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Parallel changes in genital morphology delineate cryptic diversification of planktonic nudibranchs

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The relative roles of geographical and non-geographical barriers in the genesis of genetic isolation are highly debated in evolutionary biology, yet knowing how speciation occurs is essential to our understanding of biodiversity. In the open ocean, differentiating between the two is particularly difficult, because of the high levels of gene flow found in pelagic communities. Here, we use molecular phylogenetics to test the hypothesis that geography is the primary isolating mechanism in a clade of pelagic nudibranchs, Glaucinae. Our results contradict allopatric expectations: the cosmopolitan *Glaucus atlanticus* is panmictic, whereas the Indo-Pacific *Glaucus marginatus* contains two pairs of cryptic species with overlapping distributions. Within the *G. marginatus* species complex, a parallel reproductive change has occurred in each cryptic species pair: the loss of a bursa copulatrix. Available *G. marginatus* data are most consistent with non-geographical speciation events, but we cannot rule out the possibility of allopatric speciation, followed by iterative range extension and secondary overlap. Irrespective of ancestral range distributions, our results implicate a central role for reproductive character differentiation in glaucinin speciation—a novel result in a planktonic system.

1. Introduction

Open-ocean planktonic communities confound evolutionary paradigms. They are composed of vast, passively drifting populations that span enormous geographical ranges [1,2]. Yet, in the absence of geographical barriers, regional populations and cryptic species abound [2,3]. How is pelagic biodiversity generated? Proposed speciation mechanisms in pelagic sibling species complexes are essentially variations on two classic themes: the geographical isolation of populations (allopatry) or non-geographical genetic isolation mechanisms (non-allopatry) [3,4]. 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 difficulty identifying species [5,6].

The marine neuston, the community of organisms associated with the ocean's air–water interface [7], is a promising system for investigating planktonic speciation mechanisms. In warm-water subtropical gyre systems, the base of the neustonic food chain is formed by a mutualism involving photosymbiotic dinoflagellates (zooxanthellae) and their porpitid cnidarian hosts [8]. The porpitids are preyed upon by two co-occurring gastropod mollusc lineages: the bubble-rafting snail family Janthinidae [9] and the nudibranch subfamily Glaucinae [10].

Glaucinin nudibranchs are the only truly pelagic members of their predominantly benthic suborder, Aeolidina [10]. 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 [10]. 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

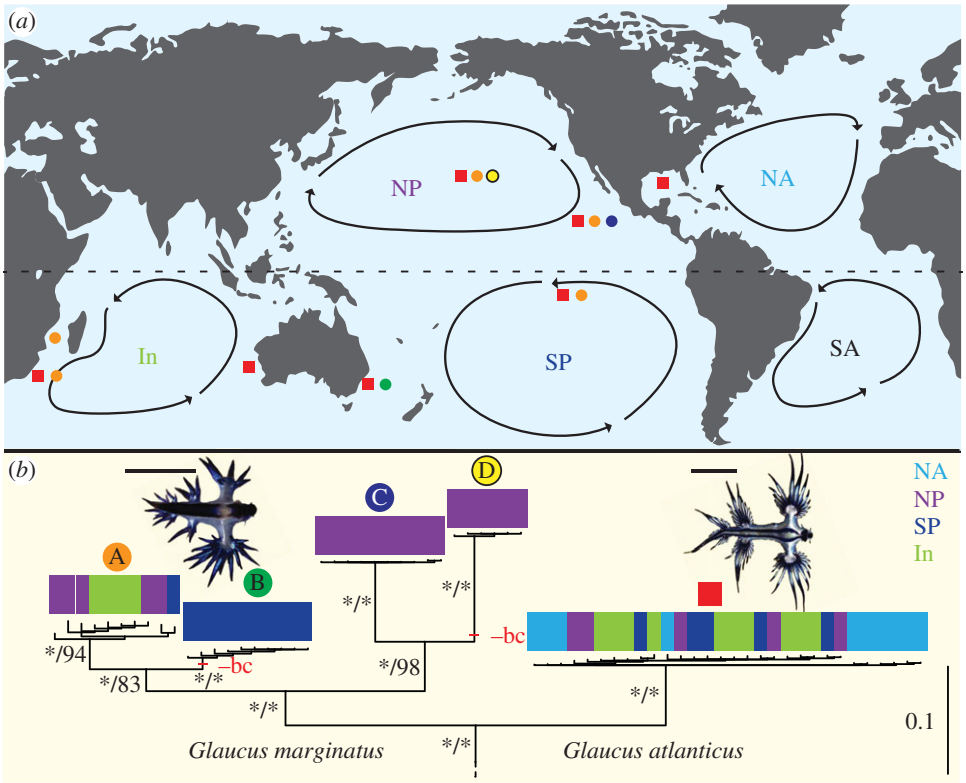

Figure 1. Biogeography and genetic structuring of two *Glaucus* morphospecies. (*a*) Ranges and sampling map of cosmopolitan *G. atlanticus* and Indo-Pacific *G. marginatus*. Sampling locations are represented on the map by circles (*G. marginatus*) and squares (*G. atlanticus*), which are colour-coded by molecular lineage. Five subtropical gyre systems are labelled: 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 2921 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 colour-coded by subtropical gyre system. 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 coloured circles and letters: A, Indo-Pacific; B, South Pacific; C, Eastern North Pacific; D, Central North Pacific.

surface (e.g. prey item, driftwood), from which larvae emerge after three days [10]. Glaucinae contains a single genus, *Glaucus*, which is thought to include two valid species, *Glaucus atlanticus* and the considerably smaller *Glaucus marginatus* [10]. Their geographical distributions differ: *G. atlanticus* is circumtropical (figure 1*a*), whereas *G. marginatus* has only been reported in the Pacific basin [10] and the Indian Ocean (this study; figure 1*a*; electronic supplementary material, table S1). 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.

2. Material and methods

(a) Sample collection

Glaucus spp. were collected as part of a global sampling effort of neustonic taxa via neuston tow and beach collection (see figure 1 and electronic supplementary material, table S1). Benthic aeolids were collected via scuba or snorkel, or from tidepools (Los Angeles County Museum and California Polytechnic State University samples), or loaned from museum collections (see the electronic supplementary material, table S2). All tissues were fixed and preserved in greater than or equal to 70% ethanol. *Glaucus* spp. were identified by external morphology [10].

(b) DNA extraction, amplification and sequencing

Whole genomic DNA was extracted from one to five cerata, specimen size permitting, using the EZNA Mollusc DNA Kit (Omega Bio-Tek) or the DNEasy Blood and Tissue Kit (Qiagen). A total of 2921 aligned nucleotides were amplified from four molecular markers. One thousand three hundred and ninety-one nucleotides 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′) [11] or by pairing the previous primers with the internal aeolid-specific primers D23Faeolid (5′-GAAAGTTTGAGARTAGGWC-3′) and D4RBaeolid (5′-CGYCR GACTCCTTGGTCCGTGT-3′), whose positions correspond to previously published primers D23F/D4RB [12]. All 28S amplifications were performed with an annealing temperature of 50°C. Three hundred and twenty-eight nucleotides of nuclear Histone-H3 were amplified using universal primers HexAF/HexAR [13] and an annealing temperature of 53°C. Five hundred and forty-four nucleotides of mitochondrial (mt) 16S rRNA were amplified using universal primers 16Sar/16Sbr [14] and an annealing temperature of 49°C. Six hundred and fifty-eight nucleotides of mt COI were amplified using universal primers LCO1490/HCO2198 [15] 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 s; X°C, 30 s 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 v. 3.7.1.1 (CodonCode Corporation) and verified by eye. Accession numbers for all sequences generated for this study are listed in electronic supplementary material, table S2.

(c) Phylogenetic analyses

Best-fit models of nucleotide substitution were selected statistically by Bayesian information criterion (BIC) in jMODELTEST v. 0.1.1 [16,17] 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 v. 3.1.2 [18] (four chains, 10 million generations) with a concatenated dataset; 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 v. 2.0 [19], 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 less than 0.4 lnL. 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* v. 4.0 [20].

(d) Anatomical and histological examination

Ethanol-preserved specimens (at least 12 of *G. atlanticus* and of each *G. marginatus* lineage except D, Central North Pacific, $n = 6$) were morphologically characterized using either anatomical dissection or histological examination. All of the specimens so examined were sexually mature, as revealed by the presence of a fully developed female gland complex, the last part of the nudibranch reproductive system to develop [21]. See electronic supplementary material, note S1 for a functional description of the complex, hermaphroditic glaucinin reproductive system.

Dissected individuals (greater than or equal to 10 of *G. atlanticus* and of each *G. marginatus* lineage except D, Central North Pacific, $n = 4$) had a transverse incision made on their dorsal side 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 (see the electronic supplementary material, figure S1).

Two individuals each of the five *Glaucus* lineages (*G. atlanticus* and *G. marginatus* A–D) were characterized histologically at the University of Michigan Medical School Microscopy and Image Analysis Laboratory (MIL) Biomedial 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 transverse and sagittal plane 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 (see the electronic supplementary material, figure S2).

3. Results and discussion

Figure 1*b* shows a detail of the first molecular phylogeny including *Glaucus* spp. and representatives of 16 other aeolid genera (see the electronic supplementary material, table S2, and figure S3 for the complete analysis). Our results contradict hypothesized geographical 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 (figure 1*b*). 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.

Although our molecular phylogenetic results (figure 1*b*) are consistent with the presence of a cryptic species complex within the *G. marginatus* morphospecies, they stem from variation in the two mitochondrial markers (mt 16S recovers *G. marginatus* lineages A–D; mt COI recovers A, C, D), not from the more conserved nuclear gene fragments (see the electronic supplementary material, figures S3 and S4 for details). Unlike many pelagic taxa [3,6], for which morphological data are unavailable, aeolid nudibranch morphology (including *Glaucus* spp.) has been studied in detail [10,21–27]. To test for anatomical corroboration that these four *G. marginatus* mt molecular clades 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 jaws) and the reproductive system. Using dissections and histology, we compared the morphologies of at least 12 representatives of each lineage except clade D ($n = 6$). We did not find any consistent differences in either external or radular morphologies; however, in dissections (see the electronic supplementary material, figure S1) and in histological sections (see the electronic supplementary material, figure S2) of the reproductive system, we found one morphological character that is consistently lineage-specific among each sister species pair: the presence or the absence of a bursa copulatrix (figure 2; see electronic supplementary material, note S1 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 [21,22] and may also have a gametolytic function; but this function was not observed in *G. atlanticus* [23] or *Glaucus* spp. (this study). It is present in *G. atlanticus* and in two (A, C) of the four *G. marginatus* cryptic lineages (electronic supplementary material, figures S1 and S2). Coding the gain/loss of the bursa copulatrix on our tree topology (figure 1*b*) shows that members of each cryptic *G. marginatus* tip clade differ in the presence/absence of this structure, and that the two cryptic lineages (B, D) lacking the bursa copulatrix have lost it independently. Loss of this structure has been previously observed in phylogenetically derived species of aeolids [21], and its presence/absence has been shown to be a species-delimiting character in nudibranchs, not an indication of the state of sexual maturation [21,24]. On the basis of this morphological corroboration of the molecular

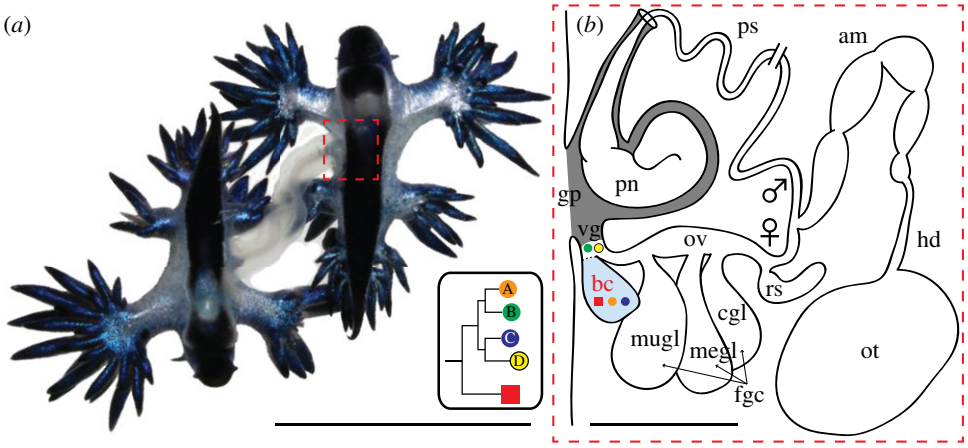

Figure 2. Two reproductive morphologies in the *G. marginatus* cryptic species complex. (*a*) Ventral view of *G. marginatus* mating pair (unassigned cryptic species from Queensland, Australia) engaged in reciprocal copulation via everted, intertwined penes. A dashed red box drawn on one individual indicates the approximate position of the internal reproductive system. Topology of *Glaucus* clade is inset. (*b*) Diagrammatic representation of glaucinid reproductive system (with inverted penis) based on dissections and histology (see the electronic supplementary material, figures S1 and S2). The bursa copulatrix (bc) is highlighted with red letters and blue shading, and colour-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. 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.

data, we conclude that the four cryptic *G. marginatus* lineages have speciated.

Our results show that in Glaucinae, a nominal taxon's geographical range is a poor predictor of its degree of genetic structuring or of its propensity to form cryptic species complexes. Both pairs of *G. marginatus* sister taxa co-occur in at least one gyre system (figure 1), a distribution pattern consistent with sympatric speciation origins. Nevertheless, we lack precise information on the actual geographical ranges of all four cryptic taxa (e.g. *G. marginatus* was unrecorded from the Indian Ocean prior to this study, and we do not know how stable these individual ranges are over evolutionary time scales). We therefore cannot rule out the possibility of allopatric or parapatric speciation, followed by iterative range extension and secondary overlap.

The loss of a bursa copulatrix in parallel *G. marginatus* clades is of particular interest because rapid divergence in genital morphology has long been considered an evolutionary result of selection [28,29], and this repeatedly evolved internal reproductive apomorphy is the only morphological change we observed among the two pairs of cryptic sister species. It may have served as the underlying speciation mechanism in both cases, and we hypothesize that sexual selection for different mating behaviours may be involved in maintaining sympatric (within same subtropical gyre system) sister *G. marginatus* lineages. If this hypothesis is correct, it would argue against an allopatric speciation scenario because it is difficult to envisage how one member of each cryptic sister species pair could have independently lost this reproductive structure in the absence of a unified mechanism of selection.

Glaucinid copulation requires participants to align their ventral surfaces, evert and intertwine their penes, and reciprocally transfer sperm to their partner's genital aperture (figure 2) [25,26]. One study has documented highly distinctive mating behaviours in *G. atlanticus* versus *G. marginatus*. Coitus in the former involves a penial spine and is much more prolonged (43–59 min) than in *G. marginatus* (50–70 s; unknown cryptic lineage sampled off Sydney, Australia) [26]. The behavioural consequences of losing a bursa copulatrix have not been studied in nudibranchs [21,22], and it is unknown if all four *G. marginatus* cryptic species share an abbreviated mating behaviour. We hypothesize that the presence or the absence of a bursa copulatrix at the genital aperture does affect mating behaviour between *G. marginatus* lineages 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 [26].

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 [3–6], 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; $BS_{ML} = 72$; electronic supplementary material, figure S3). More extensive sampling is necessary to identify the benthic sister lineage of *Glaucus*; however, the ecology of *Learchis 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 [27]. 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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Data accessibility. DNA sequences from the molecular phylogeny have been deposited with GenBank (NIH genetic sequence database) under accession numbers JQ699293–JQ699636.

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