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# Assessing species boundaries in the open sea: an integrative taxonomic approach to the pteropod genus *Diacavolinia*

ALICE K. BURRIDGE<sup>1,2\*,</sup>, REMY VAN DER HULST<sup>1</sup>, ERICA GOETZE<sup>3</sup> and KATJA T. C. A. PEIJNENBURG<sup>1,2\*</sup>

<sup>1</sup>Naturalis Biodiversity Center, P.O. Box 9517, 2300 RA Leiden, The Netherlands

<sup>2</sup>Institute for Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, P.O. Box 94248, 1090 GE Amsterdam, The Netherlands

<sup>3</sup>Department of Oceanography, University of Hawai'i at Mānoa, Honolulu, Hawaii 96822, USA

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To track changes in pelagic biodiversity in response to climate change, it is essential to accurately define species boundaries. Shelled pteropods are a group of holoplanktonic gastropods that have been proposed as bio-indicators because of their vulnerability to ocean acidification. A particularly suitable, yet challenging group for integrative taxonomy is the pteropod genus *Diacavolinia*, which has a circumglobal distribution and is the most species-rich pteropod genus, with 24 described species. We assessed species boundaries in this genus, with inferences based on geometric morphometric analyses of shell-shape variation, genetic (cytochrome *c* oxidase subunit I, 28S rDNA sequences) and geographic data. We found support for a total of 13 species worldwide, with observations of 706 museum and 263 freshly collected specimens across a global collection of material, including holo- and paratype specimens for 14 species. In the Atlantic Ocean, two species are well supported, in contrast to the eight currently described, and in the Indo-Pacific we found a maximum of 11 species, partially merging 13 of the described species. Distributions of these revised species are congruent with well-known biogeographic provinces. Combining varied datasets in an integrative framework may be suitable for many diverse taxa and is an important first step to predicting species-specific responses to global change.

ADDITIONAL KEYWORDS: biogeography – gastropoda – geometric morphometrics – mtCOI – 28S rDNA – zooplankton.

## INTRODUCTION

Integrative taxonomy aims to rigorously delimit species and prevent under- or overestimation of species numbers by statistically testing species hypotheses with diverse character and data types (Edwards & Knowles, 2014; Karanovic *et al.*, 2016). An increasing number of species is described each year as a result of the incorporation of new tools for species discovery, including virtual access to museum collections, advances in DNA sequencing, morphometric methods and geographic information systems (Vogler & Monaghan, 2006; Knapp, 2008). Such tools have enabled integrative taxonomic approaches,

in which species are described based on congruence between morphological and genetic information, with additional supporting characteristics such as behaviour, ecology or geography (McManus & Katz, 2009; Padial *et al.*, 2010; Smith & Hendricks, 2013; Edwards & Knowles, 2014). Statistical identification of genetic lineages plays a pivotal role in species detection and satisfies multiple species concepts, because it treats species as hypotheses using objective tests (De Queiroz, 2007; Hausdorf, 2011; Morard *et al.*, 2016). However, the sole use of genetic information in species delimitation may fail to detect the same number of species as methods that combine multiple lines of evidence (Edwards & Knowles, 2014; BurrIDGE *et al.*, 2015; Karanovic *et al.*, 2016). Species may go undetected based on a limited set of selected genetic markers, because they may be

\*Corresponding authors. E-mail: [akburrIDGE@yahoo.co.uk](mailto:akburrIDGE@yahoo.co.uk); [k.t.c.a.peijnenburg@uva.nl](mailto:k.t.c.a.peijnenburg@uva.nl)

distinct only in other genes, morphology or ecological niche space, or species numbers may be overestimated by the use of highly variable genetic markers. Geometric morphometric methods, in which quantitative differences in organismal shape and size are used to distinguish between taxa without requiring analysis of soft tissue, may especially strengthen studies based on limited datasets, such as those containing fossil taxa and valuable museum specimens for which genetic information cannot be obtained (Karanovic *et al.*, 2016). Developing integrative taxonomic frameworks for assessing species boundaries enables the inclusion of museum specimens originally used to describe species in studies that also incorporate fresh samples, given that there are enough specimens available for connecting different data types.

Tracking changes in marine biodiversity in response to climate change on a global scale requires accurate assessment of species boundaries and distributions (e.g. Goetze & Ohman, 2010; Churchill *et al.*, 2014a, b; Burridge *et al.*, 2015; Wall-Palmer *et al.*, 2018). Range shifts of planktonic taxa have been among the most rapid, have occurred over the largest spatial scales in comparison to other marine and terrestrial groups, and may affect higher trophic levels in the marine food web (Richardson, 2008; Beaugrand *et al.*, 2012, 2014, 2015; Parmesan *et al.*, 2013; Brown *et al.*, 2016). Plankton distributions are often concordant with biogeochemical provinces at the level of species and communities (e.g. Valentin & Monteiro-Ribas, 1993; Longhurst, 1998; Reygondeau *et al.*, 2013; Dolan *et al.*, 2016; Burridge *et al.*, 2017a,b), as well as at the level of population genetic structure within species (e.g. Norton & Goetze, 2013; Goetze *et al.*, 2015, 2017; Hirai *et al.*, 2015). Persistent dispersal barriers may limit range shifts of some taxa in response to changing ocean conditions, while other taxa may be able to adapt and occupy new ecological niches. Integrative taxonomy will improve the accuracy of marine species delimitation, enable the identification of rare taxa and provide insights in current species distributions, with the potential to predict future range shifts.

Pteropods are a group of holoplanktonic gastropods that has been identified as exceptionally vulnerable to ocean acidification (Fabry *et al.*, 2008; Bednaršek *et al.*, 2016; Manno *et al.*, 2017). Pteropods comprise the Thecosomata (Euthecosomata and Pseudotheosomata), shelled or semi-shelled pteropods commonly known as 'sea butterflies' and the Gymnosomata, shell-less pteropods known as 'sea angels'. Thecosome pteropods have aragonitic shells, and are a diverse group of organisms that are common in marine zooplankton from polar to equatorial habitats (Lalli & Gilmer, 1989). Shelled pteropods have an extensive fossil record (Janssen, 2007a, b, 2012;

Janssen & Peijnenburg, 2017), and are commonly used to examine the effects of ocean acidification on marine life (Roger *et al.*, 2011; Comeau *et al.*, 2012; Bednaršek & Ohman, 2015; Maas *et al.*, 2016; Moya *et al.*, 2016). However, their usefulness as bio-indicators of the effects of ocean acidification is compromised by limited historical context for understanding species-specific long-term exposure to variations in ocean chemistry. Accurate knowledge of their taxonomy, genetic diversity and biogeography is the essential first step to predicting ecological and evolutionary responses to climate change.

We illustrate an integrative taxonomic approach using the genus *Diacavolinia*, shelled pteropods with particularly problematic systematics that are usually not identified below genus level, or are listed as *Cavolinia* sp. or *Diacavolinia longirostris* (Blainville, 1821) in recent studies (Jennings *et al.*, 2010; Roger *et al.*, 2011; Corse *et al.*, 2013). A new taxonomic assessment of species boundaries in *Diacavolinia* pteropods is important, because they are morphologically diverse, and some taxa occur in low pH ocean regions, including the California Current coastal upwelling ecosystem (Maas *et al.*, 2013). A study of *Diacavolinia* pteropods from Australian tropical waters found a significant increase in shell porosity along with a 10% local decline in the aragonite saturation level between the 1960s and 2000s (Roger *et al.*, 2011), suggesting sensitivity of this taxon to contemporary changes in the aragonite saturation state of the ocean.

Previously known as a single species of *Cavolinia* [*Cavolinia longirostris* (Blainville, 1821)], *Diacavolinia* was described as a separate genus by Van der Spoel (1987) on the basis of a distinct shell shape and shell growth compared to *Cavolinia* taxa. *Diacavolinia* is the most species-rich genus of pteropods with a total of 24 extant species, of which 18 were introduced by Van der Spoel *et al.* (1993). Species boundaries were primarily based on shell size and small variations in shell shape that were sometimes found among sympatric taxa. *Diacavolinia* occurs in tropical and subtropical waters between ~17 and 28 °C in the Atlantic, Pacific and Indian Oceans, as well as in the Red Sea, at depths of ~200 m by day and ~75 m at night (Wormelle, 1962; Van der Spoel, 1967; Bé & Gilmer, 1977). They have complex, bilaterally symmetrical shells (0.4–1.2 cm adult size; Van der Spoel *et al.*, 1993). This shell morphology enables detailed geometric morphometric analyses of shell shape, which can be a powerful tool to distinguish taxa with hard parts (Mitteroecker & Gunz, 2009; Klingenberg, 2010; Burridge *et al.*, 2015). Maas *et al.* (2013) distinguished four *Diacavolinia* species from the north-east Atlantic and one from the eastern Tropical Pacific (ETP) based on morphological

characteristics. However, they observed that Atlantic specimens were not genetically distinct (<3% divergence), whereas specimens from the Atlantic and Pacific were much more divergent (~19%), based on a fragment of the cytochrome *c* oxidase subunit I mitochondrial gene (*COI*). [Maas \*et al.\* \(2013\)](#) concluded that broader geographic sampling and a combination of genetic and morphological information are needed to resolve species boundaries in this genus.

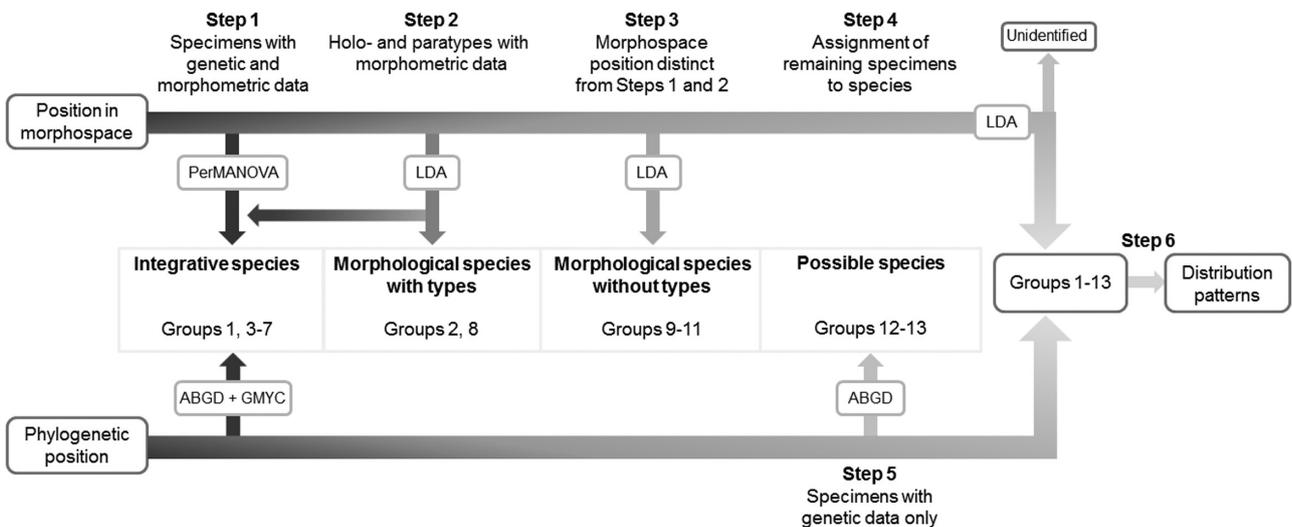
We apply an integrative approach to assess species boundaries in *Diacavolinia*, with inferences based on compatibility between genetic, geometric morphometric and geographic data ([Fig. 1](#); [Padial \*et al.\*, 2010](#)). To identify species as accurately as possible across our global collection of material, we link datasets comprising fresh specimens for which both genetic and morphometric information is available to morphometric information from museum specimens (969 specimens), including holo- and/or paratype specimens for 14 described species. We aim to (1) develop an objective method for identifying species boundaries by combining incomplete and varied datasets, (2) assess species boundaries and distribution patterns of *Diacavolinia* taxa by applying an integrative framework of genetic, morphometric and geographic information, and (3) examine consistency of results obtained with this framework across the 24 *Diacavolinia* taxa as described by [Van der Spoel \*et al.\* \(1993\)](#). We find evidence to support a reduction in the number of *Diacavolinia* species, with at least eight of the species described by Van der Spoel likely representing taxonomic over-splitting. We

provide systematic and biogeographic descriptions of the global component of species in this complex genus.

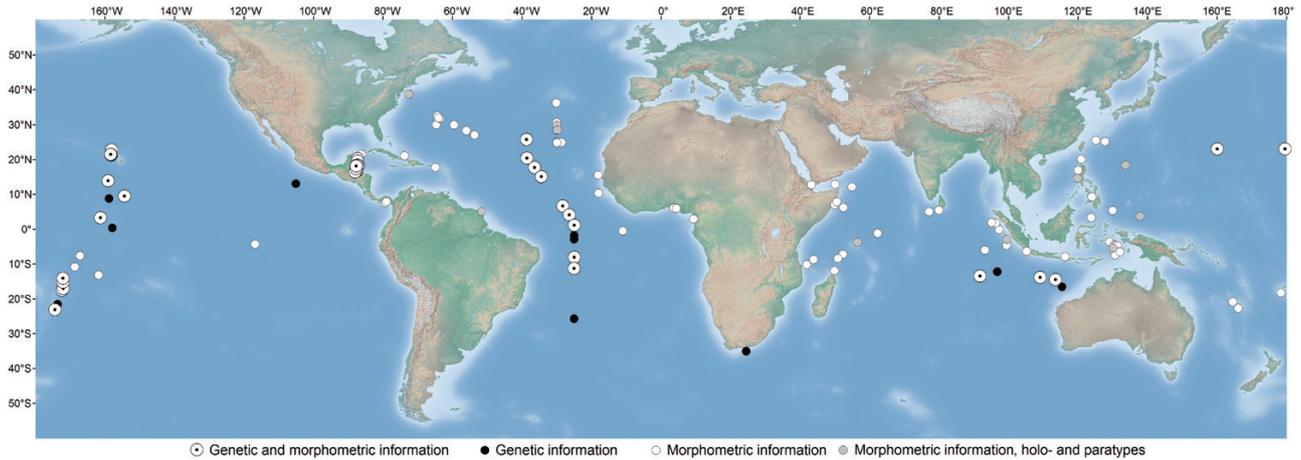
## MATERIAL AND METHODS

### SPECIMENS

We included 969 *Diacavolinia* specimens in this study, with collections from 152 locations between 40°N and 35°S in the Atlantic, Pacific and Indian Oceans ([Fig. 2](#)). Of these, 263 fresh specimens suitable for genetic analysis were obtained from 40 Atlantic, 27 Pacific and seven Indian Ocean locations ([Table 1](#)). Our fresh material was collected during nine oceanographic expeditions between 2001 and 2012 (Supporting Information, [Supplementary Information S1](#)). Plankton nets used across expeditions varied, but our study did not require quantitative sampling and identical net mesh sizes. For example, paired bongo (200- $\mu$ m, 333- $\mu$ m mesh) and Rectangular Midwater Trawl (RMT1, 333- $\mu$ m mesh) nets were used in the epipelagic and upper mesopelagic zone during night time on the AMT22 (Atlantic Meridional Transect) expedition in 2012. Specimens from the VANC10MV expedition in 2001 were collected using a bongo net (333- $\mu$ m mesh) and a 1-m ringnet (333  $\mu$ m) was used during the COOK11MV and COOK14MV expeditions in 2001. This information is not available for all collected material. All fresh specimens were preserved in 96% ethanol and stored at -20 °C. We also examined 706 specimens from museum collections at the Naturalis Biodiversity Center, Leiden, The Netherlands (NBC) and Zoological



**Figure 1.** Schematic overview of the integrative taxonomic approach as applied to *Diacavolinia* pteropods. Distinct groups that are identified in each step are indicated. Phylogenetic position was determined based on cytochrome *c* oxidase I (*COI*) and 28S molecular markers. LDA = Linear Discriminant Analysis; PerMANOVA = non-parametric permutational multivariate analysis of variance; ABGD = Automatic Barcode Gap Discovery; GMYC = Generalized Mixed Yule Coalescent.



**Figure 2.** Geographic overview of collection locations for all *Diacavolinia* specimens used in this study, with the type of information obtained from specimens at each location.

Museum of the University of Copenhagen, Denmark (ZMUC). These museum specimens were collected at 78 locations during ten expeditions between 1909 and 1993, and stored in 70% ethanol following initial fixation in formaldehyde (Table 1). Most of the museum specimens ( $N = 425$ ) were collected during the Danish DANA expeditions between 1921 and 1932 (Supporting Information, [Supplementary Information S1](#)). The available museum specimens were identified by [Van der Spoel \*et al.\* \(1993\)](#) as 23 of the 24 described *Diacavolinia* taxa, including holo- and/or paratype specimens ( $N = 79$ ) of 14 taxa. By examining and including all museum specimens used by [Van der Spoel \(1993\)](#) that could be retrieved, we provide a critical link between prior and current work (Table 2). Use of this historical material enabled us to make direct comparisons of species boundaries as identified by our methods versus those considered by [Van der Spoel \*et al.\* \(1993\)](#). All taxa were represented by the NBC and ZMUC collections, except *Diacavolinia robusta* [Van der Spoel \*et al.\*, 1993](#).

#### INTEGRATIVE APPROACH TO ASSESSING SPECIES BOUNDARIES

We combined genetic, geometric morphometric and geographic observations on single *Diacavolinia* specimens wherever possible, following the approach outlined in [Figure 1](#). The information obtained for each specimen varied, but this framework allowed for the combination of partial observations from each specimen. Morphometric measurements consisted of a partial shell shape outline of 49 landmarks (LMs) in lateral orientation and/or 23 LMs in a ventral orientation per specimen (for photographs and geometric morphometric data see: Supporting Information, [Supplementary Information S2–3](#)). For

some specimens it was possible to obtain an additional 15 ventral LMs to outline the shape of the ventral lip, in cases where the soft tissue did not obscure the ventral lip (also see next paragraph; [Fig. 3E](#); [Table 1](#)). Phylogenies were inferred from cytochrome *c* oxidase subunit I mtDNA (*COI*; 658 bp) and from nuclear large ribosomal subunit 28S (901 bp) gene fragments ([Table 1](#)).

Our approach included six steps for the discovery of, and assignment to, species ([Fig. 1](#)). The first step consisted of identifying integrative groups by linking genetic and morphometric information using fresh specimens ( $N = 173$ ). To test for significant morphometric differences between genetic clades with >five specimens, we applied non-parametric permutational multivariate analyses of variance to shell shape or size parameters (PerMANOVA based on Euclidean distances; [Anderson, 2001](#)) in PAST v.2.17c ([Hammer \*et al.\*, 2001](#)). The PerMANOVA *F*-statistic was tested against  $10^4$  non-parametric permutations. A significantly different result provided evidence for the presence of distinct species, with concordance observed among genetic and morphometric characters. In the second step, we examined the morphospace position of the holo- and paratype specimens identified by [Van der Spoel \*et al.\* \(1993\)](#) for which no genetic information is available due to specimen fixation in formaldehyde. Geometric morphometric measurements were obtained for 79 type specimens from 14 described taxa ([Tables 1, 2](#)). We applied Linear Discriminant Analysis (LDA) in R v.3.0.1 ([R Development Core Team, 2013](#)) to identify morphological species based on types, merge different types into a single morphological species or merge types with integrative species identified in Step 1. We performed separate LDAs for shape and size data of the different orientations to include as many specimens as possible and to limit the presence

**Table 1.** Overview of *Diacavolinia* specimens used in this study. For ventral and lateral geometric morphometrics, numbers of specimens for which morphometric data was obtained are indicated per number of landmarks (LM). See Figure 1 for explanation of the identification steps (Steps 1–5). Some museum specimens could not be identified due to lack of morphological data, because shells were too damaged for geometric morphometric analyses. Some fresh specimens could not be identified due to lack of genetic and/or morphological data

	Total	Atlantic	Pacific	Indian
<b><i>Diacavolinia</i> specimens (museum and fresh)</b>	<b>969</b>	<b>374</b>	<b>421</b>	<b>174</b>
Ventral photographs	920	368	381	171
of which Ventral geom. morph. 23 LMs	646	268	260	118
of which Ventral geom. morph. 38 LMs	314	140	115	59
Lateral photographs	903	363	376	164
of which Lateral geom. morph. 15 LMs	752	325	292	135
of which Lateral geom. morph. 49 LMs	549	267	190	92
<b><i>Diacavolinia</i> fresh specimens*</b>	<b>263</b>	<b>109</b>	<b>136</b>	<b>18</b>
Sequenced reference (Steps 1 and 5)*	176	65	100	11
of which <i>COI</i> (Steps 1 and 5)*	89	56	25	8
of which 28S (Step 1)	138	35	94	9
Identified by LDA (Step 4)	48	33	15	0
Unidentified	39	11	21	7
<b><i>Diacavolinia</i> museum specimens</b>	<b>706</b>	<b>265</b>	<b>285</b>	<b>156</b>
Holo- and paratype reference (Step 2)	79	34	36	9
Added reference, no types (Step 3)	26	0	15	11
Identified by LDA (Step 4)	423	195	139	89
Unidentified	178	36	95	47
<b>Outgroup sequences (<i>COI</i> and 28S)†</b>	<b>4</b>	<b>2</b>	<b>2</b>	<b>0</b>
<i>Cavolinia uncinata</i> †	4	2	2	0
<b><i>Diacavolinia</i> locations*</b>	<b>152</b>	<b>67</b>	<b>56</b>	<b>29</b>
<i>Diacavolinia</i> fresh locations*	74	40	27	7
<i>Diacavolinia</i> museum locations	78	27	29	22

These bold values are totals of non-bold values below. For example, the row for “*Diacavolinia* locations” should be in bold and is the sum of “*Diacavolinia* fresh locations” and “*Diacavolinia* museum locations”, which are listed below “*Diacavolinia* locations”

\*Includes 3 Pacific sequences / 1 location from Maas *et al.* (2013).

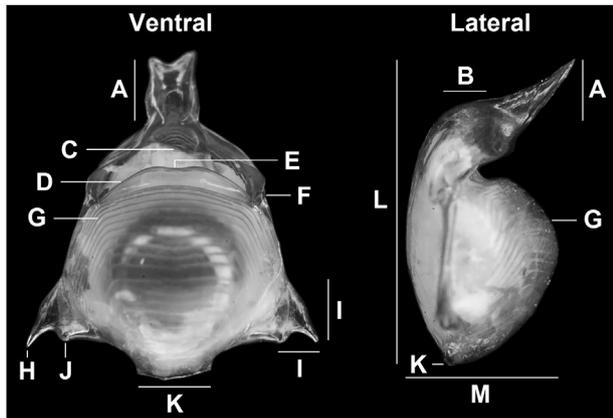
† Outgroup sequences from BurrIDGE *et al.* (2017).

of correlated lateral and ventral size variables in the same LDA. Morphometric assignment criteria for synonymization with integrative groups and/or conservation of holo- and paratypes as distinct morphological species were: at least 80% confidence of belonging to a group for lateral 49 LMs and/or 95% for ventral 23 LMs and/or 85% for ventral 38 LMs and no contradictory assignment between orientations of a specimen. These cut-off values were chosen to reflect the relatively higher information content of the shell outline of the lateral compared to the ventral orientation. If types were synonymized, i.e. merged with groups identified in Step 1 or with another group identified in Step 2, we did so for all type specimens of the same species, if the majority of these types were identically assigned, and we included any remaining unassigned types, because they were always from the same location. In this way, we reduced the number of distinct groups identified in Step 2. The third step

was to identify morphological species without holo- or paratypes, based on distinct positions in morphospace not covered by specimens from Steps 1 and 2, using LDA results as support for this distinction. The fourth step was the LDA assignment of the remaining specimens for which morphometric information was available to the groups identified in Steps 1–3. Remaining specimens were either non-sequenced fresh or non-type specimens from museum collections. Individuals remained unidentified (Table 1) if they did not meet the assignment criteria or were assigned ambiguously between orientations, which may indicate possible additional species. In the fifth step, we identified possible species based on individuals with genetic, but without morphometric, information. These are treated as separate groups, because their genetic data could not be linked to morphological data from other groups, although they may be synonymous to groups identified in Steps 2–3. Finally, in the sixth step, we

**Table 2.** Overview of 24 *Diacavolinia* taxa according to Van der Spoel *et al.* (1993) and holo- and paratypes used in this study

<i>Diacavolinia</i> taxon	Type locality	Type stored at	Types included in this study	Taxonomic description/ amendment
<i>D. angulata</i>	Indian	Laboratoire de Malacologie, Muséum d'Histoire Naturelle, Paris		Gray, 1850; Souleyet, 1852
<i>D. aspina</i>	Indian: 2°57'S 99°36'E	ZMUC, Copenhagen; NBC, Leiden	Holotype	Van der Spoel <i>et al.</i> , 1993
<i>D. atlantica</i>	Atlantic: 28°23.5'N 29°55.9'W	NBC, Leiden	Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. bandaensis</i>	Pacific: 4°36.4'S 130°21.7'E	NBC, Leiden	Holotype, Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. bicornis</i>	Indian: 3°45'S 56°33'E	ZMUC, Copenhagen; NBC, Leiden	Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. constricta</i>	Atlantic: 28°27.5'N 29°51.2'W	NBC, Leiden	Holotype	Van der Spoel <i>et al.</i> , 1993
<i>D. deblainvillei</i>	Atlantic: 38°45'N 72°20'W	NBC, Leiden	Holotype, Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. deshayesi</i>	Atlantic: 5°06'N 51°35'W	ZMUC, Copenhagen; NBC, Leiden	Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. elegans</i>	Pacific: 3°40.5'N 137°53'E	ZMUC, Copenhagen; NBC, Leiden	Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. flexipes</i>	Red Sea	Department of Zoology, University of Tel Aviv, ZMA	Holotype, Paratypes	Van der Spoel, 1971; Van der Spoel <i>et al.</i> , 1993
<i>D. grayi</i>	Pacific: 5°22.8'S 130°05'E	NBC, Leiden	Holotype, Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. limbata f. africana</i>	Atlantic: 29°48.1'N 29°57.5'W	NBC, Leiden	Holotype, Paratypes	D'Orbigny, 1836; Van der Spoel <i>et al.</i> , 1993
<i>D. limbata f. limbata</i>	Atlantic: between 24–22°N and 30–33°W	Laboratoire de Malacologie, Muséum d'Histoire Naturelle, Paris		D'Orbigny, 1836
<i>D. longirostris</i>	Atlantic: 22°N or S	Could not be located		De Blainville, 1821
<i>D. mcgowani</i>	Pacific: 6°49'N 80°25'W	ZMUC, Copenhagen		Van der Spoel, 1973; Van der Spoel <i>et al.</i> , 1993
<i>D. ovalis</i>	Atlantic: 38°50'N 72°25'W	NBC, Leiden	Holotype	Van der Spoel <i>et al.</i> , 1993
<i>D. pacifica</i>	Pacific: 6°29.9'S 131°09.2'E	NBC, Leiden	Holotype, Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. robusta</i>	Pacific: 2°52'N 87°38'W	ZMUC, Copenhagen; NBC, Leiden		Van der Spoel <i>et al.</i> , 1993
<i>D. souleyeti</i>	Indian: 2°57'S 99°36'E	ZMUC, Copenhagen; NBC, Leiden		Van der Spoel <i>et al.</i> , 1993
<i>D. strangulata</i>	Probably in the Indo-Pacific	Could not be located		Deshayes, 1823
<i>D. striata</i>	Indian: 2°57'S 99°36'E	ZMUC, Copenhagen; NBC, Leiden	Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. triangulata</i>	Pacific: Philippine Sea	NBC, Leiden	Holotype, Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>D. vanutrechtii</i>	Pacific: 18°53'S 163°02.5'W	ZMUC, Copenhagen		Van der Spoel <i>et al.</i> , 1993
<i>f. meisenheimeri</i>				
<i>D. vanutrechtii</i>	Pacific: 14°37'N 119°52'E	ZMUC, Copenhagen; NBC, Leiden	Paratypes	Van der Spoel <i>et al.</i> , 1993
<i>f. vanutrechtii</i>				



**Figure 3.** Morphology of *Diacavolinia* shells in ventral and lateral orientations, with (A) rostrum, (B) outer hump, (C) aperture, (D) ventral lip, (E) median lip depression, (F) lock area, (G) ventral ribs, (H) spine tip, (I) spine surface, (J) gutter corner, (K) caudal joint, (L) dorsal side and (M) maximum shell width as marked. Example specimen from AMT22 St. 29, 15°03'N, 34°28'W (Group 1).

plotted sampling locations of all identified species and possible species of *Diacavolinia* in order to assemble biogeographic distributions for each species based on all extant information.

#### GEOMETRIC MORPHOMETRICS

For quantitative analyses of *Diacavolinia* shell shapes and sizes, fresh and museum specimens were photographed in lateral ( $N = 904$ ) and ventral ( $N = 919$ ) orientations using a Nikon D100 6 mpx camera (Micro-Nikkor lens 55 mm/3.5, aperture  $f/11$ , shutter speed 1/1.3 s, ISO 200, fixed zoom) attached to a stand. To standardize the ventral orientation, specimens were mounted on photographic film using 60% methyl glucose. For lateral standardization, we used fine black sand, free from organic material. For geometric morphometric analyses, we selected photographs of all fully developed adults and excluded specimens that were not well-focused or in standard orientation. We also excluded specimens that were damaged or obscured at relevant positions by soft tissue that could not be removed without damaging the shell. Selected photographs were compiled for further analysis using tpsUtil software (Rohlf, 2006).

We used a combination of landmarks and semi-landmarks for partially outlining shell shapes in tpsDig (Rohlf, 2006) to cover as much shell-shape variation as possible for as many specimens as possible (Gunz & Mitteroecker, 2013). To assess the shape variation in the laterally photographed shells, two partial outlines were created, which were connected at the caudal joint between the ventral and dorsal

parts at the bottom of the shell (for shell anatomy see: Fig. 3). The first partial outline started at the position of maximum width of the ventral part, ended at the caudal joint and was standardized per specimen to 15 LMs separated by equal length. The second partial outline started at the top of the shell rostrum, ended at the caudal joint via the dorsal part of the shell and was standardized to 35 LMs. Because of the mutual landmark at the caudal joint, this resulted in a total lateral outline of 49 LMs. Creating the first partial outline of 15 LMs was possible for 752 specimens and creating both (49 LMs) was possible for a subset of 549 specimens (Table 1). To assess shape variation in a ventral orientation, two LMs were placed at the left and right gutter corners and a third LM was placed at the caudal joint. Subsequently, left and right partial outlines of ten LMs each were generated from the lock areas left and right of the shell aperture downwards to the closest position without overlap between ventral and dorsal shell parts, the upper start of the dorsal spine surface (see Fig. 3). This resulted in a total of 23 LMs for 646 specimens. No landmarks were created on the two spine tips because these were often damaged. For a subset of 314 specimens it was possible to create an additional 15 LM outline of the ventral lip, resulting in a total of 38 LMs. We used tpsRelw software (Rohlf, 2006) to rotate, translate and scale LM coordinates through generalized least-square Procrustes superimposition (GLS; Kendall, 1977). This provided centroid sizes, a size measure depending on the surface area within all LMs and multiple relative warp (RW) axes containing information on shape variation, with the first RW containing most information. The morphospace of the lateral orientation was represented by 26 relative warps (RWs) for 15 LMs and 94 RWs for 49 LMs. In ventral orientation, there were 42 RWs for 23 LMs and 72 RWs for 38 LMs.

To test for repeatability of RWs, a selection of 19 museum specimens was photographed in two subsequent series for lateral 15 LMs and 49 LMs and ventral 23 LMs, of which ten could also be used for ventral 38 LMs. Intra-class correlation coefficients (ICCs) between the two series were calculated for the centroid sizes and first ten RWs in PAST v.3.0 (Hammer *et al.*, 2001). RWs were considered repeatable when  $ICC > 0.80$ , and only repeatable RWs were used in further analyses of shell shape. Centroid sizes were always repeatable ( $ICC > 0.99$ ). For 15 LMs, the first two RWs were repeatable ( $ICC > 0.94$ ) and contained 95.96% of the shape variation for this part of the shell. For 49 LMs, RWs 1–8 and 10 were repeatable ( $ICC > 0.91$ ), accounting for 98.29% of the shell-shape variation. In a ventral orientation, repeatable RWs for 23 LMs were 1–5 and 8 ( $ICC > 0.83$ ; 92.36% of shape variation) and 1–5 ( $ICC > 0.81$ ; 83.17%) for 38 LMs, respectively.

## GENETICS

To assess genetic diversity and phylogenetic relationships in *Diacavolinia*, we obtained 86 *COI* mtDNA (GenBank accession numbers MF974762–MF974847) and 138 28S DNA (GenBank accession numbers MF974624–MF974761) sequences from a total of 173 specimens, following photography of shells of the same individuals for morphometric measurements. Tissue fragments of one mm<sup>3</sup> for DNA extraction could only be obtained by damaging the shells. DNA extraction was performed using the EZNA Mollusc DNA Kit (Omega Biotek, 2013), as recommended by Maas *et al.* (2013). We followed the manufacturer's recommended methods without freeze-drying of tissue before DNA extraction.

A 658 bp fragment of *COI* was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3'; Folmer *et al.*, 1994). Reactions were run in 25- $\mu$ L volumes consisting of 15.75  $\mu$ L MilliQ water, 2.5  $\mu$ L 10 $\times$  PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen), 0.5  $\mu$ L MgCl<sub>2</sub> (25 mM), 2.0  $\mu$ L dNTPs (2.5 mM per nucleotide), 0.25  $\mu$ L Taq (Qiagen), 1.0  $\mu$ L (10 mM) of each primer and 2.0  $\mu$ L DNA template. A 901 bp fragment of the nuclear 28S ribosomal gene was amplified using the primers 28SC1F (5'-ACCCGCTGAATTTAAGCAT-3') and 28SD3R (5'-GACGATCGATTTGCACGTCA-3'; Dayrat *et al.*, 2001). The 25- $\mu$ L reaction consisted of 15.25  $\mu$ L MilliQ water, 2.5  $\mu$ L 10 $\times$  PCR buffer containing 1.5 mM MgCl<sub>2</sub> (Qiagen), 0.5  $\mu$ L MgCl<sub>2</sub> (25 mM), 2.5  $\mu$ L dNTPs (2.5 mM per nucleotide), 0.25  $\mu$ L Taq polymerase (Qiagen), 1.0  $\mu$ L (10 mM) of each primer and 2.0  $\mu$ L DNA template. PCR was performed applying an initial denaturation of 4 min at 94 °C, 35 cycles of 1 min at 94 °C, 30 s at 50 °C, 1 min at 72 °C and finally 5 min at 72 °C for final extension, followed by ~1 h at 12 °C. If the initial PCR failed, an anti-inhibitor treatment (PCR Inhibition Removal Kit, Zymo Research, USA) was applied to the PCR solution, followed by the same PCR cycle as the initial PCR. Sanger sequencing of PCR products was performed using an ABI3730XL sequencer (Life Technologies).

Forward and reverse *COI* and 28S sequences were assembled in MEGA v.6.0 (Tamura *et al.*, 2013) and CodonCode Aligner v.4.1 (CodonCode Corporation, USA, 2013). For 28S, double peaks were registered as ambiguous when apparent in both forward and reverse sequences and with a secondary peak that was at least one-third of the height of the primary peak. Assembled sequences were aligned using the MAFFT algorithm (MAFFT v.7, 2013) and their identities as shelled pteropods were checked by BLAST against the NCBI nt database (Altschul *et al.*, 1997). Three Pacific *Diacavolinia* sequences from Maas *et al.* (2013; GenBank accession numbers JX183614–JX183616) were included in the *COI* alignment, as well as

two Atlantic and two Pacific *Cavolinia uncinata* (d'Orbigny, 1836) specimens (Burrige *et al.*, 2017c; GenBank accession numbers MF048915–MF048918 for *COI* and MF048968–MF048971 for 28S) in both alignments.

Maximum likelihood (ML; Felsenstein, 1981) was used to infer phylogenetic relationships among specimens for both the *COI* and 28S alignments and to distinguish clades with high bootstrap support. For *COI*, we used a General Time Reversible (GTR) substitution model with different evolutionary rates for the three codon positions (CP), because this is a biologically realistic model for protein coding sequences (Shapiro *et al.*, 2006). GTR with a proportion of invariable sites (I) and gamma distributed rate variation among sites ( $\Gamma$ ) was selected from 24 models using the Akaike Information Criterion (AIC) in JModelTest v.2.1.7, in which CP-models were not available (Darriba *et al.*, 2012). For 28S, the most appropriate substitution model selected using AIC was GTR + I. Molecular phylogenies were inferred using maximum likelihood followed by non-parametric bootstrap analysis with 1000 replicates in RaxMLGUI v.1.3 (Stamatakis, 2006; Silvestro & Michalak, 2012).

We identified molecular operational taxonomic units (MOTUs) based on *COI* and 28S sequences separately, using the online version of ABGD (Automatic Barcode Gap Discovery; Puillandre *et al.*, 2012) and the K2P evolutionary model (Kimura, 1980), the most complex model available in the online version. We also applied the Generalized Mixed Yule Coalescent (GMYC) approach to delimiting species (single threshold; Drummond *et al.*, 2012; Fujisawa & Barraclough, 2013), using an ultrametric tree inferred in BEAST v.2.3.3. Trees were inferred using the HKY substitution model, relaxed log normal clock, Yule model and with an MCMC run for 50 million generations (ten initialization attempts, sampling every 2000 steps for *COI* and every 5000 steps for 28S, 25% burn-in). To quantify differences between and within MOTUs and genetic clades, we calculated pairwise genetic distances between *COI* haplotypes as well as 28S alleles using the K2P +  $\Gamma$  distance model of evolution that assumes equal evolutionary rates among all transitions as well as among all transversions (Kimura, 1980) in MEGA v.6.0 (Tamura *et al.*, 2013). Among the available models in MEGA v.6.0, this model most closely represented our sequence data according to the AIC for *COI* and 28S. We reconstructed alleles from 28S genotypes using the PHASE algorithm (Stephens *et al.*, 2001; Stephens & Donnelly, 2003) in DnaSP v.5 (Librado *et al.*, 2009).

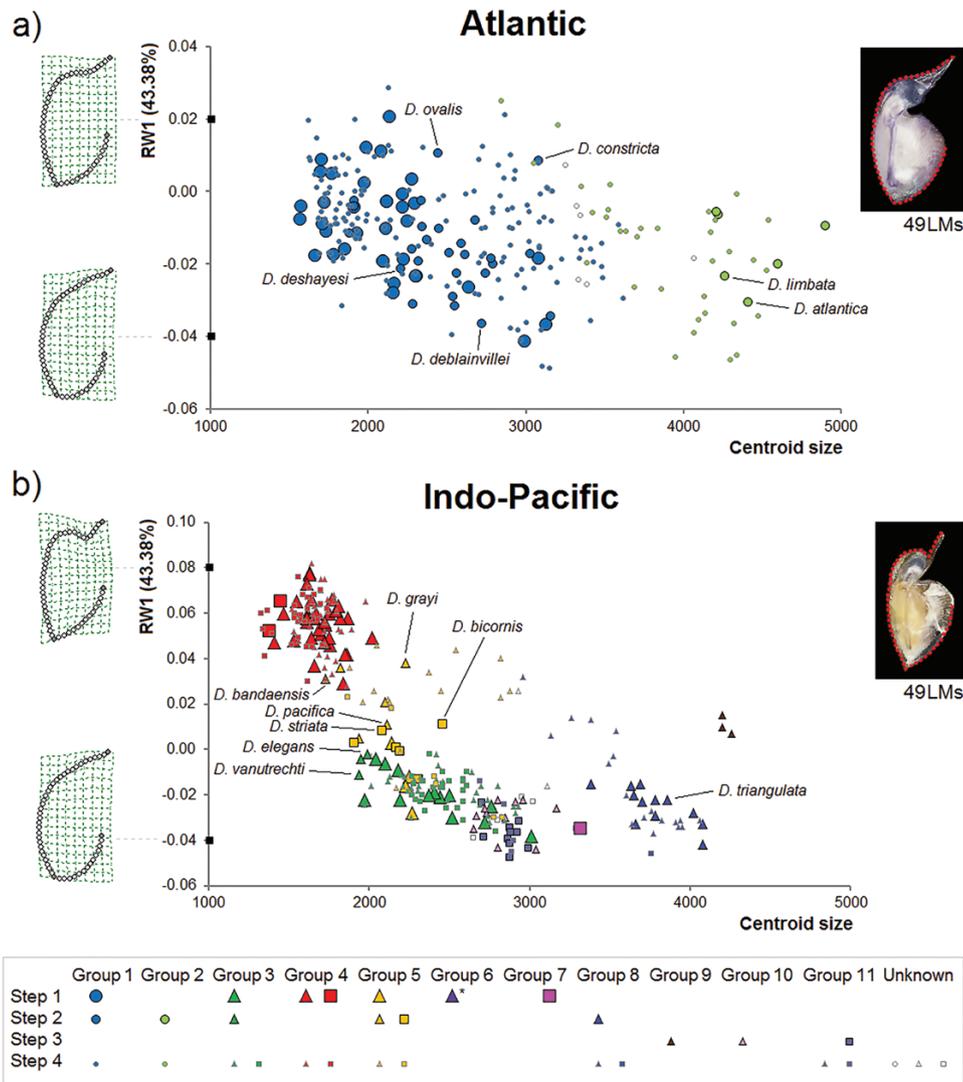
Integrative species were separated based on MOTUs with a pairwise genetic distance of >2%

divergence for *COI*, combined with the ability to distinguish groups morphologically using geometric morphometric methods. If *COI* sequences were not available, additional groups were identified based on the presence of unique mutations in 28S, a much more slowly evolving gene (e.g. BurrIDGE *et al.*, 2015). If morphologies between different MOTUs, as identified through ABGD and GMYC, could not be distinguished, they were treated as a single group with the notice of possible cryptic species within this group.

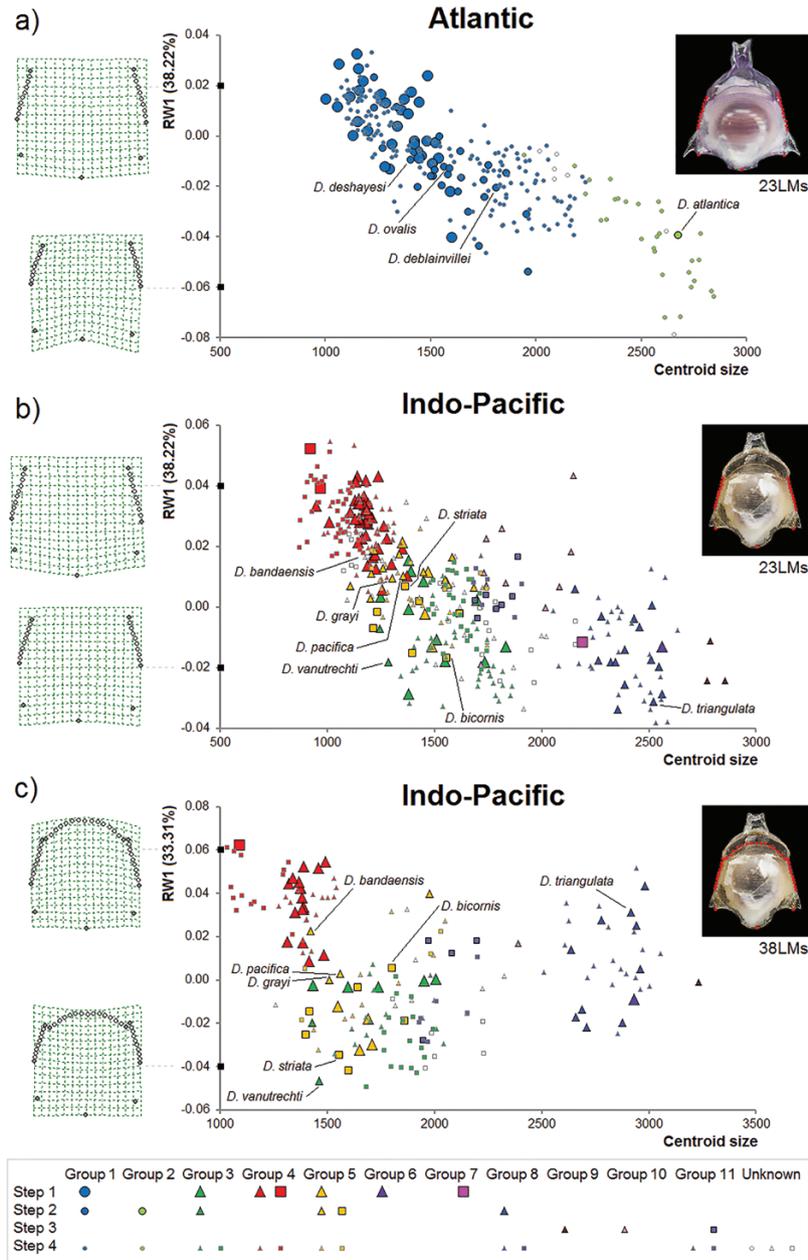
RESULTS

INTEGRATIVE APPROACH TO ASSESSING SPECIES BOUNDARIES

Geometric morphometric data demonstrate large variation in shell sizes and shapes among *Diacavolinia* specimens, especially in the Indo-Pacific (Figs 4, 5; Supporting Information, Supplementary Information S4). The first three lateral RWs captured 43.38, 21.95 and 14.97% of the total shell-shape variation, respectively (Fig.



**Figure 4.** Ordination of centroid sizes and the first Relative Warp (RW1) axis of *Diacavolinia* in a lateral orientation with 49 landmarks (LMs) for (A) Atlantic specimens and (B) Indo-Pacific specimens. The morphospace positions are indicated as circles for Atlantic ( $N = 267$ ), triangles for Pacific ( $N = 190$ ) and squares for Indian Ocean ( $N = 92$ ) specimens. Colours indicate distinct groups. Symbol sizes represent the steps in the integrative taxonomic approach in which specimens were assigned to a group (see legend). Thin plate splines from the RW positions indicated with black squares are shown left of RW1 to depict the variation in shell shape, with images of shells and LM positions shown on the right. Names of holotypes, and if no data were available representative paratypes, are indicated. Groups 12 and 13 are not shown because no morphological data was available.



**Figure 5.** Ordination of centroid sizes and the first Relative Warp (RW1) axis of *Diacavolinia* in a ventral orientation for (A) Atlantic specimens with 23 landmarks (LMs,  $N = 268$ ), (B) Indo-Pacific specimens with 23 LMs ( $N = 378$ ) and (C) a subset of Indo-Pacific specimens with 38 LMs ( $N = 174$ ). See Figure 4 for symbol definitions. Thin plate splines from the RW1 positions indicated with black squares are shown left of RW1 to depict the variation in shell shape, with images of shells and LM positions shown on the right. Names of holotypes, and if not available representative paratypes, are indicated.

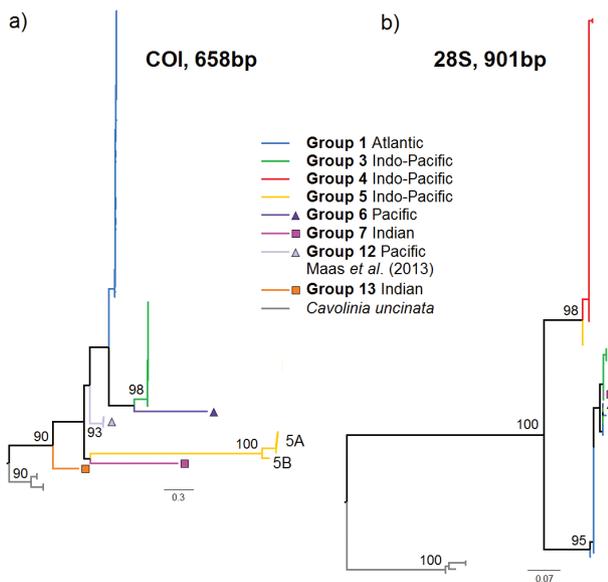
4; Supporting Information, [Supplementary Information S4A](#)). For ventral RWs (23 LMs) these results were 43.38, 27.20 and 10.79% (Fig. 5A, B; Supporting Information, [Supplementary Information S4B](#)).

There are 251 polymorphic sites in the *COI* nucleotide alignment of *Diacavolinia* ( $N = 89$ ; 658 bp; Supporting Information, [Supplementary Information](#)

[S5A](#); GenBank accession numbers [MF974762–MF974847](#)). For 28S ( $N = 138$ ; 901 bp; Supporting Information, [Supplementary Information S5B](#); GenBank accession numbers [MF974624–MF974761](#)) there are 28 polymorphic sites of which ten contained ambiguous base assignments, all based on transitions of C $\leftrightarrow$ T (Y) and A $\leftrightarrow$ G (R).

*Step 1: identification of integrative species*

We identify six integrative species based on combined genetic and geometric morphometric information of fresh specimens in Step 1: Groups 1 (Atlantic), 3–5 (Indo-Pacific), 6 (Pacific) and 7 (Indian Ocean; Fig. 6; groups numbered sequentially for Atlantic followed by Indo-Pacific throughout). The *COI* phylogeny includes Groups 1 ( $N = 56$ ; 1 MOTU based on ABGD), 3 ( $N = 21$ ; 1 MOTU), 5 ( $N = 6$ ; 2 MOTUs), 6 ( $N = 1$ ; 1 MOTU) and 7 ( $N = 1$ ; 1 MOTU; Fig. 6A). Within Group 5, a single specimen is assigned to a different MOTU based on ABGD of *COI* sequences (5B in Fig. 6), but because no geometric morphometric differences could be detected, it is not treated as a separate group. Groups 3 and 5 are also separated by GMYC (support of 1 and 0.92, respectively), as well as Group 12 (support of 1; discussed further in Step 5). Group 1 appears to have some internal structure (subclade support of 0.55), but all sequences in Group 1 are separated from



**Figure 6.** Maximum likelihood trees of (A) cytochrome *c* oxidase I (*COI*) mtDNA sequences ( $N = 89$ ) and (B) nuclear 28S rDNA sequences ( $N = 138$ ) of *Diacavolinia*. The *COI* phylogeny (A) includes Groups 1 ( $N = 56$ ), 3 ( $N = 21$ ), 5 ( $N = 6$ ), 6 ( $N = 1$ ), 7 ( $N = 1$ ), 12 ( $N = 3$ , from Maas *et al.*, 2013; GenBank accession numbers JX183614–JX183616; Pacific Ocean) and 13 ( $N = 1$ ). The 28S phylogeny (B) includes Groups 1 ( $N = 35$ ), 3 ( $N = 17$ ), 4 ( $N = 78$ ), 5 ( $N = 6$ ), 6 ( $N = 1$ ) and 7 ( $N = 1$ ). Four *Cavolinia uncinata* outgroup sequences are included to root each tree (from BurrIDGE *et al.*, 2017c). Numbers indicate bootstrap support in ML analyses (only bootstrap values  $\geq 90$  are shown). Colours indicate distinct genetic groups; symbols indicate rare genetic groups (triangle = Pacific; square = Indian Ocean). Not all groups were amplified in PCR for both markers.

any of the Indo-Pacific groups according to GMYC. The two ABGD MOTUs in Group 5 are not supported by GMYC and neither are the single specimens representing Groups 6, 7 and 13. However, this is most likely explained by the fact that the GMYC method relies on distinguishing the transition from inter- to intraspecific branching within an ultrametric tree and in the absence of multiple individuals, the method cannot detect this transition. Hence, based on results from ABGD, pairwise genetic distances and geometric morphometric information we consider these groups to be separate species. For Group 4, *COI* sequences failed to amplify in PCR. Groups 12 and 13, from which we (Group 13) and Maas *et al.* (2013; Group 12) obtained *COI* sequences, but which lack morphological data due to unavailability of shells (Group 12) or damaged shell characters (Group 13), are discussed in Step 5. Pairwise genetic distances of *COI* haplotypes between these groups are high with averages of 18.6–43.8% (Table 3). Pairwise distances are small in Groups 1 (average 1.4%, range 0.0–2.7%) and 3 (0.4%, 0.0–1.1%) and larger in Group 5 (6.3%, 0.5–15.6%). Without the single specimen assigned to another MOTU, pairwise distances in Group 5 average 2.0% (0.5–3.0%; 5A in Fig. 6). The Atlantic *COI* sequences from Maas *et al.* (2013) all correspond to Group 1 (NCBI BLAST search). Other groups with *COI* sequences, but without morphological data, are discussed in Step 5. The 28S phylogeny includes Groups 1 ( $N = 35$ ), 3 ( $N = 17$ ), 4 ( $N = 78$ ), 5 ( $N = 6$ ), 6 ( $N = 1$ ) and 7 ( $N = 1$ ) and no additional groups without morphological data are identified based on this genetic marker. Two well-supported *Diacavolinia* clades are present, each representing one 28S MOTU as identified by ABGD (Fig. 6B). GMYC distinguished 16 MOTUs and, although their composition was not incongruent with the ABGD MOTUs, they are probably an artefact of the low levels of polymorphism in this gene. The first clade contains Groups 1, 3, 6 and 7 ( $N = 54$ ) and spans all three oceans. The second clade consists of Groups 4 and 5 ( $N = 84$ ) and is restricted to the Indo-Pacific. The average genetic distance between alleles of the two clades was 2.4% (1.7–3.0%). Within the first clade, average genetic distance is 0.2% (0.0–0.6%) and within the second clade it is 0.1% (0.0–0.4%). Based on 28S, the groups in the first clade cannot be distinguished. Groups 4 and 5 of the second clade can always be distinguished from each other (0.3%, 0.2–0.4% genetic distance between alleles; Table 3).

Across all shell orientations, LDA demonstrates a 100% correspondence between geometric morphometric and genetic data for Groups 1 and 5. Accuracy of assignment is 92.9% for Group 3 and 95.9% for Group 4, with the remaining individuals not reaching all assignment criteria for unambiguous identification (Table 4). We obtained geometric morphometric measurements of

**Table 3.** Genetic distances (K2P +  $\Gamma$ ) between *Diacavolinia* COI haplotypes and 28S alleles per group identified in this study, including *Cavolinia uncinata*. *N* indicates individuals included

	Group 1 Atlantic	Group 3 Indo- Pacific	Group 4 Indo- Pacific	Group 5 Pacific	Group 6 Pacific	Group 7 Indian	Group 12 Pacific	Group 13 Indian	<i>C. uncinata</i> Atlantic	<i>C. uncinata</i> Pacific
<b>28S, between groups</b>										
<b>Group 1</b> ( <i>N</i> = 35)	0.0135	0.0036	-	0.0629	-	-	0.0083	-	0.0016	0.0077
<b>Group 3</b> ( <i>N</i> = 17)	0.0035	0.1859	-	0.4064	0.2559	0.2720	0.1903	0.2103	0.2506	0.2400
<b>Group 4</b> ( <i>N</i> = 78)	0.0241	0.0239	-	-	0.2419	0.2786	0.2070	0.2386	0.2590	0.2653
<b>Group 5</b> ( <i>N</i> = 6)	0.0241	0.0263	0.0026	0.4162	0.4379	0.3926	0.4106	0.4052	0.4052	0.3786
<b>Group 6</b> ( <i>N</i> = 1)	0.0034	0.0026	0.0242	0.0271	0.2912	0.2516	0.2655	0.2657	0.2657	0.2559
<b>Group 7</b> ( <i>N</i> = 1)	0.0036	0.0017	0.0249	0.0271	0.0083	0.2395	0.2760	0.3078	0.3078	0.3058
<b>Group 12</b> ( <i>N</i> = 3)	-	-	-	-	-	-	0.1961	0.2415	0.2415	0.2182
<b>Group 13</b> ( <i>N</i> = 1)	-	-	-	-	-	-	-	0.1914	0.1914	0.1832
<b><i>C. unc.</i> Atl</b> ( <i>N</i> = 2)	0.0450	0.0452	0.0465	0.0464	0.0460	0.0460	-	-	-	0.1082
<b><i>C. unc.</i> Pac</b> ( <i>N</i> = 2)	0.0482	0.0490	0.0503	0.0495	0.0497	0.0497	-	-	0.0073	-
<b>28S, within groups</b>	0.0011	0.0017	0.0005	0.0000	-	-	-	-	0.0033	0.0000

**Table 4.** Overview of *Diacabolinia* groups identified in this study following the integrative taxonomic steps outlined in Figure 1. The Linear Discriminant Analysis (LDA) accuracy depicts the correspondence between geometric morphometric and genetic data (Step 1, S1), the correspondence between morphospace position of holo- and paratypes and their LDA identification (Step 2, S2), or the correspondence between estimated morphospace position and their LDA identification (Step 3, S3) following the morphometric assignment criteria (see text). Step 4 (S4) depicts the numbers of remaining specimens assigned to groups identified in Steps 1–3 using LDA. A = Atlantic; P = Pacific; I = Indian Ocean. See Figure 8 for taxonomic implications

Assignment	Specimens		Stations			Steps 1 and 5		Steps 2 and 3		Step 4	
	Total	Atlantic	Pacific	Indian	Total	Ocean (N specimens)	LDA accuracy (N)	LDA accuracy (N)	Species according to Van der Spoel <i>et al.</i> (1993; Ocean, N specimens)	Species according to Van der Spoel <i>et al.</i> (1993; Ocean, N specimens)	
<b>Atlantic</b>	<b>Group 1 (G,M)</b>	<b>276</b>	<b>276</b>	<b>0</b>	<b>0</b>	<b>58</b>	S1: Atlantic (65); Atlantic sequences from Maas <i>et al.</i> (2013) (53)	100% (42/42)	S2: <i>D. deblainvillei</i> (A, 18) S2: <i>D. deshayesi</i> (A, 8) S2: <i>D. constricta</i> (A, 1) S2: <i>D. ovalis</i> (A, 1)	96.4% (27/28)	<i>D. deshayesi</i> (A, 30); <i>D. constricta</i> (A, 23); <i>D. longirostris</i> (A, 18); <i>D. angulata</i> (A, 15); <i>D. vanutrechti</i> (A, 15); <i>D. deblainvillei</i> (A, 14); <i>D. elegans</i> (Atl, 12); <i>D. strangulata</i> (A, 11); <i>D. ovalis</i> (A, 6); <i>D. limbata limbata</i> (A, 3); <i>D. vanutrechti</i> (A, 2); <i>meisenheimeri</i> (A, 2); <i>D. bicornis</i> (A, 1); Fresh specimens (A, 33)
	<b>Group 2 (M)</b>	<b>51</b>	<b>51</b>	<b>0</b>	<b>0</b>	<b>12</b>			S2: <i>D. limbata africana</i> (A, 5) S2: <i>D. atlantica</i> (A, 1)	100% (6/6)	<i>D. limbata limbata</i> (A, 31); <i>D. vanutrechti</i> (A, 7); <i>meisenheimeri</i> (A, 3); <i>D. constricta</i> (A, 2); <i>D. deshayesi</i> (A, 1); <i>D. strangulata</i> (A, 1)
	<b>Group 3 (G,M)</b>	<b>100</b>	<b>0</b>	<b>66</b>	<b>34</b>	<b>27</b>	S1: Pacific (15) S1: Indian (6)	92.9% (13/14)	S2: <i>D. vanutrechti</i> (P, 3) S2: <i>D. elegans</i> (P, 1)	75% (3/4)	<i>D. souleyeti</i> (I, 25); <i>D. vanutrechti</i> (P, 18); <i>D. vanutrechti</i> (P, 16); <i>D. flexipes</i> (P, 13); <i>D. bicornis</i> (I, 2); <i>D. limbata limbata</i> (I, 1)

**Table 4.** Continued

Assignment	Specimens			Stations			Steps 1 and 5		Steps 2 and 3		Step 4
	Total	Atlantic	Pacific	Indian	Total	Ocean ( <i>N</i> specimens)	LDA accuracy ( <i>N</i> )	Species according to Van der Spoel <i>et al.</i> (1993; Ocean, <i>N</i> specimens)	LDA accuracy ( <i>N</i> )	Species according to Van der Spoel <i>et al.</i> (1993; Ocean, <i>N</i> specimens)	
<b>Group 4 (G,M)</b> Integrative species	172	0	123	49	35	S1: Pacific (75) Indian (3)	S1: 95.9% (47/49)	<i>D. pacifica</i> (P, 11; I, 22); <i>D. angulata</i> (P, 17; I, 14); <i>D. grayi</i> (P, 5; I, 10); Fresh specimens (P, 15)			
<b>Group 5 (G,M)</b> Integrative species	64	0	48	16	18	S1: Pacific (6)	100% (5/5)	S2: <i>D. grayi</i> (P, 8); S2: <i>D. pacifica</i> (P, 15); <i>D. striata</i> (I, 7) S2: <i>D. bandaensis</i> (P, 6) S2: <i>D. pacifica</i> (P, 1); S2: <i>D. bicornis</i> (I, 2)			
<b>Group 6 (G,M)</b> Integrative species	1	0	1	0	1	S1: Pacific (1)		<i>D. longirostris</i> (P, 20); <i>D. pacifica</i> (P, 3); <i>D. triangulata</i> (P, 9; I, 1)			
<b>Group 7 (G,M)</b> Integrative species	1	0	0	1	1	S1: Indian (1)					
<b>Group 8 (M)</b> Morphospecies	49	0	48	1	5			S2: <i>D. triangulata</i> (P, 16)			
<b>Group 9 (M)</b> Morphospecies	3	0	3	0	1			S3: <i>D. strangulata</i> (P, 3)			
<b>Group 10 (M)</b> Morphospecies	12	0	12	0	2			S3: <i>D. mcgowani</i> (P, 7) S3: <i>D. longirostris</i> (P, 5)			
<b>Group 11 (M)</b> Morphospecies	19	0	1	18	7			S3: <i>D. longirostris</i> (I, 11)			
<b>Group 12 (G)</b> Possible species	3	0	3	0	1	S5: Pacific sequences from Maas <i>et al.</i> (1993) identified as <i>D. vanutrechtii</i> (3)		S3: <i>D. longirostris</i> (I, 11)			

Table 4. Continued

Assignment	Specimens			Stations			Steps 1 and 5		Steps 2 and 3		Step 4
	Total	Atlantic	Pacific	Indian	Total	Ocean ( <i>N</i> specimens)	LDA accuracy ( <i>N</i> )	LDA accuracy ( <i>N</i> )	Species according to Van der Spoel <i>et al.</i> (1993; Ocean, <i>N</i> specimens)	LDA accuracy ( <i>N</i> )	Species according to Van der Spoel <i>et al.</i> (1993; Ocean, <i>N</i> specimens)
Group 13 (G)	1	0	0	1	1	S5: Indian (1)					
Possible species											
Unknown	217	47	116	54							<i>D. aspina</i> (P, 1: ambiguous); <i>D. robusta</i> (Not available); <i>D. flexipes</i> (None from Type location: Red Sea)

shell shape and size for 112 sequenced individuals with  $N = 89$  for lateral 49 LMs,  $N = 94$  for ventral 23 LMs and  $N = 57$  for ventral 38 LMs (Supporting Information, [Supplementary Information S1](#)). The shell shapes of Groups 1 and 3–5 are significantly different in all orientations (Bonferroni-corrected  $P < 0.001$  for all orientations;  $F = 45.84$  for lateral 49 LMs;  $F = 17.49$  for ventral 23 LMs;  $F = 17.69$  for ventral 38 LMs). Some, but not all, shell sizes are significantly different based on centroid size measurements. For lateral 49 LMs, Group 4 is significantly smaller than Groups 1, 3 and 5 ( $P < 0.001$ , 0.001 and 0.01;  $F = 36.75$ , 127.1 and 59.15, respectively). The same is true for 38 LMs ( $P < 0.05$ , 0.01 and 0.01;  $F = 8.217$ , 31.74 and 35.23, same order) and for ventral 23 LMs ( $P < 0.001$  for all combinations;  $F = 27.04$ , 75.07 and 48.57). Additionally, Group 1 is significantly smaller than Group 3 in this orientation ( $P < 0.01$ ;  $F = 9.318$ ).

#### Step 2: identification of morphological species with holo- and paratypes

From the Atlantic Ocean, holo- and paratypes of *D. atlantica* Van der Spoel *et al.*, 1993 and *D. limbata* f. *africana* Van der Spoel *et al.*, 1993 are identified as Group 2 with 100% confidence. Holo- and paratypes of *D. constricta* Van der Spoel *et al.*, 1993, *D. deblainvillei* Van der Spoel *et al.*, 1993, *D. deshayesi* Van der Spoel *et al.*, 1993 and *D. ovalis* Van der Spoel *et al.*, 1993 are placed in Group 1 with a confidence of 96.4% (Figs 1, 4A, 5A; Table 4). We did not use the ventral orientation with 38 LMs for identifying Atlantic specimens because this part of the shell is always obscured by soft tissue for Group 2.

In the Pacific Ocean, *D. trangukata* Van der Spoel *et al.*, 1993 holo- and paratypes are identified as Group 8 with 100% confidence. Based on geometric morphometric data from the Indo-Pacific, seven taxa are placed into groups from Step 1 (Figs 1, 4B, 5B, C; Table 4; Supporting Information, [Supplementary Information S4](#)). *Diacavolinia elegans* Van der Spoel *et al.*, 1993 and *D. vanutrechtii* f. *vanutrechtii* Van der Spoel *et al.*, 1993 paratypes from the Pacific fit into Group 3 (75% confidence). The Pacific *D. bandaensis* Van der Spoel *et al.*, 1993, *D. grayi* Van der Spoel *et al.*, 1993 and *D. pacifica* Van der Spoel *et al.*, 1993 holo- and paratypes as well as the Indian Ocean *D. bicornis* Van der Spoel *et al.*, 1993 and *D. striata* Van der Spoel *et al.*, 1993 paratypes are identified as belonging to Group 5 (64% confidence). The higher fraction of ambiguous type specimens in Group 5 may be due to the relatively large morphospace covered by this group, in which we cannot distinguish any subgroups, and for which at least two MOTUs are identified by ABGD based on *COI* sequences.

*Step 3: identification of morphological species without types*

We distinguish three additional morphological species based on the morphospace position of non-type museum specimens from the Indo-Pacific (Groups 9–11;  $N = 26$ ; Figs 1, 4B, 5B,C; Supporting Information, Supplementary Information S4). For all three groups, there is a 100% correspondence between their estimated position in morphospace and their LDA assignment (Table 4). Non-type specimens were previously identified by Van der Spoel *et al.* (1993) as Pacific *D. strangulata* (Deshayes, 1823) (Group 9), Pacific *D. mcgowani* Van der Spoel *et al.*, 1993 and *D. longirostris* (Group 10) and *D. longirostris* from the Indian Ocean (Group 11). Group 9 ( $N = 3$  identified in Step 3) is distinguished by a strongly ventrally directed shell rostrum and large size, Group 10 ( $N = 12$ ) is identified by large spines and a triangular appearance in a ventral orientation and Group 11 ( $N = 11$ ) is recognized by a subtle outer hump combined with a ventrally directed shell rostrum.

*Step 4: assignment of remaining specimens to morphogroups*

Of our fresh specimens with morphometric, but without genetic, data, all Atlantic specimens ( $N = 33$ ) are assigned to Group 1 by LDA. Pacific specimens ( $N = 15$ ; 94.8%) are assigned to Group 4 (1 specimen was ambiguous). We thus infer that a representative number of specimens from Groups 1 and 4 were sequenced in Step 1. Of our remaining museum specimens, 423 specimens (88%) are successfully assigned to groups and 58 (12%) are not (Table 4) but may represent additional diversity.

*Step 5: identification of possible species*

Two additional groups are identified based on *COI* sequences alone, each representing one MOTU (Groups 12 and 13; Fig. 6A; Table 4). Group 12 contains all three eastern Tropical Pacific (ETP) sequences from Maas *et al.* (2013; listed as *D. vanutrechtii*). Group 13 contains a single sequence from the Indian Ocean near South Africa and its phylogenetic position is between other *Diacavolinia* groups and *Cavolinia uncinata*. No morphological information is available for these groups, because Group 12 is solely represented by sequences from Maas *et al.* (2013) and Group 13 is represented by a single juvenile specimen with a damaged shell.

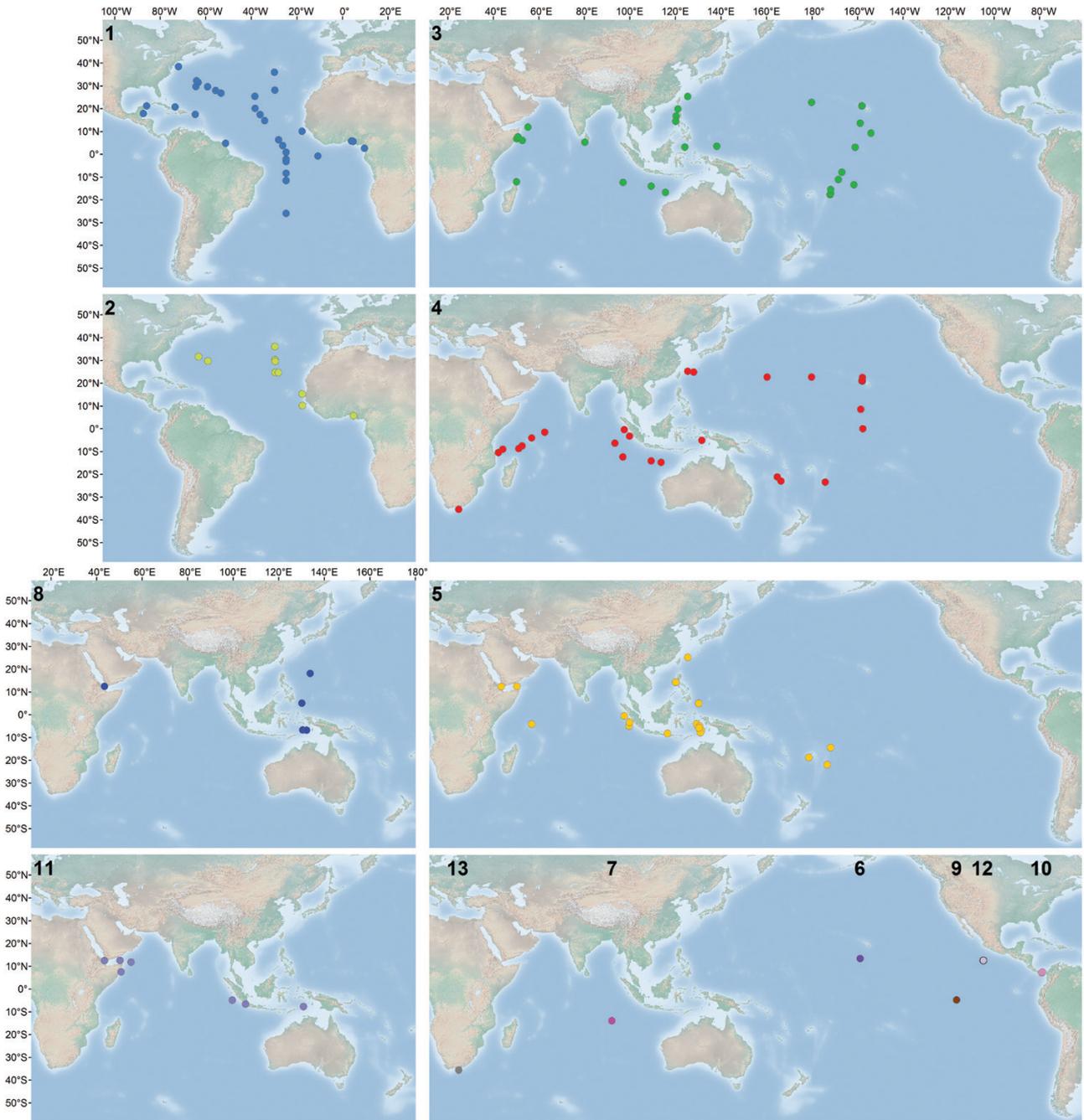
*Step 6: global biogeography*

Biogeographic ranges of the identified groups are diverse and varied in size (Fig. 7). In the Atlantic Ocean, two groups occur: Group 1 ( $N = 276$ ) is present

across the entire described range for *Diacavolinia* in the Atlantic Ocean (39°N–26°S) including the Caribbean Sea. Group 2 ( $N = 51$ ) is only found in the Northern Hemisphere and predominantly around the Azores, near the Cape Verde Islands and in the Gulf of Guinea. Groups 3–5 are the most common groups in the Indo-Pacific ( $N = 100$ ; 172; 64) and they all have wide Indo-Pacific distributions. Less common are Group 8 ( $N = 49$ ), predominantly occurring in the central Indo-Pacific but also extending west to the Red Sea, and Group 11 ( $N = 19$ ) from the southern part of the central Indo-Pacific and in and near the Gulf of Aden. Of the remaining, rare groups, Group 6 ( $N = 1$ ) is found in the Central Pacific south of Hawaii at 14°N and Groups 9 ( $N = 3$ ), 10 ( $N = 12$ ) and 12 ( $N = 3$ ) all occur in the ETP, of which Group 10 ( $N = 12$ ) was sampled near the coast of Panama. Groups 7 and 13 are each found once in the Indian Ocean, with Group 7 occurring close to the Cocos-Keeling Islands and Group 13 occurring in warm waters near South Africa (Fig. 7).

## SYNTHESIS

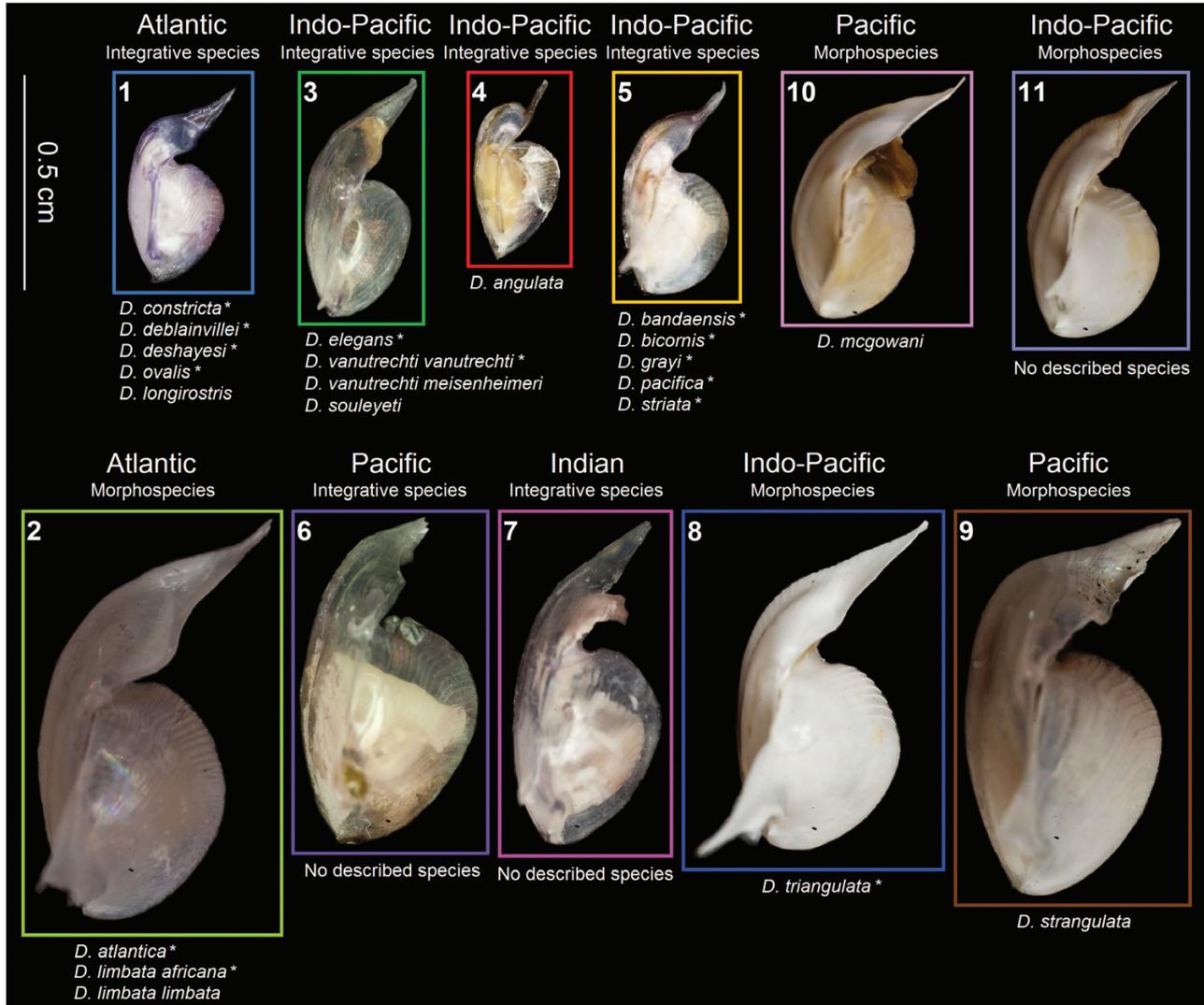
Following our integrative approach, a total of 752 specimens (77.6% of 969 available specimens) is assigned to 13 groups (Figs 1, 8; Table 4). We distinguish two groups in the Atlantic Ocean and 11 groups in the Indo-Pacific. We consider there to be sufficient genetic and morphometric evidence for the validity of the Atlantic Group 1 and Indo-Pacific Groups 3–5 as integrative species, with possible additional diversity in Group 5. The morphological variation of Atlantic Group 1 is large compared to all other groups, including Group 5, but the extensive sampling in the Atlantic Ocean, the homogeneous distribution of specimens within the morphospace and their low levels of genetic variation make it unlikely that there are additional Atlantic species. Although we had only one specimen each for Groups 6 and 7, they likely also represent separate species based on genetic and morphometric data. We identify Atlantic Group 2 and Indo-Pacific Groups 8–11 as morphological species, but they were not sampled in our recent collection and we lack genetic information for these taxa. Hence, we could not link them to possible species of Groups 12 and 13, for which we only had genetic information. However, we consider it unlikely that Group 13 is identical to any of the morphological species analysed here based on its South African locality with none of Groups 8–11 occurring nearby. Of the unassigned individuals ( $N = 217$ ), 158 specimens provide no evidence to determine their position in morphospace (no suitable photographs available), and neither can their molecular phylogenetic position be determined due to absent data. Therefore, additional diversity may be present. A further 59 specimens for



**Figure 7.** Distribution of *Diacavolinia* Groups 1–13 as identified in this study. A presence record was mapped if at least one specimen from that locality was positively identified. Group numbers are indicated at the top of each map.

which geometric morphometric measurements are available, of which 50 are from the Indo-Pacific, could not be confidently assigned to a group, and may also represent additional diversity. More molecular data are needed to establish the presence of additional diversity.

Figure 8 gives an overview of typical adult shell shapes (in lateral orientation) and sizes for Groups 1–11. Important morphological characteristics for distinguishing between *Diacavolinia* species are the presence of an outer hump (B in Fig. 3), direction of the shell rostrum (A in Fig. 3), shape of the spines (H in



**Figure 8.** Overview of revised *Diacavolinia* taxonomy with example specimens of Groups 1–11 shown in a lateral orientation. Species names as (re)described by Van der Spoel *et al.* (1993) are listed below each group based on holo- and paratype specimens. Species names are indicated with an asterisk (\*) if type specimens were included in this study. Species without type specimens are listed below the group to which the majority of specimens identified as such was assigned in Steps 3 and 4. They are listed only if the species was originally described from the same ocean basin. Photo sizes are standardized to the 0.5-cm scale bar. Groups 12 and 13 are not shown because no morphological data was available.

Fig. 3) and their angle relative to the rest of the shell, convexity of the dorsal (L in Fig. 3) and ventral parts as seen in lateral orientation and shell size.

## DISCUSSION

### INTEGRATIVE TAXONOMY AND PTEROPOD DIVERSITY

To our knowledge, this is the first time a global collection of samples, including both recent and museum-type specimens of a marine zooplankton group, are combined into a single dataset for integrative taxonomic purposes. We collected morphological and/or genetic information

from 969 specimens belonging to the pteropod genus *Diacavolinia* for which there was morphological and/or genetic information available. Our inclusion of all extant museum material also examined by Van der Spoel *et al.* (1993) allowed for direct comparison with previous taxonomies for this genus. In this way, we more accurately and objectively resolved species boundaries and species distributions than has been possible in prior work.

Our findings suggest the presence of 13 species in our available samples, while 24 taxa were originally described by Van der Spoel *et al.* (1993). Especially in

the Atlantic Ocean, the number of species should be reduced from eight to two, with one species comprising the currently described *D. constricta*, *D. deblainvillei*, *D. deshayesi*, *D. longirostis* and *D. ovalis*, and the other comprising *D. atlantica*, *D. limbata* f. *africana* and *D. limbata* f. *limbata* (d'Orbigny, 1836) (Fig. 8). New taxonomic descriptions should reflect the larger morphological variation covered by each group and follow rules of the International Commission on Zoological Nomenclature (ICZN). Although the overall morphological diversity in the Atlantic is large, we found little structure in our morphometric data and low levels of genetic diversity, in contrast to the Indo-Pacific. Although we had type specimens for only eight of the 16 original Indo-Pacific species, and no specimens at all for *D. robusta*, we found evidence for 11 species, comprising 13 of the original taxa. Based on our findings, *D. angulata* (Souleyet, 1852) (Group 4), *D. triangulata* (Group 8), *D. strangulata* (Group 9) and *D. mcgowani* (Group 10) are confirmed as valid species. Nine original taxa should be merged into two species: one species containing *D. elegans*, *D. vanutrechtii* f. *vanutrechtii*, *D. vanutrechtii* f. *meisenheimeri* Van der Spoel *et al.*, 1993 and *D. souleyeti* Van der Spoel *et al.*, 1993 (Group 3) and one containing *D. bandaensis*, *D. bicornis*, *D. grayi*, *D. pacifica* and *D. striata* (Group 5). The latter species may comprise additional taxa based on the presence of two MOTUs and a relatively large shell-shape variation compared to other taxa. Furthermore, three Indo-Pacific groups identified in this study require new taxonomic names (Fig. 8).

Despite the global distribution of our samples, some taxonomic uncertainties remain, especially in the Indo-Pacific, ETP and Red Sea. The sampling coverage in the Atlantic Ocean was higher than in the Indo-Pacific. The validity of three taxa as described by Van der Spoel (1993) remains unclear: *D. aspina* Van der Spoel *et al.*, 1993, *D. flexipes* Van der Spoel *et al.*, 1993 and *D. robusta*, described from the Indian Ocean, Red Sea and Pacific Ocean, respectively (Van der Spoel *et al.*, 1993). Material from *D. aspina* ( $N = 1$ ; non-type) could not be unambiguously placed in any group by LDA. *Diacavolinia robusta* specimens were not available. We also had no material identified as *D. flexipes* from the Red Sea, its type locality (Table 2). However, Janssen (2007b) suggested that the validity of *D. flexipes* based on subtle morphological differences is doubtful and rather represents intraspecific variation.

The higher overall species diversity in the Indo-Pacific compared to the Atlantic supports the hypothesis of an Indo-Pacific origin for *Diacavolinia* as proposed by Van der Spoel *et al.* (1993). *Diacavolinia* was already present in the Indo-Pacific in the Pliocene (Piacenzian, 3.6–2.6 Mya), based on fossils from northern Philippine sediments described as

*Diacavolinia pristina* (Janssen, 2007a). It is unknown how long *Diacavolinia* has been present in the Atlantic Ocean.

#### BARRIERS TO DISPERSAL

Persistent dispersal barriers may limit range shifts of some taxa in response to changing ocean conditions, while other taxa may be able to adapt and occupy new ecological niches. The most important biogeographic barriers for *Diacavolinia*, as inferred from distinct species assemblages, were between the Atlantic and Indo-Pacific oceans and the East and Central Pacific. Biogeographic distributions of proposed *Diacavolinia* species were as follows: Atlantic (two endemic species), warm waters south of South Africa (one endemic species), Western Indian Ocean (four non-endemic species), Red Sea and Gulf of Aden (three non-endemic species), Indo-West Pacific (six species, one endemic), Central Pacific (three species, one endemic), coastal waters of the North-West Pacific (three non-endemic species) and ETP (three endemic species). Species distributions described here are less patchy and disjunct compared to Van der Spoel *et al.* (1993) and may better reflect ecological and/or habitat preferences of *Diacavolinia* species. The distribution patterns of the revised *Diacavolinia* species are congruent with several well-known biogeographic provinces for other holoplankton or benthic species with pelagic larvae, and provide important information on the range of environmental variation experienced by each species (e.g. Kulbicki *et al.*, 2013; Bowen *et al.*, 2016; Iacchei *et al.*, 2016).

The Agulhas Current in the Indian Ocean intermittently forces warm eddies into the Atlantic (Hutchings *et al.*, 2009; Villar *et al.*, 2015). Although distributions of two *Diacavolinia* taxa extended to waters south of South Africa, we did not find evidence for recent dispersal between the Atlantic and Indian Oceans. Hence, the degree of connection of *Diacavolinia* in the Atlantic and Indo-Pacific basins appears to have been overestimated by Van der Spoel *et al.* (1993), who reported that five of the 24 originally described *Diacavolinia* taxa occurred in both the Atlantic and the Indo-Pacific. Likewise, no evidence of recent dispersal from the Indian Ocean into the Atlantic Ocean was found in pteropods of the genus *Cuvierina* (Janssen, 2005; BurrIDGE *et al.*, 2015). More rigorous molecular examination of other warm-water pteropods may identify higher numbers of endemic species in the Atlantic and Indo-Pacific Ocean basins than have been described to date. Other pelagic examples for which Atlantic taxa are isolated from the Indo-Pacific include atlantic heteropods (*Atlanta selvagensis* de Vera & Seapy, 2006 and *Protatlanta sculpta* Issel, 1911;

Wall-Palmer *et al.*, 2016, 2018), several copepods (e.g. Hirai *et al.*, 2015; Goetze, 2011) and populations of two-wing flyfish (Lewallen *et al.*, 2016). Conversely, leakage of Indian Ocean water into the Atlantic Ocean via Agulhas eddies appears to have increased over the last decade (Biaostoch *et al.*, 2009). Sporadic species dispersal through Agulhas rings has been demonstrated, e.g. for moray eels and glass-eye fish (Reece *et al.*, 2010; Gaither *et al.*, 2015). Additionally, Villar *et al.* (2015) found an overlap in MOTUs of the plankton community within Agulhas rings between the Indian and South Atlantic oceans based on metabarcoding.

We found evidence for endemism of *Diacavolinia* species in the ETP. Other genetic studies of East Pacific plankton showed that some, but not all taxa demonstrated East Pacific endemism, and it is likely that cryptic diversity is present within what are now considered single species or species complexes. Some endemic cryptic diversity within the ETP was found in the *Pleuromamma piseki* Farran, 1929–*P. gracilis* Claus, 1863 copepod species complex (Halbert *et al.*, 2013), and morphological divergence was found in *Glaucois marginatus* (Reinhardt & Bergh, 1864) sea slugs (Churchill *et al.*, 2014b). Some taxa with pelagic larval stages demonstrate East Pacific isolation, such as the reef-building coral *Porites lobata* Dana, 1846 (Baums *et al.*, 2012) and the spiny lobster *Panulirus penicillatus* (Olivier, 1791) (Iacchei *et al.*, 2016), but the echinoderm *Echinothrix diadema* (Linnaeus, 1758) does not (Lessios *et al.*, 1998). Within another Pacific group of holoplanktonic gastropods (heteropods, Pterotracheoidea), *Atlanta californiensis* Seapy & Richter, 1993 is restricted to the California Current upwelling ecosystem (Seapy *et al.*, 2003; Wall-Palmer *et al.*, 2018).

Red Sea endemism appears to have multiple causes, with a cold, nutrient-rich barrier separating the Gulf of Aden from the rest of the Arabian Sea, and a narrow strait separating the Red Sea from the Gulf of Aden. Moreover, circulation patterns and environmental gradients may provide additional isolating barriers to dispersal (DiBattista *et al.*, 2016). We found indications of isolation for Red Sea and Gulf of Aden *Diacavolinia* from the Indian Ocean based on geometric morphometric information, because specimens from the Gulf of Aden ( $N = 3$ ) and entrance of the Red Sea ( $N = 8$ ) could not be unambiguously assigned to any of the groups, although our sample sizes are low. Because no genetic information is available, we could not infer whether the Red Sea has exported *Diacavolinia* biodiversity over time, or if it is an area of ongoing speciation and local endemism due to its peripheral position and unique oceanographic conditions. Fossil records have indicated that the latter is more likely for pelagic taxa, because of the loss of plankton diversity in the central Red Sea due to low sea level stands during Pleistocene glacials, and hypersaline conditions caused by almost complete isolation from the Indian Ocean (Fenton

*et al.*, 2000). Conversely, there is also evidence that some taxa increased in abundance due to freshwater dilution in the Gulf of Aqaba, such as observed in *Creseis* pteropods and siliceous diatoms (Reiss *et al.*, 1980; Fenton *et al.*, 2000; Almogi-Labin *et al.*, 2008). Furthermore, some Red Sea lineages are older than their respective sister lineages in the Indian Ocean, suggesting export of biodiversity from the Red Sea, such as for some reef fishes (DiBattista *et al.*, 2013). Peripheral speciation was observed for the spiny lobster *Panulirus penicillatus* with a nine-month larval stage, as well as for several reef fish taxa (Liu *et al.*, 2014; Fernandez-Silva *et al.*, 2015; Iacchei *et al.*, 2016).

The high number of *Diacavolinia* species in the Indo-West Pacific (IWP) reflects the high overall marine diversity in the area (e.g. Renema *et al.*, 2008; Becking *et al.*, 2016). We found that the most common taxa were distributed across a wide range in the coastal waters of the north-west Pacific, central Pacific and Indian Oceans. Present-day waters in the Indo-West Pacific are deep enough to enable diel vertical migration in epi- and upper mesopelagic zones. However, more genetic sampling in this area is needed to further resolve species boundaries, clarify the high genetic and presumably cryptic diversity within Group 5, and explore IWP endemism.

Across all oceans, we observe no obvious equatorial dispersal barriers separating the distributions of extant *Diacavolinia* species, but our Group 2 (no genetic information available) only occurs in subtropical Atlantic waters north of the equator. We also found no evidence for equatorial genetic breaks in other *Diacavolinia* groups. Equatorial dispersal barriers may be important drivers of pelagic evolution for other taxa, such as shown for subtropical Atlantic copepods (Goetze *et al.*, 2015, 2017) and *Cuvierina* (BurrIDGE *et al.*, 2015).

## CONCLUSIONS

Combining varied datasets in an integrative taxonomic framework may be suitable for a wide array of morphologically diverse marine taxa and is an important first step to predicting species-specific responses to climate change. Museum collections prove to be an invaluable resource for assessing species boundaries and biogeographic distributions, enabling comparison of modern findings with prior works based on the same specimens. We assessed species boundaries in *Diacavolinia* pteropods based on rigorous sampling over a period of 104 years and data collection across the Atlantic, Pacific and Indian Oceans, which would have never been possible within a single research project. Our results show that taxonomic revisions of *Diacavolinia* are needed, and will be reported on in subsequent work. However, not all species boundaries were resolved in this study and some species were only sampled once or not at all in recent years. New specimens could be added to

the current framework based on new species descriptions, standardized photographs and information on *COI* or 28S. It is also remarkable that a morphospecies from the Atlantic Ocean (Group 2, suggested species name: *D. atlantica*; Fig. 8) was abundant in museum samples but has not been encountered in recent years, despite multiple sampling expeditions in this region. Although it is common to uncover new species in zooplankton when integrative approaches are applied (e.g. Wall-Palmer *et al.*, 2018), in this study, we observed fewer genetic lineages than expected based on prior taxonomic descriptions of morphological species.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

The dataset supporting the results of this work is deposited at the Dryad Digital Repository (Accession code doi:10.5061/dryad.n8h2fv0). DNA sequences have been deposited at GenBank under the following accession numbers: MF974624–MF974847.

**Supplementary Information S1.** Sample collection information, group assignment using integrative approach, data usage for geometric morphometric analyses and GenBank accession codes for each *Diacavolinia* specimen used in this study. Amb. = Ambiguous LDA assignment; Unkn. = Unknown integrative group; N.A. = Not available.

**Supplementary Information S2.** Photographs of *Diacavolinia* specimens included in this study in (A) ventral and (B) lateral orientations.

**Supplementary Information S3.** Geometric morphometric data of *Diacavolinia* specimens: centroid sizes and relative warps in ventral (A: 23 and B: 38 landmarks) and lateral orientations (C: 15 and D: 49 landmarks).

**Supplementary Information S4.** Ordination of the second and third relative warps (RWs) of *Diacavolinia* for Indo-Pacific specimens for (A) lateral orientation with 49 landmarks (LMs;  $N = 282$ ), (B) ventral orientation with 23 LMs ( $N = 378$ ), and (C) ventral orientation of a subset with 38 LMs ( $N = 174$ ). See [Figure 4](#) for symbol definitions. Thin plate splines from the RW positions indicated with black squares are shown below and left of the axes to depict the variation in shell shape, with images of shells and LM positions shown on the right.

**Supplementary Information S5.** Alignments of (A) *COI* nucleotides ( $N = 89$ ; 658bp; GenBank accession numbers MF974762–MF974847) and (B) 28S ( $N = 138$ ; 901bp; GenBank accession numbers MF974624–MF974761).