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► To cite this version:

Quentin Jossart, Marc Kochzius, Bruno Danis, Thomas Saucède, Camille V.E. Moreau. Diversity of the Pterasteridae (Asteroidea) in the Southern Ocean: a molecular and morphological approach.. Zoological Journal of the Linnean Society, 2021, 192 (1), pp.105-116. 10.1093/zoolinnean/zlaa097 . hal-03289108

HAL Id: hal-03289108

<https://hal.science/hal-03289108v1>

Submitted on 21 Feb 2024

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1 DOI: <https://doi.org/10.1093/zoolinnean/zlaa097>

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3 **Diversity of the Pterasteridae (Asteroidea) in the Southern Ocean:**
4 **a molecular and morphological approach**

5
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12
13
14 **ABSTRACT**

15 An integrative approach is crucial in discrimination of species, especially for taxa that
16 are difficult to identify on a morphological basis. In this study, we combined genetics and
17 morphology to assess the diversity of the Pterasteridae, a sea star family diversified in
18 deep-sea and polar environments. Because of their derived anatomy and the frequent
19 loss of characters during preservation, the Pterasteridae are a case in point for an
20 integrative study. The molecular identification of 191 specimens (mostly from the
21 Southern Ocean) suggested 26 to 33 species from 3 genera (*Hymenaster*, *Diplopteraster*,
22 *Pteraster*), which matched the morphological identification in 54 to 62 % of cases. The
23 mismatches were either different molecular units that are morphologically
24 indistinguishable (e.g. *Pteraster stellifer* units 2&4) or, conversely, nominal species being
25 genetically identical (e.g. *Hymenaster coccinatus/densus/praecoquis*). Several species
26 were shared between Northern and Southern Hemispheres (e.g. *Pteraster*
27 *jordani/affinis*). In conclusion, the taxonomic status of some groups was confirmed but
28 we found, for others, the need to reevaluate the taxonomy at both the genus and species
29 levels. This work significantly increases the DNA barcode library of the Southern Ocean
30 species and merged taxonomic information into an identification key that could be a
31 baseline for future studies (pterasteridae-so.identificationkey.org).

32 INTRODUCTION

33 Taxonomy has a pivotal role in biology (Costello et al. 2013). Inaccurate identifications
34 and naming lead to misunderstandings and spurious interpretations of biological
35 processes in various domains of life sciences (Dayrat 2005; Pante et al. 2015). Fifteen
36 years ago, integrative taxonomy was introduced as a promising approach to
37 complement the traditional, morphology-based taxonomy, using new data and methods
38 (Dayrat 2005). Among these, molecular markers were in the spotlight, considering the
39 simultaneous leaps achieved by new genetic methodologies such as DNA barcoding
40 (Stoeckle 2003; Hebert et al. 2003; Hebert & Gregory 2005, Ratnasingham & Hebert
41 2007; Fujita et al. 2012). Nowadays, the number of barcoded species is still low
42 compared to the total number of recognized species, less than a quarter of nominal
43 species being barcoded in most phyla (Gong et al. 2018). Nevertheless, a plethora of
44 studies have shown the importance of using genetics and morphology alongside for
45 discrimination at all taxonomic ranks (e.g. Richter et al. 2008; Laakmann et al. 2012;
46 Pante et al. 2015; Christiansen et al. 2018; Peck et al. 2018; Jossart et al. 2019). Based on
47 the data from the World Register of Marine Species (WoRMS), Appeltans et al. (2012)
48 showed that molecular methods are significantly increasing our knowledge of marine
49 biodiversity by helping in the detection of cryptic species and the establishment of
50 synonymies. This is of particular importance for under-investigated taxa, such as those
51 including numerous species that remain unrevised since their original descriptions.

52 The Asteroidea (*i.e.* sea stars or starfish) is the second most diversified class of
53 echinoderms, with around 1,900 described species assigned to 38 families (Mah & Blake
54 2012; Mah et al. 2015). They show various ecological traits and are present in a broad
55 variety of ecosystems (Mah & Blake 2012; Lawrence et al. 2013; Moreau et al. 2017). For

56 the last twenty years, considerable efforts have been made to reevaluate sea star
57 phylogeny using molecular data (Knott & Wray 2000; Janies et al. 2011; Mah & Foltz
58 2011a, 2011b; Linchangco et al. 2017; Moreau et al. 2019). However, there is a sharp
59 contrast in our knowledge of sea star diversity, namely amongst families from different
60 biogeographic regions (Feuda & Smith 2015). This is the case of the Pterasteridae
61 Perrier, 1875, the most diverse family in the Order Velatida, which includes around 130
62 nominal species and eight genera: *Amembranaster* Golotsvan, 1998, *Benthaster* Sladen,
63 1882, *Calyptraster* Sladen, 1882, *Diplopteraster* Verrill, 1880, *Euretaster* Fisher, 1940,
64 *Hymenaster* Wyville Thomson, 1873, *Hymenasterides* Fisher, 1911, *Pteraster* Müller &
65 Troschel, 1842 (Mah 2020). One unique feature of the Pterasteridae is the presence of
66 an additional (supra)dorsal membrane producing abundant quantities of mucus (Mah &
67 Blake 2012, Gale 2018). Between the dorsal and the supradorsal membranes is a
68 nidamental cavity where incubation of juveniles takes place in some species (Janies
69 1995). The taxonomy of the group is complicated for three reasons: (1) morphologies
70 are highly derived and only few diagnostic characters are recognized. Most characters
71 commonly used for species identification in other sea stars are not applicable to the
72 family (Gale 2018); (2) the few available characters are often indiscernible as specimens
73 are particularly fragile and get damaged by sampling and preservation protocols (Villier
74 et al. 2004); (3) several species are only known from their original description based on
75 few (poorly preserved) specimens (Villier et al. 2004). Consequently, the Pterasteridae
76 family is a case in point of a group that could benefit from an integrative taxonomic
77 approach.

78 The family mainly occurs in cold waters from the deep sea to the Arctic and the Southern
79 oceans (Mah & Blake 2012). Unfortunately, although genetic sequences were available

80 for specimens from the Northern Hemisphere, no public data was published for
81 Southern Ocean species (source: boldsystems.org). International initiatives of the
82 Census of Antarctic Marine Life (CAML) and of the International Polar Year (IPY) have
83 promoted sampling efforts in the Southern Ocean (Schiaparelli et al. 2013) and this
84 momentum was at the origin of many biologic campaigns until today. This significantly
85 enhanced the taxonomic and spatial coverage of the Southern Ocean biodiversity
86 inventory, including the collection of deep-sea representatives of the Pterasteridae.
87 These newly and well-preserved specimens have offered the opportunity to
88 reinvestigate the taxonomy of the family. Based on these new samples, we combined
89 morphological and molecular approaches to verify whether their joint use could better
90 assess the diversity within the Pterasteridae. After an initial morphological
91 investigation, we used a mitochondrial gene (Cytochrome c Oxidase subunit I - COI) to
92 verify how it confirms or complements the morphological identification. Subsequently,
93 we reinvestigated (*a posteriori*) specimens using a morphological approach in order to
94 identify new characters that may differentiate species. Finally, we synthesized, for the
95 first time, the revised taxonomy of the family and made it available to all potential users
96 in an open-access identification key that includes all the Southern Ocean species
97 (pterasteridae-so.identificationkey.org).

98 MATERIAL & METHODS

99 Sampling

100 Specimens were collected during seventeen international campaigns undertaken at sea
101 from 1998 to 2017 (ACE, ANDEEP-3, ANDEEP-SYSTCO, ARGOS, CEAMARC 2007-2008,
102 JR144, JR179, JR262, JR275, JR15005, MD208, MUSORSTOM 10, POKER II, PS77, PS81,
103 PS96). Available specimens cover a wide distribution within the Southern Ocean (Figure
104 1), including the Patagonian shelf, South Sandwich Islands, South Georgia, South Orkney,
105 Shag Rocks, Kerguelen, Crozet and the Antarctic continental shelf (Adélie Land,
106 Amundsen Sea, Antarctic Peninsula, Weddell Sea). A total of 174 specimens from these
107 locations were included in the analysis. Moreover, in order to increase the taxonomic
108 and geographical scope, as well as the phylogenetic resolution, 20 additional genetic
109 sequences (see below) from specimens outside the Southern Ocean were also added to
110 the dataset (*i.e.* Fiji, South Africa, Pacific and Atlantic coasts of North America, Norway,
111 Russia). Metadata documenting all the 191 samples can be found in Supplementary
112 Material 1.

113 Morphological identification

114 A total of 124 Southern Ocean individuals (preserved in ethanol or frozen) were
115 identified morphologically by the authors using both original descriptions (e.g. Sladen
116 1882, Koehler 1908), identification books (Clark 1962, Clark & Downey 1992, McKnight
117 2006) and contemporary scientific literature (Villier et al. 2004, Gale 2018).
118 Subsequently to genetic analyses (see below), an “*a posteriori*” morphological
119 investigation was carried out to look for new characters to differentiate species when
120 new species delineations and synonymies were suggested by genetic data. Finally, the
121 taxonomy of the family was synthesized and made available online, building an
122 interactive identification key through the Xper³ portal (Figure 2). Xper³ is a web portal

123 with an easy-to-use interface allowing multiple accesses (the key can be started using
124 any character; Vignes-Lebbe et al. 2016). Specimens and characters were also illustrated
125 by drawings and macro pictures (photographed using a camera with a macro lens, two
126 flashes and accessories to diffuse or reflect the light, Figure 2, Figure 3).

127 **Genetic data**

128 A fragment of the mitochondrial gene “Cytochrome c Oxidase subunit I (COI)” was
129 sequenced (601 base pairs) for the 191 individuals. These genetic sequences were
130 obtained through laboratory works in our institutes (80 individuals, see protocol
131 below), through our private Barcode of Life Data System project (BOLD; 97 individuals)
132 or mined from public BOLD projects (17 individuals).

133 DNA extractions were performed on one tube foot (podia) per sample and were based
134 on the salting-out protocol of Sunnucks and Hales (1996). Amplification step was
135 performed using the forward primers “F-COI-PTE-28” (5'-GCTGGAATGATTGGAAGTGC-
136 3') or “LCOech1aF1” (5' TTTTTTCTACTAAACACAAGGATATTGG”) and the reverse
137 universal primer “R-HCO2198” (5'- TAAACTTCAGGGTGACCAAAAAATCA'; Folmer et al.
138 1994). Each PCR mix (25 µl) included 12.5 µl of a VWR Mastermix (2.5 units of VWR Taq
139 polymerase, 0.4 mM of each dNTP and 1.5mM of MgCl₂), 10.5 µl of molecular water, 0.5
140 µl each primer (10 µM), and 1 µl of the DNA extract. PCR conditions consisted of 35
141 cycles for each of the three temperature steps [30 s at 95 °C (denaturation), 30 s at 48 °C
142 (annealing), and 30 s at 72 °C (elongation)]. These cycles were preceded by 2 min at 95
143 °C and were followed by 10 min at 72 °C. EXOSAP purification (incubation at 37 °C for 15
144 minutes followed by another at 80°C for 15 minutes) was done before the sending of
145 PCR products to the MACROGEN sequencing service. Sequence editing and alignment
146 were done using the software Geneious (Kearse et al. 2012). The absence of stop codon

147 in the sequence was checked in the same software in order to exclude the presence of
148 nuclear pseudogenes.

149 PartitionFinder2 (BIC criterion, Lanfear et al. 2016) was used within the CIPRES portal
150 (Miller et al. 2010) to select the most suitable substitution models (*i.e.* TRNEF+I+G for
151 codon position 1, HKY+I+X for codon position 2 and TRN+I+G+X for codon position 3). A
152 Bayesian phylogeny was subsequently produced using BEAST 1.8.4 (Drummond &
153 Rambaut 2007) within the CIPRES portal. Based on a previous phylogeny using multiple
154 genes, *Remaster gourdoni* Koehler, 1912 was used as the outgroup (Linchangco et al.
155 2017). Parameters of the analysis were: partitioned dataset, Yule process tree prior,
156 Markov chain Monte Carlo (MCMC) of 100×10^6 generations sampled every 10,000
157 trees. Tracer v1.6 was used to ensure an appropriate effective sampling size (ESS all
158 above 200). TreeAnnotator v1.8.4 was used to find the most likely tree that was
159 visualized in FigTree v1.4.3 (tree.bio.ed.ac.uk/software/figtree). Node support was
160 assessed through posterior probability (pp), with values lower than 0.75 not retained
161 and collapsed into polytomies (Huelsenbeck & Rannala 2004). Moreover, the software
162 DensiTree 2.2 was used to verify the potentiality of competing topologies among the set
163 of trees (Bouckaert 2010).

164 Three different methods of species delimitation were used to propose primary species
165 hypotheses: one distance-based (ABGD- Automatic Barcode Gap Discovery: Puillandre et
166 al. 2012) and two tree-based (bPTP-Bayesian Poisson Tree Process: Zhang et al. 2013
167 and GMYC-Generalized Mixed Yule Coalescent: Fujisawa & Barraclough 2013). The
168 ABGD analysis (bioinfo.mnhn.fr/abi/public/abgd) was performed with a relative gap
169 width of 1 and K80 as the genetic distance. The bPTP analysis (species.h-its.org/ptp)
170 was applied using 500,000 generations of MCMC, a thinning of 100 and a burn-in of 25
171 %. Finally, the GMYC analysis (species.h-its.org/gmyc) was performed using the single

172 threshold method. Haplotype diversity and nucleotide diversity were calculated within
173 each species using DnaSP 6 (Rozas et al. 2017).

174 RESULTS

175 Initial morphological identification

176 Among the 124 morphologically investigated individuals, 91 were identified to species
177 level. Thirty-two individuals were identified to genus level and one to family level due to
178 the small size of specimens (juveniles) or poor preservation not permitting observation
179 of diagnostic characters. Thirteen different species and three genera were identified
180 (Figure 3). Four species of *Pteraster* were found: *Pteraster affinis* Smith, 1876, *Pteraster*
181 *gibber* (Sladen, 1882), *Pteraster rugatus* Sladen, 1882, and *Pteraster stellifer* Sladen,
182 1882. The number and type of marginal oral spines were important diagnostic
183 characters to discriminate the different *Pteraster* species and these characters are
184 usually well preserved. Specimens of *Diplopteraster* were only identified up to the genus
185 level due to the absence of observable characters (e.g. between *Diplopteraster verrucosus*
186 (Sladen, 1882) and *Diplopteraster semireticulatus* (Sladen, 1882); see discussion). Nine
187 species of *Hymenaster* were identified: *Hymenaster campanulatus* Koehler, 1907,
188 *Hymenaster coccinatus* Sladen, 1882, *Hymenaster densus* Koehler, 1908, *Hymenaster edax*
189 Koehler, 1907, *Hymenaster formosus* Sladen, 1882, *Hymenaster latebrosus* Sladen, 1882,
190 *Hymenaster perspicuus* Ludwig, 1903, *Hymenaster praecoquis* Sladen, 1882 and
191 *Hymenaster sacculatus* Sladen, 1882. Some of these *Hymenaster* species were
192 discriminated based on tenuous morphological differences, such as *H. densus* and *H.*
193 *praecoquis* only differentiated based on slight variations in the morphology of the
194 segmental papillae and number of marginal spines (Clark 1962).

195 Species delimitation (COI)

196 Species delimitation methods applied to molecular data gave 26 species hypotheses for
197 ABGD and 33 for bPTP and GMYC (Figure 4). For each species hypothesis, the
198 relationship between haplotype diversity and nucleotide diversity fitted the expected

199 variation for a single species, except for *Pteraster stellifer* unit 1 (Goodall-Copestake et
200 al. 2012). Morphological species identification only matched with molecular species
201 delimitation in 54 % and 62 % of species for ABGD and GMYC/bPTP, respectively.
202 Within the genus *Pteraster*, species complexes were suggested within the nominal
203 species *P. stellifer* (3 to 5 units) and *P. affinis* (2 units). Within the genus *Hymenaster*,
204 three morphological species appeared to be undifferentiated genetically, suggesting that
205 the diversity of the genus was over-estimated (*H. coccinatus*, *H. densus* and *H.*
206 *praecoquis*). Moreover, four species are present in both the Southern and Northern
207 Hemispheres: *Pteraster jordani/affinis*, *P. militaris/affinis*, *Diplopteraster sp1* and *P.*
208 *gibber*. In every case, Northern and Southern Hemisphere specimens were closely
209 related within these species (p-distance of 0.3 % for *P. jordani/affinis* and *P.*
210 *militaris/affinis*, 0.7 % for *Diplopteraster sp1* and 1.2 % for *P. gibber*).

211 **COI phylogeny**

212 Three main groups were identified: one *Hymenaster* group (A) and two
213 *Diplopteraster/Pteraster* groups (B and C; Figure 4). The relationship of group B with the
214 two other groups was unclear, as illustrated by the low posterior probability and the
215 competing topologies from the DensiTree output (Figure 4; Supplementary material 2).
216 Within group A (*Hymenaster*), *H. campanulatus* and unidentified specimens was the
217 sister group of all other *Hymenaster* (pp = 1). Among these, *H. sacculatus* formed a
218 subclade with *H. formosus*, *H. perspicuus* and *H. pellucidus*, while *H.*
219 *coccinatus/densus/praecoquis* formed another subclade with *H. edax* and *H. latebrosus*
220 (pp = 0.85). Group B (*Diplopteraster/Pteraster*) included *Pteraster rugatus* as well as the
221 *Diplopteraster/Pteraster stellifer complex* (pp = 1). Within group C
222 (*Diplopteraster/Pteraster*), *Pteraster gibber* was close to *P. obscurus*, *P. tessellatus units*

223 1&2 and *Diplopteraster sp1* (pp = 1); while the other subclade included the *Pteraster*
224 *affinis* and *P. militaris* complexes (pp = 1).

225 ***A posteriori* morphological re-investigation**

226 Subsequently to species delimitation and phylogenetic analysis, new morphological
227 investigations were performed to clarify the mismatch between morphological and
228 molecular data and the status of ambiguous taxa. For unrecognized molecular units, new
229 discriminant morphological characters were potentially identified. First, we found a
230 different number of marginal oral spines (5 *versus* 3) in two genetic entities initially
231 identified under the same species name *P. affinis* (*i.e.* *P. jordani/affinis* and *P.*
232 *militaris/affinis*). Secondly, the presence of a large, clavate suboral spine was found in
233 several specimens of the *Diplopteraster/P. stellifer* complex, all belonging to *P. stellifer*
234 *unit 1* and *P. stellifer unit 5*. Thirdly, the morphological re-examination of two closely
235 related molecular units (*Pteraster gibber* and *Diplopteraster sp1*) showed that a
236 character state was shared by all specimens of these units. In fact, these specimens had a
237 single web (for two oral plates) among marginal oral spines while the other
238 *Pteraster/Diplopteraster* specimens harbored free marginal oral spines or a separate
239 web for each plate.

240 **Xper³ identification key**

241 The Xper3 identification key included 33 distinct species (Figures 2 and 3), namely all
242 the species currently accepted in the Register of Antarctic Marine Species (RAMS; Jossart
243 et al. 2015; De Broyer et al. 2020). Moreover, an asterisk (and related comment) was
244 attached to each species name, for which there was a mismatch between genetic and
245 morphological identification (*i.e.* *Pteraster affinis*, *P. stellifer*, *Diplopteraster clarki*, *D.*
246 *hurleyi*, *D. peregrinator*, *D. semireticulatus*, *D. verrucosus*, *Hymenaster coccinatus*, *H.*
247 *densus*, *H. praecoquis*). Fourteen characters were selected, namely the number of arms,

248 the type and number of paxillar spines, the type and number of adambulacral spines, the
249 number of rows of tube feet, the type and number of suboral/marginal oral spines, the
250 morphology of the segmental papillae, the presence of granular bodies in the
251 supradorsal membrane, the opacity of the supradorsal membrane ,the presence of
252 muscle fibres holding the supradorsal membrane and the number of oscular spines.
253 Several previously used descriptors were evaluated but not retained, because they were
254 not accurately quantifiable (e.g. osculum diameter, density of spiraculae) or
255 undistinguishable in most specimens (e.g. body convexity). Potential new diagnostic
256 characters that could be used in the *Pteraster affinis* (different number of marginal oral
257 spines) and *Diplopteraster/Pteraster stellifer* (clavate suboral spine) complexes were
258 mentioned as comments within the key. A particular attention was devoted to make this
259 identification key as user-friendly as possible: (1) the Xper³ platform allows the user to
260 easily detect the remaining taxa and characters throughout the identification process
261 (Vignes-Lebbe et al. 2016); (2) Any number of characters can be used in any order
262 (multiple accesses key); (3) Numerous macro-pictures and drawings are available,
263 illustrating both whole specimens, characters and character states. This identification
264 key is accessible through the following link: pterasteridae-so.identificationkey.org.

265 **DISCUSSION**

266 Our integrative approach was successful to revise species identity and phylogenetic
267 relationships. The results call for a revision of the taxonomic status of both species and
268 genus within the family Pterasteridae. Such a revision would not be possible without a
269 joint use of morphological investigations and molecular analyses. We identified three of
270 the four genera of Pterasteridae documented in the Southern Ocean: *Diplopteraster*,
271 *Hymenaster* and *Pteraster*, with *Calyptraster* the only genus not being encountered (Mah
272 2020). Thirty-three species were identified by the bPTP and GMYC molecular
273 approaches, which was concordant with 54 to 62 % of the morphology-based
274 identifications. Mismatches between morphological and genetic identifications were
275 either due to different molecular units that are morphologically similar or, conversely, to
276 morphological species being genetically identical (see below). Several cases of species
277 shared between high latitudes of the Northern and Southern Hemispheres were found,
278 which could correspond either to cosmopolitanism or bipolarity (species with disjunct
279 distribution *sensu* Darling et al. 2000). Following the molecular analyses, the return to
280 morphological samples allowed identification of potential new characters that can be
281 used as diagnostic features to define molecular species that were previously
282 undifferentiated based on morphology alone. Merging available morphological and
283 molecular results, we synthesized the taxonomy of the Pterasteridae from the Southern
284 Ocean and built a synthetic, polytomous and open-access identification key that is
285 intended to serve as a precious baseline for future taxonomic and ecological studies.
286 Finally, this work significantly increases the DNA barcode library for Southern Ocean
287 species. Prior to this study, no genetic sequence of Southern Ocean Pterasteridae was
288 publicly available on GenBank or BOLD platforms. Moreover, the addition of 174
289 sequences extends by more than three times the public barcode library of the whole

290 family, which also represents a 2.5 % extension for the Asteroidea class and a 0.6 %
291 extension for the Echinodermata phylum (source: boldsystems.org).

292 **Species delimitation**

293 The molecular identification confirmed the species status of several species previously
294 described on a morphological basis only (e.g. *Hymenaster campanulatus*, *H. sacculatus*,
295 *Pteraster rugatus*). On the other hand, discrepancies between morphological and genetic
296 delineations were observed. These are not surprising and illustrate taxonomic
297 uncertainties of the family already discussed in other studies (Clarke & Downey 1992;
298 McKnight 2006). For example, Clark & Courtman-Stock (1976) highlighted the
299 'ludicrous' situation in *Hymenaster*, to which more than 50 nominal species have been
300 ascribed over the last 100 years. This is well illustrated in our study by the two genetic
301 entities gathering distinct nominal species: the unit *Hymenaster*
302 *coccinatus/densus/praecoquis* and the unit *Diplopteraster*
303 *peregrinator/semireticulatus/verrucosus*. Such a taxonomic issue can be related to the
304 inadequacy of morphological characters used to discriminate species, to diagnostic
305 characters between species rather corresponding to intraspecific variations or to the
306 lack of taxonomic investigations of these species since they were originally described
307 (Clark 1962; Clark & Downey 1992). On the other extreme, an interesting case study is
308 provided by the species *Pteraster stellifer* that corresponds to a species complex (3 to 5
309 species). Variations within *P. stellifer* were already noticed by Clark (1962) who had
310 proposed to distinguish two subspecies, "*Pteraster stellifer stellifer* Sladen, 1882" and
311 "*Pteraster stellifer hunteri* Koehler, 1920", distinct from each other by their geographic
312 distribution and the shape of the paxillar spinelets. Our results also suggest that
313 *Pteraster affinis* should encompass several distinct species that, interestingly, are closely
314 related to species recorded in the Northern Hemisphere (see below). Some species

315 within these complexes correspond to unrecognized diversity as we found (*a posteriori*)
316 potential diagnostic characters (e.g. clavate suboral spines in *Pteraster stellifer* units
317 1&5). Some others might represent true cases of cryptic diversity as the morphological
318 re-investigation did not reveal any diagnostic character (e.g. among *Pteraster stellifer*
319 *unit 2 and 4*).

320 **Phylogenetic relationships**

321 As expected for a single gene phylogeny, some relationships remained partially
322 unresolved (Gontcharov et al. 2004; Christiansen 2008; Sands et al. 2008), but most of
323 them are supported by high posterior probabilities. Considering the uncertainty
324 associated with the placement of group B, on the one hand, the relationship between the
325 *Hymenaster* genus and the two other genera remains unresolved. On the other hand,
326 both *Diplopteraster* and *Pteraster* were retrieved in groups B and C. This was also found
327 in previous multiple-gene phylogeny based on Northern Hemisphere species of these
328 two genera (Mah & Foltz 2011b). This also matches our morphological observations that
329 diagnostic characters of the genus *Diplopteraster*, are doubtful (Clark & Downey 1992,
330 Villier et al. 2004). First, the presence of four rows of tube feet per arm might be
331 incorrect. The close examination of all specimens at hand reveals that they rather
332 possess two rows of overlapping tubefeet. Secondly, the alternating arrangement of
333 adambulacral plates is very difficult to observe. Finally, the presence of an enlarged
334 central paxillar spinelet could not be observed on most specimens. Therefore, we
335 recommend a taxonomic revision of these two genera using both genetic and
336 morphological data. Finally, we did not find any species belonging to the genus
337 *Calyptraster* in our collection. According to previous studies, it is the sister taxon of the
338 genus *Hymenaster*, but only a small number of species, records and diagnostic characters
339 are recognized (Clark & Downey 1992; Villier et al. 2004). Moreover, there is no genetic

340 data available (regardless of the gene) for a *Calyptroaster* species. We thus recommend a
341 thorough re-investigation of this genus in order to verify whether it should not be
342 synonymized with *Hymenaster*.

343 The molecular phylogeny confirmed the relationships of species already recognized as
344 closely related species such as *Hymenaster sacculatus* and *H. perspicuus* (Clark 1962).
345 Moreover, the phylogeny highlighted species relationships that were previously
346 unknown, such as the close relationships between *Pteraster gibber* and *P. affinis*, rather
347 than with *P. stellifer*. Another unprecedented result is the affinity of the species *P.*
348 *jordani/affinis*, *P. militaris/affinis*, *Diplopteraster sp1* and *P. gibber*, recorded both in the
349 Northern and Southern Hemispheres. Depending on the species, this could correspond
350 either to cosmopolitanism or bipolarity. This might indicate a recent migration between
351 the two hemispheres, which would notably be facilitated by deep-sea dispersal routes
352 (Strugnell et al. 2008, 2011; Laakmann et al. 2012). In fact, the Pterasteridae are known
353 to be highly diverse and abundant in the deep sea, being one of the most represented sea
354 star families in abyssal basins worldwide (Sibuet 1979; Danis et al. 2012).

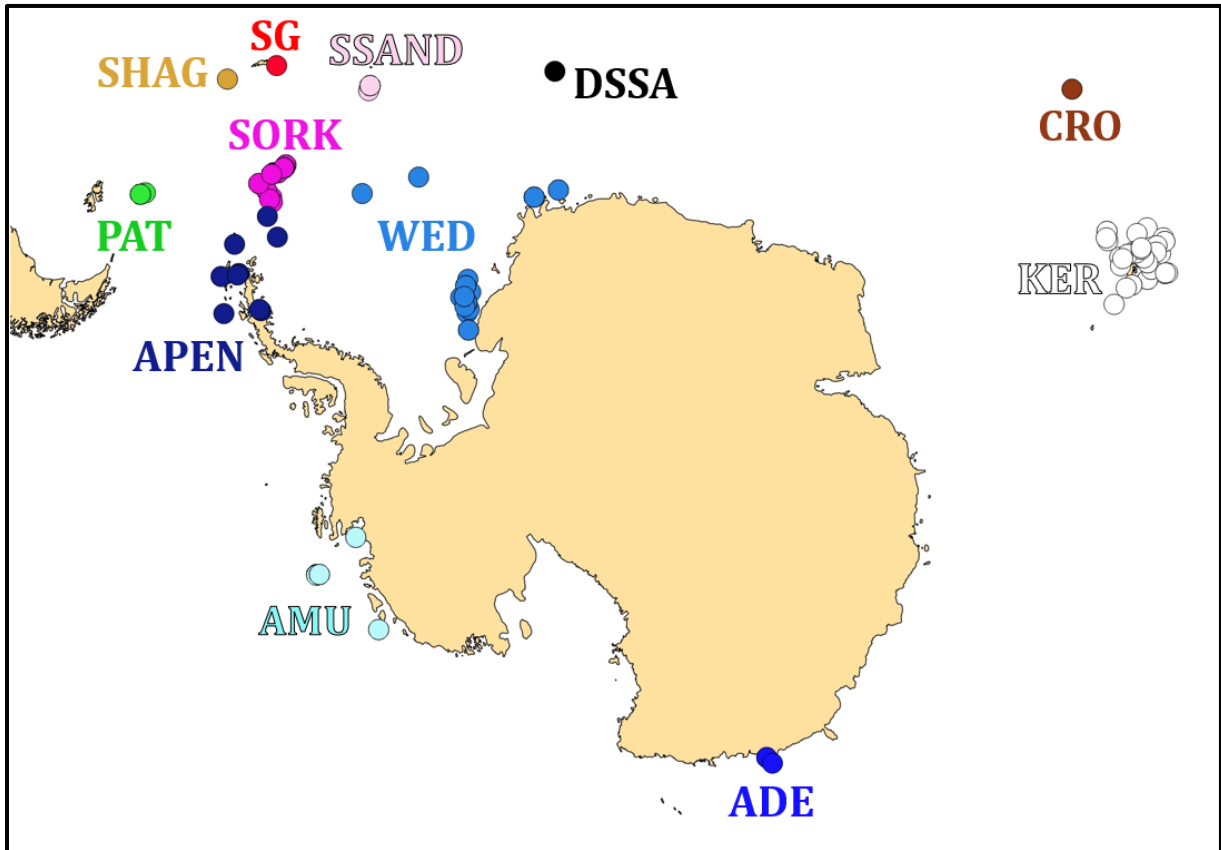
355 **CONCLUSION**

356 Our work has confirmed the relevance of using molecular tools to complement
357 morphology-based taxonomy. This is especially true for taxa that are complex to identify
358 on a morphological basis such as the Pterasteridae. In every genus investigated, we
359 found several species for which the taxonomy should be reevaluated and revised. These
360 taxonomic issues generate either unrecognized or conversely, overestimated diversity.
361 Formal taxonomic revision of these species and genera would be premature at this
362 stage. Further analyses are therefore needed to obtain a better picture of the diversity of
363 the family and precise phylogenetic relationships. This implies an extensive
364 investigation of numerous specimens, a thorough taxonomic revision and morphological

365 survey of holotypes. Some characters seem promising as new diagnostic features of
366 species (*e.g.* oral spines), while other characters currently used should be abandoned
367 (*e.g.* opacity of the supradorsal membrane), considering their intraspecific variability
368 and their problematic preservation in collection specimens. The investigation of arm
369 ossicles through X-ray photography and electron microscopy (SEM) also constitutes a
370 promising prospect. Former studies of ossicle arrangement and morphology provided
371 useful taxonomic information in different asteroid groups (Gale 2018, Fau & Villier
372 2019). In the case of Pterasteridae, primary radials (supporting the osculum) and
373 adambulacrals seem the most pertinent ossicles to be investigated (Gale 2018). Besides
374 morphological studies, the use of additional nuclear genes would be interesting to
375 properly turn the primary species hypotheses proposed here into secondary species
376 hypotheses (Sands et al. 2008; Abdelkrim et al. 2018). In addition, the use of multiple
377 genomic markers would be key to fully resolve phylogenetic relationships within the
378 family and analyze phylogeographic patterns within species. This is, for example, a
379 necessary condition to test different colonization scenarios between the two
380 hemispheres for cosmopolitan or bipolar species.

381 **FIGURES**

382 **FIGURE 1** - Sampling locations of the Pterasteridae specimens from the Southern Ocean.
383 ADE: Adelie Land, AMU: Amundsen Sea, APEN: Antarctic Peninsula, CRO: Crozet, DSSA:
384 Deep-Sea South Atlantic, KER: Kerguelen, PAT: Patagonian shelf, SHAG: Shag Rocks, SG:
385 South Georgia, SORK: South Orkney, SSAND: South Sandwich, WED: Weddell Sea.

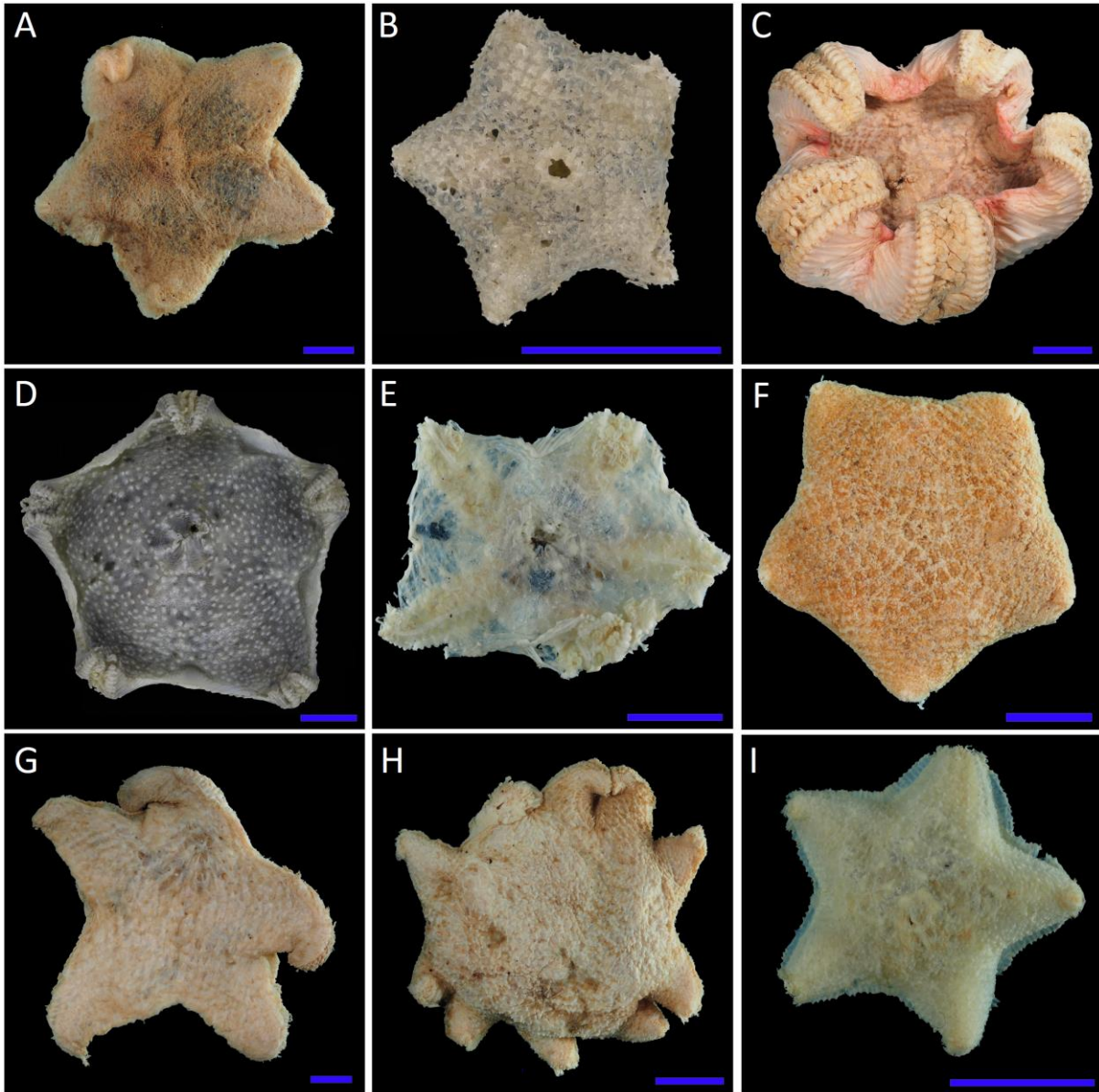


386 **FIGURE 2** - Interface of the Xper³ identification key (top) and two examples of
387 integrated drawings illustrating diagnostic characters (adambulacral spines: bottom left;
388 oscular spines: bottom right).

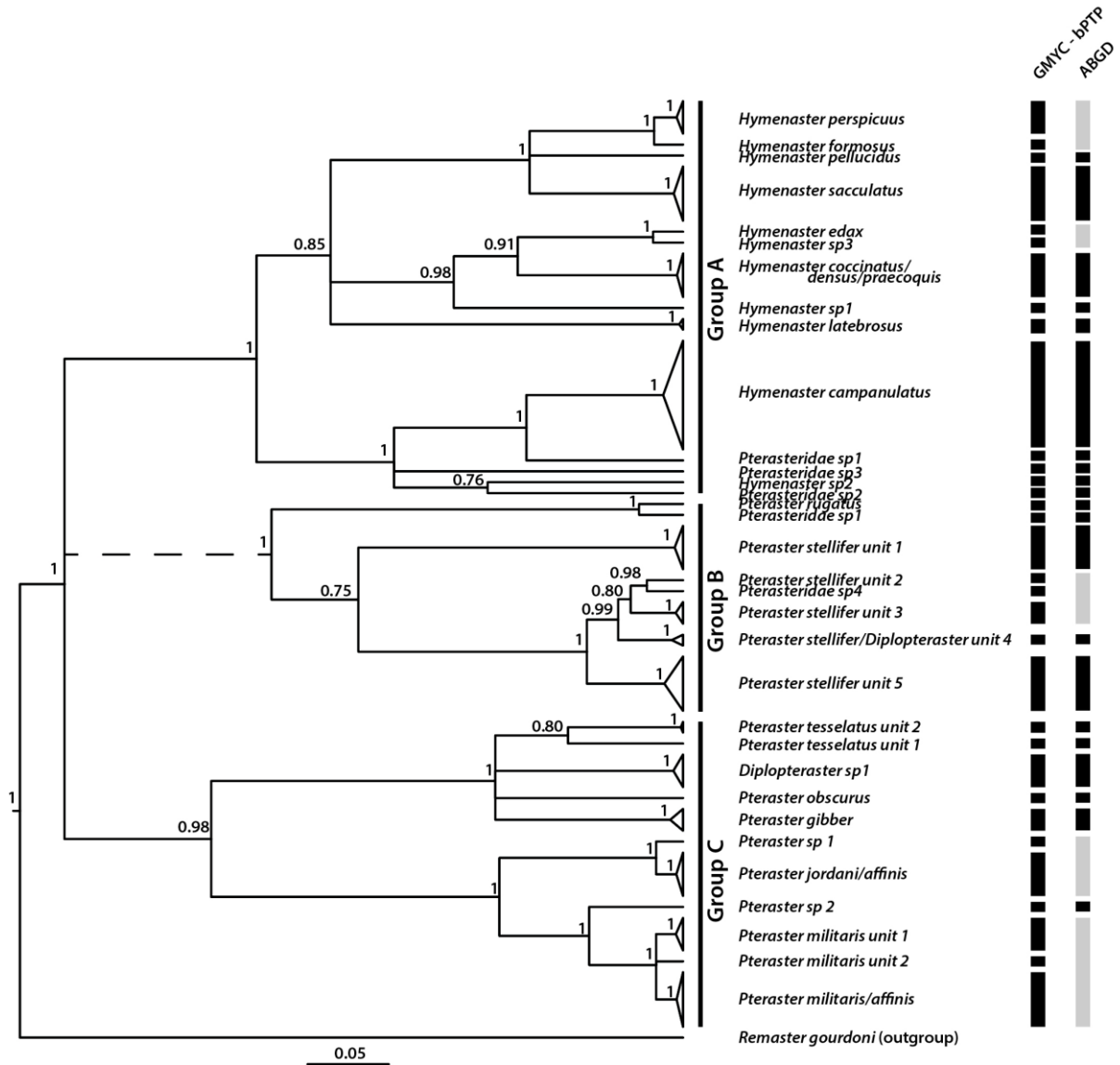
The image shows the Xper³ identification key interface. At the top, there are two selection boxes for diagnostic characters: "Adambulacral spines on most plates (Number)" and "Marginal oral spines (Number)". To the right of these boxes is a list of species names: *Pteraster robertsoni*, *Pteraster rugatus*, *Pteraster spinosissimus*, *Pteraster stellifer**, *Diplopteraster clarki**, and *Diplopteraster hurleyi**. Below the selection boxes are two integrated drawings. The left drawing shows a detailed view of adambulacral spines with labels: "Podia", "Webbed adambulacral spine", and "Adambulacral plate". The right drawing shows a detailed view of oscular spines with labels: "Oscular spines", "Oscular valve", and "Osculum".

389
390

391 **FIGURE 3** – Aboral view of Pterasteridae species illustrating their Southern Ocean
392 diversity. A: *Diplopteraster* sp; B: *Hymenaster campanulatus*; C: *Hymenaster praecoquis*;
393 D: *Hymenaster edax*; E: *Hymenaster sacculatus*; F: *Pteraster gibber*; G: *Pteraster affinis*; H:
394 *Pteraster koelheri*; I: *Pteraster stellifer*. Scale bars : 1 cm.



395 **FIGURE 4** – Bayesian phylogeny based on mitochondrial Cytochrome c Oxidase subunit I
 396 (COI) sequences. Values at each node are the posterior probabilities (nodes with support
 397 lower than 0.75 were collapsed into polytomies). The dashed line (associated to the
 398 group B) indicate competing topologies from the software DensiTree. Assignments for
 399 each species delimitation method are reported as black bars (GMYP-bPTP on the left and
 400 ABGD on the right). Distinct GMYP-bPTP and ABGD assignments are highlighted by grey
 401 bars for the ABGD assignments.



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