

Chapter 6

Spineless sex and speciation: Four cryptic species of neustonic nudibranch (Glaucinae)¹

Introduction

Glaucinin nudibranchs are the only lineage of truly pelagic sea slugs (Valdés & Angulo Campillo, 2004). Floating upside-down at the surface of the planet's subtropical oceans, glaucinins are not good swimmers, and thus are transported by wind-driven surface currents (Thompson, 1976; Lalli & Gilmer, 1989). They maintain buoyancy by holding swallowed air in their chitinized gastric cavities (Valdés & Angulo Campillo, 2004; Thompson, 1976; Thompson & McFarlane, 1967). Like bubble-rafting janthinid snails, glaucinins are neustonic predators, and primarily consume the drifting hydrozoan cnidarians *Porpita porpita*, *Velella velella*, and *Physalia physalis* (Lalli & Gilmer, 1989; Churchill *et al.*, 2011). Although glaucinins display several unique adaptations to a neustonic ecology, including countershading, an expanded, flattened body shape, and a muscular posterior sphincter in the gastric cavity for air bubble retention, many aspects of glaucinin morphology have not changed appreciably from their benthic counterparts (Thompson, 1976). The presence of cnidosacs, muscular repositories of undischarged cnidarian nematocysts, in the tips of the cerata (finger-like projections of the body),

¹ This chapter comprises an unpublished manuscript draft. The authors (in order) are: Celia K. C. Churchill, Ángel Valdés & Diarmaid Ó Foighil.

characterizes glaucinins as members of the suborder Aeolidacea (Thompson & McFarlane, 1967; Martin *et al.*, 2009).

Since the first published record of a glaucinin in 1705 (Breyn, 1705 as *Hirudinis marina*; see Thompson & McFarlane, 1967), there have been many published and unpublished descriptions of these charismatic sea slugs. Based on both anatomical and external differences, two genera have been traditionally recognized: *Glaucus* Forster, 1777 and *Glaucilla* Bergh, 1860, with one species each (Macnae, 1954; Thompson & Bennett, 1970); however, based on morphological examination of material from the Pacific Ocean, Valdés & Angulo Campillo (2004) concluded that *Glaucilla* should be synonymized with *Glaucus*, which contains two valid species *Glaucus atlanticus* Forster, 1777 and *Glaucus marginatus* (Bergh, 1860) (Fig. 6-1). Notwithstanding its derived ecology, *Glaucus* is the type genus (and *G. atlanticus* the type species) of the large family Glaucidae. Glaucid synapomorphies include simple oral glands, and the distinctive morphologies of the feeding structures (jaws and sharp radular teeth) and reproductive systems (Miller, 1974), but family level aeolid relationships remain unresolved (Valdés & Angulo Campillo, 2004).

A recent multigene molecular phylogeny of glaucinins and benthic aeolids reveals the inadequacy of current aeolid taxonomy at the family level and, when combined with morphological data, within *Glaucus* (Churchill *et al.* unpubl.; Fig. 5-2). The Indo-Pacific *G. marginatus* is a cryptic complex of four distinct lineages: ((Indo-Pacific, South Pacific) (Eastern North Pacific, Kona)) (Fig. 5-2). In two of the lineages, South Pacific and Kona, a repeated change in reproductive morphology has occurred: the loss of the bursa copulatrix. Here, we first redescribe the phylogenetically informative glaucinin

reproductive system based on histology. We also examine the level of genetic differentiation within *Glaucus*. Based on these data, we conclude that all four *G. marginatus* cryptic lineages have speciated; thus, we redescribe *G. atlanticus*, *G. marginatus* (Indo-Pacific) and three new species within the *G. marginatus* complex.

Materials and methods

Source of specimens

A total of 143 glaucinin specimens are included in this study, from various sources: a global sampling of neustonic invertebrates collected over six years (2006-2012) (Churchill *et al.* unpubl.), donations by colleagues, or museum collections. Table 5-2 shows their respective locality data, museum catalog numbers, and GenBank accession numbers for sequences generated in this study. Figure 6-2 shows examined type material from the Zoological Museum of the University of Copenhagen, Denmark (ZMUC). Sequences used in the previously published molecular phylogeny are available online (Churchill *et al.* unpubl.). Glaucinins were initially fixed and preserved in 95% EtOH. All the specimens are deposited at one of the following institutions: University of Michigan Museum of Zoology (UMMZ), Australian Museum Sydney (AMS), Los Angeles County Museum of Natural History (LACM), the Department of Invertebrate Zoology and Geology, California Academy of Sciences, San Francisco (CASIZ), the Western Australia Museum (WAM), and the Natal Museum (NM).

Morphological examination and histology

A total of 45 specimens, representing *Glaucus atlanticus* (N=10) and all four molecular lineages of *G. marginatus* (N=10 for all lineages except Kona, N=5), were examined by either dissection or histology (Table 5-2). Reproductive systems were dissected as in Churchill *et al.* (unpubl.). A representative sampling of glaucinin radulae, jaws, and penial spines (*G. atlanticus* only) were dissected for scanning electron microscopy (SEM). For the feeding structures, the buccal bulb was dissected by making a dorsal incision above the anterior gastric cavity; the penis was dissected by making a dorsal incision on the right side of the body, posterior to the anterior peduncle. To isolate hard parts, tissue was digested in a 10% household bleach solution for about 30 minutes until only chitinous structures remained. Jaws, radulae, and penial spines were then rinsed with water, dried, mounted, and sputter coated for examination with a scanning electron microscope Hitachi S-3000N at the Natural History Museum of Los Angeles County.

DNA Extraction, PCR amplification, sequencing, and phylogenetic analysis

DNA extraction was performed as in Churchill *et al.* (unpubl.) using the E.Z.N.A. Mollusc DNA Kit (Omega-Biotek). Up to 50mg of pedal or ceratal tissue was cut into fine pieces before tissue digestion; thereafter the manufacturer's protocol was followed. PCR reactions were prepared as in Churchill *et al.* (unpubl) for four molecular markers: nuclear 28S rRNA and Histone H3, and mitochondrial 16S rRNA and cytochrome oxidase I (COI). The phylogenetic analysis was performed with the same parameters as in Churchill *et al.* (unpubl.).

Genetic distances

Genetic distances for the 16S and COI haplotypes of the *Glaucus marginatus* morphospecies was calculated in Arlequin 3.5.12 (Excoffier & Lischer 2010). Samples were allocated to populations according to their geographical distribution and position in the phylogenetic tree. The following four sample classes were defined: Indo-Pacific, South Pacific, Eastern North Pacific, and Central North Pacific. All codon positions for mt COI were considered for the analysis.

Results

Redescription of glaucinin reproductive system

Proximal reproductive system: Glaucinids are simultaneous hermaphrodites. The gonad is located on the right side of the body, immediately posterior to the gastric cavity. Unlike Thompson and McFarlane (1967), we did not observe a mediolateral gradient of male and female acini, respectively. In all specimens examined here, smaller female acini are interspersed between larger male acini. The former contain few numbers of oocytes at both previtellogenic and vitellogenic developmental stages, while the latter contain bunches of spermatozoa, most of which are oriented with their heads toward the acinar walls (Fig. 6-3). Ductules within the gonad transport female and male gametes to the hermaphroditic duct, which extends to the distal reproductive system

Distal reproductive system: Glaucinins have diallic distal reproductive systems. The hermaphroditic duct connects to the ampulla, a long muscular tube that appears very convoluted when empty. The ampulla branches into the male and female reproductive tracts. On the male side, a long, thin, convoluted prostate connects to the protrusible penis.

On the female side, a series of blind reproductive organs connect with the oviduct. The most proximal is a receptaculum seminis, originally observed by Thompson and McFarlane (1967) in *Glaucus atlanticus*, which is often impossible to view in dissections of smaller specimens. It is shown here in histological sections only (Fig. 6-3). The receptaculum is oblong, fluid-filled, and contains oriented spermatozoa. In some specimens it appears dark purple or black. Fertilization occurs in the oviduct around and distal to the receptaculum seminis. Zygotes continue distally into the female gland complex, which consists of membrane, mucus, and capsule glands as described in other aeolids (Klussmann-Kolb, 2001). Finally, the oviduct terminates at the vaginal opening, which is located posterior to the penial opening, inside the gonopore. In *G. atlanticus* and two *G. marginatus* morphospecies lineages (Indo-Pacific & Eastern North Pacific) a blind bursa copulatrix branches off the oviduct immediately proximal to the vaginal opening; no bursa copulatrix is present in the other two *G. marginatus* morphospecies (South Pacific & Kona) (Figs. 5-3, 5-4).

Genetic distances

Pairwise genetic distance values are summarized in Table 6-1. All distances among clades were > 10% for mt COI and > 2% for mt 16S. These distances are all above those suggested for species delimiting in mollusks (Davison *et al.*, 2009; Malaquias & Reid, 2009) and particularly in nudibranchs (Pola *et al.*, 2012).

Systematics

Family GLAUCIDAE

Genus *Glaucus* Forster, 1777

Type species. Glaucus atlanticus Forster, 1777

Glaucus atlanticus Forster, 1777: Fig. 6-4.

Material examined. **UMMZ:** 302975-302983; **AMS:** C.462956; **WAM:** S59354; **NM:** W7469; **LACM** 111410.

Diagnosis. This species can be distinguished by uniseriate ceratal arrangement, presence of a penial spine, and by the presence of a longitudinal, medial silver stripe on the sole of the foot.

Description. External anatomy. Body slender and elongate. Head short and not distinctly separated from body. Cephalic tentacles and rhinophores short and smooth. Dorsal coloration white with silver reflective pigment. Ventral coloration blue and brownish on the lateral foot sole, with a medial longitudinal silver stripe. Lateral sides of the body colored silver. Large, broad foot with rounded anterior edges. Four clusters of cerata on each side of the body. Three anterior ceratal groups pedunculate, posterior group sessile. Cerata conical, multiseriate, bluish silver at base, dark blue at tips. Cleioproct anus, situated between tops of second and third ceratal groups. Renal pore at the same level, in front of second ceratal group. *Internal anatomy.* Diaulic reproductive system as described above. Penis is muscular and possesses a spine, a single strong curved and hollow hook. The vagina connects to a small bursa copulatrix.

Informal clade “Marginatus”

Remarks. We suggest the informal clade name, “Marginatus,” for the cryptic species complex of the *Glaucus marginatus* morphospecies. *Glaucus marginatus* is both the oldest name for this morphospecies, and the cryptic lineage with the widest range (Indo-Pacific).

Glaucus marginatus (Bergh, 1864); Indo-Pacific lineage A: Figs. 6-5, 6-6

Holotype. *Glaucilla marginata* (Bergh, 1864), ZMUC GAS-2158.

Material examined. **UMMZ:** 302984-302987.

Diagnosis. This species can be distinguished geographically (if collected in the Indian Ocean), or phylogenetically by membership in the Indo-Pacific clade. It differs morphologically from its sister species, *Glaucus bennettiae*, in having a bursa copulatrix.

Description. External anatomy. Body elongate but relatively wider than *G. atlanticus*, widest behind the head. Cephalic tentacles and rhinophores short and smooth. Dorsal coloration white with silver reflective pigment. Ventral coloration blue and brownish on the foot sole, white with silver reflective pigment around foot. Large, broad foot with rounded anterior edges. Four clusters of cerata on each side of the body. Anterior ceratal group angled toward head, three posterior groups perpendicular to midline of the body. Two anterior ceratal groups pedunculate, two posterior groups sessile and merged. Cerata conical, multiseriate, blue at base and white or transparent at tips. Cleioproct anus, situated between tops of second and third ceratal groups. Renal pore at the same level, in front of second ceratal group.

Internal anatomy. Diaulic reproductive system as described above. Penis is muscular, lobed, and devoid of a spine. The vagina connects to a small bursa copulatrix.

Radular formula 15 x 0.1.0 in a 6 mm long specimen (sample code 8GMnpko) and in a 4 mm long specimen (sample code 22GMNPKo). Radular teeth elongate with a robust, conical central cusp, flanked by 3-9 denticles on each side. Denticles elongate and slightly curved with a sharp tip. Some specimens with small denticles on one of the sides of the central cusp. Masticatory border of the jaw with a single row of denticles. Denticles simple, conical with one or two small tubercles on some of them.

Glaucus bennettiae sp. n.; South Pacific lineage B: Figs. 6-7—6-8

Holotype. UMMZ 400001 (Fig. 6-7A)

Locality. South Pacific subtropical gyre system

Etymology. Named after the marine biologist and Australian Academy of Science member Isobel Bennett (1909-2008), who wrote the first monograph on Australian plankton, and observed Australian glaucnins (Thompson & Bennett, 1970).

Other material examined. AMS C.462957 (holotype is from this lot).

Diagnosis. This species can be distinguished phylogenetically by membership in the South Pacific clade and differs morphologically from its sister species, *Glaucus bennettiae*, by lacking a bursa copulatrix.

Description. External anatomy. External anatomy identical to *G. marginatus*.

Internal anatomy. Diaulic reproductive system as described above. Penis is muscular, lobed, and devoid of a spine. No bursa copulatrix present; the vagina connects proximally to the oviduct. Radular formula 19 x 0.1.0 in a 14 mm long specimen (AMS C.462957). Radular teeth elongate with a conical, relatively short and robust central cusp, flanked by 4-9 denticles on each side. Denticles elongate and slightly curved. Masticatory border of the jaw with a single row of denticles. Denticles short, and widen covered by a number of small tubercles.

Glaucus thompsoni sp. n.; Eastern N. Pacific lineage C: Figs. 6-7, 6-10, 6-11

Holotype. UMMZ 400003 (Fig. 6-7B)

Locality. Northern Pacific subtropical gyre system

Etymology. Named after Thomas E. Thompson, the prolific nudibranch biologist who coauthored several papers on glaucinins (e.g. Thompson & Bennett, 1970; Thompson & McFarlane, 1967).

Other material examined. UMMZ 302988

Diagnosis. This species can be distinguished phylogenetically by its membership in the Eastern North Pacific clade and morphologically from its sister species, *Glaucus mcfarlanei* sp. n., by the presence of a bursa copulatrix.

Description. External anatomy. External anatomy identical to *G. marginatus*.

Internal anatomy. Diaulic reproductive system as described above. Penis is muscular, lobed, and devoid of a spine. The vagina connects to a small bursa copulatrix.

Radular formula 13 x 0.1.0 in a 4 mm long specimen (UMMZ 302988). Radular teeth elongate with a conical, very long, delicate central cusp, flanked by 4-6 denticles on each side. Denticles elongate and slightly curved, with a sharp apex. Masticatory border of the jaw with a single row of denticles. Denticles conical, elongate, some of them bearing small tubercles.

Glaucus mcfarlanei sp. n.; Kona lineage D: Figs. 6-7, 6-12, 6-13

Holotype. UMMZ 400005 (Fig. 6-7C)

Locality. North Pacific subtropical gyre system

Etymology. Named after Ian McFarlane, who co-authored the first histological study of a glaucinin (Thompson & McFarlane, 1967).

Other material examined. UMMZ 302990

Diagnosis. This species can be distinguished phylogenetically by its membership in the Kona clade and morphologically from its sister species, *Glaucus thompsoni* sp. n., by the absence of a bursa copulatrix.

Description. External anatomy. External anatomy identical to *G. marginatus*.

Internal anatomy. Diallic reproductive system as described above. Penis is muscular, lobed, and devoid of a spine. No bursa copulatrix present; the vagina connects proximally to the oviduct. Radular formula 16 x 0.1.0 in a 3 mm long specimen (UMMZ 302990). Radular teeth elongate with a conical, very long, delicate central cusp, flanked by 5-7 denticles on each side. Denticles elongate and slightly curved, with a sharp apex.

Masticatory border of the jaw with a single row of denticles. Denticles short, rounded to conical, lacking small tubercles.

Discussion

Nomenclatural history and species authorships

The first descriptions of glaucinin nudibranchs are pre-Linnean, non-binominal works, and therefore names introduced in those publications are not available. Bergh (1864) and Thompson & McFarlane (1967) provided an account and discussion of those early descriptions. Forster (1777) provided the first binominal description of a glaucinin nudibranch, *Glaucus atlanticus* Forster, 1777. Russell (1971) and McDonald (2006) considered Forster's description to be a *nomen dubium* and assigned the authorship of *Glaucus* to Gmelin (1791), who based the genus name on the species *Doris radiata* d'Orbigny, 1839. Forster (1777) described *Glaucus atlanticus* as follows: "We had also at various intervals, found the sea [North Atlantic] covered with animals belonging to the class of *mollusca*, one of which, of a blue colour, in shape like a snail, with four arms, divided into many branches, was named *glaucus atlanticus*; ..." This description unequivocally refers to the species recognized in this paper as *Glaucus atlanticus*, as all other recognized species are found in the Pacific and Indian Oceans. Therefore we agree with the current usage of the names *Glaucus* and *Glaucus atlanticus* in recent publications (Valdés & Angulo Campillo 2004, Churchill *et al.* unpubl.).

The original publication and authorship of *Glaucilla marginata* and *G. briareus* has also been the subject of confusion. Bergh (1860) introduced these two names for the first time (under Reinhardt's authorship), but did not provide a description of the animals;

instead he provided descriptions of the ingested, exogenous nematocysts. In a footnote in the same paper, Bergh (1860) introduced the genus name *Glaucilla* and provided a description and a comparison with *Glaucus*. Jensen (personal communication) argued that Bergh's (1860) description of *G. marginata* and *G. briareus* are *nomina nuda* because the paper contains no descriptions of the species. We agree with Jensen that Bergh's (1860) species descriptions do not meet the criteria for a valid species description under Article 12 of the Code of Zoological Nomenclature (ICZN, 1999), as no descriptions or illustrations of the animals, or indications are provided. In a subsequent paper (Bergh, 1864) provided descriptions and illustrations of both species (based on manuscript notes from Reinhardt). We consider Bergh (1864) to be the original publication of the two species, while the original publication of the genus name *Glaucilla* remains Bergh, 1860. The correct authorship of the two species names should then be *Glaucilla marginata* Reinhardt in Bergh, 1864 and *G. briareus* Reinhardt in Bergh, 1864. Bergh's (1864) paper is often erroneously cited as published in 1868 (Thompson & McFarlane, 1967; Valdés & Angulo Campillo, 2004).

Validity of previously described species

The genus name *Glaucilla* has been used for the two traditionally Pacific species with multiseriate cerata, *G. marginata* and *G. briareus* (Thompson & McFarlane, 1967). Bergh (1864) proposed that *G. marginata* was a north Pacific species, whereas *G. briareus* was found in the south Pacific. Thompson & Bennett (1970) synonymized *G. briareus* with *G. marginata* and Valdés & Angulo Campillo (2004) synonymized *Glaucilla* with *Glaucus*. The most recent published opinion on the taxonomy of this

group (Valdés & Angulo Campillo, 2004) is that there is only one valid genus of glaucinin nudibranchs with two species, the cosmopolitan *Glaucus atlanticus* and the Pacific *Glaucus marginatus*.

However, the conclusion of the present study is that four cryptic species with external morphological characteristics of *Glaucus marginatus* exist in the Indo-Pacific. Since there are two available names for these four Indo-Pacific species, it is important to determine whether these names can be assigned with confidence to any of the species recognized with the molecular information.

Reinhardt in Bergh (1864) described *Glaucus marginatus* in great detail and included descriptions and illustrations of the reproductive anatomy and the radula. We had access to the type material and dissected one of the syntypes. The dissected syntype has a bursa copulatrix and the rest of the reproductive anatomy is consistent with that of the most widespread species recognized with molecular data. Additionally Bergh's (1864) illustrations of the radula (Fig. 18 in Bergh, 1864) and reproductive system (Fig. 24 in Bergh, 1864) as consistent with those of that species. Therefore, we can confidently assign the name *G. marginatus* to this species.

In the case of *Glaucilla briareus*, it is impossible to determine the identity of this species. Although the holotype of this species still exists (Fig. 6-2) we could not obtain permission to dissect it. Also Reinhardt in Bergh (1864) did not provide descriptions of the internal anatomy of the species and the external morphology is not distinctive enough to recognize this species. The type locality of *G. briareus* is north of Juan Fernandez, an area in which three of the cryptic species described in the present paper could potentially be found. Therefore we consider *G. briareus* to be a *nomen dubium*.

Implications for future aeolid systematics

Aeolidacea has been an extremely difficult group to resolve systematically (Gosliner, 2011), and a full review is out of the scope of this study; however, one critical hindrance has been the lack of readily-amplifiable nuclear molecular markers that are phylogenetically informative at the family level. We suggest Churchill *et al.*'s (unpubl.) protocol for 28S rRNA as potentially useful in resolving within-aeolid relationships. In that study, the molecular phylogeny does not support the monophyly of the aeolid families Glaucidae and Aeolidiidae; members of both families are interspersed throughout a well-supported clade (Churchill *et al.*, unpubl.).

Finally, considering the repeated cryptic speciation within the *Glaucus marginatus* complex vs. the panmictic *G. atlanticus*, an obvious question is: Why hasn't speciation occurred within *G. atlanticus*? If speciation in the *G. marginatus* complex is related to reproductive morphology, then any functional differences between the reproductive systems of *G. atlanticus* and the *G. marginatus* species complex could help explain their disparate rates of speciation. The major reproductive difference between the two glaucinin morphospecies is the presence of a penial spine in *G. atlanticus* (Fig. 6-4 D, E; Valdés & Angulo Campillo, 2004); in the *G. marginatus* species complex the penis is unarmed (Valdés & Angulo Campillo, 2004). Miller (1974) considered the penial spine in *G. atlanticus* an adaptation for literally hooking individuals together in an unstable environment (the neuston) and between individuals with lateral, pedunculate cerata (a unique condition among aeolids). A single study of mating behavior in *G. atlanticus* vs. *G. marginatus* morphospecies individuals (unidentified South Pacific lineage) showed

significantly longer copulation times in *G. atlanticus* (43-59 minutes vs. 50-70 seconds; Ross & Quetin, 1990). We hypothesize that the penial spine, when present, primarily governs the mechanics of penial interaction during mating. In the *G. marginatus* morphospecies, the penis is unarmed, and thus the presence or absence of a bursa copulatrix immediately proximal to the gonopore might have a much greater effect on mating behavior and penial interaction. Aeolids have a variety of different forms of penial spines (Miller, 1974, described nine types in facelininds), and their presence or absence varies within genera. Comparing species diversity levels between taxa with or without penial spines would be one potential way to test our hypothesis of the effect of the spine on nudibranch mating.

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Figure 6-1. Live photos of two glaucinin morphospecies. A. *Glaucus atlanticus*; cosmopolitan range. **B.** *Glaucus marginatus* (unknown S. Pacific lineage); Indo-Pacific range. Scale bars = 1.0 cm. Photos by Denis Riek.

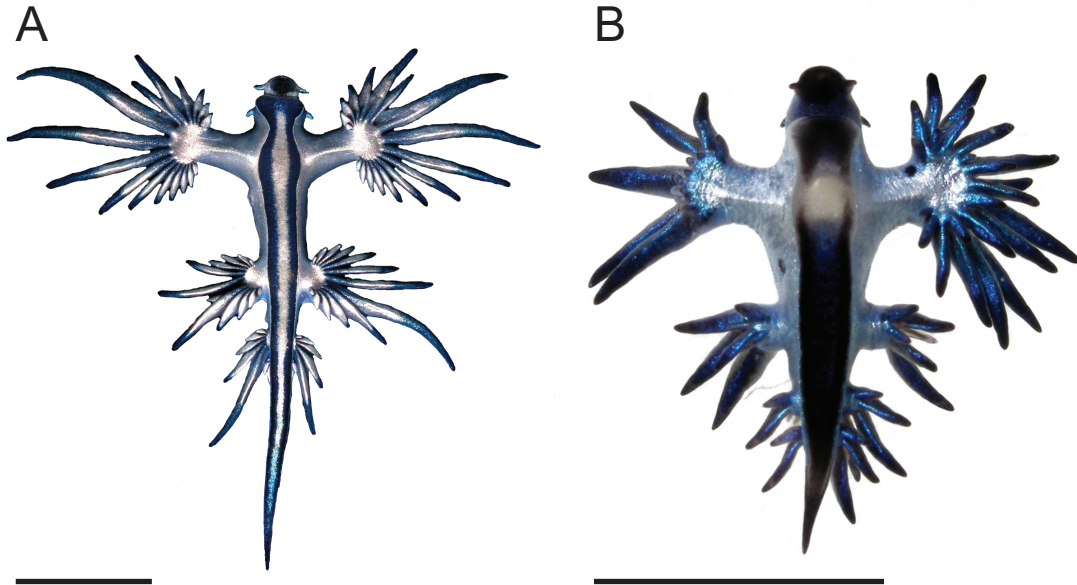


Figure 6-2. Photographs of preserved glaucinin types housed at the Zoological Museum of the University of Copenhagen (ZMUC) gastropod collection.

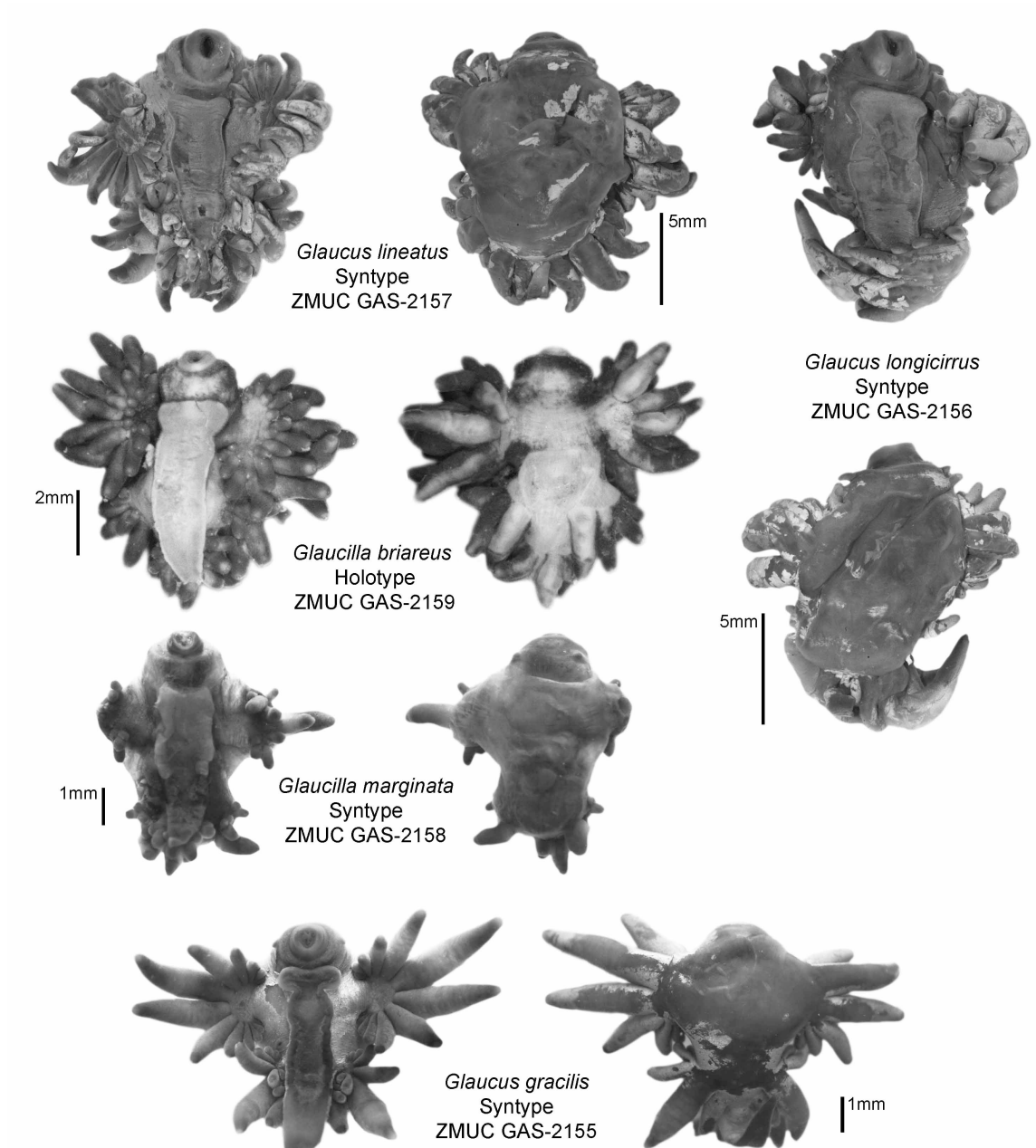


Figure 6-3. Histology of the glaucinid reproductive system emphasizing undescribed or under-described features. A. *Glaucus atlanticus*, AMS C.462956, transverse section. View of the entire reproductive system from composite slide photographs. The proximal ovotestis (ot) is composed of evenly dispersed male and female acini. The receptaculum seminis is visible as a large organ distal to the ampulla. **B.** *G. marginatus*, AMS C.462957, transverse section. View of the entire reproductive system from composite slide photographs. Ovotestis resembles *G. atlanticus* in acinar distribution. **C.** *G. atlanticus*, AMS C.462956, transverse section. Detail of receptaculum seminis containing oriented sperm. **D.** *G. marginatus*, AMS C.462957, sagittal section. Detail of ovotestis (ot) showing interspersed male (ma) and female (fa) acini. **Abbreviations:** am, ampulla; fa, female acinus; fgc, female gland complex; ma, male acinus; os; oriented sperm; ot, ovotestis; ov, oviduct; pn, penis; rs, receptaculum seminis. **Scale bars:** A, B, D = 1.0 mm; C = 500 μ m.

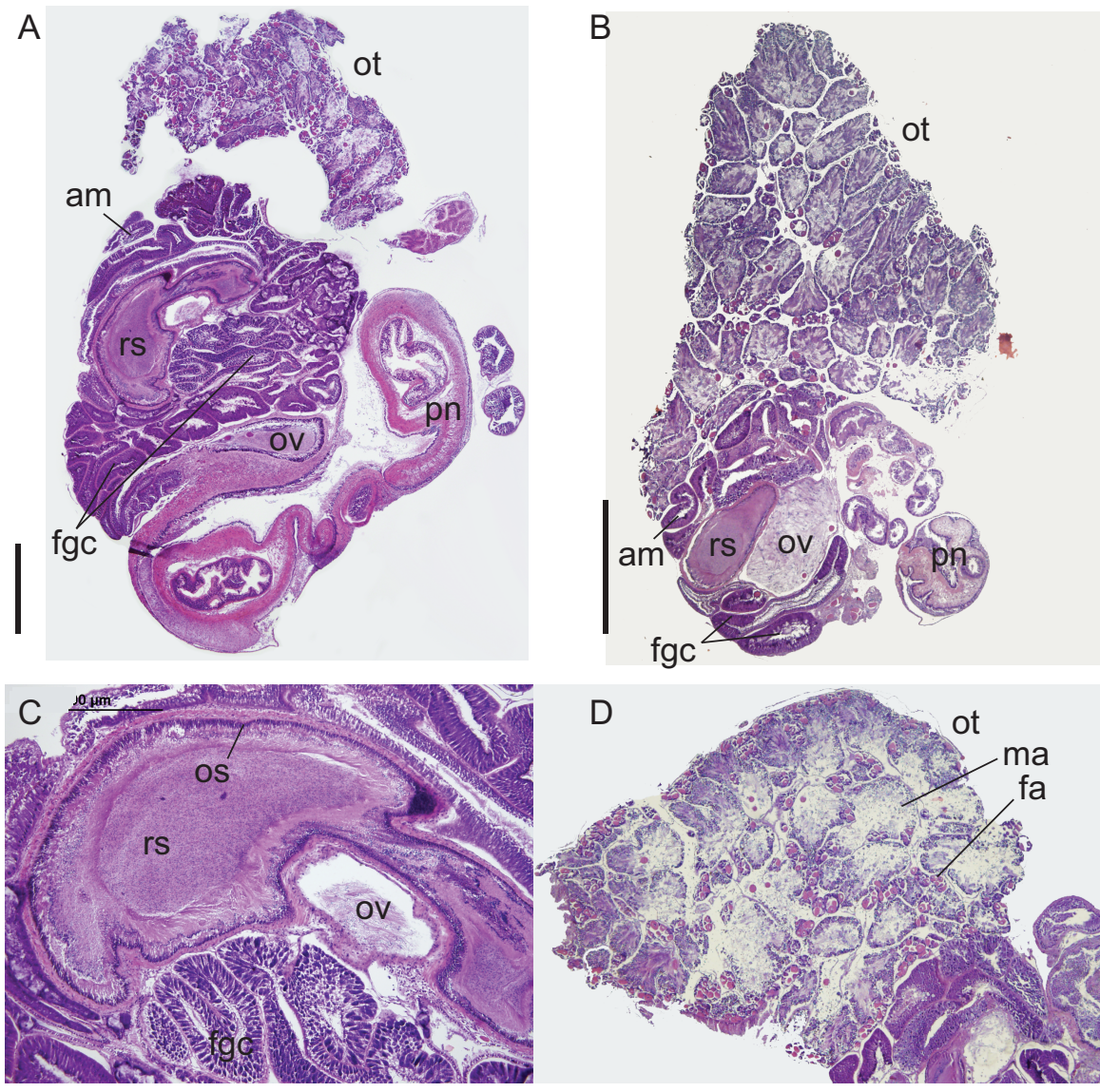
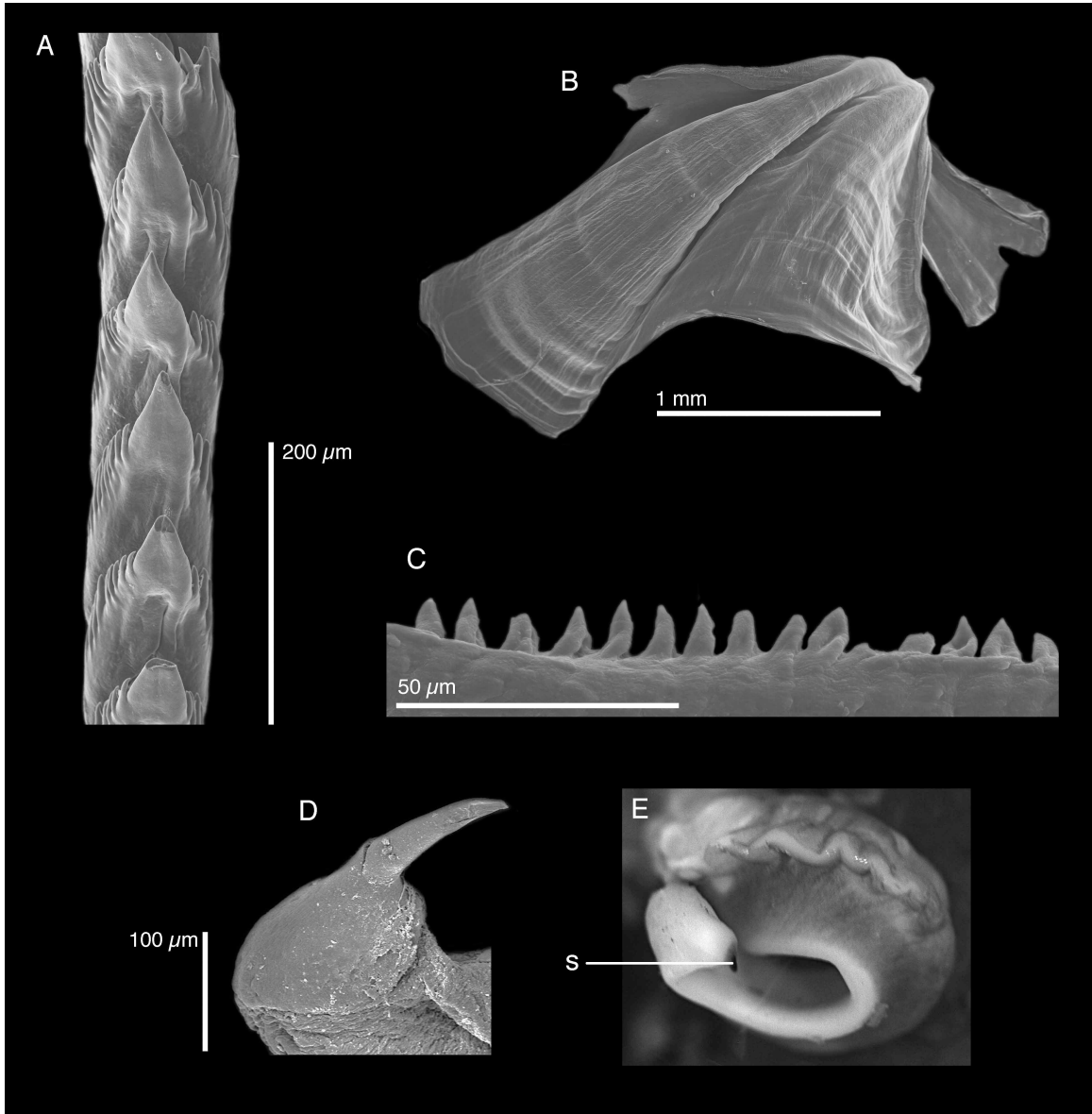
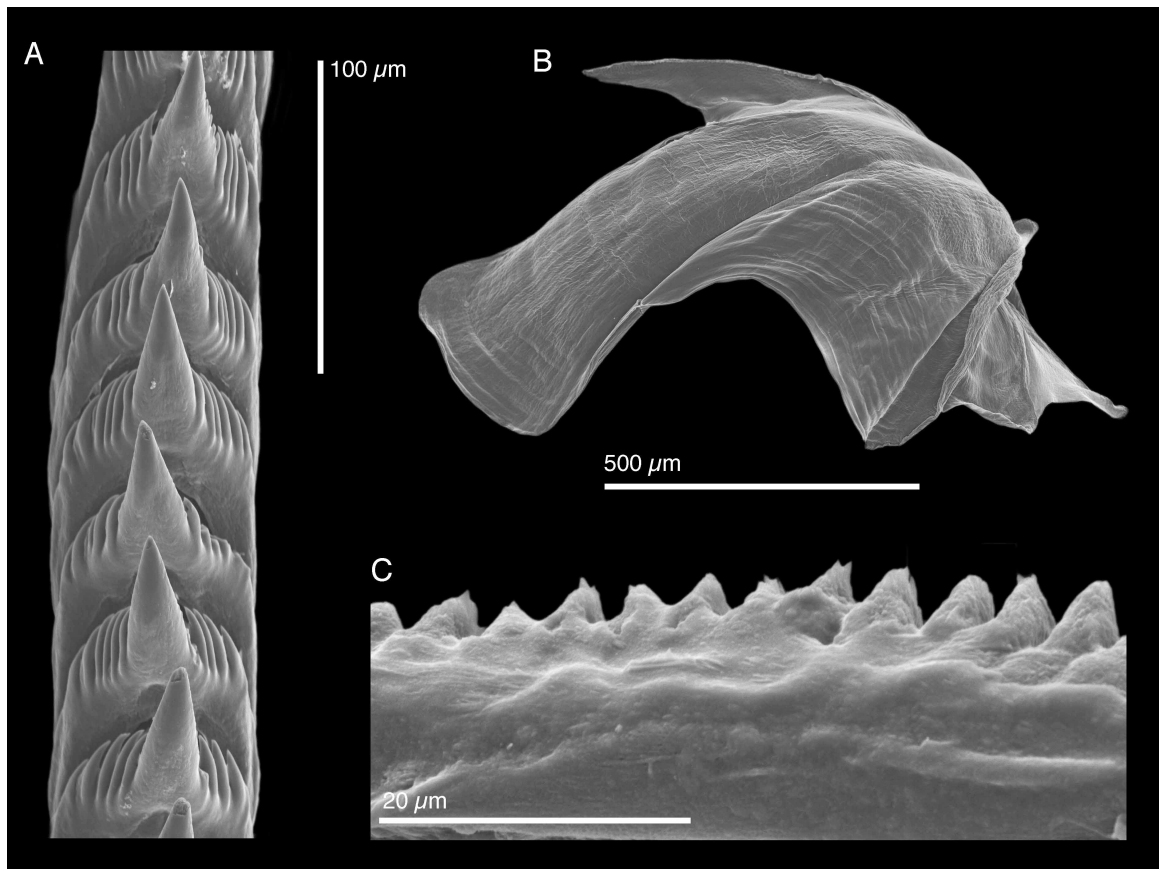


Figure 6-4. Chitinous feeding and reproductive structures of *Glaucus atlanticus* Forster, 1777.



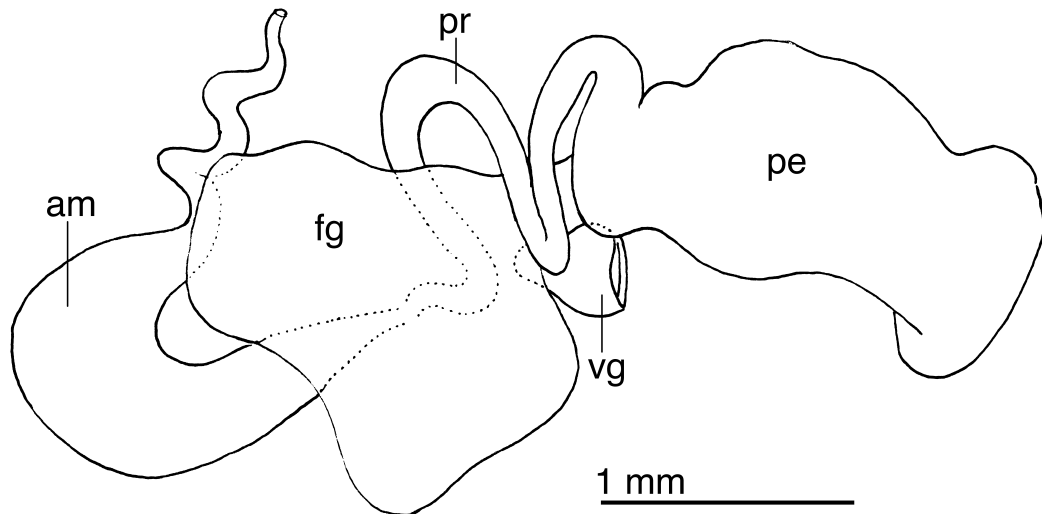
A. Scanning electron micrograph (SEM) of the radular teeth of specimen AMS C.462956. **B.** SEM of the jaw of specimen AMS C.462956. **C.** SEM of the masticatory border of the jaw of specimen AMS C.462956. **D.** SEM of the penial stylet of specimen LACM 111410. **E.** Photograph of the everted penis in preserved specimen AMS C.462956 showing the stylet (s) on the tip.

Figure 6-5. Scanning electron micrographs of the chitinous feeding structures of *Glaucus marginatus* (Bergh, 1864).



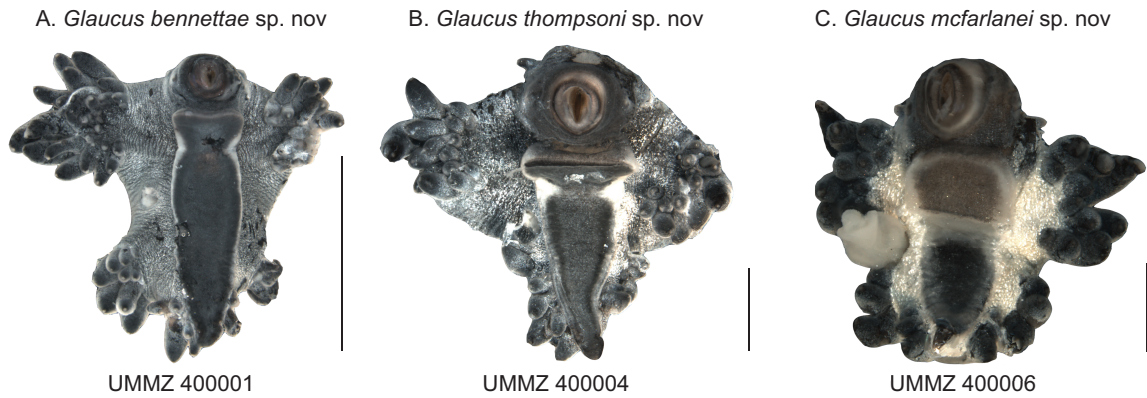
A. Radula of specimen UMMZ 302985. **B.** Jaw of specimen UMMZ 302985.
Radula of specimen UMMZ 302985. **C.** Masticatory border of the jaw of specimen UMMZ 302985.

Figure 6-6. Anatomical drawing of the distal reproductive system of *Glaucus marginatus* (Bergh, 1864); UMMZ 302985.



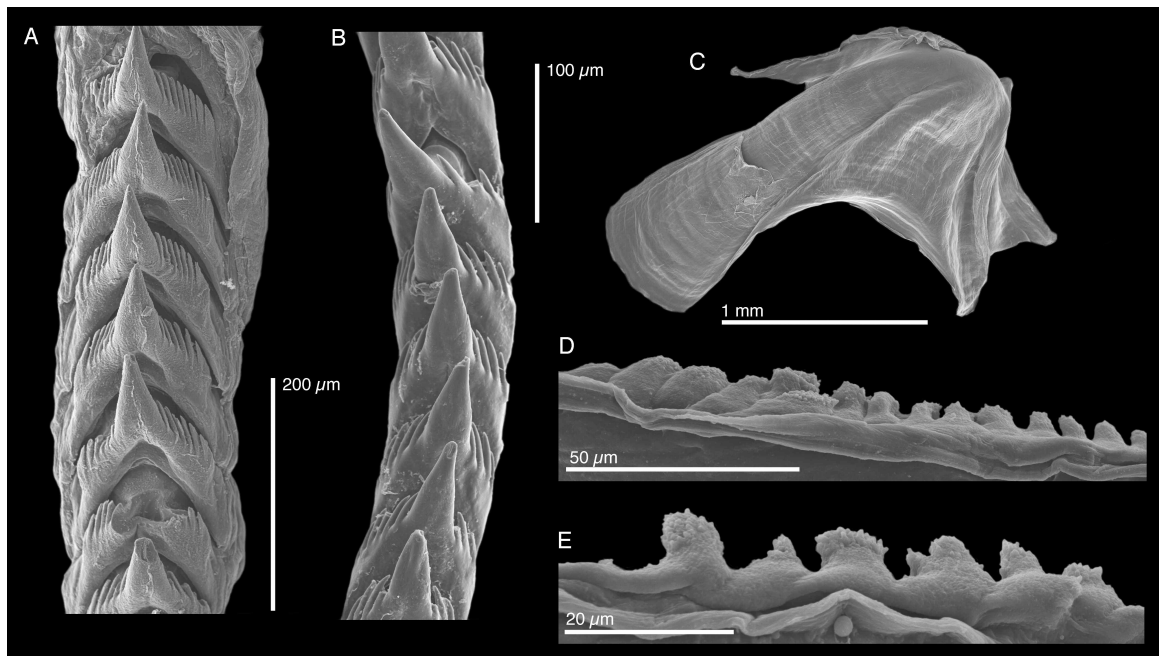
Abbreviations: am, ampulla; ag, accessory gland; fg, female gland complex; pe, penis; pr, prostate.

Figure 6-7. Photographs of the preserved holotypes of three new species of glaucinin nudibranchs (ventral views).



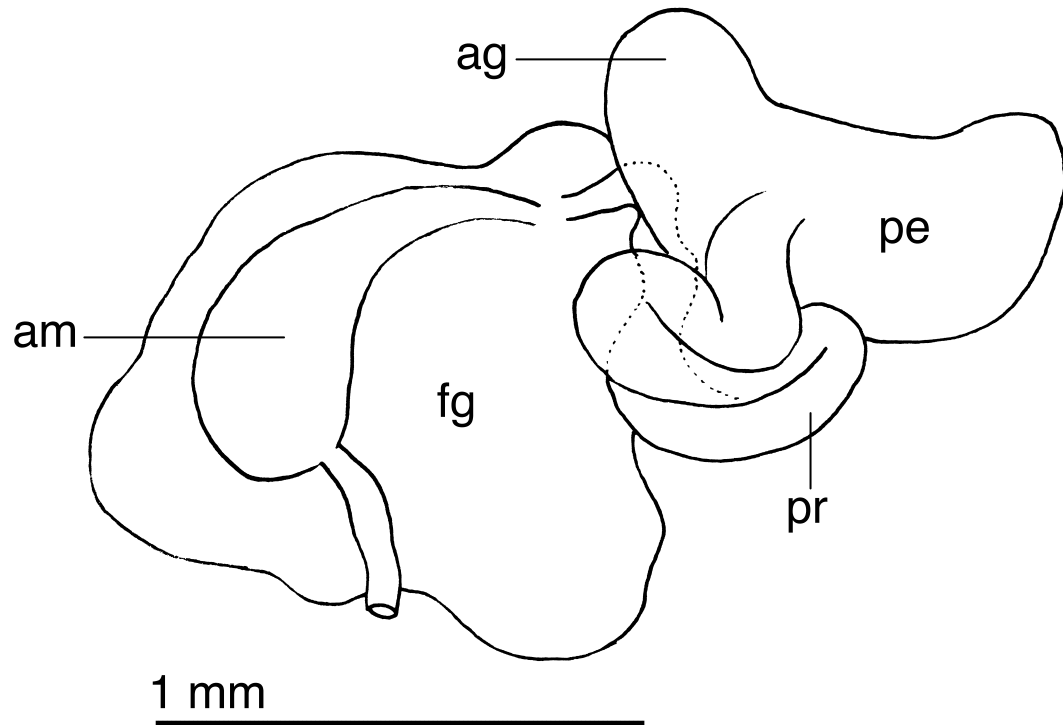
A. *Glaucus bennettiae*, sp. nov.; UMMZ 400001 **B.** *Glaucus thompsoni*, sp. nov.; UMMZ 400003 **C.** *Glaucus mcfarlanei*, sp. nov.; UMMZ 400005. **Scale bars:** A = 5.0 mm; B, C = 1.0 mm.

Figure 6-8. Scanning electron micrographs of the chitinous feeding structures of *Glaucus bennettiae* sp. nov.



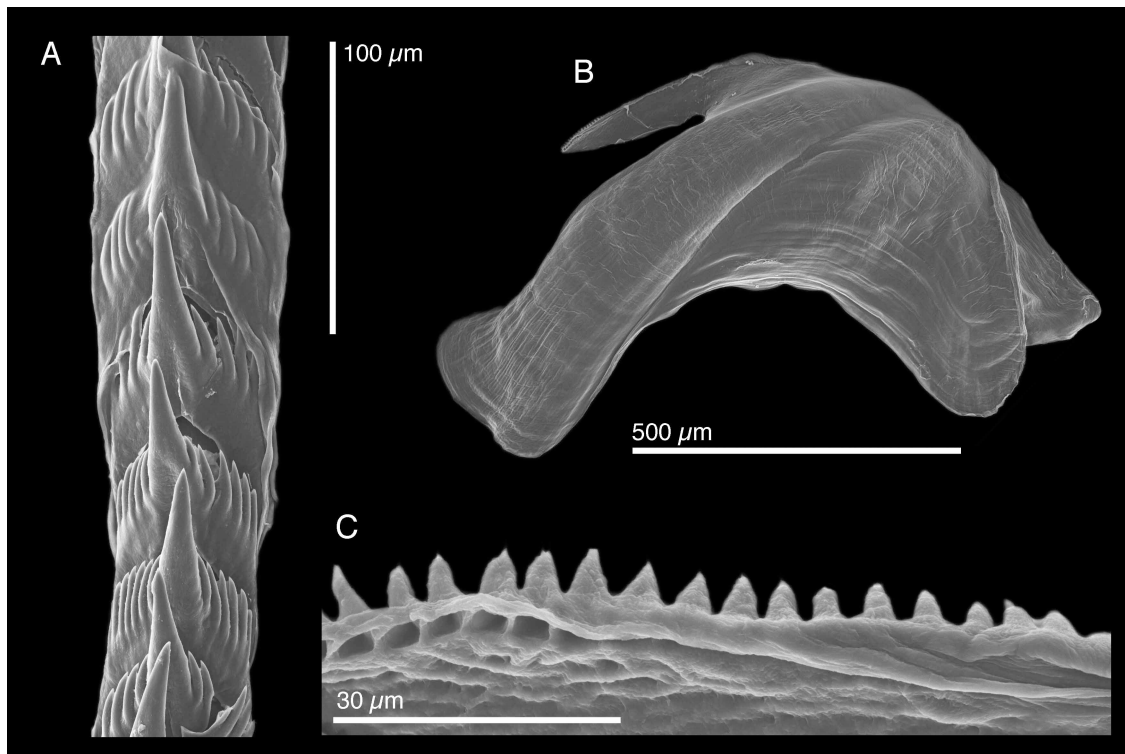
A. Radula of specimen AMS C.462957. **B.** Radula of specimen AMS C.462957. **C.** Jaw of specimen AMS C.462957. **D.** Masticatory border of the jaw of specimen AMS C.462957. **E.** Masticatory border of the jaw of specimen AMS C.462957.

Figure 6-9. Anatomical drawing of the distal reproductive system of *Glaucus bennettiae* sp. nov.; AMS C.462957.



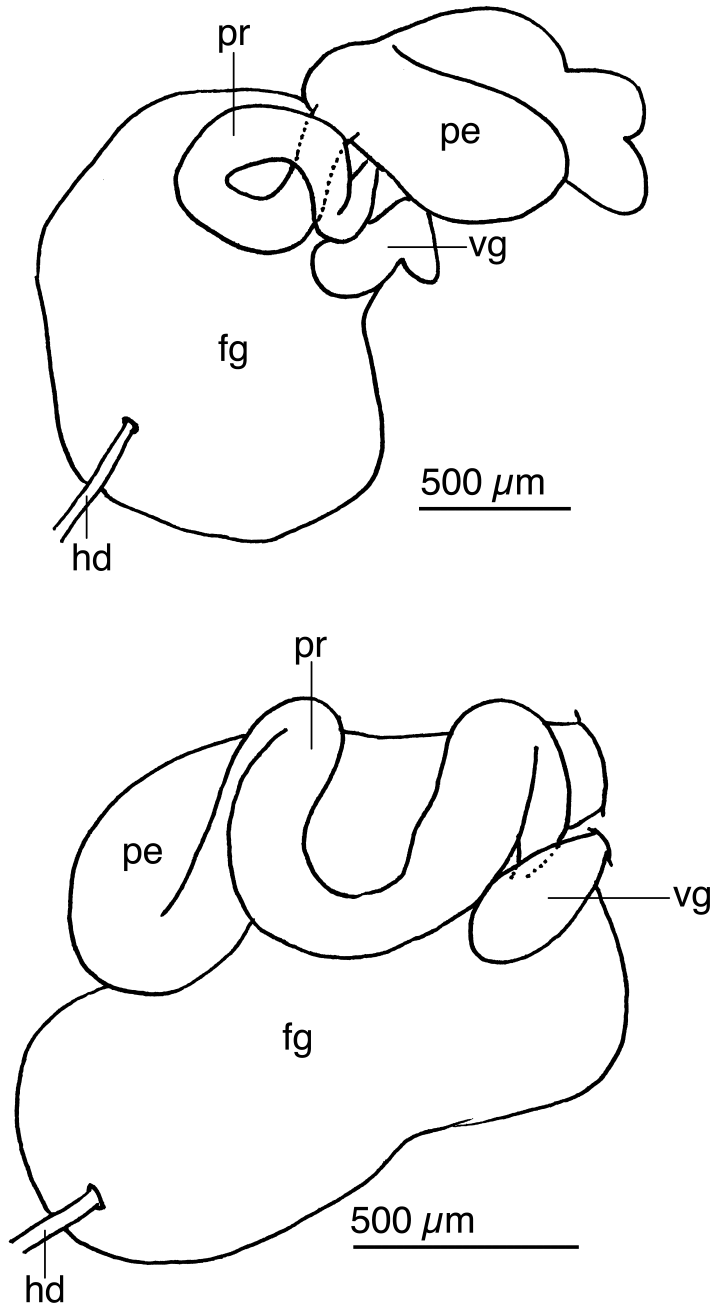
Abbreviations: am, ampulla; ag, accessory gland; fg, female gland complex; pe, penis; pr, prostate.

Figure 6-10. Scanning electron micrographs of the chitinous feeding structures of *Glaucus thompsoni* sp. nov.



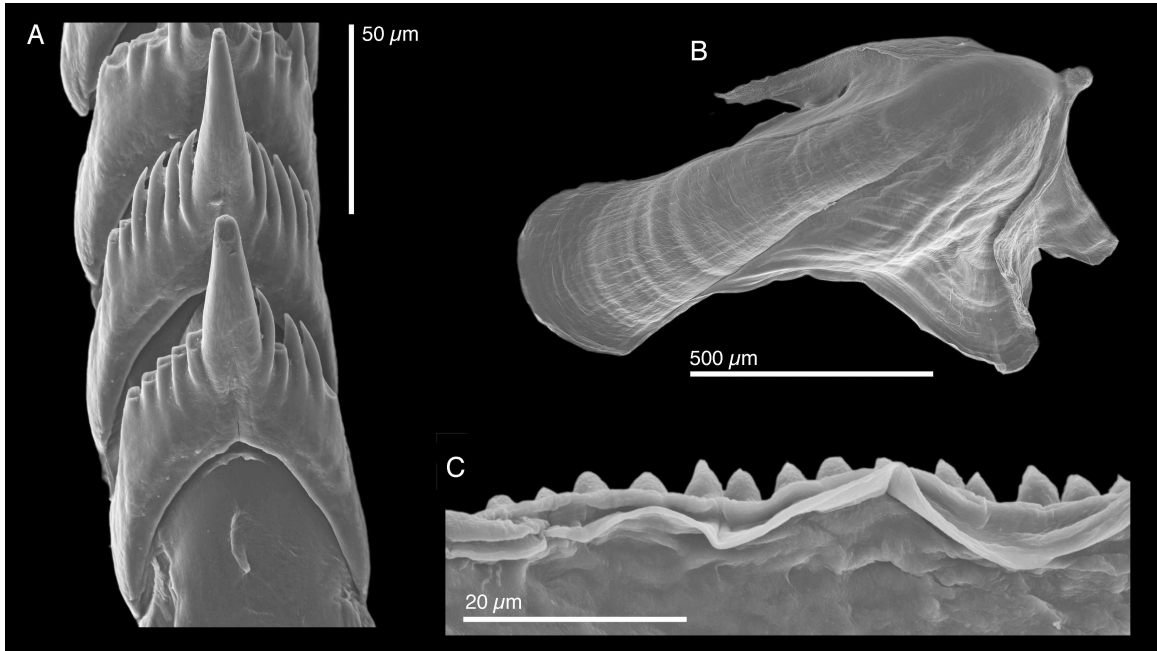
A. Radula of specimen UMMZ 302988. B. Jaw of specimen UMMZ 302988. C. Masticatory border of jaw of specimen UMMZ 302988.

Figure 6-11. Anatomical drawings of the distal reproductive system of two specimens of *Glaucus thompsoni* sp. nov.; UMMZ 302988.



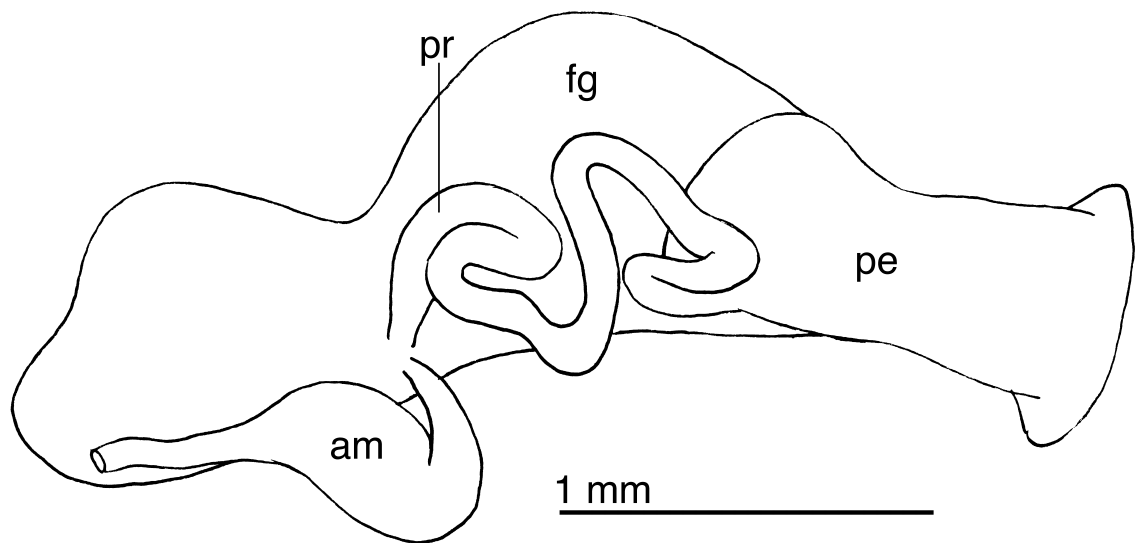
Abbreviations: fg, female gland complex; hd, hermaphroditic duct; pe, penis; pr, prostate; vg, vagina.

Figure 6-12. Scanning electron micrographs of the chitinous feeding structures of *Glaucus mcfarlanei* sp. nov.



A. Radula of specimen UMMZ 302990. B. Jaw of specimen UMMZ 302990. C. Masticatory border of the jaw of specimen UMMZ 302990.

Figure 6-13. Anatomical drawing of the distal reproductive system *Glaucus mcfarlanei* sp. nov.; UMMZ 302990.



Abbreviations: am, ampulla; fg, female gland complex; pe, penis; pr, prostate.

Table 6-1. Matrix of pairwise genetic distance percentages between *Glaucus marginatus* morphospecies for mitochondrial 16S rRNA (upper half triangle) and COI (lower half triangle).

	<i>G. marginatus</i> (Indo-Pacific)	<i>G. bennettiae</i> (South Pacific)	<i>G. thompsoni</i> (Eastern N. Pacific)	<i>G. mcfarlanei</i> (Central N. Pacific)
<i>G. marginatus</i> (Indo-Pacific)	-	2	3	5
<i>G. bennettiae</i> (South Pacific)	11	-	3	5
<i>G. thompsoni</i> (Eastern N. Pacific)	19	17	-	4
<i>G. mcfarlanei</i> (Central N. Pacific)	22	24	15	-

Chapter 7

Mitochondrial phylogeny of three cosmopolitan neustonic invertebrates

Introduction

It has been over half a century since Ernst Mayr (1954) applied terrestrial principles of geographic speciation to marine animals, and whether he was justified in doing so is still a contentious issue in evolutionary biology (Krug, 2011). Marine fauna have large population sizes, broad ranges, high dispersal, and high fecundity (Palumbi, 1992), and decades of research on marine speciation support a complex combination of geographic and biological barriers arising over vast distances; in pelagic species, especially, sampling logistics have impeded work on a global scale (Norris, 2000; Norris & Hull, 2012). Recently, phylogeographic meta-analyses of coastal marine fauna (Hellberg, 2009; Kelly & Palumbi, 2009; Dugan et al., 2010) have explored some of the first emergent properties of population connectivity in marine systems, *e. g.*, biogeographic breaks (reviewed in Carpenter et al., 2011), and modal spatial scales of exchange for multiple co-occurring species (Cowen & Sponaugle, 2009). These studies, for the most part, omit holopelagic taxa with the largest ranges and most dynamic habitats. Are populations of pelagic taxa connected in a fundamentally different way than their coastal, benthic counterparts?

This study addresses this question by focusing on a subset of pelagic taxa: three cosmopolitan members of the marine neuston, a community of passively drifting invertebrates at the surface of the planet's subtropical oceans (Marshall & Burchardt, 2005). *Velella velella* and *Porpita porpita* are monotypic sister species of porpitid cnidarians. They float using a chitinized "endoskeleton" of gas-filled chambers, and they gain nutrition from endosymbiotic zooxanthellae housed within their gastric cavities and from prey capture of zooplankton (Hyman, 1940). They are preyed upon by the third species investigated here, *Glaucus atlanticus*, a neustonic nudibranch that floats by storing gulped air inside its muscular stomach (Lalli & Gilmer, 1989).

The predominant view over the past century has been that pelagic marine taxa typically occupy vast, genetically interconnected, geographic ranges in an essentially homogeneous environment, for example, the ubiquity hypothesis for microbial eukaryotic plankton (Finlay, 2002), thereby posing severe problems for allopatric speciation models (Palumbi, 1994; Dawson & Hamner, 2008). Population genetic data for some pelagic microorganisms are broadly consistent with this view (de Vargas et al., 1999; Darling, et al., 2000; Bucklin et al., 2003; Taniguchi et al., 2004; Ely et al., 2005; Goetze, 2005). However, other pelagic microorganisms show evidence of either subtle (Cowen et al., 2007), or pronounced (de Vargas, 1999; Darling et al., 2000; Goetze, 2003, 2005; Selje et al., 2004; Dawson et al., 2011) genetic structuring within and among ocean basins and subtropical ocean gyres (Fig. 1-1). These new data are providing a much more nuanced understanding of pelagic diversification processes and it is now clear that allopatric speciation is common among subsets of marine pelagic taxa (Dawson & Hamner, 2008). A particularly interesting aspect of these new data is that within- and among-basin water

mass boundaries may be differentially permeable to particular taxa, including sister taxa (Goetze, 2005), due to variation in details of ecology and life history, as predicted by Gaylord & Gaines (2000) dispersal models.

What about genetic differentiation and speciation in the neuston? To what degree have these trophically-coupled lineages cnidarians and mollusks experienced a shared history of gene flow and cladogenesis across the five subtropical gyres? Here, three general biogeographic hypotheses are tested for each taxon: *Global Panmixis (null hypothesis)*. This hypothesis states that rates of global historical gene flow have been sufficient to result in the absence of genetic structuring among the 5 gyres (Fig. 7-1A). Allopatric speciation is therefore not possible. *Ocean Basin Panmixis*. In this model, neustonic exchange occurs readily among adjacent equatorial current fields, but not among ocean basins; producing three ocean basin-specific clades, each capable of allopatric speciation (Fig. 7-1B). *Within-Gyre Panmixis*. This hypothesis views each gyre population as a discrete and isolated gene pool. Prolonged isolation is predicted to produce reciprocal monophyly, and eventually speciation, of gyre populations (Fig. 7-1C).

Available data for neustonic taxa are almost completely restricted to the 5 epineustonic (above the water surface) species of *Halobates*, the oceanic sea skater (Fig. 1-2F) and their within-species phylogenetic data conforms most closely with Fig. 7-1C: strong evidence for restricted gene flow among gyres, including gyre specific reciprocal monophyly for the widespread species *H. micans* dated to 1-3 million years (Andersen et al., 2000). Inferred speciation patterns from *Halobates* gene tree topologies, and present day distributions, are not consistent with sympatric speciation. The 5 oceanic species stem from two distinct colonizations from different coastal ancestral lineages (Damgaard

et al., 2000) and there is little range overlap among recent sister taxa (Andersen et al., 2000). However, given that the differential permeability of ocean basin and gyre boundaries to pelagic microorganisms is related to life history (see above), will the hyponeustonic (below the water surface) taxa studied here, which all have pelagic larval stages that enter the water column, also show trenchant genetic structuring by ocean gyre?

Material and Methods

Sample collection, DNA extraction, amplification, and sequencing

Specimens of *Glaucus atlanticus*, *Velella velella*, and *Porpita porpita* were collected as part of a global sampling of neustonic invertebrates from 2006-2012 (Fig. 1-1). Specimens were either collected by hand as they washed up on beaches, or in the open ocean via neuston net tows. All specimens were preserved in 95% ethanol, and identified by external morphology. At least 15-20 individuals of each taxon, when possible, were chosen arbitrarily from each ocean gyre for genetic analysis.

Approximately 30 mg of tissue was sampled from the left ceratal cluster or foot (*Glaucus atlanticus*) or from the lateral part of the body, including zooids but with the chitinous float removed (porpitiids). Genomic DNA was extracted from *G. atlanticus* using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-Tek) and from porpitiids using the DNEasy Blood and Tissue Kit (Qiagen) according to manufacturers' protocols. Mitochondrial cytochrome oxidase I (COI) was amplified from *G. atlanticus* using the universal primers LCO1490 and HCO2198 (Folmer et al., 1994) using the general PCR protocol [2 min at 95 °C, 35 cycles of (30 s at 94 °C, 30 s at X °C, 1 min at 72 °C), 5 min

at 72 °C], where X = 45 °C. Mitochondrial large subunit rRNA (16S) was amplified from porpittids using the universal primers 16ar and 16br (Simon et al., 1994), using the general PCR protocol described above with X = 49 °C. PCR products were sequenced directly with PCR primers using an ABI 3730xl automated sequencer (Applied Biosystems, Inc.) by the University of Michigan DNA Sequencing Core. Forward and reverse primer chromatograms were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in CodonCode Aligner (CodonCode Corporation) and checked by eye. *G. atlanticus* COI sequences were aligned according to amino acid translations using the mitochondrial code for *Mytilus edulis* (NCBI-GenBank).

Phylogenetic analyses

The sequence matrices (658 nt of mt COI from *Glaucus atlanticus*; 586 nt of mt 16S from *Velella velella*; 564 nt of mt16S from *Porpita porpita*) were analysed in jModelTest (Posada, 2008; Guindon & Gascuel, 2003) to determine the best-fit models of nucleotide substitution by Akaike Information Criterion: HKY+G for *G. atlanticus* and HKY+I for both *V. velella* and *P. porpita*. Maximum likelihood analyses were conducted for each taxon using Garli v. 1.0 (Zwickl, 2006) implementing the respective best-fit models of nucleotide substitution. For *G. atlanticus*, analyses were conducted both with an unpartitioned matrix and with partitions by codon position (1+2, 3). All analyses were conducted using default parameters, assuming four rate categories and empirical base frequencies, and stepwise choice of starting trees for 100 heuristic searches. Bootstrap analyses were performed under the same settings with 300 bootstrap replicates.

Bayesian Markov Chain Monte Carlo (MCMC) analyses were performed using BEAST v. 1.7.2 (Drummond et al., in press). For each taxon matrix, preliminary analyses were run in order to optimize run and burn-in lengths. No within-taxon groups were constrained to be monophyletic. The phylogenetic reconstruction for *Glaucus atlanticus* was performed using the HKY model with empirical base frequencies, gamma site heterogeneity with four rate categories, and nucleotides were partitioned using the SRD06 model (Shapiro et al., 2006). The porpitud reconstructions differed in that the invariant site parameter was employed instead of gamma site heterogeneity, and the nucleotides were not partitioned. All parameters were linked, and the clock model was strict with substitution rates estimated for nudibranch COI (1%/MY; Shields, 2009) and hydrozoan 16S (2%/MY; Govindarajan et al., 2004). Starting trees were randomly generated and the tree prior assumed the coalescent process of constant size. MCMC analyses were 10 million generations, logging parameters every 1000 generations, and the burn-in comprised 2.5 million generations. Convergence was confirmed by eye using the “Trace” function in Tracer v. 1.5 (Rambaut & Drummond, 2007) and by repeating all analyses three times. Three analyses for each of the three taxon groups were combined to generate summary statistics and maximum clade credibility trees (median node heights and > 0.5 posterior probabilities).

Phylogeographic analyses

Minimum spanning trees were constructed using the MINSPNET algorithm in Arlequin, 3.0 (Excoffier et al., 2005). Any alternative connections were compared and matched to the phylogenetic reconstructions. Individual haplotypes were assigned to

samples by ocean gyre, and samples were grouped by ocean basin to test the hypothesis that either gyre or ocean basin boundaries are barriers to gene flow in neustonic populations. Regional structure was examined using analysis of molecular variance (AMOVA) in Arlequin 3.0 comparing within and among ocean gyre and ocean basin. Molecular diversity indices were calculated in Arlequin 3.0. For *Glaucus atlanticus*, two additional ϕ statistics were generated based on the molecular clades recovered in the phylogenetic reconstruction: the first with two groups corresponding to molecular clades, and the second with two groups corresponding to the Atlantic basin and (Pacific + Indian basins).

Demographic histories of all three taxa were characterized using mismatch analyses in Arlequin 3.0 and by comparing Bayes Factors for two demographic models in BEAST: coalescent with constant size, and coalescent exponential.

Results

Phylogenetic reconstruction

Figure 7-2 shows the results of the phylogenetic analyses. In general (other than arrangement of tip clades), tree topologies were identical for Bayesian MCMC and likelihood analyses for all three taxa. Both analyses reconstructed two well-supported monophyletic clades in *Glaucus atlanticus* (Fig. 7-2A), an exclusively Atlantic basin clade and a global clade, with four intermediary specimens from the Indo-Pacific (Fig. 7-2A). The global clade comprises individuals from all ocean gyres except the South Atlantic, but this is likely due to the low sample size in the South Atlantic (N=4); those individuals were collected at one site and all cluster within the Atlantic clade. For the

Atlantic clade, the estimated mean time to the most recent common ancestor (\pm standard error) was $\text{TMRC}_{\text{GaATL}} = 0.0062$ (0.0002); the Indo-Pacific clade was of similar age with $\text{TMRC}_{\text{GaGLOBAL}} = 0.0069$ (0.0001). Neither type of phylogenetic analysis recovered any well-supported monophyletic clades within *Veleva veleva*, and there is no clear geographical pattern in tip clade arrangement. The *V. veleva* species clade is well supported in the phylogenetic reconstruction with $\text{TMRC}_{\text{Vv}} = 0.0061$ (0.0003) (Fig. 7-2B). Like *G. atlanticus*, both Bayesian MCMC and maximum likelihood analyses recovered well-supported monophyletic clades within *Porpita porpita*. There are five distinct clades within *P. porpita* with some geographic structuring: an entirely North Atlantic clade ($\text{TMRC}_{\text{pp1}} = 0.0059$; 0.0004), a Pacific basin + South Atlantic clade ($\text{TMRC}_{\text{pp2}} = 0.0083$; 0.0002), an exclusively eastern North Pacific clade ($\text{TMRC}_{\text{pp3}} = 0.0083$; 0.0002), and finally two southern hemisphere clades, one found in the South Pacific and Indian gyres ($\text{TMRC}_{\text{pp4}} = 0.0039$; 0.0001), and the final clade found in the South Atlantic and South Pacific gyres ($\text{TMRC}_{\text{pp5}} = 0.0041$; 0.0001) (Fig. 7-2C).

Phylogeographic analyses

Figure 7-3 shows the minimum spanning trees portraying haplotypic relationships within each of the three taxa. In *Glaucus atlanticus*, at least 20 individuals were haplotyped from each ocean gyre except for the South Atlantic (N=4). Haplotype diversity (h) was maximal (1.00 within each ocean gyre population), and so an additional 30 individuals were genotyped from one ocean gyre (North Atlantic) to investigate whether the addition of more individuals might reach saturation, but North Atlantic haplotype diversity (h) remained at 1.00 (Table 7-1). This is almost entirely due to

synonymous substitutions in the third codon position, as evidenced by the much higher rate of evolution (2.54 vs. 0.23) of the third codon position generated in the Bayesian phylogenetic reconstruction (Fig. 7-4). Average pairwise diversity (π) was 6.37-7.96 for all gyre populations except the North Pacific, which was higher ($\pi_{\text{GaNp}} = 10.42$). Tajima's D values are negative for all gyre populations, significantly so for all except South Atlantic (Table 7-1); this further indicates an excess of rare haplotypes. In *Velevella velevella* and *Porpita porpita*, 20 individuals were haplotyped from each ocean gyre except for the Indian ocean (N = 15) (Table 7-1). Haplotype diversity within each ocean gyre population was overall lower in *V. velevella* ($h = 0.35-0.6$) than *P. porpita* ($h = 0.35-.8$). Average pairwise diversity (π) within ocean gyres was much higher in *P. porpita* ($\pi = 4-12.15$; with highest diversity in the South Atlantic) than *V. velevella* ($\pi = 1.31-1.8$) (Table 7-1). Tajima's D values for *V. velevella* populations are a mixture of positive and negative, but none is significant. Interestingly, while there were no deep, well-supported molecular clades within *V. velevella*, the minimum spanning tree shows some shallow structure corresponding to northern and southern hemispheres (Fig. 7-3B), and a modal haplotype shared by the two northern hemisphere gyres ($N_{\text{NA}} = 11 = N_{\text{NP}} = 3$). For *P. porpita*, the minimum spanning tree (Fig. 7-3C) depicts the five clades reconstructed in the phylogeny (Fig. 7-2C). Almost all Tajima's D values are significantly negative except in the North Atlantic (D = -1.19; P = 0.11) and the Indian ocean (D = 0.15; P = 0.61).

Hierarchical AMOVA analyses comparing variance between ocean gyres and ocean basins show different patterns of gene flow among the three taxa. For *Glaucus atlanticus*, ϕ_{ST} (between gyre) ϕ_{CT} (between ocean basin) values are similar and somewhat low: 0.26 and 0.25, respectively (Table 7-2; ϕ_{ST} is significant). This does not support

either gyre or ocean basin boundaries as barriers to gene flow. However, in one additional non-hierarchical AMOVA, samples were grouped by Atlantic basin vs. (Pacific + Indian basins), ($\phi_{ST} = 0.45$, $P = 0.00$), and in this case ϕ_{ST} is nearly twice as high as found in the original AMOVA. For *Veleva veleva*, both ϕ_{ST} and ϕ_{CT} are nearly zero, indicating panmixis between ocean gyres and basins (Table 7-2). For *Porpita porpita*, $\phi_{ST} = 0.42$ ($P = 0.00$), while $\phi_{CT} = 0$. This corroborates the phylogenetic reconstruction and haplotype networks showing distinct molecular clades corresponding to ocean gyres (Table 7-2).

Analyses of mismatch distributions for all three taxa generated in Arlequin resulted in θ_1 upper limit values of 99,999 for all populations (ocean gyres) and in each overall data set. This indicates that the population sizes are so large that there are no recent coalescent events, and that demographic history cannot be estimated precisely by this method with these data (Schneider & Excoffier, 1999); thus, those results are not shown.

Discussion

Potential sources of error

The primary aim of this study is to test hypotheses of population connectivity in cosmopolitan neustonic invertebrates, and thus the timings of divergence dates estimated in the phylogenetic reconstructions are not emphasized. Without external calibration points for neustonic taxa (*e.g.*, given global surface current connectivity, it is impossible to say certainly whether the closure of the Panamanian Isthmus separated North Atlantic and North Pacific neuston populations), the chronogram is only as accurate as the molecular clock estimates. Shields (2009) reviews the support for using 1%/MY in

nudibranchs from the Ross Sea—the rate is based upon a fossil-calibrated bivalve phylogeny of trans-isthmanian species pairs (Marko, 2002), it has also been recommended for circumtropical gastropods in a fossil-calibrated phylogeny (Frey & Vermeij, 2008) and for invertebrate mt COI in general (Dawson et al., 2011). For the porpitids, the molecular clock estimate is derived from a study reviewing molecular clocks from the hydrozoans *Obelia geniculata* and *Hydractinia* spp. based on biogeographic calibration points (Govindarajan et al., 2004). Neither of these estimates is ideal, and thus no conclusions are based upon divergence times other than presence/absence of well-supported, deep molecular clades. Furthermore, the failure of the mismatch distribution analysis is unsurprising because all three of these taxa probably have extremely large effective population sizes.

*Cape of Good Hope disjunction in *Glaucus atlanticus**

One unexpected pattern found in *Glaucus atlanticus*, and not in either porpitid lineage, is the clustering of South Atlantic specimens (sampled in Cape Town, South Africa), exclusively in the Atlantic molecular clade vs. the Global clade, which are found only about 1,850 km away in Durban. Above the subtropical convergence zone, the Agulhas current flows southwest down the eastern side of South Africa and enters a retroflexion region off the Cape of Good Hope. On the western side, the Benguela current flows northeast from the retroflexion region (Walker, 1989). Either the South Atlantic sample size was too low (N=4) to recover expected global clade lineages, or this is further support that physical models, in this case of ocean surface currents, are inadequate in accurately predicting neustonic gene flow. Data from an additional

cosmopolitan species of neustonic mollusks, the viviparous bubble-rafting snail species *Janthina janthina*, will be compared to *Glaucus atlanticus* to test whether the Cape of Good Hope disjunction applies to multiple predatory neustonic mollusks.

Different evolutionary histories of the two porpitid lineages

The most surprising result of this study is that the two porpitid species, *Verella velella* and *Porpita porpita*, have very different evolutionary histories, representing the highest (global panmixis) and lowest (regional and within-gyre panmixis) levels of hypothetical population connectivity. One obvious ecological difference separating *Verella velella* from *Porpita porpita* is its direct exposure to wind. Tacking behavior has been observed in *V. velella* (Francis, 1991), which in combination with the molecular results presented here, indicates that the two porpitid genera may be ecologically quite different although they have traditionally been considered identical (Hyman, 1940). A hydrodynamic analysis of porpitid floating behavior coupled with a physical model of ocean circulation is underway to test whether individual *Verella velella* and *Porpita porpita* escape gyre boundaries at the same rate.

Ocean gyre and basin boundaries vary as barriers to neustonic gene flow

The original hypothesis that ocean gyre or ocean basin boundaries might present barriers to gene flow appears to be differentially supported for different taxa, even in an isobathic, drifting ecosystem like the neuston. None of the hyponeustonic taxa here shows the same genetic structuring as *Halobates*—reciprocal monophyly by oceanic gyre (Anderson, 2000). The regional genetic structuring in *Porpita porpita* most closely

resembles *Halobates*, but three porpitid clades (Fig. 7-2C) consist of individuals from multiple gyres. These results indicate that hyponeustonic populations may be connected in fundamentally different ways than their immediate neighbors in the epineuston. With that in mind, one essential factor in developing future hypotheses of marine population connectivity is the different life histories of the focal taxa. For the three neustonic taxa studied here, little is known about their pelagic larval stages, or the porpitid medusoid stage.

Recent marine comparative phylogeographies vary in recovering concordant patterns; for example, seven species of benthic invertebrates in the Coral Triangle show clear phylogeographic breaks in the western and southern regions, but Coral Triangle fish and some invertebrates show no phylogeographic breaks even though they have similar ecologies (reviewed in Carpenter et al., 2011). Carpenter et al. suggest that the typical model for mapping marine dispersal patterns into “ecoregions” by physical barriers like geography and ocean currents may be inadequate when investigating patterns of population connectivity. Long-distance dispersal events, while relatively rare, are still much more common than in terrestrial organisms (Palumbi, 1992; Norris, 2000), which would disproportionately affect population genetics analyses relative to actual demography. The only solutions to these problems are to sample multiple unlinked molecular markers (this is planned for the neustonic taxa studied here) and to develop statistical tests tailored to marine demographic models (*e.g.* soft vicariance models using HABC; Hickerson & Meyer, 2008).

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Figure 7-1. Hypothetical networks of global subtropical gyre genetic structuring. A, Global panmixis; **B,** Panmixis only within ocean basins; **C,** Panmixis only within ocean gyres. Abbreviations: NA, North Atlantic; SA, South Atlantic; NP, North Pacific; SP, South Pacific; I, Indian.

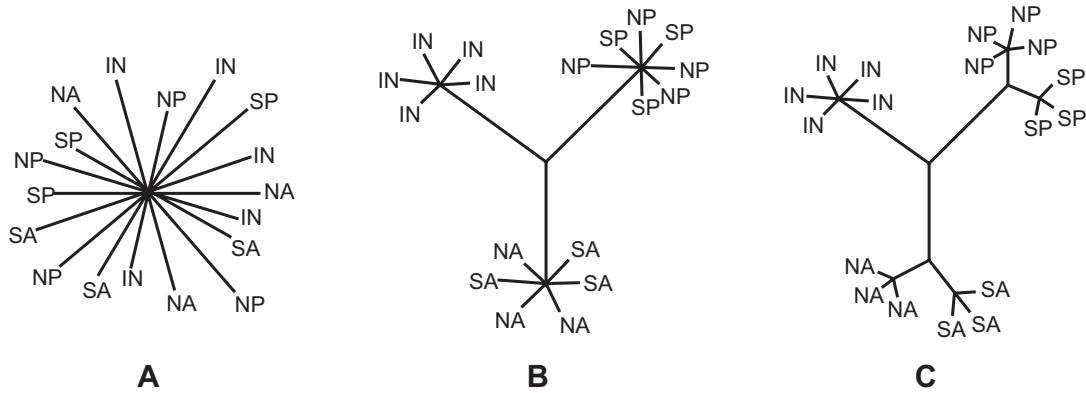


Figure 7-2. Bayesian MCMC chronograms of mitochondrial markers for three neustonic taxa. **A**, *Glaucus atlanticus* (mt COI) with two well-supported molecular clades (Atlantic, Global); **B**, *Verella verella* (mt 16S) with relatively shallow genetic structure and no well-supported clades; **C**, *Porpita porpita* (mt 16S) with five well-supported molecular clades (North Atlantic, Pacific + South Atlantic, Eastern North Pacific, South Pacific + Indian, South Atlantic + South Pacific). Intraspecific clades have been collapsed and are proportionate within-species. Numbers above branches represent Bayesian posterior probability percentages followed by Maximum Likelihood bootstrap percentages > 50. Bars to the right of the topologies correspond to ocean gyre sampling locations: red, North Atlantic; yellow, South Atlantic; blue, North Pacific; green, South Pacific; orange, Indian.

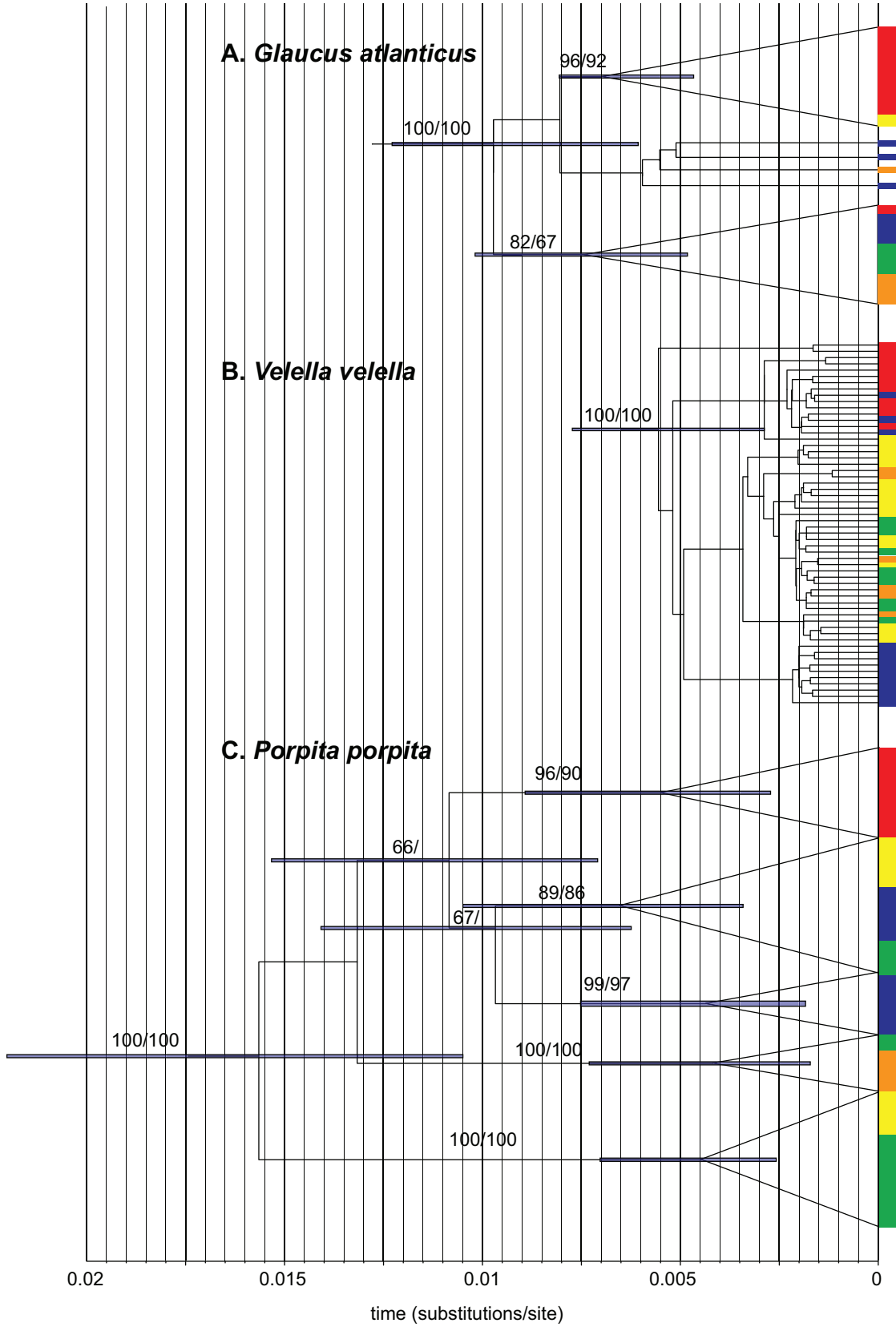


Figure 7-3. Minimum spanning trees of mitochondrial markers for three neustonic taxa. Steps are indicated by hash marks on branches. **A**, *Glaucus atlanticus* (mt COI); **B**, *Veella veella* (mt 16S); **C**, *Porpita porpita* (mt 16S). Colors correspond to ocean gyre sampling locations: red, North Atlantic; yellow, South Atlantic; blue, North Pacific; green, South Pacific; orange, Indian.

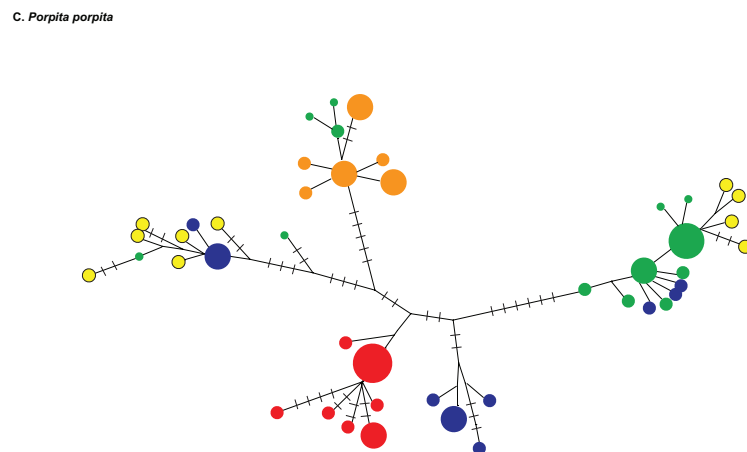
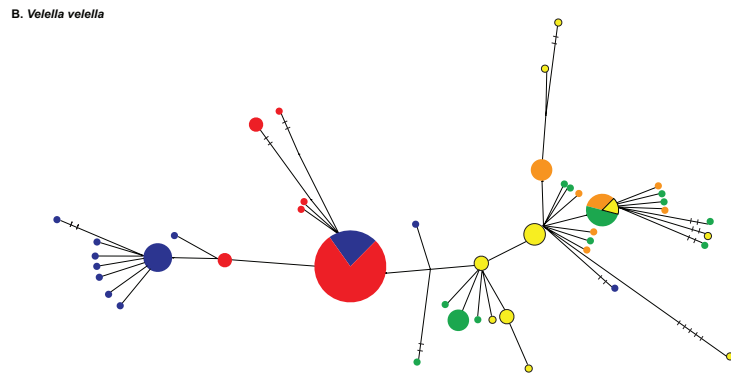
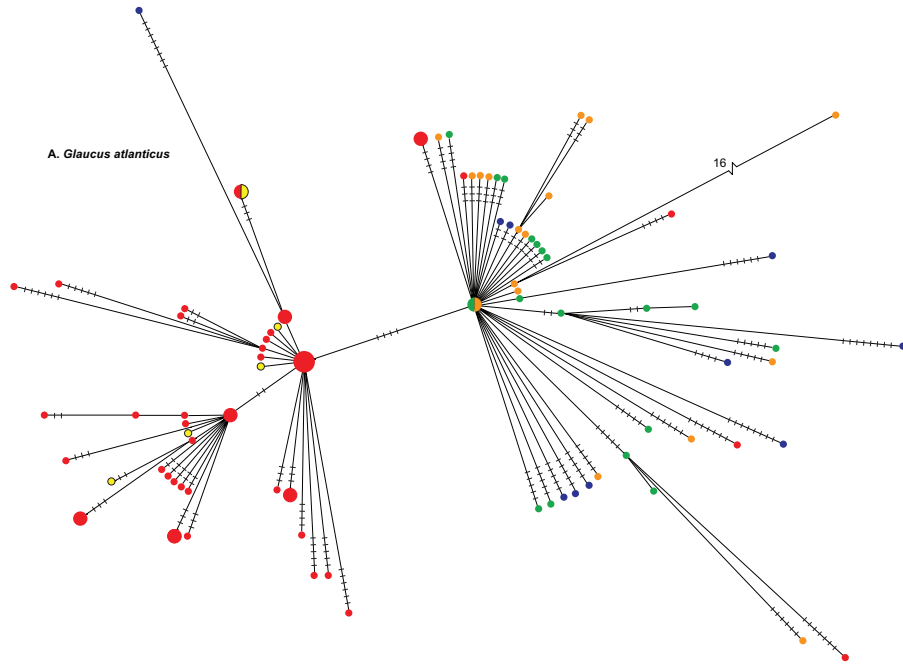
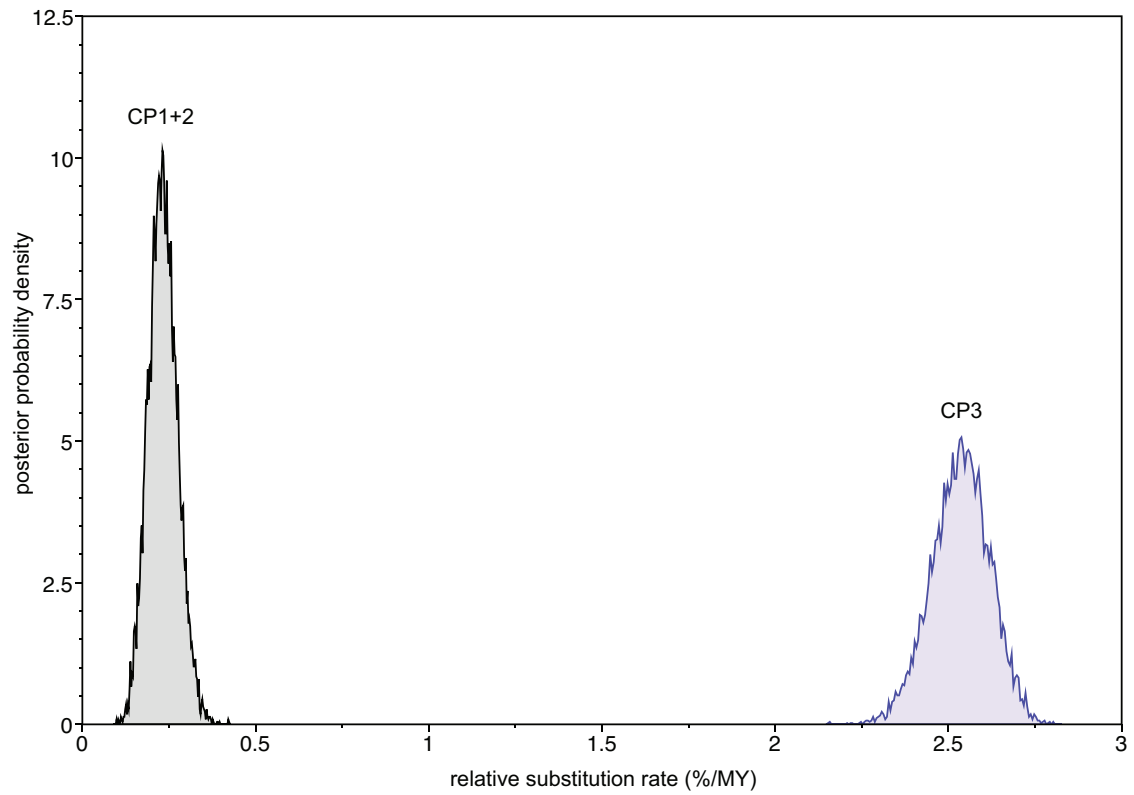


Figure 7-4. Relative substitution rates of the two codon position partitions in the phylogenetic reconstruction of *Glaucus atlanticus* mt COI.



Posterior probability density vs. relative substitution rate for codon positions 1 and 2 (CP1+2 = 0.023) and codon position 3 (CP3 = 2.54).

Table 7-1. Molecular diversity indices for ocean gyre populations of three cosmopolitan neustonic taxa. Columns represent: N, sample size; *h*, haplotype diversity; π , nucleotide diversity; Tajimas D and associated P-value.

	N	<i>h</i>	π	Tajima's D	P-value
<i>Glaucus atlanticus</i>					
North Atlantic	50	1	6.8	-2.24	P = 0.00
South Atlantic	4	1	7	-0.2	P = 0.47
North Pacific	20	1	10.42	-1.68	P = 0.02
South Pacific	20	1	6.37	-2.01	P = 0.01
Indian	20	1	7.96	-1.92	P = 0.00
<i>Veleva veleva</i>					
North Atlantic	20	0.35	1.31	-1.05	P= 0.21
South Atlantic	20	0.5	1.43	0.52	P = 0.74
North Pacific	20	0.55	1.33	0.15	P = 0.62
South Pacific	20	0.6	1.8	-1.11	P = 0.14
Indian	15	0.6	1.78	0	P = 0.81
<i>Porpita porpita</i>					
North Atlantic	20	0.35	4	-1.19	P = 0.11
South Atlantic	20	0.55	12.15	-1.73	P = 0.03
North Pacific	20	0.8	7.42	-1.59	P = 0.05
South Pacific	20	0.65	5.01	-1.9	P = 0.01
Indian	15	0.4	3.33	0.15	P = 0.61

Table 7-2. AMOVA tests of regional (ocean basin) and population (ocean gyre) genetic structure in three cosmopolitan neustonic taxa. Columns represent: d.f., degree of freedom; Sum of sq., sum of squares; Var. com., variance components, % Var., percentage of total molecular variation. ϕ_{ST} , correlation of random haplotypes within ocean gyres, and ϕ_{CT} , correlation of random haplotypes within ocean basins, are shown with P-values below each taxon.

	d.f.	Sum of sq.	Var. com.	% Var.
<i>Glaucus atlanticus</i>				
Among ocean basins	2	81.58	1.26	25.25
Within ocean basins	2	7.938	0.02	0.49
Within ocean gyres	92	340.64	3.7	74.26
f_{ST}	0.26	P = 0.00		
f_{CT}	0.25	P = 0.13		
<i>Verella verella</i>				
Among ocean basins		1.137	0.00041	0.08
Within ocean basins		1.193	0.0096	1.93
Within ocean gyres		22.474	0.49	97.99
f_{ST}	0.02	P = 0.02		
f_{CT}	0	P = 0.58		
<i>Porpita porpita</i>				
Among ocean basins	2	7.11	-0.01	-1.3
Within ocean basins	2	20.729	0.76	55.99
Within ocean gyres	46	35.06	0.66	44.31
f_{ST}	0.42	P = 0.00		
f_{CT}	0	P = 0.73		

Chapter 8

Conclusions and future directions

Although the neuston is arguably the most geographically vast ecosystem on the planet, before this research studies of neustonic taxa have been sporadic and often descriptive, lacking evolutionary or community-based perspectives. The ecological relevance of this community is likely severely underestimated, because while its species diversity is low relative to coral reefs, neuston community members comprise the base of pelagic food webs including macrofauna of conservational interest (Chapter 1). In light of the research presented here, several themes and avenues for future research have emerged.

Planktonic and neustonic macroinvertebrates should increasingly be the focus of marine evolutionary and population genetics studies. Like microscopic plankton, they are drifters, but unlike the microinvertebrates, morphological data is much easier to generate. The importance of morphological data is evident in Chapters 4-6; in this thesis it has been employed to study the evolutionary transition from benthos to neuston in Janthinidae (Chapter 4) and to delineate species in the *G. marginatus* cryptic species complex (Chapters 5 and 6). Certainly it represents an under-utilized resource, and enables hypothesis testing that would not be possible in microscopic taxa. The type of multi-pronged approach employed here required collaboration between oceanographers,

evolutionary biologists, and systematists, which adds a uniquely organismal perspective versus studies that focus solely on generating molecular data.

Another recurring theme throughout this thesis points to the general lack of ecological data from neustonic taxa. Some, like *Recluzia* cf. *jehennei*, are so rare that describing preserved specimens from a single wash-up has historically been (and still is) reason for publication (Chapter 3). The greatest gap in knowledge for all taxa centers on the earliest life history stages: what are the morphological, behavioral, and ecological characteristics of neustonic larvae? How long is pelagic larval duration? These questions must be addressed in light of the differential population connectivities of neustonic taxa (Chapter 7). Ecological and behavioral studies of live neuston are planned as part of future SEA Semester and marine lab-based projects.

One promising avenue of future research based on the data collected for this thesis is to examine the temporal stability of surface convergence zones. Surface convergence zones are areas of local down-welling where neustonic taxa gather as they are funneled by surface currents. Within three more years data will be available for 10 years of neuston sampling in the central and eastern North Pacific. This presents a unique opportunity to study whether convergence zones persist over time (they are known to change with climate events, *e.g.* ENSO processes).

Finally, as ultra high-throughput sequencing techniques become more commonly applied to non-model organisms (*i.e.*, cheaper), the possibilities increase dramatically. On the evolutionary front, for example, comparing the transcriptomes of the pedal glands of epitoniids, *Recluzia*, and *Janthina* at varying life history stages could shed some light on the genetic basis of the evolution of quick-hardening mucus and float-building. The

phylogeographic analyses would also be improved by the identification of rapidly-evolving nuclear markers (*e.g.* microsatellites) *via* genomics. Establishing baseline levels of genetic variation for neustonic populations (as discussed in Chapter 2) is imperative in the face of anthropogenic climate change.

Appendix I

DNA sequence of partial small subunit nuclear 18S rRNA (529 nt) obtained from porpitid zooxanthellae in this study.

>Scrippsiella_velellae18S

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ATGGATAACTGTGGTAATTCTAGAGCTAATACATGCGCCCAAACCCGACT
CCGTGGAAGGGTTGTGCTTATTAGTTACAGAACCAACCCAGGCTCTGCCT
GGTTTTGTGGTGATTTCATAATAACTCAACGAATCGCATGGCATCCGCCGG
CGATGAATCATTCAAGTTTCTGACCTATCAGCTTCCGACGGTAGGGTATT
GGCCTACCGTGGCAATGACGGGTAACGGAGAATTAGGGTTCGATTCCGGA
GAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGC
AAATTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAATAACAATACA
GGGCATCCATGTCTTGTAATTGGAATGAATAGAATTTAAATCCCTTTACG
AGTATCGATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC
TCCAATAGCGTATATTAAGTTGTTGCGGTTAAAAAGCTCGTAGTTGGAT
TTCTGCCTAGGACGACCGGTCCGCCCTC
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Appendix II

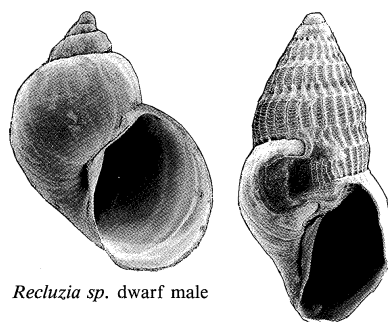
Observations on *Recluzia* (P. Colman)

Australian Shell News, No. 53, January 1986.

Page 3

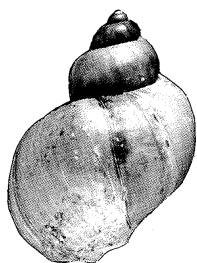
OBSERVATIONS ON *RECLUZIA*

Specimens of rare member of the Janthinidae, *Recluzia* sp., (possibly *R. rollandiana* Petit de la Saussaye, 1853) were recently forwarded to the Australian Museum. They had been collected on Bundagen Beach, Bellinger River heads, northern N.S.W., by Mr. David Tarrant, whose interest was aroused by the "four juveniles attached to the float". The "juveniles" were badly decomposed, and it was not possible to study the soft parts. It seems, however, less likely that *Recluzia* supplies the young with a float than that the small specimens are dwarf males, living on the float of the female. If this is the case, 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*.



Recluzia sp. dwarf male

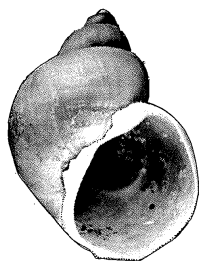
Litiopa sp.



Recluzia sp. adult



Recluzia sp. radula



Recluzia sp. adult

It is well documented in the literature that the Janthinidae are protandric hermaphrodites (that is, starting as males and ending as females), but as far as I am aware dwarf males have never before been recorded as being associated with the float of a large female. Within the genus *Janthina* the males have their own float.

Also on the float was a specimen of *Litiopa*, a minute species belonging to the family Litiopidae. As bacteria were no doubt present on the mucous float, the *Litiopa* was probably feeding on the bacteria.

The accompanying photographs show dorsal and ventral views of the *Recluzia* sp. (registered Aust. Mus. C.145648) plus Scanning Electron Microscope photos of the dwarf males and the *Litiopa*. A radula was extracted from the badly decomposed body of the *Recluzia*, and an SEM shot of this is also included.

The help of Dr. Anders Waren is gratefully acknowledged in supplying the SEM photographs.

Phil Colman