



Dwarf brooder versus giant broadcaster: combining genetic and reproductive data to unravel cryptic diversity in an Antarctic brittle star

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Abstract

Poecilogony, or multiple developmental modes in a single species, is exceedingly rare. Several species described as poecilogenous were later demonstrated to be multiple (cryptic) species with a different developmental mode. The Southern Ocean is known to harbor a high proportion of brooders (Thorson's Rule) but with an increasing number of counter examples over recent years. Here we evaluated poecilogony vs. crypticism in the brittle star *Astrotoma agassizii* across the Southern Ocean. This species was initially described from South America as a brooder before some pelagic stages were identified in Antarctica. Reproductive and mitochondrial data were combined to unravel geographic and genetic variation of developmental modes. Our results indicate that *A. agassizii* is composed of seven well-supported and deeply divergent clades (I: Antarctica and South Georgia; II: South Georgia and Sub-Antarctic locations including Kerguelen, Patagonian shelf, and New Zealand; III-VI-VII: Patagonian shelf, IV-V: South Georgia). Two of these clades demonstrated strong size dimorphism when in sympatry and can be linked to differing developmental modes (Clade V: dwarf brooder vs. Clade I: giant broadcaster). Based on their restricted geographic distributions and on previous studies, it is likely that Clades III-VI-VII are brooders. Clade II is composed of different morphological species, *A. agassizii* and *A. drachi*, the latter originally used as the outgroup. By integrating morphology, reproductive, and molecular data we conclude that the variation identified in *A. agassizii* is best described as crypticism rather than poecilogony.

Introduction

Marine invertebrates display a wide diversity of developmental modes, with development occurring in both pelagic and non-pelagic habitats (Levin and Bridges 1995; Poulin and Féral 1996; Marshall et al. 2012). It is generally accepted that developmental mode has an influence on dispersal distance, and that brooding species have a shorter distance of dispersal than broadcasters (Kelly and Palumbi 2010; White et al. 2010; Selkoe and Toonen 2011; Moreau

et al. 2017). Developmental mode strongly affects connectivity and demographic patterns within or among populations and, consequently, how independently these populations evolve (Kelly and Palumbi 2010; Allcock and Strugnell 2012; Riesgo et al. 2015).

Marine invertebrates have long been a model for studying life-history evolution because of the diversity of their developmental modes (Emler et al. 1987; McEdward 1995), with phylogenetic analyses in some taxa showing rapid transition in developmental mode (Hart et al. 1997, 2003; Duda and Palumbi 1999; Collin et al. 2007). Poecilogony, defined as an “intraspecific variation in developmental mode” (Knott and McHugh 2012), is a rare life-history strategy that has been reported in a variety of marine invertebrates, primarily polychaetes and molluscs (e.g., see Blake and Arnofsky 1999; Krug 2007). Typically, this involves a species producing both planktotrophic and lecithotrophic larvae, but under Levin and Bridges' (1995) classification scheme for invertebrate larval development, a shift in the site of development (e.g., pelagic to benthic lecithotrophy) would also be

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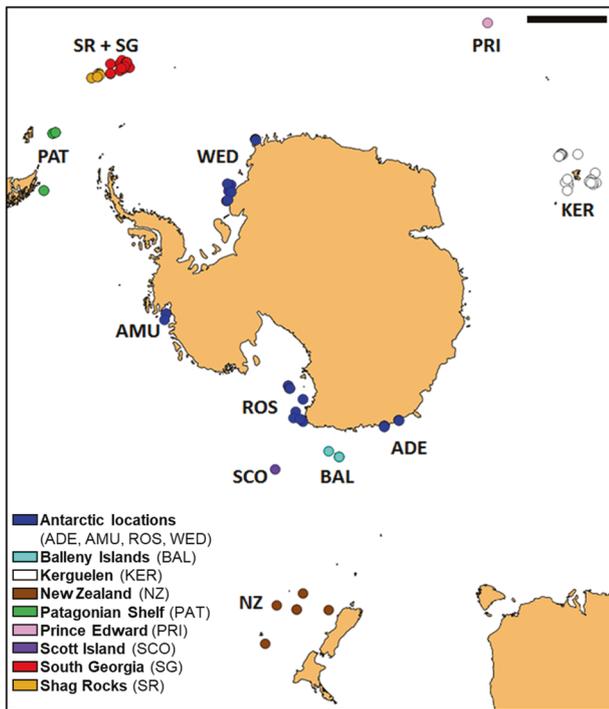


Fig. 1 Sampling locations of *Astrotoma* samples in the Southern Ocean. Projection Reference System is South Pole Azimuthal Equidistant. Scale bar: 1000 km

considered a variation in developmental mode. In many cases, species described as poecilogenous have later been shown with molecular analyses to be morphologically cryptic species (Byrne et al. 2003; Hart et al. 2003; Knott and McHugh 2012). Differentiating between cases of true poecilogony and the presence of cryptic species is critical to understanding evolutionary transitions in developmental mode and the implications this has for the dispersal potential of a single species (Knott and McHugh 2012; Moon et al. 2017).

A case in point is the brittle star *Astrotoma agassizii* Lyman, 1875 (Euryalida: Gorgonocephalidae), which has a southern circumpolar distribution (Antarctic and sub-Antarctic through to southern temperate regions) at depths ranging from 40 to 1700 m (Bernasconi 1965; Sands et al. 2013; Berecoechea et al. 2017) and has been recognized as a morphologically uniform species throughout its range (Hunter and Halanych 2008; Galaska et al. 2017). This species was first described as a brooder using a small number of South American specimens (three to four individuals/study; Bernasconi 1965; De La Serna De Estaban 1966). A more large-scale study from several Antarctic and sub-Antarctic locations (806 specimens from Antarctic Peninsula, Ross Sea, South Georgia, and Patagonia) revealed brooding specimens in each region, but with a very low frequency in Antarctica (2 individuals out of 325

investigated; Monteiro and Tommasi 1983). Molecular analyses—using mitochondrial genes (Hunter and Halanych 2008) and RAD-seq data (Galaska et al. 2017)—have revealed three clades in *A. agassizii*, one from the Antarctic continent (Antarctic Peninsula) and two from the Patagonian shelf of South America. Recent evidence using DNA barcoding of plankton samples has shown that *A. agassizii* from the Ross Sea region of Antarctica has a pelagic lecithotrophic larva (Heimeier et al. 2010) and all sequences were within the Antarctic clades presented by Hunter and Halanych (2008). This suggests that *A. agassizii* might be composed of cryptic species with distinct developmental modes: a broadcasting species in Antarctica and two brooding species on the Patagonian shelf. None of the previous studies focused on both genetic and reproductive data, so the potential for poecilogony at some locations cannot be excluded.

In the current work, we investigated the likelihood of poecilogony in *A. agassizii* by examining the relationship between genetic diversity and developmental mode in specimens collected across the Southern Ocean (Fig. 1). Genetic variation was evaluated using the mitochondrial gene Cytochrome c Oxidase subunit I, whereas developmental mode was investigated through direct (brooding in mature individuals) and indirect (egg size) evidence. This combination of distinct methods allowed us to address two evolutionary questions: (1) Can we identify different developmental modes in *A. agassizii*? (2) Can these modes be linked to genetic clades or is this a rare example of poecilogony? (3) When compared with the previous Antarctic Peninsula focus of Hunter and Halanych (2008), and Galaska et al. (2017), is there further genetic structure in *A. agassizii* when taking into account the wider distribution of the species? (4) When compared with the related species *Astrotoma drachi* (Guille 1979), is the variation among clades equivalent to that seen between the ingroup clades and *A. drachi*?

Material and methods

Sampling

Specimens were collected during several international expeditions (ACE, JR144, JR179, JR262, TAN0402, TAN0602, TAN0705, TAN0802, TRIP, POKER II, PS77, PS82, and REVOLTA I-II-III) from 2004 to 2017. Samples were immediately frozen or stored in 96% ethanol. In total, 298 specimens were sampled to obtain both reproductive and genetic data. Sampling locations (Fig. 1) include Antarctic locations (Adélie Land: 17, Amundsen Sea: 15, Balleny Islands: 5, Ross Sea: 26, Scott Island: 2, and Weddell Sea: 38), Patagonian shelf (33), South Georgia

(78) and Shag Rocks (50), Kerguelen (26), Prince Edward (1), and Subantarctic New Zealand (7). All the samples were identified as *A. agassizii* by Sadie Mills, Rafael Martin-Ledo, and Chester Sands, except the New Zealand specimens that were identified as *A. drachi*.

Genetic data

Cytochrome oxidase 1 (CO1) sequences (603 base fragment) were obtained for 227 individuals. DNA extractions were performed on arm tip tissues and were based on the salting-out protocol from Sunnucks and Hales (1996). Amplification used the primers “F-LCO1490-OPIUR-OID” (5′-TTTCAACNAAACAYAAGGAYATWGG-3′) and “R-HCO” (5′-TAAACTTCAGGGTGACCAAAAAA TCA-3′; Folmer et al. 1994). Each PCR mix (10 µl) included 8.5 µl of Mastermix (dNTP 25 mM, ammonium sulfate 0.1 M, Tris HCl 1 M, bovine serum albumin 25 mg/ml, β-mercaptoethanol 1 M, MgCl₂ 50 mM, H₂O), 0.5 µl of Taq Polymerase (5 U/µl), 0.5 µl each primer (10 µM), and 0.5 µl of the DNA extract. PCR conditions consisted of 35 cycles for each of the three temperature steps [30 s at 94 °C (denaturation), 30 s at 48 °C (annealing), and 45 s at 72 °C (elongation)]. These cycles were preceded by a step of 2 min at 94 °C and were followed by a step of 10 min at 72 °C. PCR products were then sent to the MACROGEN sequencing service for purification and sequencing. Sequence editing and alignment were performed using BioEdit 7.0 (Hall 1999) and MEGA7 (Kumar et al. 2016). The absence of stop codon in the sequence was checked in MEGA7, whereas the absence of base compositional heterogeneity was examined in SeqVis v1.5 (Ho et al. 2006). A TCS (Templeton Crandall Singh) haplotype network was produced in PopART 1.7 (Leigh and Bryant 2015). Molecular diversity indices were calculated using Arlequin 3.5 (Excoffier and Lischer 2010), including the number of haplotypes, the haplotype diversity, the number of polymorphic sites, the nucleotide diversity, and Tajima’s *D* and Fu’s *F_s*. Significance of *D* and *F* were evaluated through a permutation test (10,000 randomizations). Only clades with at least six individuals were considered for the calculations of these statistics, as it has been demonstrated that lower sampling sizes might lead to inaccurate estimates (Goodall-Copestake et al. 2012). JModeltestv2 (AIC - “Akaike information criterion”, Darriba et al. 2012) was used to determine that GTR + G was the most suitable substitution model for our dataset. A Bayesian phylogeny was then produced in MrBayes 3.2 (Ronquist and Huelsenbeck 2003) using 100 million generations. Every 100,000 generations, the tree was sampled, thus obtaining a total of 1000 trees. Node support was assessed through posterior probability values. Initially, the closely related species *A. drachi* was

used as the outgroup but our first results showed that it was in fact an ingroup. We therefore selected another outgroup, namely *Ophiura lymani* and other more closely related taxa (*Asteronyx*, *Astrochlamys*, *Astrohamma*, *Gorgonocephalus*). Mean pairwise distances between clades (*p*-distances, corrected, and uncorrected) were calculated between clades using the software Mega X (Kumar et al. 2018).

Two methods of “automatic” single locus species delimitation were used to assess how clade divergence matched with theoretical views on the speciation process. The multirate General Mixed Yule Coalescent (GMYC) was implemented using the Splits package (Ezard et al. 2009; Fujisawa, Barraclough 2013) with R (V 3.5.1, R Core Team 2016). First, an ultrametric phylogeny was produced in BEAST 2.4 (Bouckaert et al. 2014) using a GTR model, a relaxed uncorrelated molecular clock, and a yule process prior. Several runs were conducted and after each run prior value performance was assessed in TRACER V1.6; prior values were then “tweaked” until performance was deemed satisfactory. The final run was 10⁸ generations with 25% burnin, sampling every 10⁵ generations. Sampled trees were combined in LogCombiner V2.4 and summarized in TreeAnnotator V2.4, both part of the BEAST 2 package. Species delimitation was also explored using the Poisson Tree Process (PTP; Zhang et al. 2013). Although GMYC uses branch coalescing patterns to group putative species, PTP delimits species by judging relative branch lengths within and among putative groups. We used the more sophisticated multirate (mPTP) package of Kapli et al. (2017), using the maximum likelihood tree and a maximum likelihood heuristic search, taking into account minimum branch lengths.

Reproductive data

A total of 233 specimens were dissected and the disc diameter was measured using Vernier calipers. For each individual we described the presence of gonads, as well as any direct evidence of brooding, such as the presence of juveniles inside the bursae. In order to compare brooding and sexual maturity at different locations, we used the definition of Monteiro and Tommasi (1983) as sexually mature “individuals incubating juveniles or harboring ovaries with distinguishable eggs” and extracted some reproductive data from Monteiro and Tommasi (1983). In ten specimens from different locations/clades, we also measured the size of the largest eggs from the ovaries using a binocular microscope. Egg diameter was determined from digital photographs using the software ImageJ (Schneider et al. 2012). Statistical tests were performed using STATISTICA 7.0 (statsoft.com) or in R 3.3.2 (R Core Team 2016) using the Vegan package (Oksanen et al. 2010).

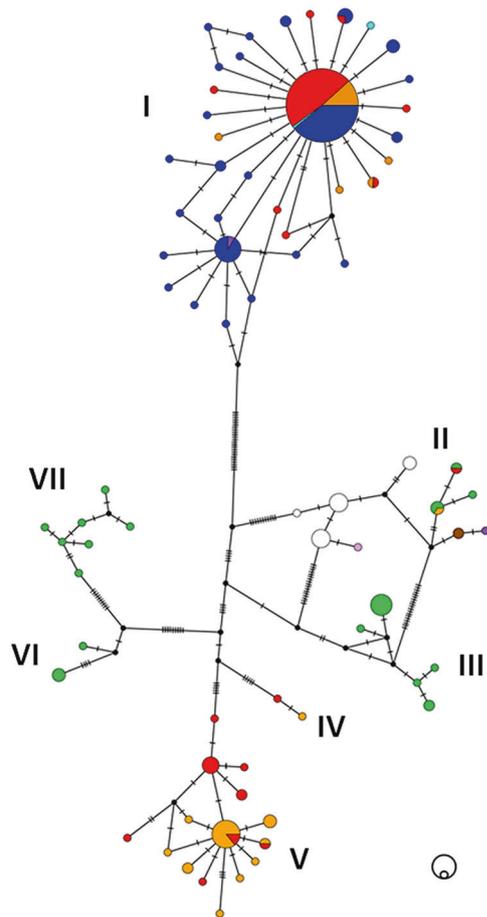


Fig. 2 Haplotype network based on 227 CO1 sequences (603 bp) of *Astrotoma*. Different colors denote different locations (see legend in Fig. 1). Circle sizes at the bottom right denote the number of samples exhibiting the haplotype (outer circle: 10 samples, inner circle: 1 sample). Clades I–VII are based on the mPTP analysis (species delimitation method)

Results

Genetic data

We successfully obtained a 603 base alignment of 227 CO1 sequences resulting in 75 haplotypes distributed among 7 discrete genetic lineages (posterior probabilities = 1, Figs. 2 and 3). These seven lineages are based on the mPTP analysis, whereas the other species delimitation method (GMYC) provided a number of 35 hypothesized species. The largest clade (Clade I, 128 individuals) included samples from both the Antarctic continental shelf and from around the shelf of South Georgia and Shag Rocks. All other clades formed a polytomy and were sister to Clade I (Fig. 3). Clade II (divided into three geographically specific sub-clades) included samples from the Kerguelen Plateau and Prince Edward Island (sub-clade A), South Georgia, Shag Rocks and the Patagonian Shelf (sub-clade

B), and the “outgroup” taxon *A. drachi* from New Zealand with one specimen from Scott Island (sub-clade C). Clade III was exclusively from the Patagonian Shelf. Clades IV and V share a common ancestor and are exclusively from the South Georgia/Shag Rocks shelf region, whereas Clade VI and VII, also sharing a common ancestor, are exclusively from Patagonian Shelf. The degree of divergence between Clade I and the other six clades is considerable (>10% average corrected pairwise distance, >6% uncorrected *p*-distance; Supplementary Material 1), and the corrected distances between the other clades ranged between 2.4% (Clades IV and V) and 8.3% (Clades II and VII), respectively (2.1% and 5.0% uncorrected *p*-distances, Supplementary Material 1). Within clade, distances ranged between 0.1% (Clade I) and 0.5% (Clade VII), demonstrating a clear diversity “gap” within and among clades.

The relationship between haplotype diversity and nucleotide diversity in the combined dataset (all samples in one group) was high compared with that expected from a panmictic species (see Fig. 3 from Goodall-Copstake et al. 2012), indicating that it is likely there are cryptic species present. When analyzed on a clade-by-clade basis, haplotype diversity vs. nucleotide diversity fits within the expected variation of a panmictic species in each clade (Table 1). Nucleotide diversity of Clade I was, however, low compared with haplotype diversity (Table 1). As a result, both Tajima’s *D* and Fu’s *S* significantly deviated from neutral expectations, indicating either selection or population expansion (Table 1). When separated out into the two geographic components “Antarctic Clade I” and “South Georgia Clade I,” the result for each group was similar (Tajima’s *D* and Fu’s *S* significantly negative, data not shown). Clade V, also found on South Georgia, was significantly different from neutral expectations, suggesting a common mechanism or shared history with Clade I (Table 1).

Reproductive data

Among the 233 specimens dissected in our study, 173 were assigned to a genetic clade (116 individuals to Clade I, 19 to Clade II, 1 to Clade III, 2 to Clade IV, 19 to Clade V, and 4 to Clade VII). Thirty-eight percent of specimens were observed to be mature with a disc diameter (at maturity) ranging between 1.3 and 6.4 cm (Table 2). Mature specimens were present in Clade I in February (Antarctica), April (South Georgia, Antarctica), and November (South Georgia); Clade V had mature specimens in April and November (Supplementary Material 3). Only 2% of the total specimens were observed to carry young in the bursae, much fewer than expected (following Monteiro and Tommasi (1983), the expectation was closer to 11%) (Table 2). Brooding was only recorded from five specimens from South Georgia/Shag Rocks (4% of total individuals in this region, Table 2).

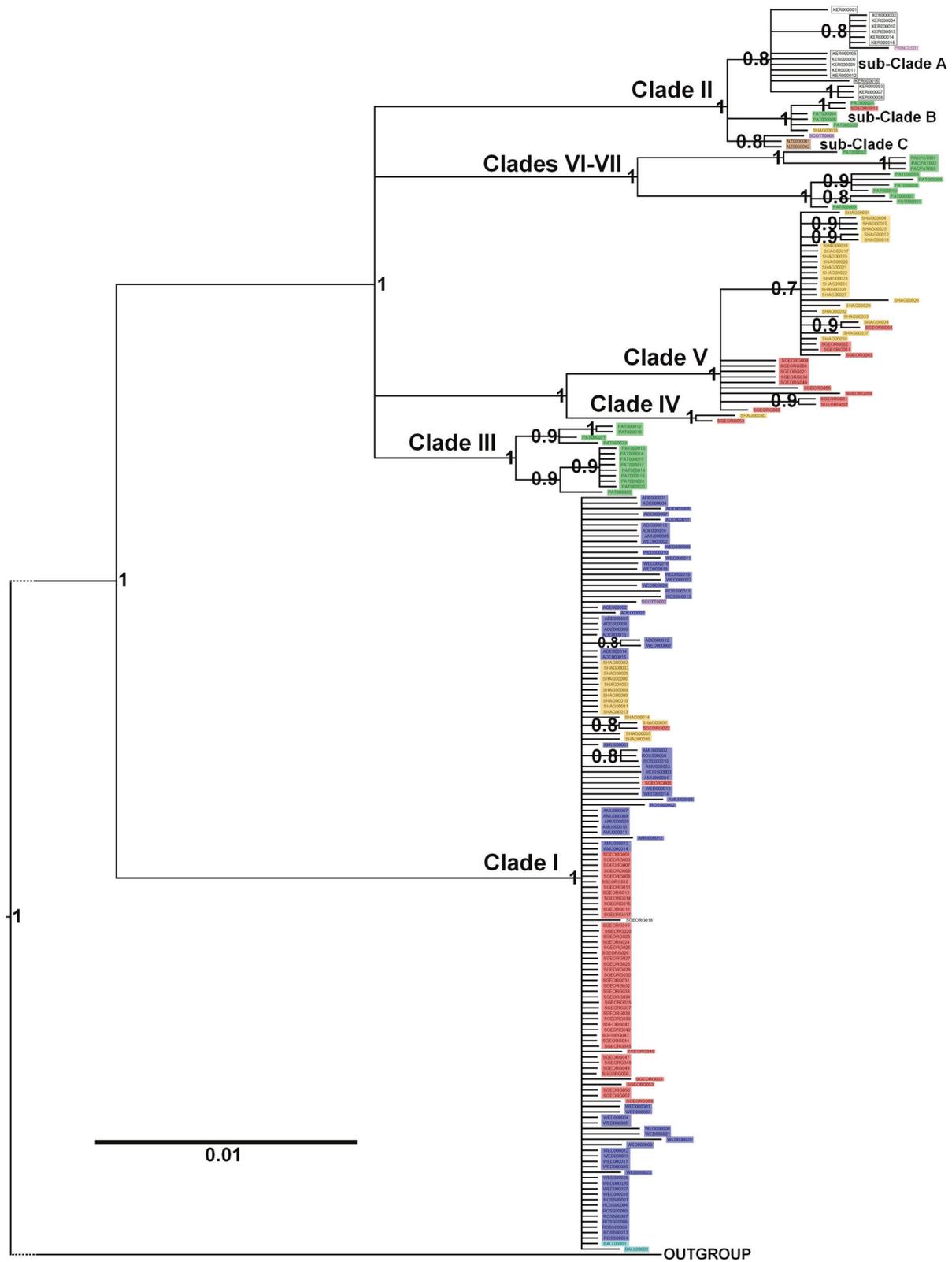


Fig. 3 Bayesian phylogeny of 227 *Astrotoma* CO1 sequences. Values at each node are the posterior probabilities. Nodes with support less than 0.75 were collapsed. Different colors denote different locations (see legend in Figure 1). For better visualization, some outgroups were removed and the branch closest to the basal node was shortened (a link to the complete and unmodified phylogeny can be found in Data availability section). Clades I to VII are based on the mPTP analysis (species delimitation method)

Monteiro and Tommasi (1983) found evidence of brooding on the Patagonian shelf (22%), in South Georgia/Shag Rocks (14%) and in Antarctica (0.6 %) (Table 2).

We focused our quantitative comparisons using specimens from Clade I (Antarctica, South Georgia) and Clade V (South Georgia). From the Antarctic continental shelf region, the mean disc diameter of *A. agassizii* was 3.9 cm (range 2.1–5.1, SE \pm 0.2), whereas from the shelf areas around South Georgia and Shag Rocks there was a larger range in disc diameter (mean: 3.5 cm, 1.3–6.4, SE \pm 0.2). Disc diameter frequency showed a bimodal distribution around South Georgia, suggesting either separate cohorts or distinct size-specific populations on the South Georgia shelf (Fig. 4). As molecular data indicated that there were distinct clades on the South Georgia shelf, we investigated whether the size categories identified were related to the genetic lineages: this analysis showed a clear size bimodality within the South Georgia specimens: the smaller morphotype is associated with Clade V, whereas the larger morphotype is associated with the Antarctic Clade I (Mann–Whitney test, $p < 0.001$). Within Clade I, we also observed a size

dimorphism between Antarctic and South Georgia specimens (Fig. 4, Mann–Whitney test: $p < 0.001$).

Monteiro and Tommasi (1983) also had a bimodal distribution in disc diameter of South Georgia specimens, although it was not so pronounced as in Fig. 4, because they had fewer large specimens. A Mann–Whitney test indicated that their South Georgia collections were not directly comparable to ours (disc diameter significantly different, $p < 0.0001$). However, their collection did not significantly differ in disc diameter from our Clade V specimens. Monteiro and Tommasi (1983) found that only individuals with small disc size (<3 cm) were brooders. All the brooding individuals from our collection had a considerably smaller disc diameter (mean 1.82 cm, range 1.6–2 cm) than expected from the average of our South Georgia collection ($p < 0.05$) but their disc size did not deviate significantly from the Monteiro and Tommasi (1983) collection.

Due to the condition of many of the ethanol samples, egg size measurements were only possible on very few individuals. Of these six were from Clade I (mean 452 μ m, SE \pm 20), and four were from Clade V (mean 749 μ m, SE \pm 41), indicating even with this small dataset that eggs from Clade V are considerably larger than those from Clade I (Mann–Whitney test, $p < 0.015$).

Table 1 Molecular diversity statistics for each CO1 clade and combined dataset

	<i>N</i>	<i>N_H</i>	<i>h</i>	<i>S</i>	π	<i>D_T</i>	<i>F_s</i>
Clade I	138	34	0.6140	28	0.0015	−2.40*	−31.56*
Clade II	26	10	0.8831	12	0.0053	0.082	−1.58
Clade III	13	5	0.6282	6	0.0034	0.17	−0.033
Clade IV	2	2	-	-	-	-	-
Clade V	37	15	0.8393	16	0.0026	−1.93*	−10.93*
Clade VI	4	2	-	-	-	-	-
Clade VII	7	7	1	8	0.0046	−0.81	−4.66*
Combined	227	75	0.8508	92	0.0362	1.29	−8.36

D_T Tajima's *D*, *F_s* Fu's *F_s*, *h* haplotype diversity, *N* number of individuals, *N_H* number of haplotypes, *S* number of polymorphic sites, π nucleotide diversity. *A significant p-value (<0.01)

Discussion

Developmental modes and their genetic distinctness

Although historically identified as a brooding species (Bernasconi 1965), Heimeier et al. (2010) found strong evidence for a dispersing larva in Antarctic *A. agassizii*. The previous work of Monteiro and Tommasi (1983) was able to identify brooding individuals around South Georgia and the Patagonian Shelf with moderate frequency (14% and 22%, respectively) but found brooding to be exceedingly rare

Table 2 Summary of main reproductive data obtained in the current study [1] and in Monteiro and Tommasi 1983 [2]

Location/Clade	<i>N</i>	% Maturity	% Brooding	DD at maturity	Source
ANT	79	22%	0%	4 cm (2.1–5.1)	[1]
ANT	325	25%	0.6%	1 to 4–5 cm	[2]
SG + SR	122	54%	4%	3.5 cm (1.3–6.4)	[1]
SG + SR	185	43%	14%	<1 to 6–7 cm	[2]
PAT	298	68%	22%	<1 to 2–3 cm	[2]
ALL	234	38%	2%	3.6 cm (1.3–6.4)	[1]
Clade I	116	40%	0%	4.7 cm (2.3–6.4)	[1]
Clade II	19	21%	0%	3.3 cm (2.2–4.7)	[1]
Clade V	30	77%	17%	1.8 cm (1.3–2.4)	[1]

ALL all locations merged, ANT Antarctica, DD disc diameter, PAT Patagonian shelf, SR + SG South Georgia/Shag Rocks. Clade I includes ANT and SR + SG samples, whereas clade V is restricted to SR + SG

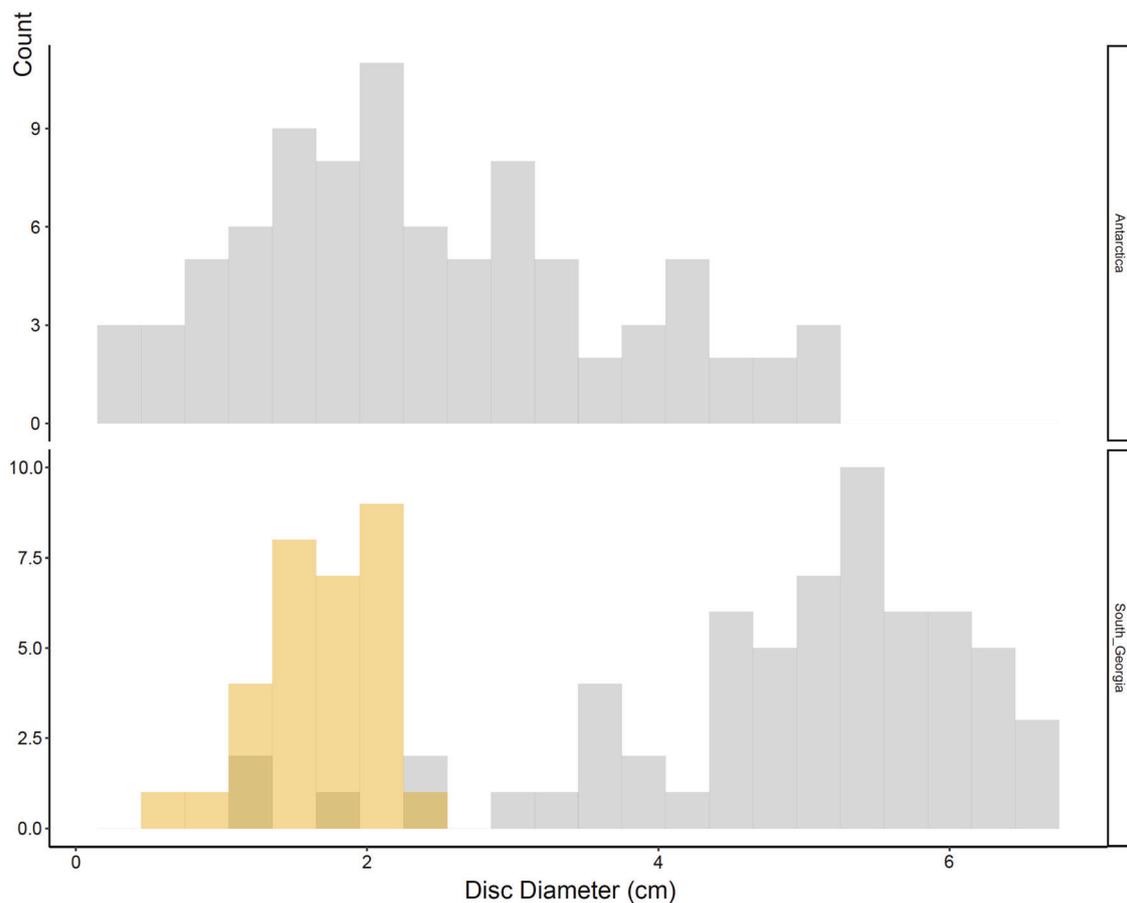


Fig. 4 Distribution of disc diameter (cm) for specimens in Antarctic (top) and South Georgia (bottom). Genetic Clade I is in gray, whereas genetic Clade V is in dark yellow

around the Antarctic continental shelf with only 2 specimens out of 325 investigated (0.6%). We were unable to find any evidence for brooding among the 79 specimens dissected from around the Antarctic Continental Shelf, supporting the idea that Antarctic *Astrotoma* tend toward a dispersive larval stage.

Further support for this hypothesis was provided by the egg size measurements obtained from the few well-preserved mature *Astrotoma* specimens. It is generally accepted that smaller eggs result in a feeding pelagic larval stage, whereas larger eggs produce non-feeding larvae or are brooded and develop directly into young adults (Stearns 1977), a pattern supported by empirical data on ophiuroid egg sizes (Sewell and Young 1997). The ovarian egg size that we measured from Clade I Antarctic individuals of $\approx 450 \mu\text{m}$ suggests a non-feeding larva and is in the size range of DNA-barcoded *A. agassizii*-fertilized eggs collected from the plankton (mean $463 \mu\text{m}$, range $436\text{--}513 \mu\text{m}$, Heimeier et al. 2010). Species with a planktonic larva, which usually indicates substantial dispersal ability, are expected to have few barriers to gene flow (although this varies depending on the species, see Shanks 2009; Kelly

and Palumbi 2010; Selkoe and Toonen 2011; Faurby and Barber (2012)). Our molecular data (Antarctic shelf individuals all belonging to Clade I), as well as that of Galaska et al. (2017), show no population structure across the whole of the sampled range, again strongly supporting a clade with a planktonic larval stage.

Brooding was detected in our study in a few individuals from around South Georgia and Shag Rocks, but at a considerably lower prevalence (4%) than reported by Monteiro and Tommasi (1983) from South Georgia (14%). Our brooded specimens had much larger eggs ($\approx 750 \mu\text{m}$), and in both our dataset and Monteiro and Tommasi (1983), brooding was detected in the smaller mature individuals. This in accordance with the generally accepted hypothesis that correlates brooding with small adult size (e.g., Strathmann and Strathmann 1982).

Detailed analysis showed that our South Georgia specimens had a bimodal size-frequency distribution in disc diameters, with the brooding individuals always within the smaller size class, and from Clade V, whereas the larger mode represented Clade I, but with a considerably larger disc diameter on average than Clade I individuals from the

Antarctic continental shelf (Fig. 4). We hypothesize that the size-frequency patterns in *A. agassizii* in South Georgia represent a case of character divergence, as described by Darwin (1859) or character displacement as refined by Brown and Wilson (1956); both refer to accentuated change in a character state (here the disc size) between two closely related species in a zone of sympatry that is not observed outside of the zone of overlap (Brown and Wilson 1956). Although earlier research focused on resource (ecological) or sexual selection (reinforcement) as driving character displacement (Brown and Wilson 1956), more recent studies have found subtle variations and combinations of these themes to play a part (see review in Table 1 of Stuart et al. 2017). Here, as in situ observations of *A. agassizii* are extremely difficult due to their depth distribution (200–1500 m), we will avoid speculation on the reasons for character displacement, but instead focus on the implications.

If we accept that character displacement occurs between closely related species (Brown and Wilson 1956), this suggests that Clade I and V of *A. agassizii* represent cryptic (unrecognized) sympatric species, although because of overlapping reproductive seasons there is, as yet, no obvious barrier to interbreeding and we are uncertain how species cohesion and morphological uniqueness are maintained. We find little support for poecilogony as the two developmental modes (pelagic lecithotrophy, brooding) occur in separate clades, rather than within clades (e.g., Ellingson and Krug 2006).

The North Atlantic ophiuroid *Ophioderma longicauda* tells a very similar story to *A. agassizii* (Stöhr et al. 2009; Boissin et al. 2011; Weber et al. 2014). Originally thought to have a dispersive pelagic larva, further research revealed brooding individuals in a monophyletic clade. In the area of sympatry between brooding and non-brooding forms, the mature brooding individuals (<13 mm) were smaller than the non-brooding individuals (20–26 mm). The authors concluded that the brooding form is a cryptic species in what is likely to be a species complex. Similarly, *Ophiacantha wolfarntzi* (Martín-Ledo et al. 2013), another ophiuroid found around the South Georgia/Shag Rocks continental shelf, is sympatric with a sister clade, which is yet to be formally described but appears to be identical to *O. wolfarntzi* (Martín-Ledo et al. 2013). It is likely that if the two clades do represent different diverging species, some morphological shift might become apparent once sufficient samples are examined.

Although the most parsimonious hypothesis for the presence of two developmental modes within *A. agassizii* is the presence of cryptic species, the report by Monteiro and Tommasi (1983) of two brooding specimens of *A. agassizii* in Antarctica (northern Ross Sea) is perplexing. These 2 specimens (disc diameter <3 cm) were found to be

brooding 182 and 923 juveniles in the bursae. Although rare (2 out of 235 Antarctic specimens), records of such large numbers of young within the bursae make it likely that this is a true record of *A. agassizii* brooding in the Ross Sea. How might this be explained? Three non-mutually exclusive answers are proposed as follows: (1) that there is more than one species of *A. agassizii* on the Antarctic continental shelf: one that broadcasts lecithotrophic larvae (Heimeier et al. 2010) and a rarer species that broods (this was, however, not revealed in our genetic data); (2) that, rarely, some individuals of Clade I are poecilogenous with a dual developmental mode (broadcasting-brooding) as reported or suggested for other echinoderms (McClary and Mladenov 1989; Mercier and Hamel 2008; O'Hara et al. 2014); or (3) that there is connectivity of adult specimens between a brooding *A. agassizii* species in South America and/or the Subantarctic with the Antarctic continent, through rafting. Connectivity by rafting has been previously proposed for *A. agassizii* within Antarctica by Hunter and Halanych (2008), and across the Antarctic Polar Front by Galaska et al. (2017), to explain a South American haplotype being found on the Antarctic Peninsula.

High levels of diversity across the sub-Antarctic

It is clear from the previous research of Hunter and Halanych (2008) and Galaska et al. (2017) that there is considerable genetic variation within *A. agassizii* between Antarctic and Patagonian shelf samples, as well as among Patagonian Shelf individuals. The divergence between the Antarctic and Patagonian shelf samples is, however, maintained, despite some indication of gene flow, and there were few putative hybrids between two identified Patagonian shelf populations, despite overlap of distributions (Hunter and Halanych 2008, Galaska et al. (2017).

Our sampling of *A. agassizii* extended more broadly around Antarctica and to sub-Antarctic regions, and unsurprisingly identified further deep genetic differentiation with what appears to be a rapid radiation of the sub-Antarctic clades. This pattern bears some resemblance to the polytomy identified by Sands et al. (2015) in the similarly distributed Southern Ocean ophiuroid *O. lymani*, where there was strong support for each geographically specific clade, but low support for timing of divergence order. In addition, both species show a low diversity Antarctic-specific clade, although in *Astrotoma* the Antarctic clade is shared with South Georgia, whereas the Antarctic clade in *Ophiura* is specific to the Antarctic continental shelf (Sands et al. 2015). A third similarity between studies is that in both cases the chosen “outgroup”, or assumed sister species, has ended up being an “ingroup”. In the case of *Ophiura*, the Antarctic clade is better known as *Ophiura carinifera* and is morphologically distinct from *O. lymani*, but

apparently nested within the group's genetic variation. In *Astrotoma*, *A. drachi* from southern New Zealand waters nests in Clade II.

A. drachi was initially described in the Philippines by Guille (1979) who noted the absence of multiple conical spines on the aboral areas (the opposite of *Astrotoma manilense*, also described from the Philippines, which harbors such spines). Despite numerous surveys, there is no recorded specimen between the Philippines and New Zealand (disjunct distribution), meaning that the New Zealand specimens are unlikely to be conspecific with any species from the Philippines (O'Hara and Harding 2014). After further morphological investigations of *A. drachi* from New Zealand waters and *A. manilense* from Tasmanian waters, O'Hara and Harding (2014) were hesitant to synonymize these two species and suggested the need for molecular data to investigate species boundaries inside the genus. Our molecular data from *A. drachi* (New Zealand) groups the species with an individual sampled from Scott Island (Northern Ross Sea, Antarctica) in what we have labeled Clade II sub-clade C. The three sub-clades of Clade II are also a polytomy with sub-clade A, restricted to islands of the Indian Ocean sector of the Southern Ocean (Kerguelen and Prince Edward Islands), sub-clade B from the Atlantic region of the Southern Ocean (Patagonian Shelf and South Georgia/Shag Rocks), and sub-clade C from the Pacific region of the Southern Ocean (New Zealand and Scott Island). Uncorrected among group genetic differences were <1% (Supplementary Material 1).

Of interest is the identification of four distinct genetic clades on the Patagonian Shelf (Clades III, VI, VII, and representatives of Clade II sub-clade B), where other studies have only identified two (Hunter and Halanych 2008; Galaska et al. 2017). A closer inspection a posteriori may nevertheless bring out the existence of the other two groups in the previous studies (as suggested by Figs. 3 and 4 from Galaska et al. 2017). The holotype of *A. agassizii*, described from the Strait of Magellan (Lyman 1875), is likely to belong to one of these four clades. We have found between group genetic-corrected distances between 5% and 8% for these Patagonian clades (Supplementary Material 2). The discrete genetic groupings in sympatry may indicate species boundaries, despite limited gene flow as identified in Hunter and Halanych (2008).

Galaska et al. (2017) used 2bRAD to generate genomic-wide single-nucleotide polymorphisms to characterize their populations. Although a powerful technique within and between closely related populations, the power of this technique rapidly diminishes as genetic divergences increase, because the necessary restriction sites become more and more prone to mutation over time. We suggest a more conservative approach to understanding the evolutionary and demographic history of *Astrotoma*, particularly

the Patagonian Shelf populations, would be to use more conserved sequencing techniques such as the exon capture system recently developed by Hugall et al. (2015). Exon capture would still bring the power of many genomic markers, but have the advantage of these markers being homologous across multiple divergent populations and species.

Conclusions

Following the suggestion of "species as hypotheses" proposed by Pante et al. (2015), our work combined with the historic papers cited above lead us to propose that *A. agassizii* is a species complex, where Clade I to VII are hypothesized species that encompass both brooding and dispersing developmental modes. We can be confident that Clade I, distributed across the Antarctic continental shelf and in South Georgia, is a genetically discrete entity with a dispersing pelagic larva, whereas Clade V, endemic to South Georgia, is a smaller brooding species. It is clear from Monteiro and Tommasi (1983) that some or all of the other clades found on the Patagonian Shelf may also brood; however, sample preservation problems and sample size erosion as a consequence of many divergent lineages has made further meaningful comment on clade-specific developmental mode or poecilogony impossible.

We do acknowledge that our suggestion of cryptic species is based on a single mitochondrial marker, and that caution is advised when using mitochondrial markers as a proxy for species evolution (Ballard and Whitlock 2004; Weber et al. 2019). However, such markers can be a good predictor of cryptic species where multiple strongly supported clades are found in morphologically uniform species (Held 2003, 2005; Linse et al. 2007; Leese et al. 2008; Janosik and Halanych 2010; Hemery et al. 2012; Sands et al. 2015; Verheye et al. 2016; Brasier et al. 2017; Jossart et al. 2017). Between Clades I and V, the genetic differentiation is corroborated by a morphological differentiation that we hypothesize as a case of character displacement. Further research using nuclear markers will be needed to further clarify the species status of the other clades.

This research also has two broader implications. First, Clade II of *A. agassizii* includes the recognized outgroup *A. drachi*. If included as an ingroup, then we must reconsider *A. agassizii* as a complex in which *A. drachi* (*A. manilense*) are elements in much the same way as Sands et al. (2015) found that *O. carinifera* is an element of the *O. lymani* complex (Sands et al. 2015). Another similarity with *O. lymani* is the large diversity of *A. agassizii* haplotypes and clades identified from Patagonian Shelf and South Georgia (the Atlantic sector of the Southern Ocean), which hints at a South Georgia/Patagonian shelf origin for the *A. agassizii*

complex. It was proposed that *O. lymani* originated in the sub-Antarctic and expanded its range onto the Patagonian Shelf in more recent times, and it may be that *A. agassizii*, having shared geographic range and some congruence in mitochondrial phylogeny, had a similar (shared) evolutionary history (Sands et al. 2015).

Finally, the identification of multiple hypothetical species has consequences for conservation management, particularly in South Georgia where Clade V appears to be endemic, but one of three distinct clades present. If all three of these clades are later recognized as distinct species, then South Georgia now has an increased biodiversity (1 species to 3) but shifts in terms of management from a single robust faunal element (wide distribution, high connectivity, and large population size) to three separate evolutionary units, one with no connectivity and lower population size. Further detailed testing is thus required of the specific species hypotheses we propose here.

Data archiving

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.n7s0g10>.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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