

**Evolutionary History and Global Phylogeography of the Neuston**

by

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## **Dedication**

This thesis is dedicated to my advisor and mentor, Diarmaid Ó Foighil, my parents, Muriel and George Christianson, and my husband, Chris Churchill.

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## Abstract

The neuston comprises a community of interacting species that drift at the water/atmosphere interface of the planet's subtropical gyres and play an important role in open-ocean epipelagic food webs. The ecological base of the neuston community is an endosymbiosis involving cnidarian hosts (Porpitidae) and their dinoflagellate photosymbionts. Porpitids are preyed upon by a variety of predators, including two specialized gastropods (Janthinidae and Glaucinae). This prominent open-ocean community has been poorly studied, apart from its resident insect genus *Halobates*.

Based on a global sampling of neuston conducted between 2005-2012, this research begins by focusing on the evolutionary history of neustonic taxa across three trophic levels (photosymbionts, porpitud hosts, predatory gastropods) and all 5 subtropical gyre systems. First, photosymbionts genotyped from exemplars of both porpitud genera are identified as the same genus, *Scrippsiella* (Peridiniales), in every ocean gyre. Second, a molecular phylogeny including bubble-rafting janthinids shows that they are nested within the benthic family Epitoniidae, or wentletrap snails. Using morphological and ecological data from epitoniids, the common violet snail genus *Janthina*, and the rare brown janthinid genus *Recluzia*, two hypotheses for the evolutionary origins of bubble floats are tested. The data support the evolution of the janthinid float from an epitoniid egg mass. Third, the differential distributions of the two species of glaucinin nudibranchs (*Glaucus atlanticus*, global; *G. marginatus*, Indo-Pacific) presented an opportunity to test

whether the establishment of geographic vs. biological barriers is the primary speciation mechanism. Comparing genetic structuring between the two congeners shows that the global species, *G. atlanticus*, is panmictic whereas the Indo-Pacific *G. marginatus* is a complex of four overlapping cryptic species in two clades. A repeated reproductive change has occurred once in each clade: the loss of the bursa copulatrix, which is hypothesized to be a biological speciation mechanism in this clade.

Finally, mitochondrial phylogeographies of three global neustonic taxa, *Glaucus atlanticus* (Glaucinae), *Veella veella*, and *Porpita porpita* (Porpitidae) are compared to test three hypotheses of within-species genetic structuring: global panmixis; ocean basin panmixis; within-gyre panmixis. The results show that ocean basin and ocean gyre boundaries vary by taxon as barriers to gene flow.

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## Chapter 1

### Introduction

The famous last paragraph of *The Origin of Species* presents an evocative view of a natural species assemblage “so different from each other, and dependent upon each other in so complex a manner” that seamlessly melds evolutionary and ecological perspectives (Darwin, 1859). A similar integration lies at the root of the modern concept of biodiversity, a now ubiquitous term that was first coined in 1985 and that entered the literature a few years later (Wilson, 1988). Biodiversity has been defined in a variety of ways (Gaston, 1996), but all widely used definitions integrate both ecological and evolutionary perspectives, incorporating all living organisms on the planet, the evolutionary histories they embody and the ecosystems they form.

Although possessing enormous innate appeal, this concept is not without its shortcomings, especially for empiricists. At its most expansive (incorporating all hierarchical levels from genes to ecosystems), biodiversity runs the risk of simply becoming all of biology (Sarkar, 2005). Even when restricted to specific ecosystems, the sheer number of interacting taxa in, for instance, coral reef communities (Reaka-Kudla, 1997; Bouchet *et al.*, 2002), poses levels of complexity that border on the intractable. Nevertheless, significant progress has been made in biodiversity studies of natural communities in recent decades. On the ecological front, experimental, observational and theoretical studies demonstrate that ecosystem properties are greatly influenced by the

functional attributes of their constituent organisms, as well as by the distribution of these organisms in time and space (Hooper *et al.*, 2005; Worm *et al.*, 2006; Duffy & Stachowicz, 2006; Stachowicz *et al.*, 2007). Our understanding of the historical complexity of these spatiotemporal patterns has greatly benefited from the now routine application of phylogenetics to species distributional datasets (Cracraft, 1994) and of phylogeographic studies to intraspecific variation (Avise, 1998). However, a large fraction of such studies has narrow taxonomic foci and, although there has been increasing recognition of the importance of evolutionary history in ecology (Brooks & McLennan, 1993; Ricklefs & Schluter, 1993; Losos, 1996; Thompson *et al.*, 2001; Riddle *et al.*, 2008), relatively little integration of historical biogeographical and ecological datasets has occurred (Webb *et al.*, 2002; Wiens & Donoghue, 2004).

In the past decade, a new focus at the interface of ecology and evolution has developed: the investigation of how communities are structured, and what processes drive community structure (Emerson *et al.*, 2011). Initial efforts primarily involved the community structure of rainforest trees (*e.g.*, Webb, 2000), but these have since expanded to include other terrestrial communities of plants, insects, and some vertebrates (reviewed in Cavender-Bares *et al.*, 2009; Emerson *et al.*, 2008; Johnson & Stinchcombe, 2007; Vamosi *et al.*, 2009), and have been termed “community phylogenies.” There is as yet no comprehensive example of a marine community phylogeny. Perhaps the closest approximation is collectively formed by the numerous individual studies of the North Atlantic near-shore biota (and its peripheral Baltic Sea offshoot; Johannesson & André, 2006), a large fraction of which consists of Trans-Arctic migrants (Palumbi & Kessing, 1991; van Oppen *et al.*, 1995; Collins *et al.*, 1996; Wares & Cunningham, 2001; Wares,

2002; Riginos *et al.*, 2004; Addison & Hart, 2005; Nikula *et al.*, 2007; Dodson *et al.*, 2007). More recently, comparative phylogeography has also been proposed as a means of investigating community assembly (Dick *et al.*, 2004; Emerson, 2011; Hickerson *et al.*, 2010); however, statistical methods are still in developmental stages and are not necessarily community-focused (Hickerson *et al.*, 2010). With both the community phylogenetic and comparative phylogeographic approaches, critical gaps remain for marine systems, *e.g.*, life in the ocean is dominated, both in terms of biomass and metabolism, by pelagic microorganisms (Karl, 2007). Their absence from otherwise admirably comprehensive studies (Wares, 2002) is of course perfectly understandable because unavoidable pragmatic impositions often limit the inclusion of multiple trophic levels in biodiversity/ecosystem studies (Hooper *et al.*, 2005).

How then might the integrative promise of characterizing community structure by phylogenetic methods be achieved for any marine ecosystem? A logical prerequisite would be to identify candidate marine communities that have the following set of salient characteristics: 1) be one of the planet's most prominent/extensive biological communities; 2) occur in a distinctive, easily defined, environment; 3) have low species diversity with clearly defined trophic relationships; 4) occur in a small number of spatially discrete populations; 5) contain lineages that have phylogenetic roots in different communities and that display putative functional trait synapomorphies relative to presumed sister lineages in the ancestral environment(s). As detailed in the following pages, the focal marine community of this dissertation, the open-ocean neuston, possesses arguably all of these desired attributes, although only one constituent taxon, the neustonic insect genus *Halobates*, has been subjected to detailed study in recent decades (Cheng,

1985; Andersen, 1999; Andersen *et al.*, 2000; Andersen & Cheng, 2005). The strategic goal of this research is to construct a comprehensive phylogeny/phylogeography of the neuston across three trophic levels and all five of the planet's subtropical gyre systems.

### **Study system**

The marine environment is comprised of three primary ecological domains. Two of these are familiar and relatively tractable: the pelagos, composed of pelagic organisms that live in the water column, and the benthos, encompassing benthic organisms living in/on the sea floor. The third is much less familiar and its open-ocean taxa are rarely sampled at first hand except when they are occasionally cast ashore. This is the neuston (a.k.a. pleuston), a community of approximately 100 highly specialized oceanic species that drift at the water/atmosphere interface of the planet's subtropical gyres (Cheng, 1985; Zaitsev, 1997; Marshall & Burchardt, 2005). The neuston community plays an important role in open ocean epipelagic food webs and represents a major food source for, amongst others, loggerhead turtles (Parker *et al.*, 2005; Revelles *et al.*, 2007) sunfishes (Fraser-Bruner, 1951) and open-ocean seabirds (Gould *et al.*, 1997). Despite its remoteness, this ecosystem is heavily impacted by anthropogenic pollution in the form of floating plastic particles that often exceed local biomass (Moore *et al.*, 2001), pose a threat to marine life (Bugoni *et al.*, 2001), and act as an entrée point for persistent organic pollutants into oceanic food chains (Rios *et al.*, 2007).

### **Subtropical gyres**

Global oceanic surface circulation patterns are dominated by five enormous

subtropical gyres: North Pacific, South Pacific, Indian Ocean, North Atlantic and South Atlantic (Fig. 1-1), that collectively occupy 40% of the surface of the planet (McClain *et al.* 2004). Driven by tropical trade winds and deflected by the planet's rotation, gyre surface water moves continuously in a circular motion: clockwise in the northern hemisphere, counterclockwise in the southern (Mann & Lazier, 2006), and they are separated longitudinally by continents and latitudinally by strong equatorial currents (Pinet, 2003). They are major players in shaping the planet's climate (Huang & Qiu, 1994) and, in addition to transporting vast quantities of heat poleward (Klinger & Marotzke, 2000), each gyre also defines a discrete spatial domain characterized by a deep pycnocline (rapid change in water density) at its center and strong horizontal gradients of temperature and salinity at its fringes (McClain *et al.*, 2004). Subtropical gyres typically exhibit low nutrient and biological productivity levels (Marañón, 2005) although their immense size means that they still contribute significantly to global primary productivity (Martin *et al.*, 1987). They are dynamic entities that exhibit variability on seasonal, interannual and paleontological timescales (Slowey & Curry, 1992; Karl *et al.*, 2001; McClain *et al.*, 2004; Clauzet *et al.*, 2007). Subtropical gyres appear to be expanding at present in association with global warming processes and this has important implications for future ocean productivity levels (Polovina *et al.*, 2008).

### **Neustonic cnidarians (Porpitidae)**

Each subtropical gyre (Fig. 1-1) entrains a spatially discrete segment of the neuston. The base of the neustonic food chain is formed by a mutualism involving photosymbiotic dinoflagellates (zooxanthellae) and their porpitid (formerly known as

chondrophoran) hosts, *Porpita porpita* and *Velella velella* (Hyman, 1940) (Fig. 1-2A, B); now thought to represent monotypic genera (Bouillon & Boero, 2000). Porpitids also prey on zooplankton (Bieri, 1961, 1970) and with their dual mode of nutrition, they effectively represent open-ocean ecosystem equivalents of hermatypic corals (Muscatine & Porter, 1977; Venn *et al.*, 2008). These highly specialized cnidarians are poorly studied but are known to represent each other's closest living relatives (Dunn *et al.*, 2005), secrete chitinous gas-filled chambered floats (Rudall, 1955; Bouillon & Boero, 2000) and form vast populations (Annandale, 1904; Larson, 1980) of floating translucent zooxanthellar culture chambers that are continuously circulated around each gyre. Porpitud life cycles are incompletely documented, primarily through laboratory culture attempts. The neustonic hydroid life history stage gonozooids are known to bud off large numbers of tiny medusae (containing zooxanthellae) that enter the water column, produce gametes, and are presumed to reproduce sexually (Brinckmann, 1964; Larson, 1980; Bouillon, 1984). *V. velella* medusae have been very rarely observed in nature, but one mature specimen captured in the open ocean occurred at a depth of 10 m, contained dense concentrations of zooxanthellae, and is presumed to have had an epipelagic existence (Larson, 1980). Cultured *P. porpita* medusae appear yellow-brown due to their large complement of photosymbionts (Bouillon, 1984).

### **Neustonic gastropods**

In all five subtropical gyres, the two chondrophores are preyed upon by two highly specialized co-occurring neustonic gastropod mollusk lineages: the bubble-rafting snail family Janthinidae (Fig. 1-2C) and the nudibranch subfamily Glaucinae (Fig. 1-2D)

Abbott, 1963; Bayer, 1963; Thompson & McFarlane, 1967; Valdés & Angula Campillo, 2004). Members of both gastropod lineages float upside down at the ocean/atmosphere interface where they drift passively, congregating with their prey in vast numbers at surface convergence zones (Franks, 1992; Dandonneau *et al.*, 2004, 2008). Both taxa maintain buoyancy using air bubbles: Glaucinae swallow air that they store in their specialized gastric cavities (Thompson & McFarlane, 1967; Thompson & Bennett, 1970; Lalli & Gilmer, 1989), and Janthinidae use rapid foot movements, combined with quick-hardening mucus production, to trap air bubbles in a remarkable float from which they hang (Fraenkel, 1927; Bayer, 1963; Wilson & Wilson, 1956; Lalli & Gilmer, 1989). Both taxa also produce planktotrophic larvae that enter the water column, although little is known about their larval ecologies (Laursen, 1953; Ross & Quetin, 1990). Glaucinae is understood to comprise two congeneric species: *Glaucus atlanticus* present in all five gyres, and *G. marginatus* restricted to the two Pacific gyres (extended to include the Indian Ocean, see Chapter V) (Thompson & Bennett, 1970; Valdés & Angula Campillo, 2004). Janthinidae contains two globally-distributed genera: *Recluzia* (Abbott, 1963), and *Janthina*, a genus composed of five globally distributed species, according to the last taxonomic revision (Laursen, 1953). The type species, *J. janthina*, is unambiguously distinct in that it is ovoviviparous, the other four nominal congeners (*J. pallida*, *J. prolongata*, *J. exigua*, and *J. umbilicata*) are oviparous, attach egg capsules to their floats, as does *Recluzia* (Abbott, 1963), and are distinguished primarily by minor conchological features (Laursen, 1953), some of which are quite variable (Bayer, 1963). Neustonic gastropods have been poorly studied in recent decades; before this research, a Web of Science online search returned 6 records for Janthinidae and 11 (molluscan) records for

*Glaucus*; there were no GenBank submissions for either taxon.

### **Dissertation overview**

The following six chapters collectively address a number of key events in the evolutionary history of this community and can be partitioned into two sections: phylogenetics of individual neustonic lineages (Chapters 2-6) and global phylogeography (Chapter 7). Chapter 2 focuses on the porpitid symbionts, and asks whether porpitids from all five subtropical gyres have the same genus of zooxanthellae. Dinoflagellates were genotyped for nuclear large subunit 18S rRNA, a commonly used dinoflagellate barcoding marker, and analyzed in a molecular phylogenetic reconstruction with zooxanthellae from both benthic and pelagic hosts. The results show that porpitid symbionts share the same lineage of endosymbiont (Peridinales: *Scrippsiella* sp.), which is more closely related to the zooxanthellae of pelagic radiolarians (also *Scrippsiella*) than of pelagic foraminiferans and benthic corals. Pelagic foraminiferans have a much more recent benthic ancestor than radiolarians, and in spite of their close relationship (and also the relatively closer relationship of porpitids and corals), it appears that zooxanthellar associations are driven more by the historical ecology of the host than the host's taxonomy.

Chapter 3 is a morphological, conchological and ecological study of the extremely rare brown bubble-rafting snail, *Recluzia* cf. *jehennei*. A mature female *R.* cf. *jehennei* was collected with four tiny conspecific individuals attached to the float and/or egg capsules. This presented an opportunity to corroborate and extend a previous observation of attached conspecifics on *Recluzia* (Colman, 1986; Appendix I). The method of

attachment was photographed and described, and the shells of attached individuals were photographed and confirmed as planktotrophic *via* scanning electron microscopy (meaning that they had spent time in the plankton as larvae instead of developing directly from the female's egg capsules). Since janthinids are protandrous hermaphrodites, a life history stage including dwarf males could explain how *R. cf. jehennei* has managed to persist while remaining very rare. This hypothesis was tested by examining serial sections from two of the attached individuals, but both were juveniles. However, live photographs of another *R. cf. jehennei* female with a larger attached individual (presumably male) support the hypothesis that in *R. cf. jehennei*, float building may be associated with the female life cycle phase.

Chapter 4 investigates the evolutionary origins of Janthinidae, the bubble-rafting snails. The primary data generated in this study is a multigene molecular phylogeny of Janthinidae and potential sister gastropod families. The phylogenetic reconstruction shows that Janthinidae is nested within Epitoniidae (“wentletraps”), which are benthic ectoparasites of sea anemones and corals. Confirmation that epitoniids are the ancestors of janthinids allowed for the development of hypotheses explaining the origin of the bubble float (and rafting) based on morphology and life history data. These hypotheses were tested by mapping traits on the molecular phylogeny. The data support that *Recluzia* is a transformative janthinid lineage, and that bubble floats originated from epitoniid egg masses.

Chapter 5 compares the relative roles of geographic and biological barriers as mechanisms of genetic isolation in two species of neustonic nudibranchs. In the open ocean, differentiating between the two is particularly difficult because of the high levels

of gene flow found in pelagic communities. Here, molecular phylogenetics are employed to test the hypothesis that geography is the primary isolating mechanism in Glaucinae. The results are the inverse of allopatric expectations: the cosmopolitan species *Glaucus atlanticus* is panmictic, whereas the Indo-Pacific *G. marginatus* contains four species in two clades with overlapping distributions. Within the *G. marginatus* species complex, a parallel reproductive change has occurred in each cryptic species pair: the loss of a bursa copulatrix. The data not only reject allopatry, but support biological isolation as the primary driver of speciation—a novel result in a planktonic system.

Chapter 6 is essentially a systematic revision of Glaucinae based on the molecular and morphological data generated in Chapter 5. Because of the importance of the bursa copulatrix as a species-delimiting character in *Glaucus*, the glaucinin reproductive system is redescribed based on histology. Tests of the genetic structure of the *G. marginatus* complex show that pairwise differences between lineages all exceed 10% (mt COI) or 2% (mt 16S), which have been suggested as species-delimiting levels for aeolid nudibranchs. Based on the presence of well-supported molecular divergences and morphological synapomorphies, the four *G. marginatus* lineages are herein considered cryptic species. A review of the literature and available type material was conducted to determine the valid name for each of the four species. The conclusion of this revision is that the valid names of the species are *Glaucus marginatus* (Bergh, 1868) – Indo-Pacific, *Glaucus bennettiae* sp. nov. – South Pacific, *Glaucus thompsoni* sp. nov. – Eastern North Pacific, *Glaucus mcfarlanei* sp. nov. – Kona. Finally, a hypothesis is posed to explain the difference in genetic structuring between *G. atlanticus* versus the *G. marginatus* complex: the lack of a penial spine in *G. marginatus*.

Finally, Chapter 7 is a traditional mitochondrial phylogeographic study of three neustonic taxa: *Glaucus atlanticus*, *Veella veella*, and *Porpita porpita*. Three hypotheses of within-species genetic structuring are tested: global panmixis, ocean basin panmixis, and within-gyre panmixis. The results show that ocean gyre and ocean basin boundaries act as genetic barriers to varying degrees for each species. Within *G. atlanticus* there are two well-supported molecular clades: an Atlantic basin clade and a global clade. Within *V. veella*, there was no evidence of trenchant genetic structuring (global panmixis could not be rejected), although haplotype networks show some structure between northern and southern hemisphere. Conversely, genetic structuring in *P. porpita* shows multiple molecular clades corresponding roughly to oceanic gyres. Even though these three taxa are ecologically linked and passively drifting, these data indicate that neustonic organisms have differential migration across the planet.

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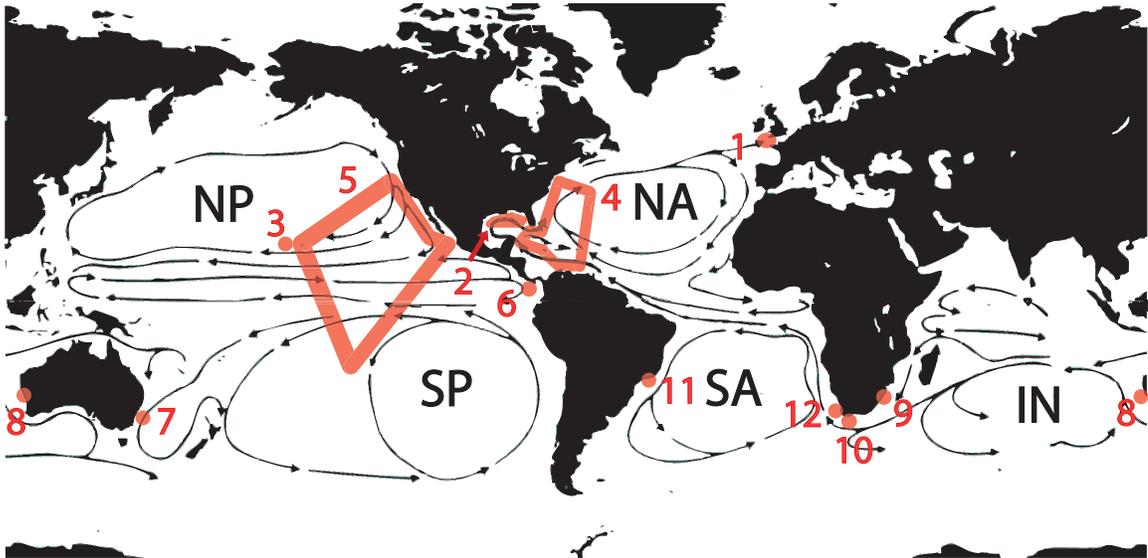
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**Figure 1-1. Map of ocean surface currents (subtropical gyres) and neuston sampling.**



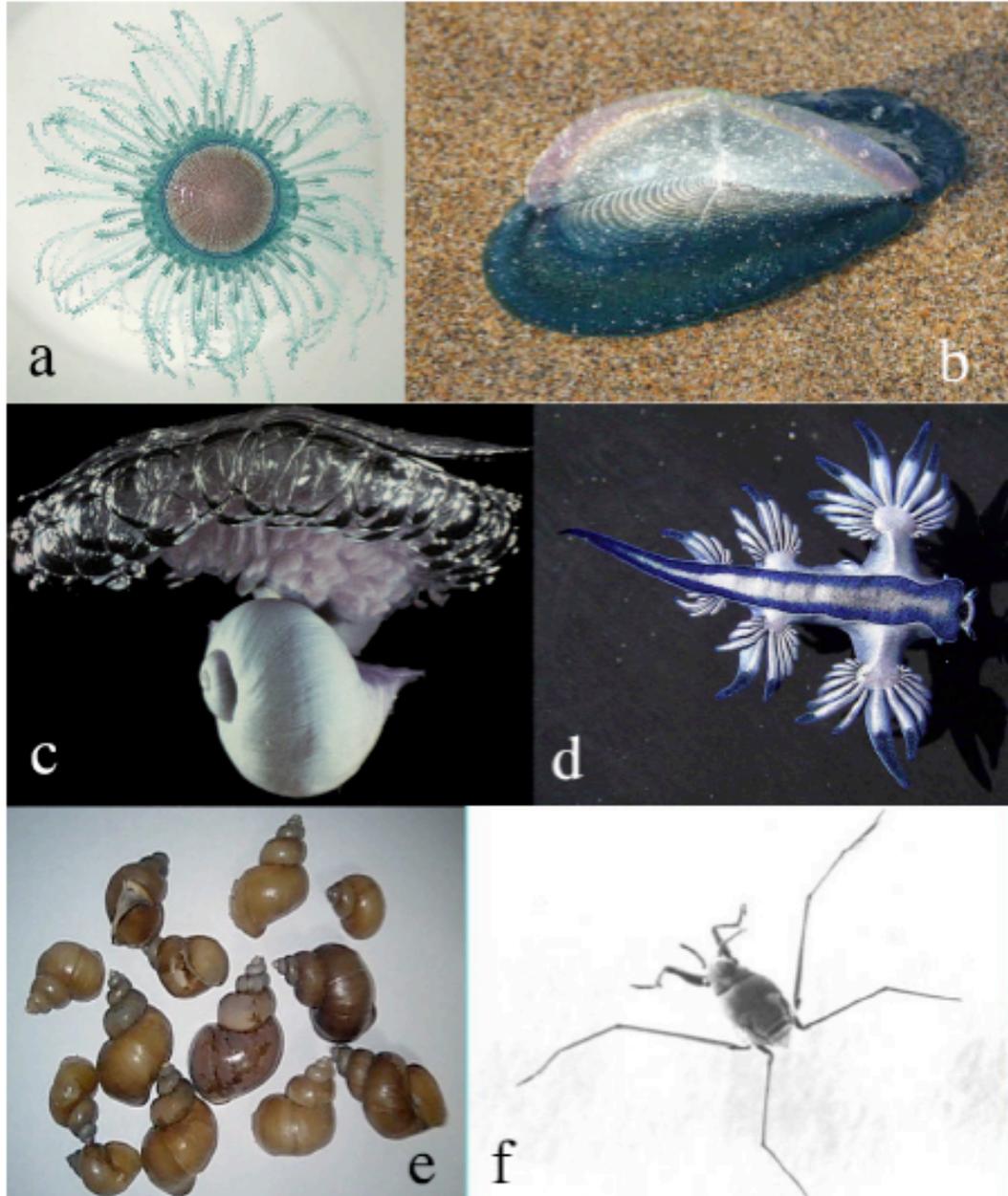
Map of the World's Oceans, modified from Garrison (2004), showing the major surface currents and the location of the North Pacific (NP), South Pacific (SP), North Atlantic (NA), South Atlantic (SA), and Indian Ocean (IN) subtropical gyres. Neuston sampling locations are indicated by numbers: **1**, Cornwall, UK (M. Byrne); **2**, Gulf of Mexico (NOAA); **3**, Kona, Hawaii (NOAA); **4**, North Atlantic Ocean (SEA Semester); **5**, Pacific Ocean (SEA Semester); **6**, Western Panama (R. Collin); **7**, Sydney, Australia (P. Colman); **8**, Fremantle (C. K. C. Churchill); **9**, Kwazulu-Natal, South Africa (C. K. C. Churchill); **10**, Cape Town, South Africa (C. K. C. Churchill); **11**, Sao Paulo, Brazil (A. C. Marques); **12**, Cape Town, South Africa (M. Gibbons).

Figure 1-2. Neuston collecting flier.

## Neuston Watch List

Celia K. C. Churchill, University of Michigan

*If sampling, please preserve in 95% ethanol (EtOH) and change to fresh ethanol after 24 hours*



Constituent neuston taxa: a) *Porpita porpita* (NOAA Ocean Explorer); b) *Verella verella* (MarLIN); c) *Janthina* sp. showing bubble raft and egg capsules (Lalli & Gilmer, 1989); d) *Glaucus atlanticus* (Hartley's Undersea Walk); e) *Recluzia* sp. (So-Net) f) *Halobates* sp. (Scripps Institute of Oceanography)

Sample collection flier distributed to NOAA SEAMAP, SEA Semester, and individual collectors as part of neuston sampling efforts, 2006-2012.

## Chapter 2

### Zooxanthellae of cosmopolitan neustonic cnidarians (Porpitidae) are *Scrippsiella* (Peridinales)

#### Introduction

The keystone species concept, originally articulated by Paine (1966), has become one of the principal foci of research in conservation biology. Like its counterpart in masonry, a keystone species has a disproportionate effect on ecosystem structure relative to its biomass and abundance, and thus identifying these taxa is essential to preserving biodiversity. Zook (2001) proposed that microbial symbionts, while largely ignored in conservation research, unambiguously fit the definition of keystone species. They have relatively low biomass, but when associated with other organisms, microbial symbionts have a synergistic effect on biodiversity levels (Zook, 2001).

Although some objections to the keystone species model have been posed for terrestrial ecosystem conservation (Simberloff, 1998), in the marine realm the majority of conservation efforts have relied upon “focal species” concepts, including keystone species, umbrella species, and nursery-role habitat identification (reviewed in Zacharias & Roff, 2001; Beck *et al.*, 2001). Most marine focal species are macroscopic heterotrophs, but one marine microbial symbiont has received considerable attention: the phototrophic algae (zooxanthellae, genus *Symbiodinium*) housed within reef-building (hermatypic) corals (Zook, 2001; Venn *et al.*, 2008). Hermatypic corals are mixotrophs,

obtaining some of their fixed carbon from heterotrophic sources such as particulate organic matter, bacteria and zooplankton, but typically most of their energy budget is sourced autotrophically from endosymbiotic zooxanthellae (Palardy *et al.*, 2008).

Zooxanthellae are endosymbiotic dinoflagellates housed in a variety of marine invertebrates (reviewed in Trench, 1987). The zooxanthellar life cycle, in general, consists of a motile free-living sexual stage and a “vegetative” symbiotic asexual (mitotic) stage (Banaszak *et al.*, 1993). The biology of the vegetative states confounded early zooxanthellar taxonomy for several reasons: there are few informative morphological characters in the symbiotic life history stage (Rowan & Powers, 1991), and many symbionts cannot be easily cultured *in vitro* (Schoenberg & Trench, 1980). In the past two decades, several molecular studies of *Symbiodinium* clades (*e.g.* Venn *et al.*, 2008) have yielded complex results. Multiple *Symbiodinium* clades occur in association with coral hosts (Rowan & Powers, 1991; LaJeunesse, 2005), sometimes together (Rowan *et al.*, 1997) but often exhibiting niche partitioning, especially with depth (LaJeunesse, 2002) and they may be transmitted vertically (Richmond, 1997) or horizontally (Szmant, 1986) and the latter mode may result in rapid turnover of symbiont assemblages during ontogeny (Little *et al.*, 2004).

Coral-associated *Symbiodinium* spp. have received considerable attention for decades (Muscatine & Porter, 1977) due to the ecological and commercial importance of coral reefs in nearshore tropical ecosystems. Moreover, coral bleaching, the phenomenon in which a coral expels its zooxanthellae, is a well-known reef health indicator (Barber *et al.*, 2001). On the other hand, there is another, much larger, oligotrophic ecosystem that also depends upon zooxanthellar photosynthates: the marine neuston, a community of

passively-drifting organisms at the air-water interface of the planet's tropical and temperate seas (Marshall & Burchardt, 2005). The base of the neuston ecosystem is formed by a mutualism between zooxanthellae and porpitid hydrozoan cnidarians (Hyman, 1940). The cnidarian hosts comprise two monotypic genera, *Porpita porpita* and *Veleva veleva* (Hyman, 1940; Bouillon & Boero, 2000). Porpitids also prey on zooplankton (Bieri, 1961, 1970) and with their dual mode of nutrition, effectively represent the open-ocean equivalent of hermatypic corals.

Very few details are known about the ecology and host-specificity of the porpitid-zooxanthellar symbiosis. Based on their morphology, specifically the pattern of their thecal plates, zooxanthellae from *Veleva veleva* are placed in the genus *Scripsiella* (Peridinales) (D'Onofrio *et al.*, 1999; Huan *et al.*, 2007). The morphology of *Porpita*'s zooxanthellae has never been described. Banaszak *et al.* (1993) morphologically distinguished *Scripsiella ex Veleva veleva* from the North Pacific (*S. velevae*) and the Mediterranean (*S. chattonii*) based upon their organellar composition: presence or absence of trichocysts, respectively, and different numbers of pyrenoids. Banaszak *et al.* (1993) also noted the morphological similarities between *V. veleva* symbionts and those of pelagic radiolarians. This linkage was later corroborated phylogenetically by Gast & Caron (1996) in the first (and to-date only) molecular analysis including porpitid zooxanthellae: from a single specimen of *V. veleva* sampled in the tropical North Atlantic.

Here, zooxanthellae from a global sampling of porpitids were genotyped and added to the existing molecular phylogeny to: 1) corroborate this initial result (Gast & Caron, 1996); 2) test if it applied to both porpitid hosts across in all five global subtropical gyre systems.

## Material and methods

### *Sample Collection*

Specimens of *Velella velella* and *Porpita porpita* come from a global sampling of neustonic invertebrates collected from 2003-2012. Table 2-1 shows locality data for porpitud specimens whose symbionts were analyzed in the molecular phylogeny.

Specimens will be housed in the cnidarian collection at the Smithsonian Museum of Natural History (USNM).

### *DNA Extraction and Amplification*

Zooxanthellae were dissected from the gastric cavity of the central gastrozoid of preserved specimens of *Velella* and *Porpita*. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit spin column protocol. 529 nt of the small nuclear ribosomal RNA (18S) were amplified from three porpituds from each genus, from each subtropical gyre, for a total of 30 samples (Table 2-1). PCRs were performed using a standard PCR cycle [95° 2:00, (94° 0:30, 58° 0:30, 72 1:00) x 40 cycles, 72 5:00] with previously published primers: 18ScomF1 (Zhang & Lin, 2005) and Dino18SR1 (Lin *et al.*, 2006). PCR products were sequenced directly using an ABI 3730xl (Applied Biosystems, Inc.) automated sequencer at the University of Michigan DNA Sequencing Core. Sequences were aligned using the MUSCLE alignment method (Edgar, 2004) implemented in CodonCode Aligner 3.7.1.1 (CodonCode Corporation) and verified by eye.

### *Phylogenetic analysis*

For the phylogenetic analysis, previously published sequences from Gast & Caron's (1996) phylogeny, including *Scrippsiella velellae*, and other symbiotic and free-living dinoflagellate sequences were included (Table 2-2). The outgroup, *Amphidinium carterae*, was chosen based on a recent three-gene phylogeny of dinoflagellates, including 18S rRNA (Zhang *et al.*, 2007). The best-fit model of nucleotide substitution (Trn+I+Γ) was selected statistically by Akaike Information Criterion in jModelTest 0.1.1 (Posada, 2008, Guindon & Gascuel, 2003). Bayesian phylogenetic analysis was conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) (4 chains, 5 million generations); the model of nucleotide substitution chosen was the closest approximation to the AIC best-fit model available in MrBayes. Convergence was estimated by plotting the average sums of split frequencies every 1000 generations. Bayesian posterior probabilities were calculated after a burn-in of 25%. Maximum likelihood phylogenetic analysis was conducted in PAUP\* 4.0 (Swofford, 2003), using the AIC best-fit models of nucleotide substitution for respective markers. Likelihood bootstrap values were also calculated with PAUP\* 4.0 (Swofford, 2003) with 200 replicates.

## Results

All of the 30 zooanthellae amplifications, obtained from both porpitid taxa and from all gyre systems, shared a single 18S genotype with Gast & Caron's (1996) *Scrippsiella velellae* sample and with radiolarian zooxanthellae (Figure 2-1; Appendix I). They collectively formed a well-supported clade distinct from free-living *Scrippsiella* and other dinoflagellates and from coral and foraminiferan endosymbionts. In the previous study, the authors amplified an additional 1,273 nt of 18S rRNA from *S. velellae*, which

contained all of the variation they observed between *S. veillelae* and pelagic radiolarian zooxanthellae (4 substitutions of a total 1,802 nt). Efforts to amplify the variable region of 18S were unsuccessful with the neuston samples from this study; here, *S. veillelae* and the radiolarian zooxanthellae were monotypic.

## Discussion

This study corroborates and extends Gast & Caron's (1996) conclusion that pelagic radiolarians and a porpitid share similar symbionts; here, zooxanthellae from a global sampling of two porpitid genera also group within the radiolarian/porpitid symbiont molecular clade.

It has been suggested that there has been no coevolution of host and symbiont in pelagic sarcodines (foraminiferans + radiolarians) because of the lack of genetic diversity of zooxanthellae within each host lineage (Gast & Caron, 1996; Fig. 2-1), considering that both sarcodine lineages have speciated many times. The data here support that hypothesis and emphasize the importance of time and space vs. host specificity in the holobiont mutualism. Porpitids and radiolarians share similar symbionts, and both have been members of the plankton (or neuston) since the Precambrian (Stanley, 1982; Stanley & Kanie, 1985) and Cambrian (Lipps, 1970), respectively. Pelagic foraminiferans have a much more recent benthic ancestor, having joined the plankton almost 400 MY later in the Upper Jurassic (Lipps, 1970), and their symbionts form a molecular clade with the benthic *Symbiodinium* spp. (Fig. 2-1). Additional support for the importance of host ecology in zooxanthellar lineage comes from *Symbiodinium* itself, which can be found within hosts from at least five different benthic phyla (Stat *et al.*, 2006). Studies of the

variation in *Symbiodinium* lineages among oceans (LaJeunesse *et al.*, 2003; LaJeunesse *et al.*, 2004; LaJeunesse, 2005), among regional reefs (LaJeunesse *et al.*, 2004; Loh *et al.*, 2001), and among individual coral colonies within reefs (Stat *et al.* 2008; Stat *et al.*, 2011) present a complex picture of host-symbiont specificity, environmental modulation, and stochastic processes (Stat *et al.*, 2011).

There are several interesting avenues for potential future research in light of this study. First, several new molecular barcoding and phylogeographic markers have recently been developed (or are in stages of development) for dinoflagellates (*e.g.* mt COI, Stern *et al.*, 2010; ITS2, Stat *et al.*, 2011), and with low tissue volume and/or single-cell DNA extraction techniques, it is becoming increasingly feasible to study zooxanthellae *in situ* without first making time- and space-consuming cell cultures. These techniques may also help reduce one potential source of error in the current dataset: PCR bias. In corals, one colony may have multiple *Symbiodinium* strains (Stat *et al.*, 2011), and although only one zooxanthellar morphology has been reported from porpitids (Banaszak *et al.*, 1993), it is possible that individual hydrozoans may contain multiple strains of *Scrippsiella*. Preliminary mt COI sequences from porpitid symbionts are not monotypic (Churchill, unpubl.), and a phylogeographic study of *S. velellae* is underway. Second, considering the massive diversity in zooxanthellar hosts, including within a single clade (*e.g.* *Gymnodinium/Symbiodinium*) one obvious question is, what makes Chromalveolates (vs. other photosynthetic eukaryotic algae) so prone to symbiotic ecologies? Chromalveolates, including dinoflagellates, comprise the vast majority of eukaryotic endosymbionts, and the application of genomics and transcriptomics to symbiotic and free-living dinoflagellates will undoubtedly shed light on the physiology of endosymbiosis (Nowack

& Melkonian, 2010). Finally, for porpitid symbionts, many of the basic questions already researched for coral symbionts remain uninvestigated; for example, what fraction of the host's nutrition comes from the symbiont and how are symbionts transferred? Although the neuston is inherently more difficult to study ecologically than a coral reef, it covers a much larger area—about 40% of the planet's surface, and neustonic endosymbionts are the primary producers in a much wider pelagic ecosystem including species of conservational interest like sunfish, sea turtles, and seabirds (Marshall & Burchardt, 2005). Using studies of *Symbiodinium* as forerunners (LaJeunesse, 2004; Yang *et al.*, 2012), baseline *Scrippsiella* diversity should be established, as well as its response to the environmental stressors that will inevitably come with anthropogenic climate change.

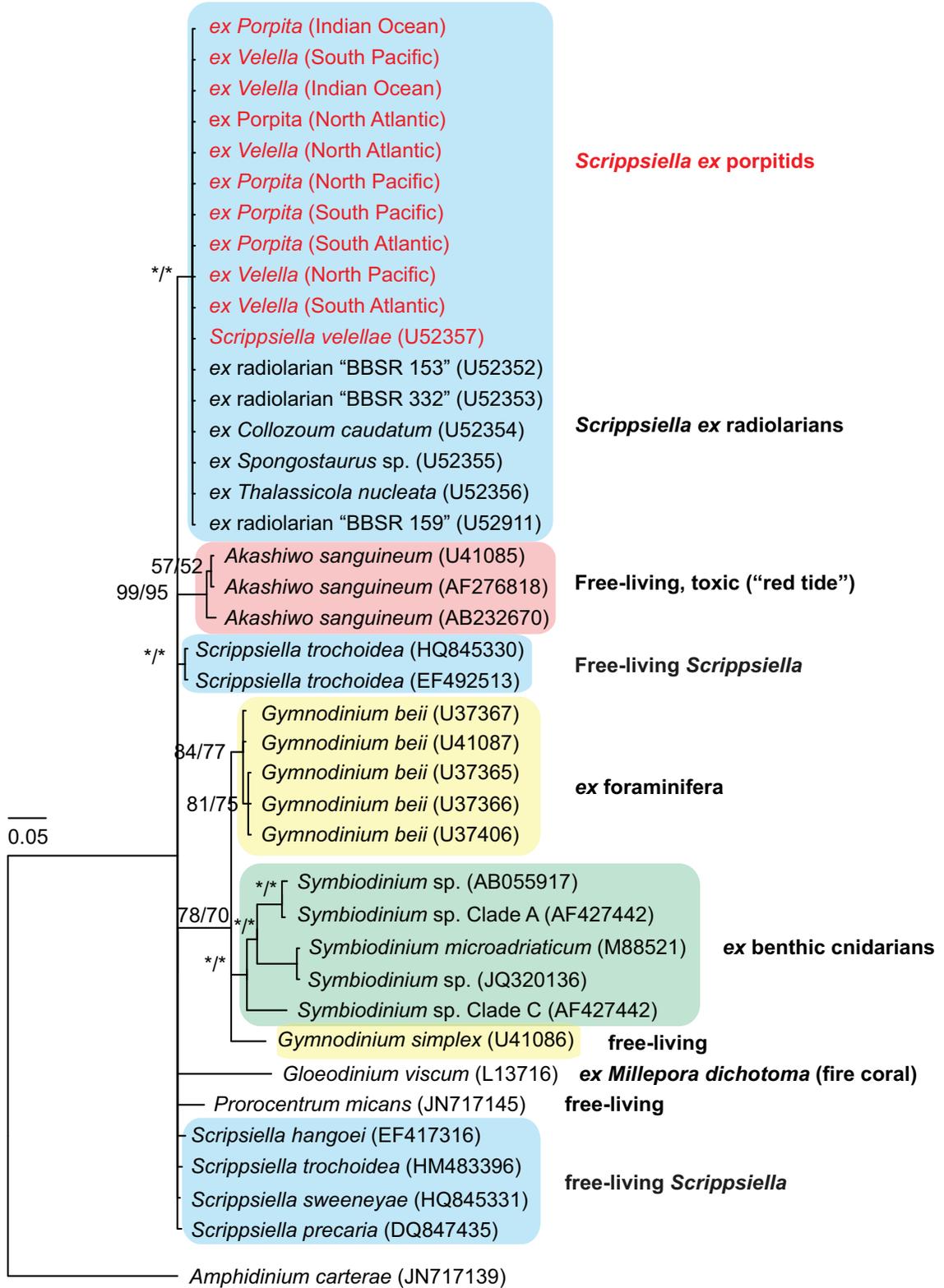
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**Figure 2-1. Bayesian phylogram of nuclear small subunit 18S rRNA from free-living and symbiotic dinoflagellates.** *Amphidinium carterae* is the outgroup. Bayesian posterior probability percentages appear before maximum likelihood bootstrap percentages (only > 50 are shown) at nodes. Four major genera of dinoflagellates are indicated by color: *Scrippsiella* (blue), *Akashiwo* (red); *Gymnodinium* (yellow), and *Symbiodinium* (green). Sequences generated for this study are shown in red type. The ecology of each member of the ingroup is indicated in bold to the right of the topology.



**Table 2-1. Locality data for porpitud specimens included in the molecular phylogeny.**

<b>Species</b>	<b>Latitude (° decimal)</b>	<b>Longitude (° decimal)</b>
<i>Verella verella</i> (North Atlantic)	28.53	-78.34
<i>Verella verella</i> (South Atlantic)	-34.1323	18.44
<i>Verella verella</i> (North Pacific)	33.79333	-160.91167
<i>Verella verella</i> (South Pacific)	-33.738	151.31
<i>Verella verella</i> (Indian)	-29.865879	31.04688
<i>Porpita porpita</i> (North Atlantic)	28.53	-78.34
<i>Porpita porpita</i> (South Atlantic)	-34.1323	18.44
<i>Porpita porpita</i> (North Pacific)	37.145	-159.72333
<i>Porpita porpita</i> (South Pacific)	-33.738	151.31
<i>Porpita porpita</i> (Indian)	-29.865879	31.04688

**Table 2-2. Taxonomy, GenBank accession numbers, and ecologies of previously published dinoflagellate sequences included in the molecular phylogeny.**

<b>Species</b>	<b>Order</b>	<b>Accession No.</b>	<b>Ecology</b>
<i>Akashiwo sanguinea</i>	Gymnodiniales	U41085	Free-living, toxic ("red tide")
<i>Akashiwo sanguinea</i>	Gymnodiniales	AF276818	Free-living, toxic ("red tide")
<i>Akashiwo sanguinea</i>	Gymnodiniales	AB232670	Free-living, toxic ("red tide")
<i>Amphidinium carterae</i>	Gymnodiniales	JN717139	Free-living (outgroup)
<i>Gymnodinium beii</i>	Gymnodiniales	U37367	Symbiont of <i>Globigerinoides ruber</i>
<i>Gymnodinium beii</i>	Gymnodiniales	U41087	Symbiont of <i>Globigerinoides ruber</i>
<i>Gymnodinium beii</i>	Gymnodiniales	U37365	Symbiont of <i>Globigerinoides conglobatus</i>
<i>Gymnodinium beii</i>	Gymnodiniales	U37366	Symbiont of <i>Globigerinoides sacculifer</i>
<i>Gymnodinium beii</i>	Gymnodiniales	U37406	Symbiont of <i>Orbulina universa</i>
<i>Gymnodinium simplex</i>	Gymnodiniales	U41086	Free-living
<i>Prorocentrum micans</i>	Peridinales	JN717145	Free-living
<i>Scrippsiella hangoei</i>	Peridinales	EF417316	Free-living
<i>Scrippsiella precariae</i>	Peridinales	DQ847435	Free-living
<i>Scrippsiella sweeneyae</i>	Peridinales	HQ845331	Free-living
<i>Scrippsiella trochoidea</i>	Peridinales	HQ845330	Free-living
<i>Scrippsiella trochoidea</i>	Peridinales	EF492513	Free-living
<i>Scrippsiella trochoidea</i>	Peridinales	HM483396	Free-living
<i>Scrippsiella veillelae</i>	Peridinales	U52357	Symbiont of <i>Veillela veillela</i>
Unidentified	Peridinales*	U52352	Symbiont of unidentified radiolarian
Unidentified	Peridinales*	U52353	Symbiont of unidentified radiolarian
Unidentified	Peridinales*	U52354	Symbiont of <i>Collozoum caudatum</i>
Unidentified	Peridinales*	U52355	Symbiont of <i>Spongostaurus</i> sp.
Unidentified	Peridinales*	U52356	Symbiont of <i>Thalassicola nucleata</i>
Unidentified	Peridinales*	U52911	Symbiont of unidentified radiolarian
<i>Gloeodinium viscum</i>	Phytodinales	L13716	Symbiont of <i>Millepora dichotoma</i>
<i>Symbiodinium microadriaticum</i>	Suessiales	M88521	Symbiont of <i>Cassiopea xamachana</i>
<i>Symbiodinium</i> sp.	Suessiales	AB055917	Symbiont of <i>Clavularia viridis</i>
<i>Symbiodinium</i> sp.	Suessiales	JQ320136	Symbiont of unidentified coral
<i>Symbiodinium</i> sp. Clade A	Suessiales	AF427442	Symbiont of <i>Cassiopea xamachana</i>
<i>Symbiodinium</i> sp. Clade C	Suessiales	AF427442	Symbiont of <i>Cassiopea xamachana</i>

### Chapter 3

#### Hitchhiking juveniles in the rare neustonic gastropod *Recluzia cf. jehennei* (Janthinidae)<sup>1</sup>

Most species in nature are rare, and their ecologies are poorly known (Lyons *et al.*, 2005). Our lack of knowledge concerning the fundamental biodiversity of rare species in natural ecosystems impairs our ability to develop effective conservation programs and to test hypotheses of community assembly. Although the term “rare” is qualitative, ecologists traditionally determine rarity using range and abundance, combined with guild-level comparisons (Kunin & Gaston, 1996). Ecological studies of rare marine species lag behind their terrestrial counterparts because they are more difficult to sample, and as a result, taxonomic ambiguities abound (Jones, Caley & Munday, 2002). In fact, we know so little about the ecological roles and contributions of rare marine species that it isn’t farfetched to ask, “Are rare species [ecologically] boring?” (R.T. Paine in Schindler *et al.*, 2002). Here we present evidence to the contrary in a rare species of pelagic bubble-rafting snail, *Recluzia cf. jehennei* Petit, 1853.

Members of the genus *Recluzia* Petit, 1853 are also known as brown janthinas, because of their close relationship to the much more ubiquitous violet snails of the genus *Janthina* (Lalli & Gilmer, 1989; see *Journal of Molluscan Studies* cover, volume 77).

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<sup>1</sup> Churchill, C. K. C., Strong, E. E. & Ó Foighil, D. 2011. Hitchhiking juveniles in the rare neustonic gastropod *Recluzia cf. jehennei* (Janthinidae). *Journal of Molluscan Studies*, **77**: 441-444.

These two genera comprise the family Janthinidae, and share a most unusual ecology: they drift passively in the neuston, the vast ecosystem at the surface of the planet's subtropical oceans, which occupies 40% of the Earth's surface (McClain, Signorini & Christian, 2004). They achieve floatation via a remarkable synapomorphy: using quick-setting mucus and rapid foot movements, they construct a bubble raft from which they are suspended (Lalli & Gilmer, 1989). *Recluzia* are much more poorly known than *Janthina* (Fretter & Graham, 1962), and there is a large discrepancy in publishing effort between the two genera. *Janthina* has been comprehensively revised (Laursen, 1953; 60 named species synonymized to five), has been the topic of several anatomical works (*e.g.* Cuvier, 1817; Graham, 1965), and has featured in several comparative morphological studies of caenogastropods (Collin, 2000; Golding, Ponder & Byrne, 2009a, b). *Recluzia*, on the other hand, has never received a formal taxonomic treatment. There are 15 nominal species, all but two described in the nineteenth century, and usually based on few (often single) specimens. A Web of Science search for "*Recluzia*" yields a single record (*vs.* 10 for *Janthina*) of a beach stranding, in which the author noted that he could "now discontinue a search [that] lasted for 25 years" (Poorman, 1980). Five years of neuston sampling by the first author in the North and South Pacific gyre systems has yielded hundreds of *Janthina*, but no *Recluzia*.

Janthinids are highly specialized predators of neustonic cnidarians: the porpitiids *Velevella velevella* and *Porpita porpita*, the siphonophore *Physalia physalis*, and the actiniarian *Mynias* spp. (Abbott, 1963; Lalli & Gilmer, 1989). They are protandrous hermaphrodites (Laursen, 1953) and most adult female janthinids cement egg capsules to their floats, which develop and hatch as planktotrophic veligers. *Janthina janthina*,

however, is ovoviviparous and broods pre-veliger stages in the gonad. Observations of *Recluzia* come from few preserved specimens (Thiele, 1928; Abbott, 1963, Poorman, 1980), as a result their ecology has largely been inferred from *Janthina*. *Recluzia* is known to be oviparous (Poorman, 1980); the only record distinguishing its life history from that of *Janthina* comes from a gray literature note by Colman (1986, Appendix II). Colman observed an adult female, (*Recluzia* sp., Australian Museum #C.145648; here tentatively identified as *Recluzia* cf. *jehennei* Petit, 1853) from Bundagen Beach, New South Wales, Australia, with four conspecific individuals attached to the float. Colman hypothesized that they were dwarf males, and that, “they may live all their life on the float, or, after some time, make their own float and change sex to female, to complete the same sexual cycle as *Janthina*” (Colman, 1986). This proposed early life history is radically different from that of *Janthina* species. Juvenile *Janthina* float autonomously by creating a mucus stalk with a terminal bubble (Simroth, 1895) and there is no evidence that males live in association with adult females (*e.g.*, Laursen, 1953; C.K.C.C. pers. obs.).

During a morphological and molecular systematics study of Janthinidae, we obtained on loan a specimen of *Recluzia* cf. *jehennei* collected stranded in the intertidal in Moreton Bay, Queensland, Australia (Field Museum of Natural History #328104) and preserved in 95% ethanol. This represented a rare opportunity to corroborate and extend Colman’s (1986) observations. In particular, Colman’s samples were too decomposed to study the soft anatomy of the juveniles to look for evidence of autonomous float formation or sexual maturity. If juvenile *R.* cf. *jehennei* remain associated with the female until sexual maturity, it would provide an evolutionary means of achieving a functionally

simultaneous hermaphroditic unit (Ghiselin, 1969). Such unions are selectively advantageous in populations with low densities; when males have limited reproductive opportunity, remaining with one female may be the best strategy (Charnov, 1979) and highly specialized dwarf males are known to occur in a variety of molluscan groups (Turner & Yakovlev, 1983; Ó Foighil, 1985; Warén, 1983; Voight, 1997).

Initial inspection confirmed the specimen to be a large, mature female, with associated float and egg capsules containing early embryonic stages, although both capsules and float had partially dried prior to preservation (Fig. 3-1A). Further examination revealed four associated post-larval individuals (Fig. 3-1A). Three of the individuals had between 0-1 whorls of teleoconch growth (Fig. 3-1A: I-III), and the fourth appeared to be newly metamorphosed (Fig. 3-1A: IV). Each of the four small individuals was located in close proximity to the large female; two were attached to the float near the female's propodium (Fig. 3-1B: I-II), and two were attached to a single egg capsule near the metapodium (Fig. 3-1B: III-IV). The method of attachment was the same for all four individuals: a flat patch of mucus cemented to the shell on the side of the spire. The two individuals (I and II) attached to the float fell off as the float was removed, but the individuals on the egg capsule remained firmly attached (Fig. 3-1C), which is significant considering the specimen had previously been stranded, preserved, and shipped. None of the small individuals showed any sign of creating an autonomous float.

Scanning electron micrographs of the smallest post-larval individual, IV (Fig. 3-1D, E) show a typical janthinid protoconch (see Robertson, 1971, Pls. V-VI). All *Janthina* spp. have obligate planktotrophic larval development (Laursen, 1953; Robertson, 1971) but their morphologies (Simroth, 1895, Laursen, 1953; Robertson,

1971) are not completely consistent with Thorson's (1950) "shell apex theory:" they are low-spired, and the line of demarcation between embryonic (P1) and postembryonic (P2) protoconch growth may be unclear (*e.g.* Robertson, 1971, Pl. V, Fig. 20). Otherwise, janthinid protoconchs are consistent with Thorson's model: they have a small, smooth P1 and a large, multispiral P2 with increasingly prominent axial plicae (Robertson, 1971). Additionally, there is a large size difference between late-stage embryos and fully-grown larvae in *Janthina* (Laursen, 1953, Wilson & Wilson, 1956; Robertson, 1971). There are no late-stage embryos present for comparison in the specimen of *Recluzia cf. jehennei* examined here, but interestingly, the protoconch of individual IV (Fig. 3-1D, E) greatly resembles that of the oviparous *J. pallida* (Robertson, 1971). They have similar shell lengths (0.45 mm *vs.* a mean of 0.42 mm) and numbers of whorls (3.625 *vs.* 3.25); a smooth P1 with faint axial plicae, an unclear line of demarcation between P1 and P2 (between 1-1.25 whorls *vs.* 1 whorl), and a P2 with regular axial plicae (5-10  $\mu$ m apart *vs.* 3-9  $\mu$ m) (Fig. 3-1E). These similarities support an inference of planktotrophic larval development in *Recluzia cf. jehennei*.

Furthermore, the presence of a thick varix on the protoconch of individual IV (Fig. 3-1D) indicates that it is newly metamorphosed. Fully-grown *Janthina* spp. larvae have a continuous thickened peritreme forming a varix at the end of P2, which is further defined by a change in the axis of coiling of between 10-50° with the onset of teleoconch growth (Robertson, 1971). The axis of teleoconch growth appears to change in *Recluzia cf. jehennei*, but we cannot measure the precise angle because the protoconch apex is broken in the large female, and there is < 1 whorl of teleoconch growth in the small individuals I-III. From the available material, it appears consistent with *Janthina*. Given

that there was no evidence of float formation in any of the small *R. cf. jehennei*, the varix and lack of teleoconch on individual IV, in particular, supports recruitment of larvae directly onto conspecific female floats or egg capsules, followed by a prolonged association with the host female while the teleoconch grows.

After removing the shells from the largest post-larval individuals (I and II; 2 mm shell height), there was no external evidence of gonadal tissue. Histological sections (sectioned at 5  $\mu\text{m}$ , stained with haematoxylin and eosin-phloxine; Humason, 1967) show very early stages of gonadal development: developing oogenic follicles with oogonia (Fig. 3-1E). These are immature female reproductive structures, but because there has been no study of gonadal development in janthinids, we hesitate to identify the juveniles as female. Sections of male *Janthina* at various stages of development also show inactive female gonadal tissue (pre-vitellogenic oocytes) (C.K.C.C. unpubl. data). Our sections of *Recluzia cf. jehennei* indicate that the female part of the reproductive system develops first, irrespective of the order of maturation. There is no evidence of male testis development or of sperm production. The pallial reproductive tract is undifferentiated and too undeveloped to be identified conclusively as male or female. In short, neither individual was sexually mature, which is not surprising given their size (2 mm).

Although there is no evidence of sexual maturity in individuals I and II, several lines of circumstantial evidence support an extended association between mature female *Recluzia cf. jehennei* and smaller conspecifics. First, there has been no record of a free-living *R. cf. jehennei*, or of any *Recluzia*, as small as autonomously floating *Janthina* spp. (< 4 mm, although this may be partially due to size bias in sampling). Second, Colman's (1986) auxiliary specimens, although too decomposed for anatomical study, were much

larger than the juveniles we observed, and if *Recluzia*'s growth is similar to *Janthina*'s, they were presumably sexually mature (as Colman supposed). Finally, a recent (2008) photograph of *R. cf. jehennei* from Hastings Point, New South Wales, taken by Denis Riek (<http://www.roboastra.com>) clearly shows a much larger auxiliary individual (shell height ~ 5 mm) associated with the float of a large female (Fig. 3-1G).

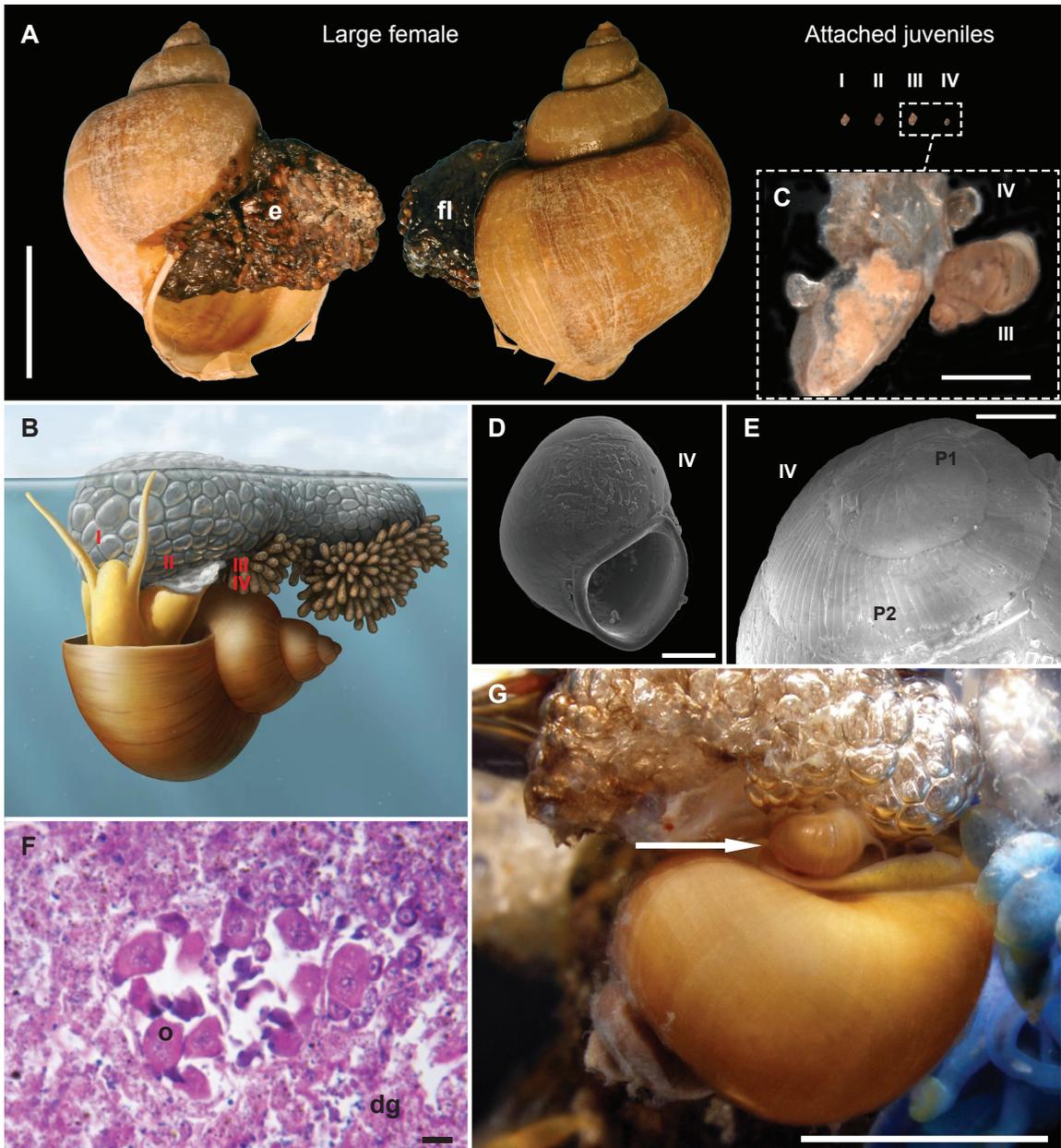
Larval recruitment onto a conspecific float may be key in casting light on the evolutionary transition of ancestral janthinids from the benthos to the neuston. Interestingly, associations between mature females and smaller males are found in several species of the protandrous Epitoniidae (*e.g.* Robertson, 1983), the suspected sister group to the Janthinidae (Ponder *et al.*, 2008). If juvenile *R. cf. jehennei* do not build floats, then float formation is a post-juvenile trait in this species, and possibly associated with the mature female phase of its life cycle. Conspecific larval recruitment, in addition to suspected dwarf/complemental males, are life history traits that have likely allowed *R. cf. jehennei* to persist while remaining extremely rare, and could be tested directly by taking histological sections of larger hitchhiking *R. cf. jehennei*. The results presented here not only provide ecological information about a scarcely recorded species, but they will also be part of a larger study examining Janthinidae's transition to the neuston.

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**Figure 3-1. Hitchhiking juveniles, protoconch sculpture, and histology of *Recluzia cf. jehennei*.** **A-F.** *Recluzia cf. jehennei*. Moreton Bay, Queensland, Australia (FMNH reg. no. 328104). **A.** Apertural and abapertural views of large female with dried float and egg capsules; abapertural views of four attached juveniles (I–IV) associated with the float (I and II) and one egg capsule (III and IV, indicated with a dashed rectangle). Abbreviations: fl, float; e, egg capsule. **B.** Artist’s reconstruction of live specimen with Roman numerals indicating locations of juveniles I–IV. **C.** Egg capsule with attached juveniles III and IV. **D.** Scanning electron micrograph of individual IV, apertural view, showing a varix and no teleoconch growth. **E.** Scanning electron micrograph of individual IV, showing differences in sculpture between embryonic Protoconch 1 (P1) and postembryonic Protoconch 2 (P2). **F.** Histological section of visceral mass showing digestive gland surrounding developing oogenic follicles with oogonia. Abbreviations: dg, digestive gland; o, oogonium. **G.** *Recluzia cf. jehennei*. Live photo from Hastings Point, New South Wales, Australia. A large female, with float, is feeding on the siphonophore *Physalia physalis*. An arrow indicates a smaller associated individual with no float. Scale bars: **A, G** = 10 mm; **C** = 1 mm; **D** = 100  $\mu$ m; **E** = 50  $\mu$ m; **F** = 10  $\mu$ m.



## Chapter 4

### Females floated first in bubble-rafting snails<sup>1</sup>

Ever since Mivart asked Darwin to explain a bird's use for half a wing, biologists have been challenged to explain extraordinary evolutionary change mechanistically. Here, we investigate the enigmatic evolutionary origins of Janthinidae, a family of marine snails with one of the most implausible ecologies of any mollusk. Janthinids raft passively in the neuston, a vast oceanic surface habitat, by constructing floats of mucus bubbles. We present the first molecular phylogeny including Janthinidae, which confirms janthinids are derived from Epitoniidae (wentletraps)—benthic predators and parasites of sea anemones and corals. Our data support the hypothesis that floats and rafting evolved *via* modified epitoniid egg masses rather than by juvenile droguing. Our phylogeny also reveals sequential modifications of float formation and function among janthinid lineages. We interpret these changes as sequential adaptations to a neustonic existence, a conclusion supported by the positive association of apomorphic janthinid traits with ecological prevalence.

The marine environment comprises three primary ecological domains: benthos (sea floor), pelagos (water column), and the rarely studied neuston (water surface). The latter contains a community of approximately 100 species drifting in subtropical oceans,

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<sup>1</sup> Churchill, C. K. C., Ó Foighil, D., Strong, E. E. & Gittenberger, A. 2011. Females floated first in bubble-rafting snails. *Current Biology*, **21**:R802-R803

a vast area covering 40% of the planet (Marshall & Burchardt, 2005). These include all members of Janthinidae, a predatory snail family that achieves buoyancy by constructing bubble floats. The family comprises two genera: *Janthina* (5 species), the violet snails, and *Recluzia* (likely 2-3 global species) the rare brown janthinas (Lalli & Gilmer, 1989).

Janthinids are classified with the large (~630 species) benthic family Epitoniidae morphological and ecological synapomorphies and (weakly) by a single cladistic analysis (Ponder *et al.*, 2008). Epitoniids are specialized predators and ecto-parasites of benthic cnidarians (Robertson, 1963). Like janthinids (Churchill *et al.*, 2011), epitoniids develop as protandric hermaphrodites (Robertson, 1980). Smaller epitoniid males associate with larger females, which remain attached to tethered egg masses by elastic mucus threads (Robertson, 1963). Relationships within Janthinoidea remain ambiguous (Ponder *et al.*, 2008; Robertson, 1963) but one morphological study supports *Recluzia* as a transitional form between Epitoniidae and Janthinidae (Thiele, 1928).

Figure 4-1 shows the first molecular phylogeny incorporating Janthinidae. We included representatives of 7 potential sister families of Epitoniidae, based on published molecular and morphological caenogastropod phylogenies (Ponder *et al.*, 2008). Bayesian and maximum likelihood analyses of one mitochondrial and three nuclear molecular markers reveal robust statistical support (PP=100, BS<sub>ML</sub>=100) for the monophyly of Janthinoidea, strongly corroborating the earlier cladistic analysis (Ponder *et al.*, 2008). Our results place Janthinidae within a paraphyletic “Epitoniidae” (hereafter enclosed in quotation marks), but support levels for the nested placement of Janthinidae are not as robust (PP=89-93, BS<sub>ML</sub>=57-63) as for other basal nodes (Fig. 4-1). A more extensive sampling of “epitoniid” diversity may be required to identify the closest living

benthic sister lineage of Janthinidae.

Confirmation of the origins of Janthinidae allows us to ask, how did a benthic “epitoniid” become neustonic? The fundamental janthinid adaptation to life in the neuston is the bubble float, and we propose two hypotheses of its evolutionary origin. First, the float may derive from a modified juvenile drogue thread. Many benthic snail species are capable, as juveniles, of short-term pelagic dispersal *via* mucus threads that may become captured by surface tension (Vermeij, 1995). Second, the float may represent a modified “epitoniid” egg mass, which typically has capsules in various stages of development, from newly encased embryos to empty husks (Robertson, 1980). These husks could trap air in an intertidal species, providing temporary buoyancy for both the egg mass and the attached female. Both scenarios would initially produce temporary periods of passive rafting, but adding air-filled mucus bubbles to the drogue thread or the egg mass would be a prerequisite to attaining a fully neustonic existence.

To test our evolutionary hypotheses, we examined the distribution of janthinid life history traits across the phylogeny (Fig. 4-1:A-D). *Janthina* spp. juveniles build floats (Laursen, 1953), consistent with the Juvenile Drogue hypothesis, but there is no record of autonomous float formation by a juvenile *Recluzia* spp. (Churchill *et al.*, 2011). Instead, a recent study documents larval recruitment and prolonged association of small-bodied *R. cf. jehennei* on the float and egg capsules of larger conspecific females (Churchill *et al.*, 2011) (Fig. 4-1B), a life history consistent with the Egg Mass hypothesis. Which neustonic genus (and float ontogeny) is plesiomorphic? *Recluzia* exclusively shares six morphological character states, independent of float ontogeny, with “epitoniids” but *Janthina* has none (Table 4-1). These data support the Egg Mass hypothesis.

Our molecular phylogeny allows us to reconstruct how an ancestral janthinoidean lineage evolved a neustonic mode of life. The proto-janthinid was benthic and females formed tethered egg masses with associated males (Fig. 4-1A, represented by an extant “epitoniid”). The key adaptation of rafting derives from an egg mass modified for buoyancy (Fig. 4-1B). Subsequent evolutionary change in the janthinid float involved a sequential loss of reproductive functions. In *Recluzia cf. jehennei* (Fig. 4-1B), the float is a female-only trait (Churchill *et al.*, 2011) and serves as a raft, a substrate for egg masses, and a platform for post-larval juveniles. The latter function is not present in the derived genus *Janthina*, in which all post-metamorphic individuals make autonomous floats. In the plesiomorphic oviparous condition, the float serves as a raft and (in females) a substrate for egg masses (Fig. 4-1C). In the most derived condition, the ovoviviparous *J. janthina*, the float functions only as a raft during all life history stages (Fig. 4-1D).

We interpret these sequential float modifications as adaptations to a neustonic existence. The evolution of autonomous float building by juvenile *Janthina* spp. removed the necessity of meeting a conspecific female before metamorphosis, allowing juveniles to exploit local resources earlier. The relatively compact and buoyant float of ovoviviparous *J. janthina* can be completely rebuilt (Wilson & Wilson, 1956) and is the most specialized for rafting, whereas oviparous species are tied to a single, trailing float encumbered with egg capsules (Fig. 4-1B, C). Our inference of adaptive change is strengthened by the observation that ecological prevalence of janthinid species is correlated with the possession of derived float traits (Lalli & Gilmer, 1989) (Fig. 4-2). Through sequential modification of float construction and function, janthinids have become increasingly successful at exploiting neustonic resources.

## Material and methods

### *Sampling*

*Janthina* spp. were collected as part of a global sampling effort of neustonic taxa via neuston tow and beach collection (Fig. 4-3). Epitoniids were collected intertidally and via SCUBA. *Recluzia* cf. *jehennei*, *Cirostrema varicosa*, and non-epitonioid caenogastropods were loaned from museum collections. Table 4-2 shows the taxonomic data, voucher numbers, and housing museums for all specimens used in this study. All tissue samples were fixed and preserved in  $\geq 95\%$  ethanol. *Janthina* spp. were identified according to Laursen's (1953) taxonomic key.

### *Molecular data*

Whole genomic DNA was extracted from dissected foot tissue when possible, or whole body extractions for very small specimens, using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-Tek). A total of 2,217 aligned nucleotides were amplified from four molecular markers using previously published primers. 536 nt of mitochondrial 16S rDNA was amplified using universal primers combination 16Sar/16Sbr (named primer pairs are in the format 5'/3') (Simon *et al.*, 1994) and an annealing temperature of 49°C. 1,192 nt of nuclear 28S rDNA (DI-DIII) was amplified using molluscan primers D23F/D6R (Park & Ó Foighil, 2000) and an annealing temperature of 50°C. 328 nt of nuclear Histone-H4 was amplified using universal primers HexAF/HexAR (Colgan *et al.*, 1998) and an annealing temperature of 53°C. 160 nt of nuclear Histone-H4 was amplified using universal primers H42FS/H4F2er (Pineau *et al.*, 2004) and an annealing

temperature of 50°C. All PCRs followed a general protocol: initial denaturation (95°C, 2 min); 35 cycles of (94°C, 30 sec; X°C, 30 sec; 72°C, 1 min); final elongation (72°C, 5 min), where X = Annealing temperature. After verifying the size of amplified fragments *via* gel electrophoresis, PCR products were directly sequenced using an ABI 3730xl (Applied Biosystems, Inc.) automated sequencer by the University of Michigan DNA Sequencing Core. Sequences were aligned using the MUSCLE alignment method (Edgar, 2004) implemented in CodonCode Aligner 3.7.1.1 (CodonCode Corporation) and verified by eye. Two sequences for *Littoraria intermedia* (mt 16S rDNA, nuclear 28S rDNA) used in this study were previously published in GenBank.

#### *Phylogenetic analyses*

For the molecular analysis, best-fit models of nucleotide substitution were selected statistically by Akaike Information Criterion in jMODELTEST 0.1.1 (Posada, 2008) for each molecular marker: mt 16S rDNA (TPM2uf+ $\Gamma$ ), nuclear 28S rDNA (TIM3+I+ $\Gamma$ ), Histone-H3 (GTR+I+ $\Gamma$ ), and Histone-H4 (SYM+I+ $\Gamma$ ). Bayesian phylogenetic analysis was conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) (4 chains, 10 million generations) with a partitioned data set; the model of nucleotide substitution chosen for each partition was the closest approximation to the AIC best-fit model available in MrBayes: mitochondrial, (GTR+ $\Gamma$ ); nuclear, (GTR+I+ $\Gamma$ ). Convergence was estimated by plotting the average sums of split frequencies every 1000 generations. Bayesian posterior probabilities were calculated after a burn-in of 25%. Maximum likelihood phylogenetic analysis was conducted in PAUP\* 4.0 (Swofford, 2002), using the AIC best-fit models of nucleotide substitution for respective markers.

Likelihood bootstrap values were also calculated with PAUP\* 4.0 (Swofford, 2002) with 300 replicates.

*Morphological data*

To confirm and add to previous studies (Robertson, 1963; Churchill *et al.*, 2011; Thiele, 1928) supporting *Recluzia* as a plesiomorphic janthinid, ethanol-preserved oviparous and ovoviviparous *Janthina* spp., and *Recluzia* cf. *jehennei*, were dissected using standard techniques (Strong, 2003). Morphological data for benthic janthinoideans (=Epitoniidae) came from previously published studies (Robertson, 1963; Thiele, 1928) (Table 4-1).

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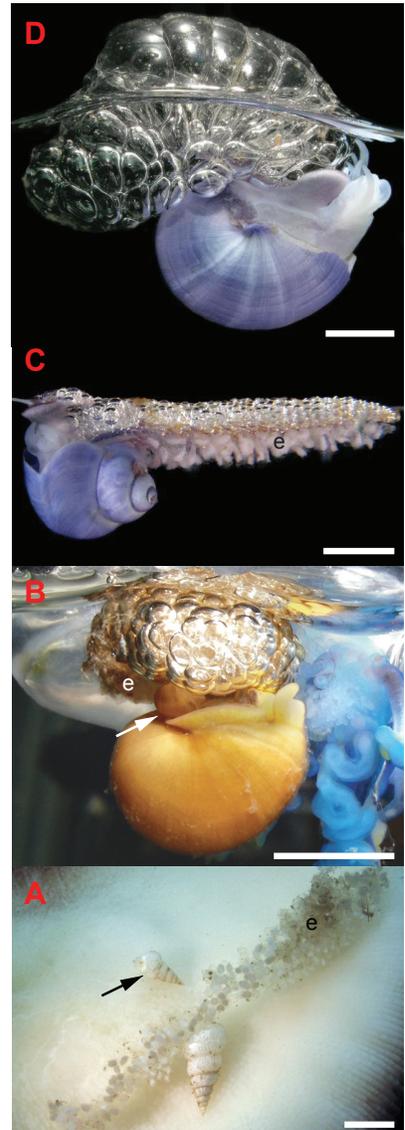
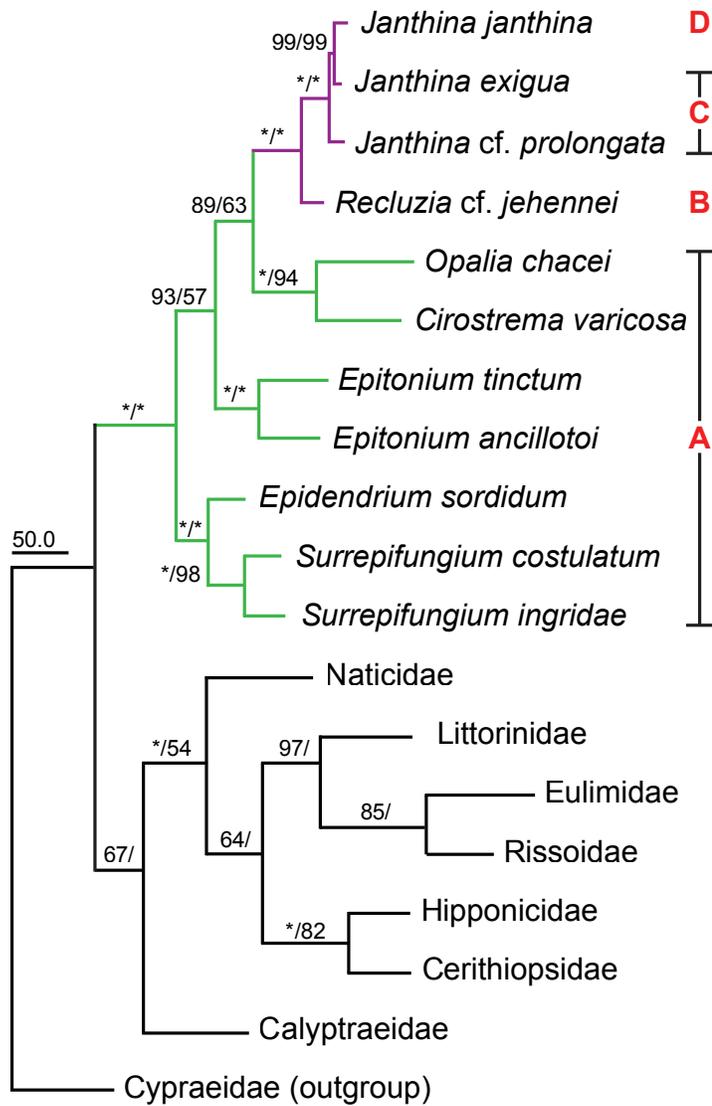
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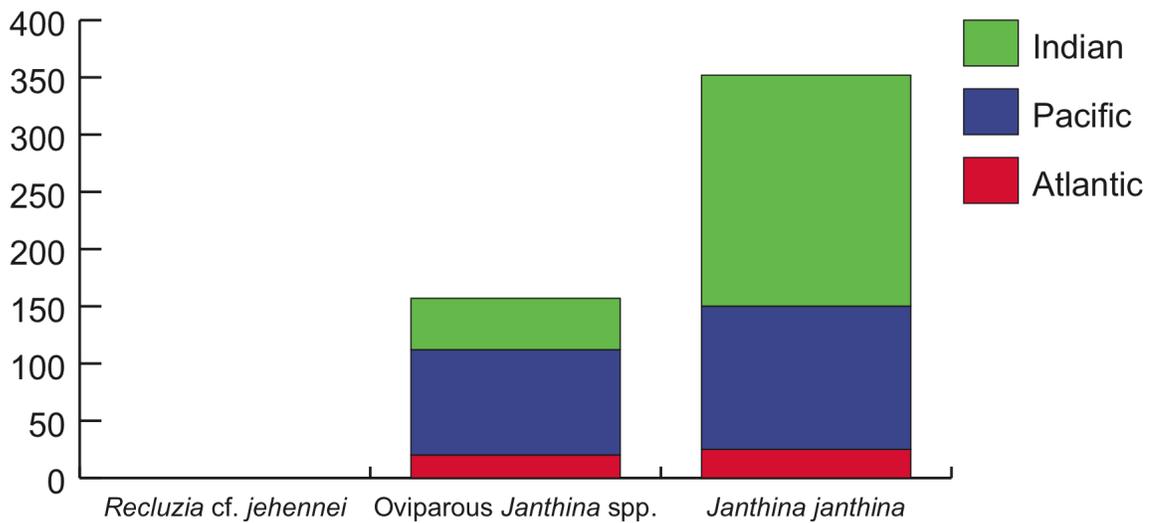
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**Figure 4-1. Proposed evolutionary steps to rafting in Janthinidae.** *Left:* Bayesian phylogram of Janthinoidea and seven potential sister families. DNA sequence data comes from four loci: mitochondrial 16S rDNA; nuclear 28S rDNA, Histone-H3, Histone-H4. Bayesian posterior probabilities are followed by maximum likelihood bootstrap percentages above internal branches. Asterisks indicate support values of 100. Janthinidae and “Epitoniidae” are represented in purple and green, respectively. Red letters indicate the evolution of four key rafting apomorphies, represented by photographs to the right of the topology. *Right:* **A**, Tethered egg masses in Janthinoidea. Mature female *Epifungium nielsi*, a benthic “epitoniid,” on its coral host with egg capsules (e). An arrow indicates a small associated male. **B**, Floating *via* modified egg masses in Janthinidae. Mature female *Recluzia* cf. *jehennei* with bubble raft and husks of egg capsules (e), feeding on the cnidarian *Physalia physalis*. An arrow indicates a small associated conspecific. **C**, Autonomous floating of juveniles and males in *Janthina*. Mature female *Janthina exigua* with bubble raft and egg capsules (E). **D**, Loss of egg capsules from the float in ovoviviparous *J. janthina*. Mature female *J. janthina* with large bubble raft. **Scale bars: A-D: 1.0 cm.**

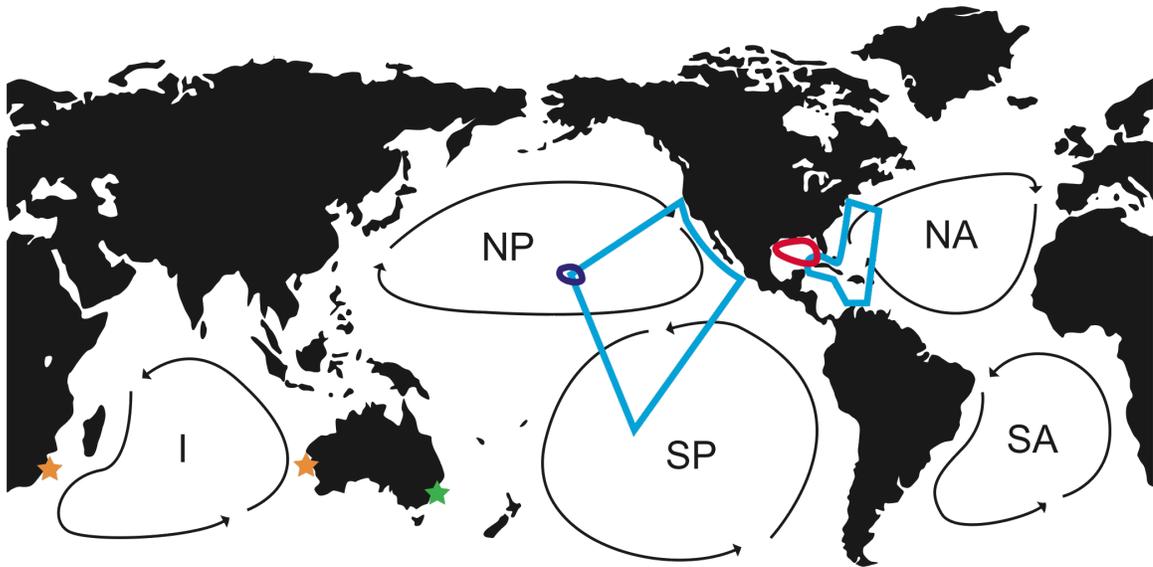


**Figure 4-2. Collection data for Janthinidae from five years of global sampling of neustonic taxa.** Janthinids have been divided into three categories corresponding to the three sequential apomorphies for a neustonic ecology (Fig. 4-1): rafting via modified egg masses (*Recluzia cf. jehennei*, N=0); autonomous juvenile rafting (oviparous species of *Janthina*, N=157); ovoviviparous reproduction (*J. janthina*, N=352). Three geographic categories, corresponding to ocean basin, are represented by color: **red**, Atlantic; **blue**, Pacific; **green**, Indian.



**Chi-Square test:**  $\chi^2 = 366.56$ ;  $df = 2$ ;  $p$ -value < 0.0001

**Figure 4-3. Sampling map for 2005-2010 global neustonic collection. Sampling sources are color-coded. Red, National Oceanographic & Atmospheric Administration (NOAA) Southeast Area Monitoring and Assessment Project (SEAMAP); dark blue, NOAA Pacific Island Fisheries Science Center; light blue, Woods Hole Oceanographic Institute SEA Semester Program; green, P. Colman; orange, C.K.C.C.**



**Table 4-1: Taxonomic distribution of janthinoidean characters used to support *Recluzia cf. jehenei* as a transformative janthinid.** References appear after each character; an asterisk indicates this study.

<b>Character</b>	<b>Benthic janthinoideans (= Epitoniidae)</b>	<b><i>Recluzia cf. jehenei</i></b>	<b><i>Janthina</i></b>
Statocysts (Thiele, 1928)	yes	yes	no
Stylets in the inner paired salivary glands (Thiele, 1928)	yes	yes	no
Cephalic tentacle structure (Thiele, 1928)	uniramous	uniramous	branched
Pre-female stages associated with egg mass or float (Churchill <i>et al.</i> , 2011; Robertson, 1980)	yes	yes	no
Unpaired labial gland*	large	large	reduced
Metapodial attachment to egg mass or float*	mucus stalk	mucus stalk	mucus sheets

**Table 4-2. Taxonomy, museum registration number of voucher specimen, and GenBank accession numbers for sequences for four molecular markers (mitochondrial 16S rDNA, nuclear 28S rDNA, Histone-H3, Histone-H4) from the caenogastropod species included in the molecular phylogeny of Janthinidae (Fig. 4-1).** Museums holding voucher specimens are identified by prefixes before catalog numbers: **FLMNH**, Florida Museum of Natural History, University of Florida, Gainesville, USA; **UMMZ**, University of Michigan Museum of Zoology, Ann Arbor, USA; **RMNH**, Netherlands Centre for Biodiversity Naturalis, Leiden, The Netherlands; **FMNH**, Field Museum of Natural History, Chicago, USA. Asterisks indicate no sequence data.

Family	Species	Voucher	16S	28S	H3	H4
Calyptraeidae	<i>Crepidula fornicata</i>	FLMNH 419266	JF750952	JF750968	JF750985	JF751004
Cerithiopsidae	<i>Cerithiopsis</i> sp.	FLMNH 424366	*	JF750972	JF750990	JF751009
Cypraeidae	<i>Cypraea labrolineata</i>	FLMNH 428603	JF750953	JF750969	JF750986	JF751005
Epitoniidae	<i>Cirostrema varicosa</i>	FLMNH 414037	JF750950	JF750966	JF750984	JF751002
Epitoniidae	<i>Epitonium tinctum</i>	UMMZ 302904	JF750948	JF750964	JF750982	JF751000
Epitoniidae	<i>Opalia chacei</i>	UMMZ 302906	JF750944	JF750960	JF750978	JF750996
Epitoniidae	<i>Surrepifungium costulatum</i>	RMNH 95226	JF750946	JF750962	JF750980	JF750998
Epitoniidae	<i>Surrepifungium ingridae</i>	RMNH 95318	JF750947	JF750963	JF750981	JF750999
Epitoniidae	<i>Epitonium ancillotoi</i>	RMNH 88484	JF750949	JF750965	JF750983	JF751001
Epitoniidae	<i>Epidendrium sordidum</i>	RMNH 95286	JF750945	JF750961	JF750979	JF750997
Eulimidae	<i>Melanella</i> sp.	FLMNH 427921	JF750955	JF750971	JF750989	JF751008
Hipponicidae	<i>Hipponix australis</i>	FLMNH 429250	JF750954	JF750970	JF750988	JF751007
Janthinidae	<i>Janthina exigua</i>	UMMZ 302912	JF750940	JF750956	JF750974	JF750992
Janthinidae	<i>Janthina</i> cf. <i>prolongata</i>	UMMZ 302910	JF750942	JF750958	JF750976	JF750994
Janthinidae	<i>Janthina janthina</i>	UMMZ 302911	JF750941	JF750957	JF750975	JF750993
Janthinidae	<i>Recluzia</i> cf. <i>jehennei</i>	FMNH 328104	JF750943	JF750959	JF750977	JF750995
Littorinidae	<i>Littoraria intermedia</i>	FLMNH 400967	U46785	FN556295	JF750987	JF751006
Naticidae	<i>Polinices lacteus</i>	FLMNH 419220	JF750951	JF750967	*	JF751003
Rissoidae	<i>Zebina</i> sp.	FLMNH 427712	*	JF750973	JF750991	JF751010