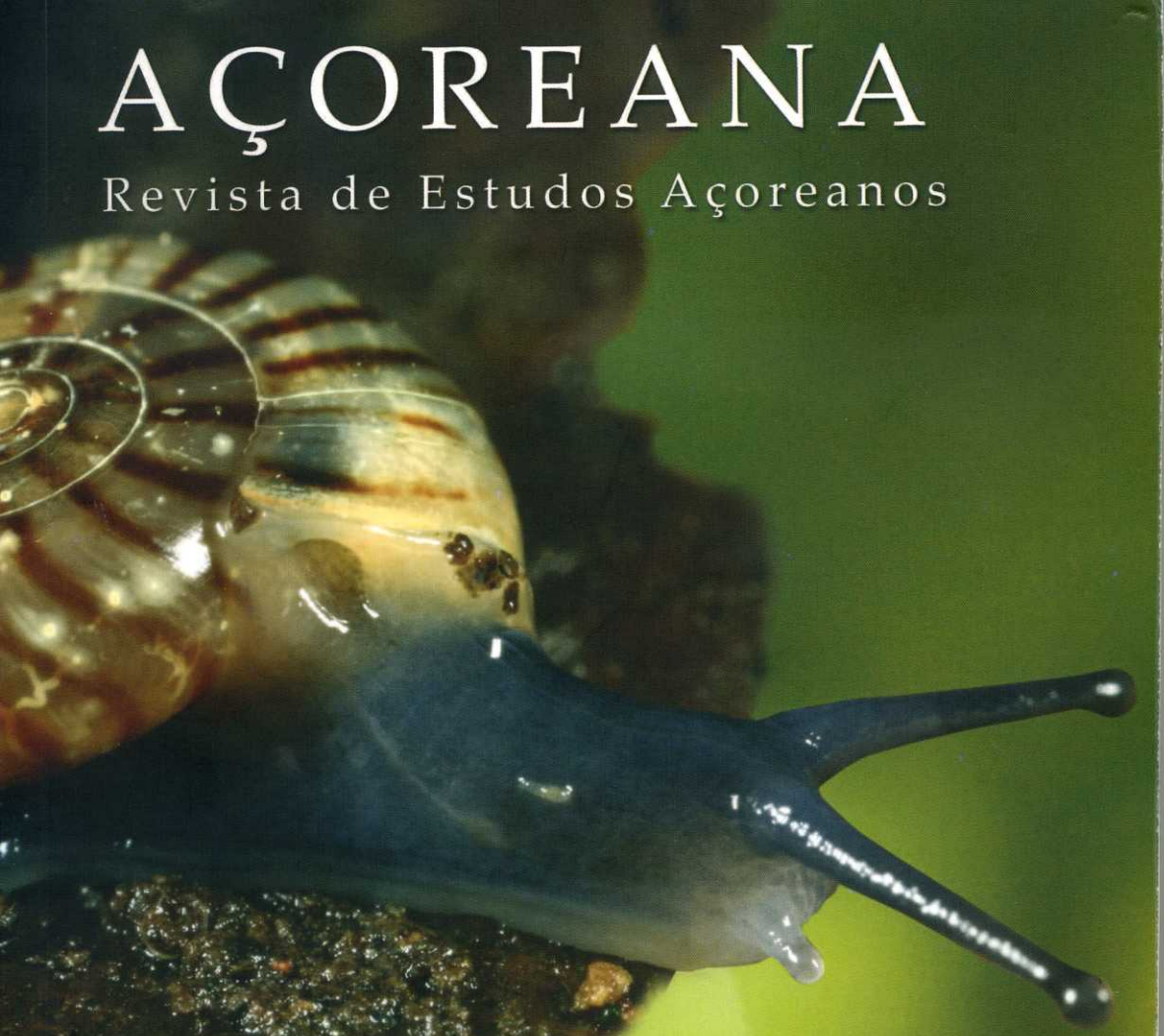

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