

Chapter 5

Parallel sexual rekeying supports non-geographic planktonic speciation¹

Introduction

Open-ocean planktonic communities confound evolutionary paradigms. They are composed of vast, passively drifting populations that span enormous geographic ranges (Palumbi, 1992; Casteleyn *et al.*, 2010). Yet in the absence of geographical barriers, regional populations and cryptic species abound (Casteleyn *et al.*, 2010; Norris, 2000). How is pelagic biodiversity generated? Proposed speciation mechanisms in pelagic sibling species complexes are essentially variations on two classic themes: the development of geographic isolation of populations (allopatry) or biological isolation (non-allopatry) (Norris, 2000; Norris & Hull, 2011). The greatest obstacles to testing these hypotheses are sampling limitations for representative collections of cosmopolitan groups, and a related paucity of morphological data for marine taxa, leading to subsequent difficulty identifying species (Knowlton, 2000; Goetze, 2003).

The marine neuston is a promising system for investigating planktonic speciation mechanisms. Located at the surface of the planet's subtropical oceans, it contains approximately 100 highly specialized passively-drifting species (Marshall & Burchardt, 2005; Churchill *et al.*, 2011), and its isobathic nature renders it exceptionally tractable

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both for sampling and for understanding localized ecological variation. The base of the neustonic food chain is formed by a mutualism involving photosymbiotic dinoflagellates (zooxanthellae) and their porpitid cnidarian hosts (Lalli & Gilmer, 1989). The porpitids are preyed upon by two co-occurring neustonic gastropod mollusk lineages: the bubble-rafting snail family Janthinidae (Churchill *et al.*, 2011) and the nudibranch subfamily Glaucinae (Valdés & Angulo Campillo, 2004).

Glaucinin nudibranchs are the only truly pelagic members of their predominantly benthic suborder, Aeolidina (Valdés & Angulo Campillo, 2004). They are highly specialized for this unusual lifestyle, floating upside-down at the subtropical ocean surface by trapping gulps of air in their modified, muscular stomachs (Valdés & Angulo Campillo, 2004). After a planktotrophic larval stage, juveniles develop as simultaneous hermaphrodites. Mated adults release strings of egg capsules either into the sea or attached to a solid surface (*e.g.* prey item, driftwood), from which larvae emerge after three days (Valdés & Angulo Campillo, 2004). Glaucinae contains a single genus, *Glaucus*, which in turn consists of two valid species, *G. atlanticus* and a considerably smaller *G. marginatus* (Valdés & Angulo Campillo, 2004). Their geographic distributions differ: *G. atlanticus* is circumtropical (Fig. 5-1A), whereas *G. marginatus* has only been reported in the Pacific basin (Valdés & Angulo Campillo, 2004) and the Indian Ocean (this study, Fig. 5-1A, Table 5-1). The overlapping but distinct ranges of these two congeners present an opportunity to test the effect of geography on genetic structuring and speciation: if isolation by distance is the primary driver of speciation, then *G. atlanticus*, the cosmopolitan species, should have more pronounced regional genetic

structuring and/or cryptic species. Using a global collection of neuston from five years of sampling, we test this hypothesis in a molecular phylogenetic framework.

Material and methods

Sample collection

Glaucus spp. were collected as part of a global sampling effort of neustonic taxa *via* neuston tow and beach collection (Fig. 5-1, Table 5-1). Benthic aeolids were collected *via* SCUBA, snorkel, or from tidepools (Los Angeles County Museum and California Polytechnic State University samples) or loaned from museum collections (Table 5-2). All tissues were fixed and preserved in $\geq 70\%$ ethanol. *Glaucus* spp. were identified by external morphology (Valdés & Angulo Campillo, 2004).

DNA extraction, amplification, and sequencing

Whole genomic DNA was extracted from 1-5 cerata, specimen size permitting, using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-Tek) or the DNEasy Blood and Tissue Kit (Qiagen). A total of 2,921 aligned nucleotides were amplified from four molecular markers. 1,391 nt of nuclear 28S rRNA (DI-DIII) were amplified either by using the primer pair 28SF4/28SR1 (named primer pairs are in the format 5'/3') (Morgan *et al.*, 2002) or by pairing the previous primers with internal aeolid-specific primers: D23Faeolid (5'-GAAAGTTTGAGARTAGGWC-3') and D4RBAeolid (5'-CGYCRGACTCCTTGGTCCGTGT-3') whose positions correspond to previously published primers D23F/D4RB (Park & Ó Foighil, 2000). All 28S amplifications were performed with an annealing temperature of 50°C. 328 nt of nuclear Histone-H3 were

amplified using universal primers HexAF/HexAR (Colgan *et al.*, 1998) and an annealing temperature of 53°C. 544 nt of mitochondrial (mt) 16S rRNA were amplified using universal primers combination 16Sar/16Sbr (Simon *et al.*, 1994) and an annealing temperature of 49°C. 658 nt of mt COI were amplified using universal primers LCO1490/HCO2198 (Folmer *et al.*, 1994) and an annealing temperature of 45°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 sequenced directly using an ABI 3730xl (Applied Biosystems, Inc.) automated sequencer by the University of Michigan DNA Sequencing Core. Sequences were aligned using CodonCode Aligner 3.7.1.1 (CodonCode Corporation) and verified by eye. Accession numbers for all sequences generated for this study are listed in Table 5-2.

Phylogenetic analyses

Best-fit models of nucleotide substitution were selected statistically by Bayesian Information Criterion (BIC) in jMODELTEST 0.1.1 (Posada, 2008) for each molecular marker: nuclear 28S rRNA (TIM3+I+ Γ) and Histone-H3 (TIM2+I+ Γ), mt 16S rRNA (TPM3uf+I+ Γ) and COI (TIM1+I+ Γ). Bayesian phylogenetic analysis was conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) (4 chains, 10 million generations) with a concatenated data set; the model of nucleotide substitution chosen was the closest approximation to the BIC best-fit model available in MrBayes (GTR+I+ Γ). 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 (ML) phylogenetic analysis was conducted in GARLI 2.0 (Zwickl, 2006), using the BIC best-fit models of nucleotide substitution for respective markers (partitioned model) and default settings. To ensure tree searches did not become trapped in local optima, five separate ML analysis repetitions were run. Final log likelihood scores between the five runs differed by $< 0.4 \ln L$. The best ML tree from all runs and the Bayesian consensus phylogram had identical topologies. ML bootstrap searches were performed using GARLI (300 replicates) and assembled with PAUP* 4.0 (Swofford, 2002).

Morphological examination

Ethanol preserved specimens (at least 10 of *G. atlanticus* and each *G. marginatus* lineage except clade D, Central North Pacific, N=4) were dissected using a Leica MZ16 stereomicroscope. A transverse incision was made on the dorsal side of each specimen at the level of the first anterior ceratal cluster. The incision inevitably punctured the gastric cavity, which is hyperinflated when animals are fixed in ethanol, often causing the penis to evert. The incision continued to the right side, under the right ceratal cluster, to the ventral foot, and posteriorly around the gonopore. Once the gonopore was freed of the outer body wall, the posterior diverticulum of the digestive gland was unwrapped from the ovotestis and the entire reproductive system was removed, studied, and photographed (with all or part of the ovotestis removed) using a Leica DFC300 digital camera with Z-stacking focus.

Histology

For each of the five *Glaucus* lineages (*G. atlanticus* and *G. marginatus* A-D), two ethanol preserved specimens were prepared for transverse and sagittal plane sections. All histology was performed at the University of Michigan Medical School Microscopy and Image Analysis Laboratory (MIL) Biomedical Research Core Facility. Specimens were processed using a Leica ASP 300 paraffin tissue processor and embedded in paraffin using a Leica Tissue-Tek paraffin embedding station. Serial sections were cut at 5-7 μm using a Leica 2155 rotary paraffin microtome. Sections were mounted and stained with haematoxylin and eosin. Slides were viewed with an Olympus BX-51 upright light microscope and photographed with an Olympus DP-70 high resolution digital camera.

Results and discussion

Figure 5-1B shows a detail of the first molecular phylogeny including *Glaucus* spp. and representatives of 16 other aeolid genera (Table 5-2; see Fig. 5-2 for full analysis). Our results are the inverse of hypothetical geographic expectations: the global species, *G. atlanticus*, exhibits no evidence for cryptic species or trenchant genetic structuring, but the Indo-Pacific congener, *G. marginatus*, contains four distinct cryptic lineages forming two robust clades with overlapping distributions (Fig. 5-1B). One of the four cryptic lineages (A) spans all three Indo-Pacific gyres and has a South Pacific sister lineage (B). The other two cryptic sister lineages (C, D) were encountered only in North Pacific samples.

Our molecular phylogenetic results are consistent with the presence of a cryptic species complex within the *Glaucus marginatus* morphospecies. Unlike many pelagic taxa (*e.g.* Casteleyn *et al.*, 2010; Goetze, 2003), for which morphological data is

unavailable, aeolid nudibranch morphology (including *Glaucus* spp.) has been studied in detail (Valdés & Angulo Campillo, 2004; Gosliner, 1994; Valdés *et al.*, 2010; Thompson & McFarlane, 1967; Thompson & Bennett, 1970; Miller, 1974; Ross & Quetin, 1990; Thompson, 1980). To investigate whether these four *Glaucus marginatus* molecular lineages represent cryptic morphospecies, we identified three anatomical areas most likely to differ in cases of recent speciation: external morphology, the chitinous feeding structures (radulae), and the reproductive system. We compared the morphologies of at least ten representatives of each lineage (except clade D: N=6). We did not find any discernible differences in either external or radular morphologies; however, in dissections (Fig. 5-3) and histological sections (Fig. 5-4) of the reproductive system, we found one morphological character that is lineage-specific: the presence or absence of a bursa copulatrix (Fig. 5-5; see Chapter 6 for functional description of glaucinin reproductive system). The bursa copulatrix is a blind-ending epithelial sac immediately proximal to the vaginal opening, which functions in short-term exogenous sperm storage (Gosliner 1994; Valdés *et al.*, 2010) and may also have a gametolytic function (but this function was not observed in *G. atlanticus* by Thompson & McFarlane, 1967, or *Glaucus* spp., this study). It is present in *G. atlanticus* and in two (B, D) of the four *G. marginatus* cryptic lineages (Figs. 5-3—5-5). Coding the gain/loss of the bursa copulatrix on our tree topology (Fig. 5-1B) shows that members of each cryptic *G. marginatus* tip clade differ in the presence/absence of this structure and that the two cryptic lineages (A, C) lacking the bursa copulatrix have lost it independently. Loss of this structure has been previously observed in phylogenetically derived species of aeolids (Gosliner, 1994) and its presence/absence is considered a species-delimiting character in nudibranchs (Gosliner,

1994). Based on this morphological corroboration of the molecular data, we conclude that the four cryptic *G. marginatus* lineages have speciated.

Our results show that details of genital morphology are better predictors of latent evolutionary relationships among glaucinid lineages than biogeography. Rapid divergence in genital morphology has long been considered an evolutionary result of selection (Eberhard, 1985; Leonard, 2006). We interpret the loss of a bursa copulatrix, a reproductive change that has occurred twice in parallel *G. marginatus* clades (and in the North Pacific clade, possibly in sympatry), as strong evidence that the evolution of biological barriers plays a greater role than geography in glaucinin speciation. We further propose that these inferred “sexual rekeying” events may have served as the underlying speciation mechanism.

Glaucinid copulation requires participants to align their ventral surfaces, evert and intertwine their penes, and reciprocally transfer sperm to their partner’s genital aperture (Fig. 5-5; Miller, 1974; Ross & Quetin, 1990). One study has documented highly distinctive mating behaviors in *Glaucus atlanticus* vs. *G. marginatus*. Coitus in the former involves a penial spine and is much more prolonged (43-59 min) than in *G. marginatus* (50-70 sec; unknown cryptic lineage sampled off Sydney, Australia) (Ross & Quetin, 1990). The behavioral consequences of losing a bursa copulatrix have not been studied in nudibranchs (Gosliner, 1994; Valdés *et al.*, 2010) and it is unknown if all four *G. marginatus* cryptic species share an abbreviated mating behavior. We hypothesize that the presence or absence of a bursa copulatrix at the genital aperture does affect mating behavior by changing the mechanics of penial insertion. The effects of any changes in

mating mechanics may be greater in glaucinins, who must copulate while drifting, versus their benthic counterparts (Ross & Quetin, 1990).

Our study adds support to the growing body of literature revealing the inadequacy of applying dispersal-limiting (*i.e.*, terrestrial) speciation models in the open ocean (Norris, 2000, Norris & Hull, 2011; Knowlton, 2000; Goetze, 2003) and is the first to reveal a specific, biologically driven isolating barrier in a planktonic group. It also provides new insights into the benthic evolutionary origins of the neustonic Glaucinae. Our gene trees consistently place *Glaucus* in an aeolidioidean clade with three other aeolid genera: *Favorinus*, *Learchis*, and *Hermosita* (PP=96; BS_{ML}=72; Fig. 5-2). More extensive sampling is necessary to identify the benthic sister lineage of *Glaucus*; however, the ecology of *L. poica* is similar to *Glaucus*: it preys upon hydroids, and it lives associated with benthic sargassum algae, which may become detached and free-floating as a result of rough wave action (Thompson, 1980). Further research on benthic sister lineages and on the reproductive biology of the *G. marginatus* sister species complex is required to flesh out the evolutionary history of this remarkable neustonic nudibranch radiation.

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Figure 5-1. Biogeography and genetic structuring of two *Glaucus* morphospecies. **A.** Ranges and sampling map of cosmopolitan *G. atlanticus* and Indo-Pacific *G. marginatus*. Sampling locations are represented on the map by circles (*G. marginatus*) and squares (*G. atlanticus*), which are color-coded by molecular lineage. Five subtropical gyre systems are labeled: NA, North Atlantic; SA, South Atlantic; NP, North Pacific; SP, South Pacific; In, Indian. **B.** Bayesian consensus phylogram of *Glaucus* clade based on four molecular markers (nuclear 28S rRNA and Histone-H3; mt 16S rRNA and COI; total of 2,921 aligned nucleotides). Statistical support percentages are shown on internal branches; Bayesian posterior probabilities precede maximum likelihood bootstrap values. Asterisks indicate values of 100. Individuals are color-coded by subtropical gyre system. Presence (+bc) or absence (-bc) of the bursa copulatrix is coded in red on the topology. Photographs of the two morphospecies are above the topology (scale bars = 1.0 cm). Four cryptic lineages of *G. marginatus* are denoted by colored circles and letters: **A**, Indo-Pacific; **B**, South Pacific; **C**, Eastern North Pacific; **D**, Central North Pacific.

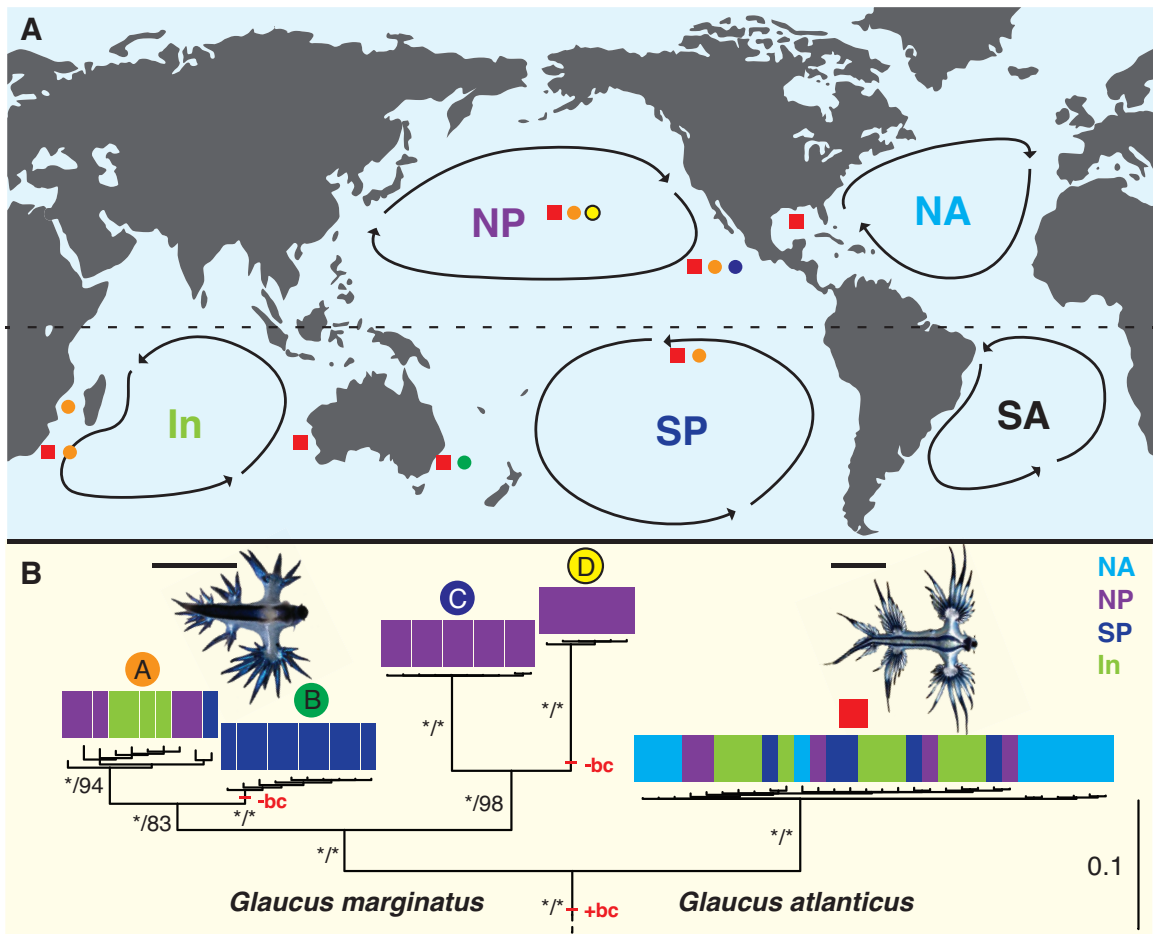


Figure 5-2. Bayesian consensus phylogram of aeolid nudibranchs based on four loci (mt 16S rDNA and COI; nuclear 28S rDNA and Histone-H3). Statistical support percentages are shown above internal branches in the form: Bayesian posterior probability/Maximum likelihood bootstrap value. Asterisks indicate values of 100. Clades Aeolidida and Doridida (outgroup) are indicated to the far right of the topology. Superfamilies Aeolidioidea (Aeolidiidae+Glaucidae, blue), Fionioidea (green), and Flabellinoidea (orange) are indicated in color and labeled near internal branches. Presence (+bc) or absence (-bc) of the bursa copulatrix is indicated in red on the topology. *Glaucus* lineage color coding appears as in Figs. 1, 2. Four cryptic lineages of *G. marginatus* are denoted by bold letters: A, Indo-Pacific; B, South Pacific; C, Eastern North Pacific; D, Central North Pacific.

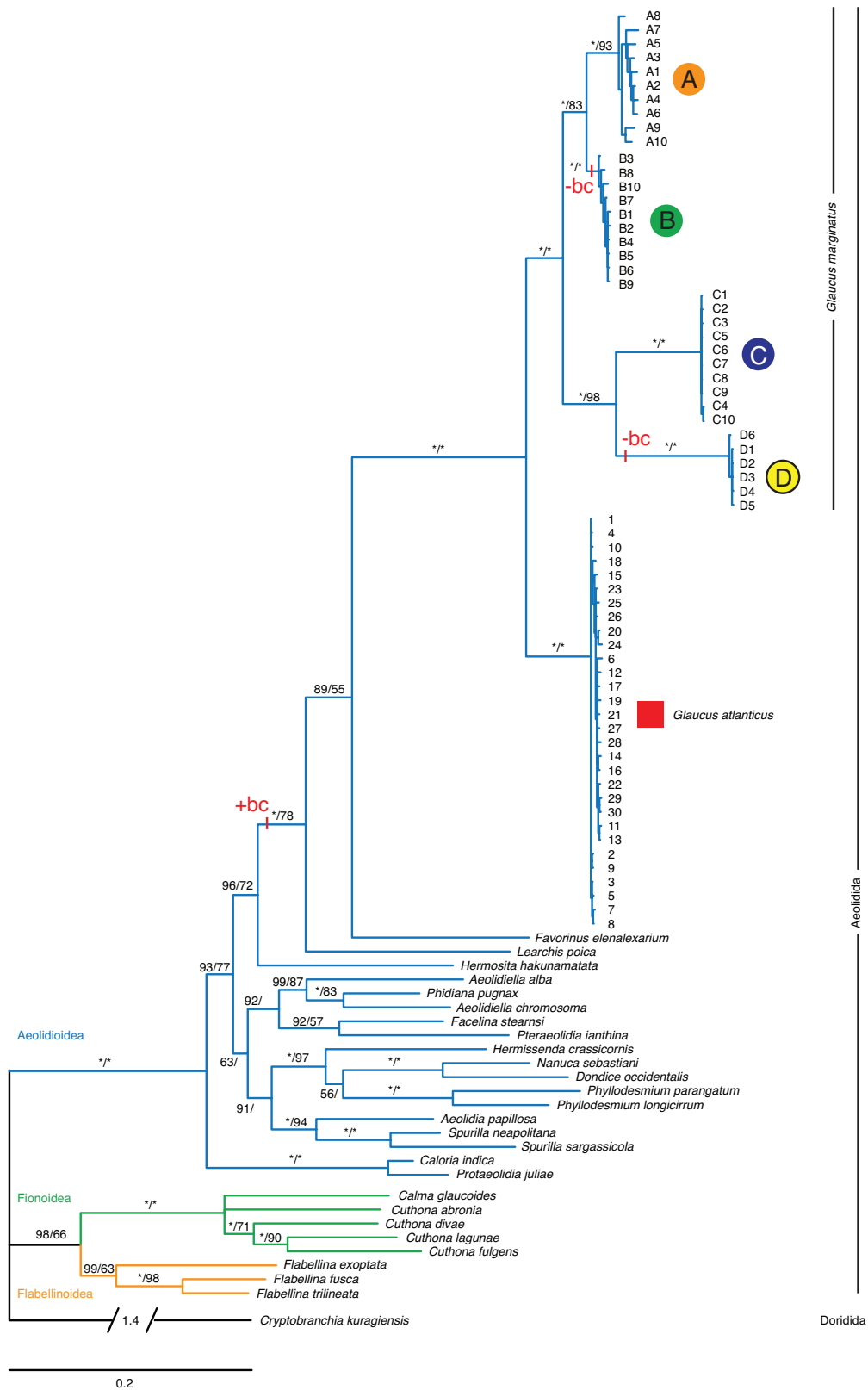


Figure 5-3. Two reproductive morphologies in *Glaucus* spp. viewed via dissections.

The topology of the *Glaucus* clade is presented at left with color-coding corresponding to four *G. marginatus* lineages (A-D) and *G. atlanticus* (E) (Figs. 5-1, 5-2). Images on the right are dorsal view photographs of glaucinid reproductive systems with most or all of the ovotestis removed. Dashed black circles outline the gonopore, black arrows indicate position of the vagina, and the bursa copulatrix (when present) is indicated with red letters. The penis is everted in all *G. marginatus* specimens (A-D) and inverted in *G. atlanticus* (E). A. *G. marginatus* (Indo-Pacific). B. *G. marginatus* (South Pacific). C. *G. marginatus* (Eastern North Pacific). D. *G. marginatus* (Central North Pacific). E. *G. atlanticus*. Abbreviations: am, ampulla; bc, bursa copulatrix; fgc, female gland complex; ot, ovotestis; pn, penis; ps, prostate; vg, vagina (dashed red arrow). Scale bars = 1.0 mm.

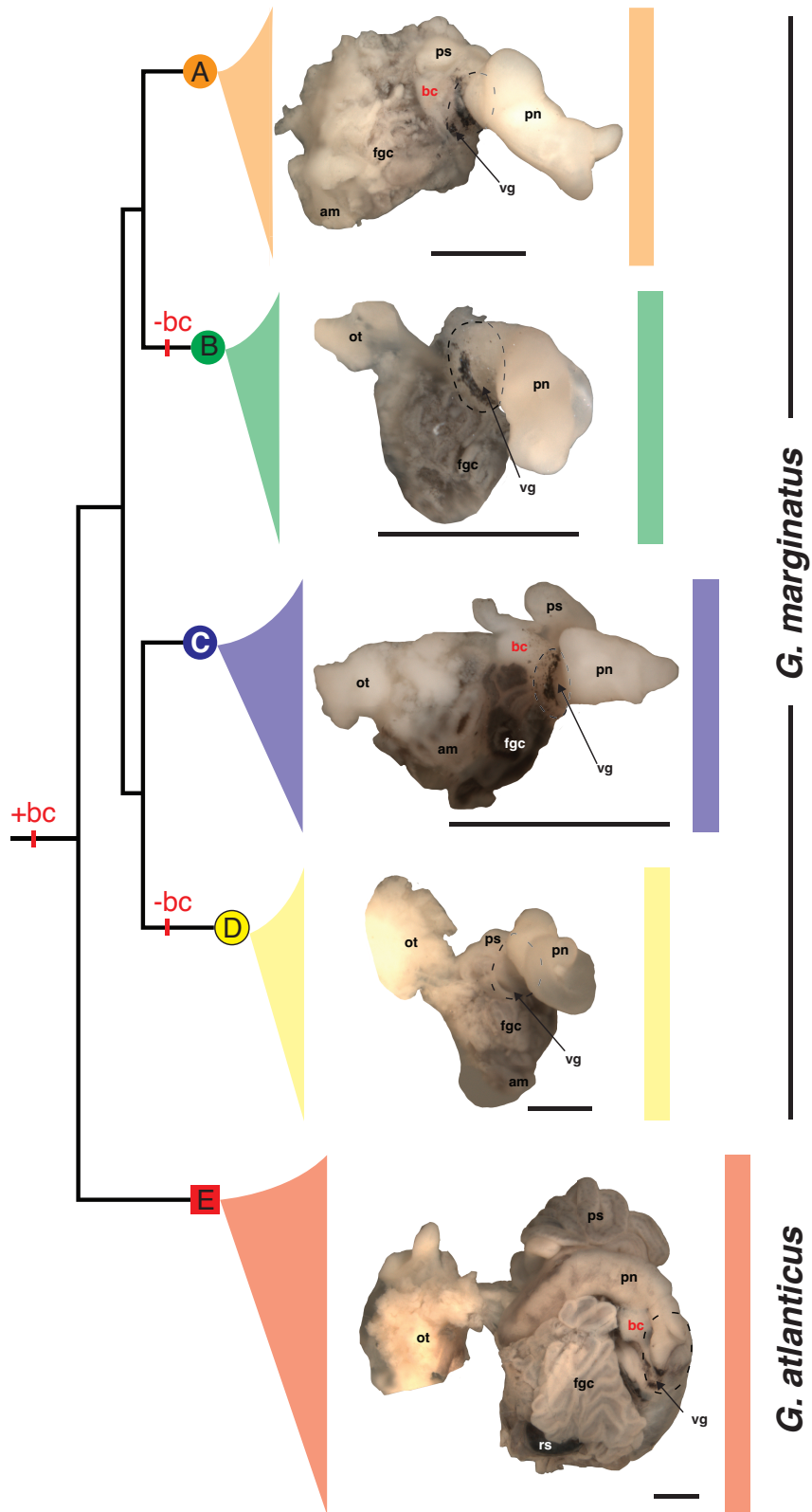


Figure 5-4. Two reproductive morphologies in *Glaucus* spp. viewed via histological sections. The topology of the *Glaucus* clade is presented at left with color-coding corresponding to four *G. marginatus* lineages (A-D) and *G. atlanticus* (E) (Figs. 5-1, 5-2). Images on the right are photographs of histological sections of distal glaucinid reproductive systems. The bursa copulatrix (when present) is indicated with red letters. **A1.** *G. marginatus* (Indo-Pacific) dorsal view, penis inverted. The bursa copulatrix is located immediately proximal to the vagina. **A2.** *G. marginatus* (Indo-Pacific) dorsal view. Detail of bursa copulatrix containing unoriented allosperm. **B1.** *G. marginatus* (South Pacific) ventral view, outer body wall removed, penis everted. No bursa copulatrix is present; proximal to the vagina, the oviduct leads to the female gland complex. **B2.** *G. marginatus* (South Pacific). Detail of oviduct. **C1.** *G. marginatus* (Eastern North Pacific) dorsal view, penis everted. The bursa copulatrix is located immediately proximal to the vagina. **C2.** *G. marginatus* (Eastern North Pacific). Detail of bursa copulatrix containing unoriented allosperm. **D1.** *G. marginatus* (Central North Pacific) dorsal view, penis everted. No bursa copulatrix is present; proximal to the vagina, the oviduct leads to the female gland complex. **D2.** *G. marginatus* (Central North Pacific). Detail of oviduct. **E1.** *G. atlanticus* (ventral view), outer body wall removed, penis inverted. The vagina opens immediately to the bursa copulatrix and the oviduct. **E2.** *G. atlanticus*. Detail of the vagina. **E3.** *G. atlanticus* sagittal section, lateral view. Detail of the bursa copulatrix containing unoriented allosperm, which is separate from the oviduct containing part of an egg string. **Abbreviations:** bc, bursa copulatrix; fgc, female gland complex; pn, penis; ps, prostate; ot, ovotestis; ov, oviduct; rs, receptaculum seminis; vg, vagina. **Scale bars:** **A1-A2** = 200 μm ; **B1** = 1.0 mm; **B2** = 500 μm ; **C1-C2** = 200 μm ; **D1- D2** = 200 μm ; **E1** = 1.0 mm; **E2** = 1.0 mm; **E3** = 500 μm .

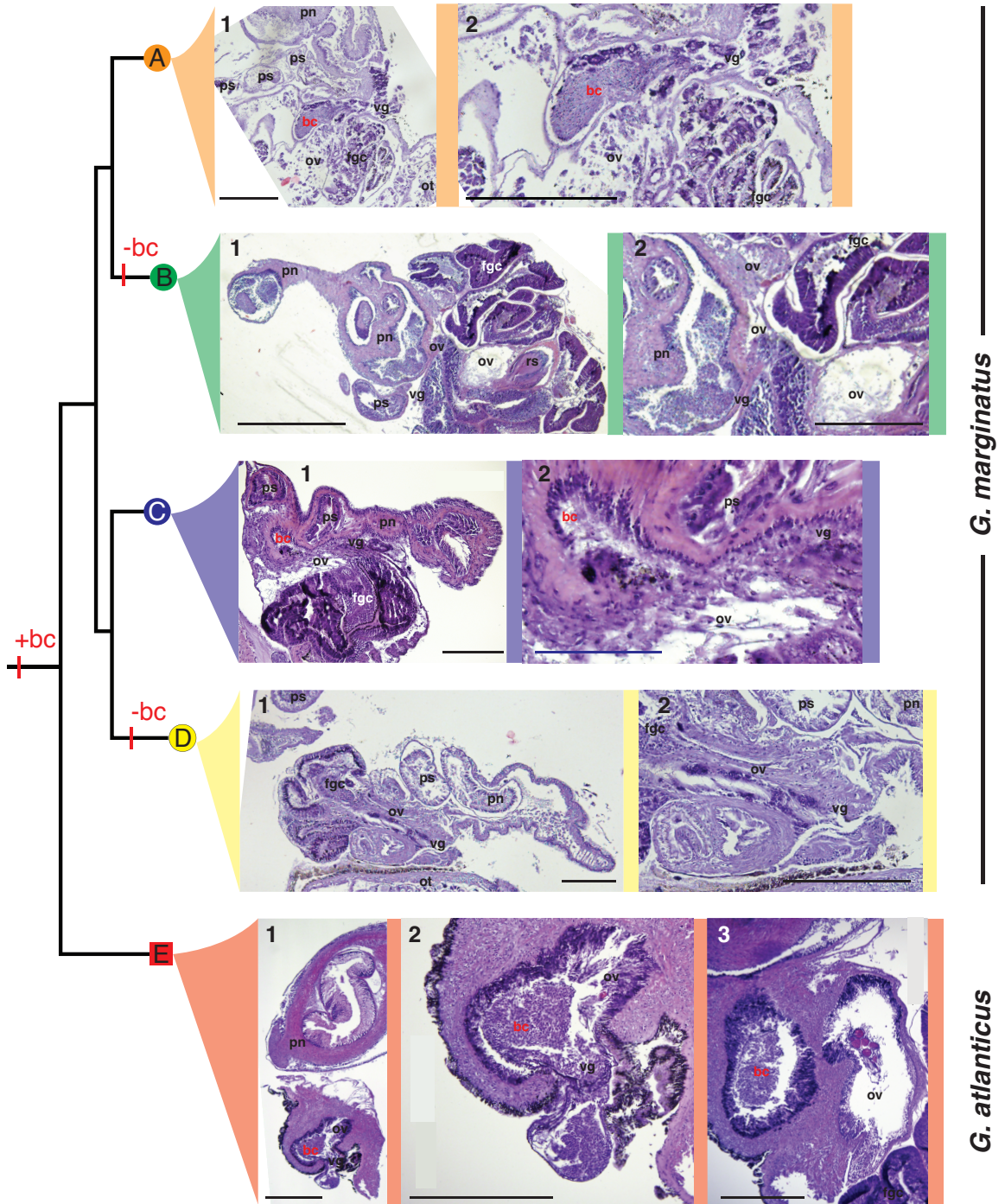


Figure 5-5. Two reproductive morphologies in the *Glaucus marginatus* cryptic species complex. **A.** Ventral view of *G. marginatus* mating pair (unassigned cryptic species from Queensland, Australia) engaged in reciprocal copulation via everted, intertwined penes. A dashed red box drawn on one individual indicates the approximate position of the internal reproductive system. Topology of *Glaucus* clade is inset. **B.** Diagrammatic representation of glaucinid reproductive system (with inverted penes) based on dissections and histology (Figs. 2, S2). The bursa copulatrix (bc) is highlighted with red letters and blue shading, and color-coding indicates presence (*G. atlanticus*, *G. marginatus* A and C) or absence (*G. marginatus* B and D). Diaulic branching of the distal ampulla is indicated by male and female gender symbols. **Abbreviations:** am, ampulla; bc, bursa copulatrix; cgl, capsule gland; fgc, female gland complex; gp, gonopore; hd, hermaphroditic duct; megl, membrane gland; mugl, mucus gland; ot, ovotestis; ov, oviduct; pn, penis; ps, prostate; rs, receptaculum seminis; vg, vagina. **Scale bars:** **A**, 1.0 cm; **B**, 1.0 mm.

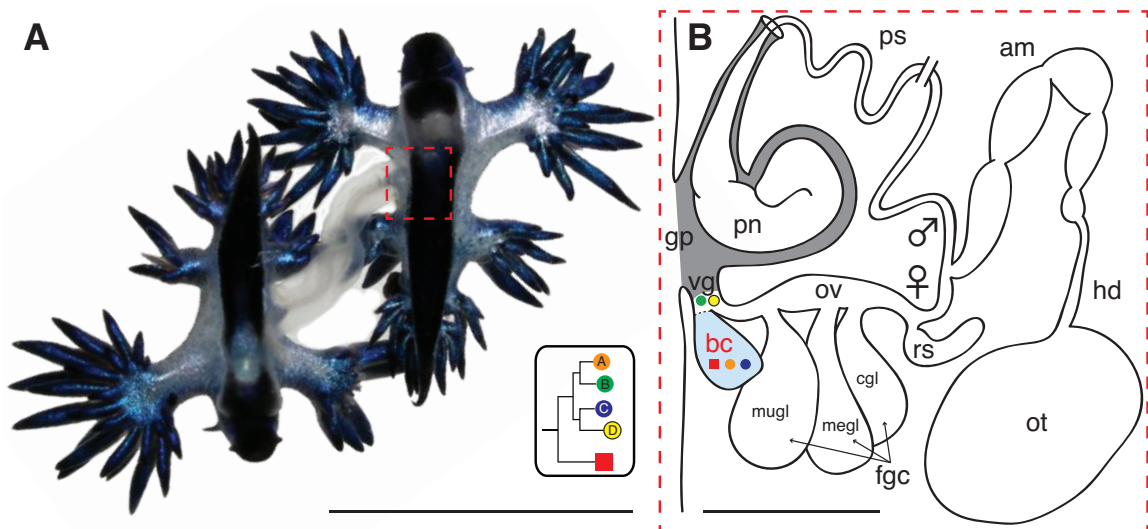


Table 5-1. Locality data for *Glaucus* spp. used in the molecular and morphological analyses. Color coding corresponds to the four glaucinin molecular lineages (Fig. 5-1B).

Species	Genus	Gyre System	Latitude (° Decimal)	Longitude (° Decimal)
<i>atlanticus</i> 1	<i>Glaucus</i>	North Atlantic	27.4965	-84.9965
<i>atlanticus</i> 2	<i>Glaucus</i>	North Atlantic	27.4965	-84.9965
<i>atlanticus</i> 3	<i>Glaucus</i>	North Atlantic	27.4965	-84.9965
<i>atlanticus</i> 4	<i>Glaucus</i>	North Atlantic	27.4965	-84.9965
<i>atlanticus</i> 5	<i>Glaucus</i>	North Atlantic	27.4965	-84.9965
<i>atlanticus</i> 6	<i>Glaucus</i>	North Atlantic	28.6627	-85.488
<i>atlanticus</i> 7	<i>Glaucus</i>	North Atlantic	27.4997	-86.0057
<i>atlanticus</i> 8	<i>Glaucus</i>	North Atlantic	28.0004	-87.4846
<i>atlanticus</i> 9	<i>Glaucus</i>	North Atlantic	24.4932	83.5035
<i>atlanticus</i> 10	<i>Glaucus</i>	North Atlantic	26.0047	86.0083
<i>atlanticus</i> 11	<i>Glaucus</i>	South Pacific	-4.265	-141.715
<i>atlanticus</i> 12	<i>Glaucus</i>	North Pacific	19.355	-156.7217
<i>atlanticus</i> 13	<i>Glaucus</i>	North Pacific	19.5212	-156.222
<i>atlanticus</i> 14	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>atlanticus</i> 15	<i>Glaucus</i>	North Pacific	19.355	-156.7217
<i>atlanticus</i> 16	<i>Glaucus</i>	North Pacific	19.355	-156.7217
<i>atlanticus</i> 17	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>atlanticus</i> 18	<i>Glaucus</i>	North Pacific	19.355	-156.7217
<i>atlanticus</i> 19	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>atlanticus</i> 20	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>atlanticus</i> 21	<i>Glaucus</i>	Indian	-32.05	115.7333
<i>atlanticus</i> 22	<i>Glaucus</i>	Indian	-32.05	115.7333
<i>atlanticus</i> 23	<i>Glaucus</i>	Indian	-32.05	115.7333
<i>atlanticus</i> 24	<i>Glaucus</i>	Indian	-32.05	115.7333
<i>atlanticus</i> 25	<i>Glaucus</i>	Indian	-32.05	115.7333
<i>atlanticus</i> 26	<i>Glaucus</i>	Indian	-32.05	115.7333
<i>atlanticus</i> 27	<i>Glaucus</i>	Indian	-30.095	30.862
<i>atlanticus</i> 28	<i>Glaucus</i>	Indian	-30.095	30.862
<i>atlanticus</i> 29	<i>Glaucus</i>	Indian	-30.095	30.862
<i>atlanticus</i> 30	<i>Glaucus</i>	Indian	-30.095	30.862
<i>marginatus</i> A1	<i>Glaucus</i>	Indian	-21.8	35.59
<i>marginatus</i> A2	<i>Glaucus</i>	Indian	-21.8	35.59
<i>marginatus</i> A3	<i>Glaucus</i>	Indian	-21.8	35.59
<i>marginatus</i> A4	<i>Glaucus</i>	Indian	-21.8	35.59
<i>marginatus</i> A5	<i>Glaucus</i>	North Pacific	19.5212	-156.222
<i>marginatus</i> A6	<i>Glaucus</i>	North Pacific	19.355	-156.7217
<i>marginatus</i> A7	<i>Glaucus</i>	North Pacific	19.355	-156.7217
<i>marginatus</i> A8	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> A9	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> A10	<i>Glaucus</i>	South Pacific	-4.265	-141.715
<i>marginatus</i> B1	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B2	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B3	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B4	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B5	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B6	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B7	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B8	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> B9	<i>Glaucus</i>	South Pacific	-33.738	151.31

<i>marginatus</i> B10	<i>Glaucus</i>	South Pacific	-33.738	151.31
<i>marginatus</i> C1	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C2	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C3	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C4	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C5	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C6	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C7	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C8	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C9	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> C10	<i>Glaucus</i>	North Pacific	25.082	-114.481
<i>marginatus</i> D1	<i>Glaucus</i>	North Pacific	19.48	-156.2883
<i>marginatus</i> D2	<i>Glaucus</i>	North Pacific	19.48	-156.2883
<i>marginatus</i> D3	<i>Glaucus</i>	North Pacific	19.48	-156.2883
<i>marginatus</i> D4	<i>Glaucus</i>	North Pacific	19.48	-156.2883
<i>marginatus</i> D5	<i>Glaucus</i>	North Pacific	19.355	-156.7212
<i>marginatus</i> D6	<i>Glaucus</i>	North Pacific	19.48	-156.2883

Table 5-2. Taxonomy, museum catalog number of voucher specimen, and GenBank accession numbers for sequences for four molecular markers (nuclear 28S rRNA, Histone-H3, mitochondrial 16S rRNA, COI) from the nudibranch species included in the molecular phylogeny (Figs. 5-1, 5-2). Museums and universities holding voucher specimens are identified by prefixes before catalog numbers: **AMS**, Australian Museum, Sydney, Australia; **CASIZ**, California Academy of Sciences; **CPIC**, California Polytechnic State University, Pomona, USA; **FLMNH**, Florida Museum of Natural History, University of Florida, Gainesville, USA; **LACM**, Los Angeles County Museum of Natural History, Los Angeles, USA; **NM**, Natal Museum, Pietermaritzburg, South Africa; **NUGB**, Nagoya University, Nagoya, Japan (outgroup previously in GenBank); **UMMZ**, University of Michigan Museum of Zoology, Ann Arbor, USA; **WAM**, Western Australian Museum, Perth, Australia. For *Glaucus* spp. with no voucher, the specimen size was so small that the whole animal was used for DNA extraction. Whole genomic DNA template for these samples is available at **UMMZ**. All accession numbers not beginning with JQ come from other studies and were previously published in GenBank. Asterisks indicate no sequence data.

Species	Genus	Family	Voucher	28S	H3	16S	COI
		Aeolidiida	CPIC				
<i>papillosa</i>	<i>Aeolidia</i>	e	00717	JQ699293	JQ699385	JQ699475	JQ699565
	<i>Aeolidiell</i>	Aeolidiida	LACM				
<i>alba</i>	<i>a</i>	e	175021	JQ699294	JQ699386	*	*
<i>chromosoma</i>	<i>Aeolidiell</i>	Aeolidiida	LACM				
	<i>a</i>	e	175027	JQ699295	JQ699387	JQ699476	JQ699566
			CPIC				
<i>glaucoides</i>	<i>Calma</i>	Calmididae	00562	JQ699296	JQ699388	JQ699477	JQ699567
			FLMNH			DQ41727	DQ41732
<i>indica</i>	<i>Caloria</i>	Glaucidae	429050	JQ699297	JQ699389	3	5
<i>kuragiensis</i>	<i>Cryptobranchia</i>	Dorididae	NUGB-	AB28277	AB54398	AB23834	AB23845
<i>s</i>	<i>nchia</i>	Dorididae	L505	9	4	6	7
		Tergipedi	CASIZ				
<i>abronia</i>	<i>Cuthona</i>	dae	174485	JQ699298	JQ699390	JQ699478	JQ699568
		Tergipedi	CASIZ				
<i>divae</i>	<i>Cuthona</i>	dae	174495	JQ699299	JQ699391	JQ699479	JQ699569
		Tergipedi	CASIZ				
<i>fulgens</i>	<i>Cuthona</i>	dae	174484	JQ699300	JQ699392	JQ699480	*
		Tergipedi	no				
<i>lagunae</i>	<i>Cuthona</i>	dae	voucher	JQ699301	JQ699393	JQ699481	*
<i>occidentalis</i>			LACM				
	<i>Dondice</i>	Glaucidae	2003-41.5	JQ699302	JQ699394	JQ699482	JQ699570
			no				
<i>stearnsi</i>	<i>Facelina</i>	Glaucidae	voucher	JQ699303	JQ699395	JQ699483	JQ699571
<i>elenalexia</i>			LACM				HM16275
<i>rum</i>	<i>Favorinus</i>	Glaucidae	153318	JQ699304	JQ699396	JQ699484	5
		Flabellini	LACM				
<i>exoptata</i>	<i>Flabellina</i>	dae	153895	JQ699305	JQ699397	JQ699485	JQ699572
		Flabellini	LACM				
<i>fusca</i>	<i>Flabellina</i>	dae	172497	JQ699306	JQ699398	JQ699486	JQ699573
		Flabellini	CPIC				GQ29202
<i>trilineata</i>	<i>Flabellina</i>	dae	00058	JQ699307	JQ699399	JQ699487	4
<i>atlanticus</i>	<i>Glaucus</i>	Glaucidae	UMMZ	JQ699308	JQ699400	JQ699488	JQ699574

1			302975				
<i>atlanticus</i>			UMMZ				
2	<i>Glaucus</i>	Glaucidae	302975	JQ699309	JQ699401	JQ699489	JQ699575
<i>atlanticus</i>			no				
3	<i>Glaucus</i>	Glaucidae	voucher	JQ699310	JQ699402	JQ699490	JQ699576
<i>atlanticus</i>			UMMZ				
4	<i>Glaucus</i>	Glaucidae	302975	JQ699311	JQ699403	JQ699491	JQ699577
<i>atlanticus</i>			UMMZ				
5	<i>Glaucus</i>	Glaucidae	302975	JQ699312	JQ699404	JQ699492	JQ699578
<i>atlanticus</i>			no				
6	<i>Glaucus</i>	Glaucidae	voucher	JQ699313	JQ699405	JQ699493	JQ699579
<i>atlanticus</i>			UMMZ				
7	<i>Glaucus</i>	Glaucidae	302976	JQ699314	JQ699406	JQ699494	JQ699580
<i>atlanticus</i>			UMMZ				
8	<i>Glaucus</i>	Glaucidae	302977	JQ699315	JQ699407	JQ699495	JQ699581
<i>atlanticus</i>			UMMZ				
9	<i>Glaucus</i>	Glaucidae	302978	JQ699316	JQ699408	JQ699496	JQ699582
<i>atlanticus</i>			UMMZ				
10	<i>Glaucus</i>	Glaucidae	302979	JQ699317	JQ699409	JQ699497	JQ699583
<i>atlanticus</i>			UMMZ				
11	<i>Glaucus</i>	Glaucidae	302980	JQ699318	JQ699410	JQ699498	JQ699584
<i>atlanticus</i>			UMMZ				
12	<i>Glaucus</i>	Glaucidae	302981	JQ699319	JQ699411	JQ699499	JQ699585
<i>atlanticus</i>			UMMZ				
13	<i>Glaucus</i>	Glaucidae	302982	JQ699320	JQ699412	JQ699500	JQ699586
<i>atlanticus</i>			AMS				
14	<i>Glaucus</i>	Glaucidae	C.462956	JQ699321	JQ699413	JQ699501	JQ699587
<i>atlanticus</i>			UMMZ				
15	<i>Glaucus</i>	Glaucidae	302983	JQ699322	JQ699414	JQ699502	JQ699588
<i>atlanticus</i>			UMMZ				
16	<i>Glaucus</i>	Glaucidae	302983	JQ699323	JQ699415	JQ699503	JQ699589
<i>atlanticus</i>			AMS				
17	<i>Glaucus</i>	Glaucidae	C.462956	JQ699324	JQ699416	JQ699504	JQ699590
<i>atlanticus</i>			UMMZ				
18	<i>Glaucus</i>	Glaucidae	302983	JQ699325	JQ699417	JQ699505	JQ699591
<i>atlanticus</i>			AMS				
19	<i>Glaucus</i>	Glaucidae	C.462956	JQ699326	JQ699418	JQ699506	JQ699592
<i>atlanticus</i>			AMS				
20	<i>Glaucus</i>	Glaucidae	C.462956	JQ699327	JQ699419	JQ699507	JQ699593
<i>atlanticus</i>			WAM				
21	<i>Glaucus</i>	Glaucidae	S59354	JQ699328	JQ699420	JQ699508	JQ699594
<i>atlanticus</i>			WAM				
22	<i>Glaucus</i>	Glaucidae	S59354	JQ699329	JQ699421	JQ699509	JQ699595
<i>atlanticus</i>			WAM				
23	<i>Glaucus</i>	Glaucidae	S59354	JQ699330	JQ699422	JQ699510	JQ699596
<i>atlanticus</i>			WAM				
24	<i>Glaucus</i>	Glaucidae	S59354	JQ699331	JQ699423	JQ699511	JQ699597
<i>atlanticus</i>			WAM				
25	<i>Glaucus</i>	Glaucidae	S59354	JQ699332	JQ699424	JQ699512	JQ699598
<i>atlanticus</i>			WAM				
26	<i>Glaucus</i>	Glaucidae	S59354	JQ699333	JQ699425	JQ699513	JQ699599
<i>atlanticus</i>			NM				
27	<i>Glaucus</i>	Glaucidae	W7469	JQ699334	JQ699426	JQ699514	JQ699600
<i>atlanticus</i>			NM				
28	<i>Glaucus</i>	Glaucidae	W7469	JQ699335	JQ699427	JQ699515	JQ699601

<i>atlanticus</i> 29	<i>Glaucus</i>	Glaucidae	NM W7469	JQ699336	JQ699428	JQ699516	JQ699602
<i>atlanticus</i> 30	<i>Glaucus</i>	Glaucidae	NM W7469	JQ699337	JQ699429	JQ699517	JQ699603
<i>marginatus</i> s A1	<i>Glaucus</i>	Glaucidae	CASIZ 176985	JQ699338	JQ699430	JQ699518	JQ699604
<i>marginatus</i> s A2	<i>Glaucus</i>	Glaucidae	CASIZ 176985	JQ699339	JQ699431	JQ699519	JQ699605
<i>marginatus</i> s A3	<i>Glaucus</i>	Glaucidae	CASIZ 176985	JQ699340	JQ699432	JQ699520	JQ699606
<i>marginatus</i> s A4	<i>Glaucus</i>	Glaucidae	CASIZ 176985	JQ699341	JQ699433	JQ699521	JQ699607
<i>marginatus</i> s A5	<i>Glaucus</i>	Glaucidae	UMMZ 302984	JQ699342	JQ699434	JQ699522	JQ699608
<i>marginatus</i> s A6	<i>Glaucus</i>	Glaucidae	UMMZ 302985	JQ699343	JQ699435	JQ699523	JQ699609
<i>marginatus</i> s A7	<i>Glaucus</i>	Glaucidae	UMMZ 302985	JQ699344	JQ699436	JQ699524	JQ699610
<i>marginatus</i> s A8	<i>Glaucus</i>	Glaucidae	UMMZ 302986	JQ699345	JQ699437	JQ699525	JQ699611
<i>marginatus</i> s A9	<i>Glaucus</i>	Glaucidae	UMMZ 302986	JQ699346	JQ699438	JQ699526	JQ699612
<i>marginatus</i> s A10	<i>Glaucus</i>	Glaucidae	UMMZ 302987	JQ699347	JQ699439	JQ699527	JQ699613
<i>marginatus</i> s B1	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699348	JQ699440	JQ699528	*
<i>marginatus</i> s B2	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699349	JQ699441	JQ699529	*
<i>marginatus</i> s B3	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699350	JQ699442	JQ699530	*
<i>marginatus</i> s B4	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699351	JQ699443	JQ699531	*
<i>marginatus</i> s B5	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699352	JQ699444	JQ699532	*
<i>marginatus</i> s B6	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699353	JQ699445	JQ699533	*
<i>marginatus</i> s B7	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699354	JQ699446	JQ699534	*
<i>marginatus</i> s B8	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699355	JQ699447	JQ699535	*
<i>marginatus</i> s B9	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699356	JQ699448	JQ699536	*
<i>marginatus</i> s B10	<i>Glaucus</i>	Glaucidae	AMS C.462957	JQ699357	JQ699449	JQ699537	*
<i>marginatus</i> s C1	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699358	JQ699450	JQ699538	JQ699614
<i>marginatus</i> s C2	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699359	JQ699451	JQ699539	JQ699615
<i>marginatus</i> s C3	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699360	JQ699452	JQ699540	JQ699616
<i>marginatus</i> s C4	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699361	JQ699453	JQ699541	JQ699617
<i>marginatus</i> s C5	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699362	JQ699454	JQ699542	JQ699618
<i>marginatus</i> s C6	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699363	JQ699455	JQ699543	JQ699619

<i>marginatus</i> C7	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699364	JQ699456	JQ699544	JQ699620
<i>marginatus</i> C8	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699365	JQ699457	JQ699545	JQ699621
<i>marginatus</i> C9	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699366	JQ699458	JQ699546	JQ699622
<i>marginatus</i> C10	<i>Glaucus</i>	Glaucidae	UMMZ 302988	JQ699367	JQ699459	JQ699547	JQ699623
<i>marginatus</i> D1	<i>Glaucus</i>	Glaucidae	UMMZ 302989	JQ699368	JQ699460	JQ699548	JQ699624
<i>marginatus</i> D2	<i>Glaucus</i>	Glaucidae	UMMZ 302989	JQ699369	JQ699461	JQ699549	JQ699625
<i>marginatus</i> D3	<i>Glaucus</i>	Glaucidae	UMMZ 302989	JQ699370	JQ699462	JQ699550	JQ699626
<i>marginatus</i> D4	<i>Glaucus</i>	Glaucidae	UMMZ 302989	JQ699371	JQ699463	JQ699551	JQ699627
<i>marginatus</i> D5	<i>Glaucus</i>	Glaucidae	UMMZ 302990	JQ699372	JQ699464	JQ699552	JQ699628
<i>marginatus</i> D6	<i>Glaucus</i>	Glaucidae	UMMZ 302989	JQ699373	JQ699465	JQ699553	JQ699629
<i>crassicornis</i>	<i>Hermisenda</i>	Glaucidae	LACM 2007-2.2	JQ699374	JQ699466	JQ699554	JQ699630
<i>hakunamata</i>	<i>Hermosita</i>	Glaucidae	LACM 175023	JQ699375	JQ699467	JQ699555	JQ699631
<i>poica</i>	<i>Learchis</i>	Glaucidae	CPIC 00137	JQ699376	JQ699468	JQ699556	JQ699632
<i>sebastiani</i>	<i>Nanuca</i>	Glaucidae	CPIC LACM 00522	JQ699377	JQ699469	JQ699557	JQ699633
<i>pugnax</i>	<i>Phidiana</i>	Glaucidae	LACM 2004-2.1	JQ699378	JQ699470	JQ699558	*
<i>longicirrum</i>	<i>Phyllodesmium</i>	Glaucidae	LACM 153823	JQ699379	JQ699471	JQ699559	JQ699634
<i>parangatum</i>	<i>Phyllodesmium</i>	Glaucidae	CASIZ 174440	JQ699380	JQ699472	JQ699560	JQ699635
<i>juliae</i>	<i>Protaeolidia</i>	Aeolidiida	LACM 153851	JQ699381	*	JQ699561	*
<i>ianthina</i>	<i>Pteraeolidia</i>	Glaucidae	LACM 153995	JQ699382	JQ699473	JQ699562	*
<i>neapolitana</i>	<i>Spurilla</i>	Aeolidiida	LACM 2004-94.3	JQ699383	JQ699474	JQ699563	JQ699636
<i>sargassicola</i>	<i>Spurilla</i>	Aeolidiida	CPIC 00718	JQ699384	*	JQ699564	*