**Shell-bound archives - uncovering nematode encapsulations in the Galapagos' largest radiation**

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**Abstract**

Understanding the interplay between macroevolution and species interactions and its consequences on biodiversity patterns is a major question in ecology. However, empirical data at the community level to test theoretical predictions and uncover new patterns remains very scarce, which limits the progress in this area. Sampling parasitic interactions in island radiations offer a unique opportunity to overcome this limitation: phylogenies are usually available for radiated groups, and rich interaction and genetic data can be obtained from parasites across their clades using last-generation genetic analysis. Here, we focus on a recently discovered interaction mechanism between snails and parasites in which hosts kill nematodes with their shells by encapsulating them within their inner layers. Because snail radiations are numerous across oceanic archipelagos across the world, this mechanism could open the possibility to obtain unparalleled insightful data that combines phylogenies and interaction data across temporal, spatial, and environmental dimensions. We focused on the largest radiation of Galapagos land snails, *Naesiotus*, examining the presence and quantity of nematode encapsulations. We also analyzed the phylogenetic signal and its relationship with ecological factors such as habitat, island characteristics, and shell adaptation to thermoregulation. Using three snail species, we demonstrated that nematode diversity can be measured via DNA sequencing. We found that encapsulations were present in X species and the X islands analyzed, that they showed phylogenetic signal, and that island age was the strongest predictor. Our findings highlight the potential of further analyses, including nematode phylogenies, and encourage further sampling in snails’ radiations distributed in archipelagos worldwide, which can offer unique information to gain insights into the interplay between macroevolution and species interactions.

**Keywords:** snail-parasite interactions, island radiation, parasitism, macroevolution, oceanic islands

**INTRODUCTION**

The intricate relationship between species' evolutionary trajectories and their biotic interactions is pivotal in elucidating macroevolutionary processes and the dynamics of ecological communities across macro temporal scales (Weber et al., 2017; Hembry et al., 2020). Several foundational theories posit that interactions among species and macroevolutionary dynamics are mutually influential, including concepts such as adaptive radiations, escape-and-radiate dynamics, the Red Queen hypothesis, and the Geographic Mosaic Theory of Coevolution (refs). However, most of the theory and empirical work has been done on species-pairs or few species systems (ref), leaving a notable gap in our understanding at the community level where species interact within complex ecological networks (ref).

Advances in computational and theoretical models are now shedding light on the critical role of interactions in driving macroevolutionary dynamics within communities (Guimarães & Thomson, 2012; Hutchinson et al., 2017, refs) and shaping their composition and structure over evolutionary timescales (Fontaine et al., 2011; Guimarães et al., 2007, 2011; Pires and Guimarães, 2013, refs). Despite this progress, the integration of macroevolution and community ecology remains constrained by limited empirical data availability. Comprehensive datasets, encompassing interaction and phylogenetic information across multiple species within communities, are essential to rigorously test predictions and advance theoretical frameworks (Weber et al., 2017, refs).

Oceanic islands, DEFINITION, provide a unique opportunity to obtain empirical data on biodiversity and evolutionary patterns due to their well-defined spatial and temporal origins, discrete and isolated communities, distinct ecological boundaries, high habitat diversity, and manageable ecosystem sizes (Gillespie 2007, Warren et al. 2015, Patiño et al. 2017). These islands have been particularly informative for studying diversification processes in numerous evolutionary radiations (ref summary of island radiations by Christine). By sampling interaction data within these radiated groups, such as mutualistic interactions in the Galapagos finch radiation (e.g., Grant & Grant 2008), competitive interactions among Caribbean anolis species (ref), and food webs of arthropods in Hawaii (ref), researchers have gained valuable insights into the interplay between macroevolution and species interactions.

Despite the fact that manny other examples of well-studied radiations on archipelagos around the world exist and continue to improve the accuracy of its evolutionary data, the examples mentioned are some of the few datasets that allow for these analyses - departing from a radiated group to sample its interactions with other(s) and evolutionary data from the other interacting guild. It remains unknown the diet of lava lizards radiation () or \_\_\_ radiation ( ), pollinators of Scalesia radiation ( ) or \_\_ plant radiation ( ), or parasites of moth radiation ( ), to cite some examples. One major cause for this is because sampling interactions across the clades of a phylogeny is still a major challenge due to the cost and complexity of intensive field campaigns across multiple islands, which are usually remote and of difficult access (ref).

Parasitic interactions present a promising avenue to overcome these challenges. Parasites, typically small organisms found within hosts, offer advantages in high-yield sampling and identification through global DNA databases. This last point is important for being able to build up phylogenies for the parasitic guild together with the host (radiated group) and compare them with network patterns of interactions (refs). In addition to the advantages for sampling, there exists extensive theoretical work exploring the effects of parasitic interactions on macroevolutionary dynamics (refs) (Nuismer et al. 2008, Buckingham & Ashby 2022). They are proposed to be the most prevalent interaction by means of obtaining food among organisms and comprise approximately 40% of the species described worldwide (the number could be much higher; Dobson et al. 2008), with the potential to affect the structure and functioning of communities and ecosystems (Lafferty et al. 2008, Wood & Johnson 2015).

Among potential hosts for studying parasitic interactions, land snails represent a particularly suitable group. Land snail radiations across oceanic islands—including those in Hawaii, Galapagos, Seychelles, Japan, Philippines, New Zealand, Caribbean islands, Mauritius, Canary islands, and Madagascar—offer insights into diversification processes. Land snails typically exhibit low dispersal and adapt to diverse habitats, making their shell characteristics a readily measurable and identifiable trait (Chiba & Cowie 2016). Moreover, they host a rich diversity of parasites, predominantly nematodes and trematodes, with well-documented interactions and life cycles that can affect broader trophic guilds, including humans (refs).

A recent discovery by Rae 2017 found an immune defense of snails against nematodes through its shell: upon an arrival of a nematode, the shell can capture, lysis the body of the nematode and encapsulate it within its layers. This mechanism lasts for one-few days and can encapsulate one to hundreds of nematodes at once, from \_\_-fold sizes including eggs or nematodes of 1cm big. This mechanism allows for the temporal screening of interactions, both for measuring them on old shells (Rae et all were able to quantify encapsulations on fossil specimens X years old from museum collections) and along the ontogeny of individuals (from early to late shell development), thereby overcoming traditional limitations of interaction sampling (ref Rae). Rae and colleagues (ref) showed that the DNA of encapsulated nematodes can be retrieved and sequenced, allowing the identification of taxa. This opens the possibility to obtain accurate interaction data and being abe to sample interactions across snail radiations by using dry collections, which confers enormous advantages such as providing rich spatial and temporal cover of data, analyzing extinct species, and avoiding killing individuals. These are crucial points for a highly threaten biodiversity in insular ecosystems. Land snail radiations are the group that suffered the most extinctions and most of its species are endangered (ref).

Rae and colleagues \_\_ showed that this mechanism should be widespread among the gastropoda group, but we don't know whether the radiated groups of snails in islands present these encapsulations and whether we can conduct DNA identification of nematodes from dry collections, which could open the opportunity to sample host-parasitic interactions in radiations offering unparalleled data to investigate the interplay between interactions and macroevolution. Motivated by the opportunities that such data could offer, and by the implication for measuring nematode encapsulations in other snail island radiations, our main objective was to find and quantify the presence of these encapsulations in one of these prominent radiations and whether it is feasible to analyze nematode DNA from dry collections.

**The Naesiotus radiation**

In this study, we investigate the presence of encapsulations in the largest radiation of the Galapagos islands, the one protagonized by the genus of land snails *Naesiotus*. It is composed of *X* species, which are island-specific. Even species are specific to certain volcanos (species and volcanos). Its radiation had very low dispersal. Species inhabit a variety of habitats and change in its shell characteristics including brightness, size, color and shape. Kraemer et al. \_\_ suggest that there is a biotic and abiotic trade-off signal in adaptation regarding its shell brightness: \_\_\_\_\_\_\_. This radiated group is therefore ideal to sample parasitic interactions and merge interaction and phylogenetic data, exploring this relationship across different community maturity stages using island chronosequences, isolation, habitat types and strength of biotic or abiotic selection.

We analyzed the dry collection of *Naesiotus* to test whether we find encapsulations and with what prevalence, and whether the DNA extraction and sequencing of encapsulated nematodes is possible. In addition, we conduct exploratory analyses to relate general ecological and evolutionary patterns. Specifically, we (1) assess the presence-absence of encapsulations in the *Naesiotus* group, (2) quantify the nematode load of *Naesiotus* species, (3) explore whether nematode loads are associated to ecological (habitat type, island characteristics) or evolutionary (phylogenetic distance) patterns, and (3) test if it is possible to amplify and analyze the DNA of encapsulated nematodes to analyze their diversity.

**METHODS**

**Quantification of nematode load**

A major advantage for this shell immune defense is that the encapsulated nematode remains in the shell and can be accessed even after hundreds of years in fossil individuals (Rae 2017). This allows for getting interaction data from dry collections (only shell preserved). We measured encapsulations in the dry collection of Naesiotus snails of the Galapagos National Park which is managed by Dr. Christine Parent at the University of Idaho.

We selected individuals randomly from each species and examined the encapsulations using an optical microscope to observe the presence and quantity of nematodes. We stopped counting when there were more than 100 nematodes, noting “>100”. We did not count nematodes in the non-visible parts including non-visible body whorl parts and inner structures of the spine, because to be able to observe these would require breaking up all shells, destroying or damaging a big portion of the collection. Instead, we limited our counts to the aperture or operculum (which includes outer lips, aperture, palatal, parietal, and columellar walls) until where is visible which corresponds with the adult phase. This implies that we are not certain about whether the nematode counts from early juvenile to adult phase could largely differ from the adult-phase only. To address this issue, at least in part for the presence/absence data, we selected 10 individuals per species for which 0 nematodes were counted by looking at the aperture, and opened their shells to examine them in full (until the protoconch, the last tip and oldest part of the shell) and test whether these were true negatives.

Our sampling effort consisted of sampling no less than 50 individuals per species, from more diverse localizations at random. Because future analysis will involve DNA sequencing of encapsulated nematodes in the species examined in this study, we increased the count of 10 species to find shells with high nematode loads that could be more useful for successful DNA extractions and used this analysis to count nematodes and add it into our data. This process also followed random selection of individuals.

**Effect of ecological features on nematode load**

**Phylogenetic signal of nematode load**

**Sequencing DNA from encapsulated nematodes**

*Sample Collection and Processing*

Dry shell material was taken from the collection of Dr. Christine Parent at the University of Idaho. We chose a total of eleven shells from individuals belonging to three different species and two different islands. Six shells belonged to *N. ochnsneri* and three to *N. hirsustus* from Santa Cruz island. We chose this island because is the most humanly populated, contains high diversity of snail species, and humid habitats, all which could promote high nematode diversity. We also analyzed two *N. bauri* shells from San Cristobal island to perform the analysis in a different island, the second most populated and containing also high diversity of snail species. The number of shells we analyzed per species responded to individuals that were readily accessible. Most of the species are endangered, and obtaining shell bits means the partial destruction or damage of the shell, which is also an important trait for analysis performed in the collection. Because the objective for this study was to ascertain whether nematode diversity could be obtained from shell samples, we performed the analyses on a small number of individuals that were no longer useful for other analysis in the collection to minimize the impact to the collection, which permits answering our question in addition to adding different species and islands.

*DNA extraction*

We cut shell bits containing encapsulating nematodes using tweezers and crushed them inside an eppendorf tube until making it shell dust. We then performed a demineralization protocol using a mild decalcifying agent (ethylenediaminetetraacetic acid, EDTA) to remove calcium and expose the internal matrix of the shell to access the DNA trapped within it (Martin et al. 2021). We added EDTA 0.5M to the crushed shell dust until covering it completely and incubated overnight at 58 degrees Celsius. After incubation with the demineralization solution, we proceeded with the Quiagen extraction (DNeasy Blood & Tissue Kit).

*Sequencing*

Extracted DNA was sent to the genomic platform CERMO-FC at the Université du Québec à Montréal, for 18S rRNA sequencing of the 4th region (Kenmotsu et al. 2020). We used primers NF1\_MiseqF (GGTGGTGCATGGCCGTTCTTAGTT) and 18Sr2b\_ExtR\_MiseqR (GGTGTGTACAAAKSGCAGGGACGTA) (Kenmotsu et al. 2020). All samples and a negative control were sequenced on a (Xbp?) paired-end Illumina Miseq platform (PE300?) lane ((Illumina Corporation, San Diego, CA, USA).

*Data Analyses*

Read processing and analyses were performed using QIIME 2 2023.5 (Bolyen et al. 2019). Raw sequence data were quality filtered using the q2‐demux plugin followed by denoising with DADA2 (Callahan et al. 2016, Kenmotsu et al. 2020)(via q2‐dada2). This left a total of (number of samples) with (number of ASVs) and (number of reads).Taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich et al. 2018a) classify-sklearn using the pre-trained classifier Greengenes2 2022.10 (McDonald et al. 2022) trained on the 515F/806R region of the 16S gene.

Alpha-diversity metrics (observed features, Faith’s Phylogenetic Diversity (Faith 1992), and Shannon’s diversity index (Shannon 1948)), beta diversity metric, Robust Aitchison distance (C. Martino et al. 2019), and Principle Coordinate Analysis (PCoA) were estimated using the plugin deicode and q2-diversity.

**RESULTS**

We analyzed a total of X individuals from X species (mean of X individuals per species), and X islands. A total of 6 species have less than 50 individuals: *N. ochsneri* (n = 45) and *N. basiplicatus* (n = 42) because they were miscounted, *N. cavagnaroi* (n = 44), *spp12* (n = 20), *spp15* (n = 19), and *spp14* (n = 17) because they had no more specimens available in the collection. Santa Cruz, San Cristobal, and Isabela accounted for X, X, and X species, respectively, whereas \_\_, \_\_, \_\_, \_\_, and \_\_ accounted for only ones species (the only one found in these islands).

**Nematode load**

Encapsulations were found in all 47 analyzed species. The mean nematode load was X (min = X, max = X) and SD = X. A total of X individuals had 0, X between 1 and 10, X between 10 and 30, X between 30 and 50, and X between 50 to more than 100. \_\_\_ was the species with the highest mean load of X (SD = X), followed by \_\_\_\_( X; SD = X) and \_\_\_( X; SD = X). Most species falled within the mean load range of X to X. Species \_\_, \_\_, and \_\_ had the lowest means with X (SD = X), X (SD = X), and X (SD = X), respectively.

**Effect of ecological features on nematode load**

**Phylogenetic signal of nematode load**

**Sequencing DNA from encapsulated nematodes**

**DISCUSSION**

[Discuss results island age/size, brightness/habitat]

[Very little is known about nematode diversity in Galapagos -> need further studies to know their diversity and abundance]

[We don't know if this is a truly parasitic interaction - some nematode species might not be parasites]

[We dont know if snails are final hosts or vectors]

[Very relevant for the impact on other species in the islands - detect changes in parasitic loads and immune depression of communities, perhaps with the invasion of exotic species or the impact of human population]

[Even if we tested false negatives, we might be underestimating the loads, especially for not checking encapsulations at juvenile phases]

[potential analysis with genetic sequencing of nematodes across all species - interaction networks, combine phylogenetic + network data, temporal networks, networks through chronosequences, specialization/generalism traits in different habitats, clades diversification and species roles in networks]

[potential for archipelago-continent and archipelago-archipelago comparisons]

**Figures**

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| **Figure X.** |