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Integrative species delimitation and phylogeny of the branchiate worm *Branchiodrilus* (Clitellata, Naididae)

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Abstract

The clitellate branchiate genus *Branchiodrilus* presently includes three nominal species with a mainly tropical distribution. A recent molecular phylogeny of the subfamily Naidinae suggested that species complexes may occur within the genus. In order to delimit species, we studied a total of 91 *Branchiodrilus* specimens collected in Asia, Africa and Europe; the genus is introduced in the latter area. We used an integrative approach, where genetic data were analysed for 42 specimens (single-locus and multiple-locus methods) and then compared with patterns shown by morphology. Ten Molecular Operational Taxonomic Units could be identified within *Branchiodrilus*, potentially representing 10 different species. Most of the delimited species are genetically well separated. In contrast, morphological scrutiny identified only three non-overlapping clusters of specimens, one of them grouping all specimens from the Palaearctic region and belonging to the same species. Other morphological groups are mostly consistent with the biogeographic distribution of species. The Oriental region harbours six species and might be the centre of origin from which *Branchiodrilus* species have dispersed and radiated. Two other species are present in the Afrotropical region, among them *B. cleistochaeta* newly obtained from the type locality and genetically characterized, but the presence of *B. hortensis* in Africa is queried. The taxonomic relevance of the morphological criteria traditionally used to distinguish nominal species is useless at the species level. *B. hortensis* and *B. semperi* are now considered as *species inquirenda*. To document the genital organs of species remains highly desirable, although hardly practicable for this species complex with a primarily asexual reproductive mode.

KEYWORDS

aquatic Clitellata, *Branchiodrilus*, integrative taxonomy, species delimitation

1 | INTRODUCTION

Some groups of clitellate worms in the family Naididae possess various types of gill-like appendages (Timm, 2012), one of them is the genus *Branchiodrilus* Michaelsen, 1900 (subfamily Naidinae). This genus presently includes three nominal species with a mainly tropical distribution, and it is easily recognized on the branchial processes (gills) that enclose the dorsal chaetae of the anterior part of the body (Figure 1). The type species of the genus, *B. semperi* (Bourne, 1890), was described from Chennai (Madras), India, and is known from South and East Asia (Naidu, 2005; Nesemann et al., 2007; Ohtaka, 2018; Ohtaka, Sudarso, & Wulandari, 2006). *Branchiodrilus hortensis* (Stephenson, 1910) was originally described from Lahore, Pakistan, and is known from South and East Asia, as well from northern Australia and

north-eastern Africa (Brinkhurst, 1966, 1971, 1984; Green, Moghraby, & Ali, 1984; Naidu, 2005; Nesemann et al., 2007); it has also been introduced to some European countries (Šporka, 2009; van Haaren, 2005). The third species, *B. cleistochaeta* Dahl, 1957, is only known from west Africa (Brinkhurst, 1966; Dahl, 1957; Hrabě, 1966; Lauzanne, 1968). Theoretically, these three morphospecies can be differentiated by the distribution and length of the gills, and the location of hair chaetae with respect to gills (Brinkhurst & Jamieson, 1971). The species of *Branchiodrilus*, like most other Naidinae, primarily reproduce asexually with budding (paratomic fission followed by regeneration of segments at the fission zones) and therefore rarely develop genital organs, which contain the most useful characters for identification of other clitellate taxa. For this reason, naidine taxonomy and identification are mainly based on external and somatic, nonsexual characters (Harman, 1980).

Cryptic species, that is, species that are morphologically indistinguishable or so similar that they have been classified under the same species name (Bickford et al., 2007) are known in this subfamily (e.g., Envall, Gustavsson, & Erséus, 2012) and other clitellate groups (see Erséus & Gustafsson, 2009), as well as other animal groups (Pfenninger & Schwenk, 2007). Many clitellates have proved hard to distinguish without the aid of molecular markers, often due to the lack of externally discernible characters or to the limited taxonomic value of such when present. Thus, the species diversity has in many cases been shown to be underestimated if based on morphology alone (e.g., Gustafsson, Price, & Erséus, 2009; Kvist, Sarkar, & Erséus, 2010; Liu, Fend, Martinsson, & Erséus, 2017; Martinsson & Erséus, 2017a). In this paper, we use the term "cryptic species" in a somewhat relaxed way, meaning species that are morphologically so similar and therefore have been or are still classified as the same species; this does not necessarily mean that they are impossible to separate using traditional morphological techniques once they are delimited.

A commonly used molecular marker for recognition of species is the mitochondrial (mt) gene *Cytochrome C Subunit I* (COI), that is, today the standard DNA barcode for animals (Hebert, Cywinska, Ball, & deWaard, 2003). However, a single marker is often insufficient for an appropriate delimitation of species (e.g., Achurra & Erséus, 2013; Dasmahapatra, Elias, Hill, Hoffman, & Mallet, 2010; Dupuis, Roe, & Sperling, 2012; Martinsson, Achurra, Svensson, & Erséus, 2013; Martinsson, Rhodén, & Erséus, 2017), for which COI data need to be complemented with other information, for example, from nuclear markers and morphology. For species of Clitellata, in particular, two nuclear loci, the Internal Transcribed Spacer (ITS) region, consisting of the highly variable ITS1 and ITS2 and the more conservative 5.8S rRNA, and Histone 3 (H3), have been investigated in several studies (e.g., Achurra & Erséus, 2013; Martinsson et al., 2013; Martinsson et al., 2017; Matamoros, Rota, & Erséus, 2012). Different molecular methods for species delimitation have been suggested (see e.g., Fujita, Leaché, Burbrink, McGuire, & Moritz, 2012; Sites & Marshall, 2003), some of which are based on the multispecies coalescent model (Rannala & Yang, 2003). In this model, genes evolve within a species phylogeny where the branches are species and the properties of the branches restrict the gene trees. One such restriction is that the divergence times between species have to be more recent than the coalescent times for any genes shared between them (Rannala & Yang, 2003). When this

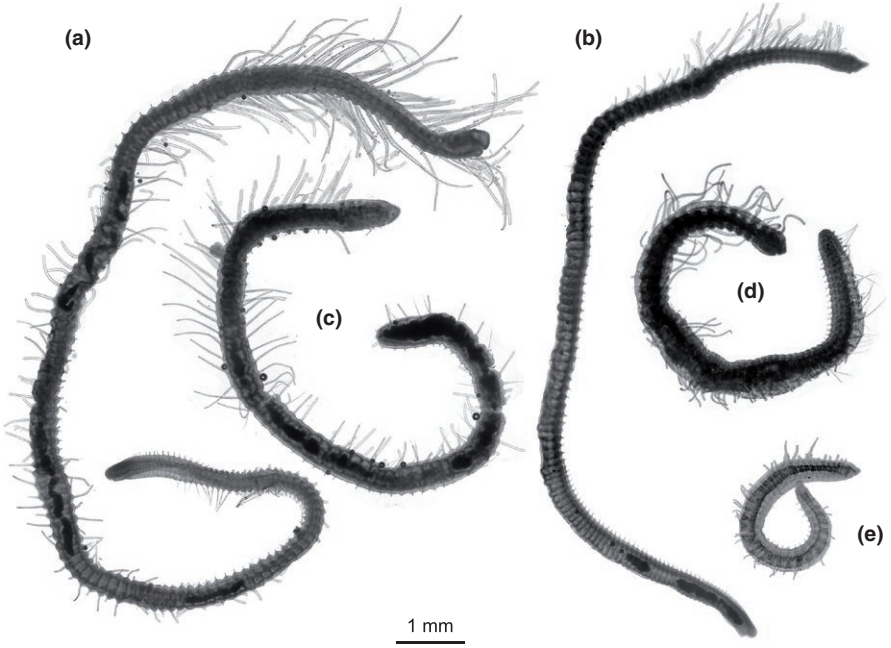

FIGURE 1 Habitus of *Branchiodrilus* specimens representative of different MOTUs identified in this study (see text and Supporting Information Table S1). A, IRSNB-16.336.01, Cambodia, M4; B, IRSNB-16.336.09, Cambodia, M6; C, IRSNB-16.336.07, Cambodia, M8; D, JW1606501, Cameroon, M2; E, IRSNB-15.065.03, China. The specimens were whole-mounted on slides with glycerine and photographed before being processed for genetic analyses, except the Chinese specimen (not sequenced although morphologically associated with M2; and whole-mounted in Canada balsam). Pictures sized to the same scale

model is used for statistical testing of species assignments, it is based on a clearly defined species concept in which a species constitutes a branch of a species tree, which is defined by abrupt speciation and no genetic exchange after the speciation event (Aydin, Marcussen, Ertekin, & Oxelman, 2014).

Species delimitations depend on the species concept used, as most concepts are both the definition of a species and the criteria by which a species is delimited. However, the unified species concept proposed by de Queiroz (2007) separates the species definition and the species delimitation. In this concept, a species is defined as a separately evolving metapopulation lineage, and other species concepts are used as secondary criteria to assess lineage separation. In this paper, we are using the unified species concept.

A recent molecular phylogeny of the subfamily Naidinae (Erséus, Envall, Wit, & Gustavsson, 2017) suggested that *B. hortensis* is actually a species complex, and the need to test the species boundaries within this genus using molecular data became apparent. A few years earlier, specimens tentatively identified as *B. hortensis* were sampled for the first time in southern France by one of us (JW). To track the exact identity and geographic origin of this invasive taxon, the material was DNA barcoded (see below) together with *Branchiodrilus* specimens from other parts of the world (Supporting Information Figure S1). The main aim of this study is to delimit species of *Branchiodrilus* using an integrative approach, where we analyse genetic data, using both single-locus and multilocus methods, and then comparing with the patterns shown by morphology. Secondary aims are (a) to assess the validity of morphological characters to discriminate between *Branchiodrilus* species and (b) to estimate the phylogeny of the genus.

2 | MATERIAL AND METHODS

A total of 91 specimens of *Branchiodrilus* were included in the study, collected at different localities in Europe, Asia and Africa, either by Jean Wuillot or collected and put at our disposal by other contributors (Supporting Information Table S1). Some of the specimens are of particular value as they were sampled from the type locality of *B. cleistochaeta*, in the river Nyong, at the height of the "Case du Nyong" as referred to by Dahl (1957) and described in Birket-Smith (1956); the ruins of the "Case du Nyong" had almost disappeared in the jungle but they could be found thanks to the obstinacy of one of us (JW). All specimens were processed without taking preliminary identification (based on existing descriptions) into account, to avoid a priori reliance on the taxonomical value of morphological characters previously used to discriminate *Branchiodrilus* species.

2.1 | Molecular analyses

In this study, we analysed COI (*cytochrome c oxidase subunit I*) barcodes in combination with another mitochondrial gene, 16S (*16S ribosomal DNA*), and the nuclear markers ITS1 (*Internal Transcribed Spacer 1*) and H3 (*Histone* 3).

2.1.1 | Specimens

Forty-two specimens of *Branchiodrilus* spp. were used for molecular analyses from the Oriental biogeographic region (Cambodia, India, Indonesia and Thailand), the Afrotropical region (Cameroon, Ghana and Senegal) and the Palaearctic region (South Korea, China), including two populations introduced in Europe (France and the Netherlands). Two species of *Dero* (*D. digitat*a and *D. furcata*) were used as outgroups. A few sequences in the dataset are from Erséus et al. (2017) (Supporting Information Table S1).

2.1.2 | DNA extraction and sequencing

DNA was extracted from a small part of the posterior or medial section of the animals using Qiagen's DNeasy Blood & Tissue Kit or Epicentre QuickExtract DNA Extraction Solution 1.0, following the manufacturer's instructions. Parts of the mitochondrial COI and, 16S, parts of the nuclear H3 and the complete ITS1 were amplified. Primers and programs for the PCRs are listed in Supporting Information Table S2, and the same primers were used for sequencing. PCR was carried out using Red Taq DNA Polymerase Master Mix (VWR, Haasrode, Belgium) in 25 µl reactions. After amplification by PCR, the existence of the target genes was tested using 1% agarose gel electrophoresis. PCR products were purified using exonuclease I (Fermentas, Burlington, Canada) and FastAP thermosensitive alkaline phosphatase (Fermentas). The sequencing was performed either by Macrogen (Geumcheon-Gu, Seoul, Korea) or by Eurofins MWG Operon (Ebersberg, Germany). Sequences were assembled and aligned in Geneious pro v. 7.1 (Biomatters Ltd., Auckland, New Zealand). Some of the specimens were length-variant heterozygotes for ITS1 and were phased using Champuru v1.0 (Flot, 2007; Flot, Tillier, Samadi, & Tillier, 2006). Alignments were performed using MAFFT v.7.017 (Katoh, Misawa, Kuma, & Miyata, 2002) as implemented in Geneious with the auto algorithm and default settings.

2.1.3 | ML Phylogeny

A phylogeny was estimated using maximum likelihood (ML) as implemented in PhyML v.3.0 (Guindon et al., 2010). The analysis was run on the South of France bioinformatics platform (https://www.atgc-montpellier.fr/phyml/) using a concatenated dataset consisting of all four markers, and

all 44 specimens (including the outgroups); for specimens with two ITS1 haplotype, one was discarded randomly, in all cases the two ITS1 haplotypes were similar to each other. The automatic Smart Model Selection (Lefort, Longueville, & Gascuel, 2017) was used with the Bayesian information criterion, and the model selected was TN93 +G. A random starting tree was used, and SPR was selected for tree improvement. The SH-like approximative likelihood ratio test (aLRT; Anisimova & Gascuel, 2006) and non-parametric bootstrap proportions (Felsenstein, 1985) were used for calculating branch support. The resulting tree was used to identify MOTUs (Molecular Operational Taxonomic Units; Blaxter et al., 2005; Vogler & Monaghan, 2007) that were further tested in a multilocus species delimitation analysis (see below).

2.1.4 | Multilocus species delimitation

The MOTUs, identified in the phylogenetic analysis described above, were used as input species in a combined Bayesian multilocus species delimitation and species tree estimation conducted with BPP v3.1. All four loci were included in the analysis. The population size parameters (θs) were assigned the gamma prior G(2, 200), with mean 2/200 = 0.01. The divergence time at the root of the species tree (τ0) was assigned the gamma prior G(2, 50), while the other divergence time parameters were assigned the Dirichlet prior (Yang & Rannala, 2010 equation 2). The analysis was run three times to confirm consistency between runs.

2.1.5 | Single-locus species delimitation

As a complement to the multilocus species delimitation described above, we also performed single-locus species delimitation on each of the four markers. This was done in ABGD (Automatic Barcode Gap Discovery; Puillandre, Lambert, Brouillet, & Achaz, 2012) using a relative gap width of 1, and simple distances. The distinctness of the delimited clusters from the ABGD analyses was tested using the species delimitation plug-in (Masters, Fan, & Ross, 2011) in Geneious pro v. 7.1. Both $P_{(\text{Randomly Distinct})}$, (Rodrigo et al., 2008) and *Rosenberg's* P_{AB} (Rosenberg, 2007) were calculated on the ML tree.

2.1.6 | Species tree estimation

We also performed a species tree estimation using the multispecies coalescent model as implemented in *BEAST. It was conducted in BEAST v1.8 (Drummond & Rambaut, 2007; Drummond, Suchard, Xie, & Rambaut, 2012); all 44 specimens were used and assigned to their respective species, as found by the molecular species identification by BPP (see Results), and all four loci were used. For species missing sequences of a marker, "dummy" sequences consisting of only "Ns" were added for that species. All other species had at least one sequence from each marker. All trees and clock models were unlinked. A HKY +Γ substitution model with empirical base frequencies was used for all markers, and exponential relaxed clocks were used, the root age was confined using a strong normal distributed prior with mean 1 and *SD* 0.01, combined with weak normal distributed priors for the clock rates with mean 0.1 for all genes. The Yule process species tree prior and piecewise linear and constant root population size model were used for all markers; the ploidy level of the mitochondrial markers was set to half of the nuclear ones, and UPGMA starting trees were used for all markers. The species popMean and Yule birthRate priors were set as exponential with mean 1, for all other priors the default values were used. The analysis was run twice for 100 million generations, sampling every 10,000th generation. The log files were checked in Tracer 1.6 (Rambaut, Suchard, Xie, & Drummond, 2014) to ensure convergence and good estimated sample sizes (ESS), to determine burn-in. Tree and log files were combined from the two runs using LogCombiner 1.8.2 discarding the first 50% as burn-in. Trees were summarized using TreeAnnotator 1.8.2, and the maximum clade credibility tree was then drawn using FigTree v. 1.4 (Rambaut, 2009).

2.1.7 | Distance analysis

Uncorrelated pairwise genetic distances were calculated for the COI dataset using MEGA 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Pairwise deletion was used for missing data. Histograms of the distances, divided into intra-, interspecific and intergeneric, were drawn using Microsoft Excel.

2.1.8 | Haplotype networks

To visualize the variation in each marker, haplotype networks were created in PopART v1 (Leigh & Bryant, 2015) using statistical parsimony (Clement, Snell, Walke, Posada, & Crandall, 2002; Templeton, Crandall, & Sing, 1992). Sites with missing data or gaps were masked and not included in the networks, and due to large amount of missing data, specimens CE1821 and CE1822 were excluded from the H3 network altogether.

2.2 | Morphological scrutiny

2.2.1 | Specimens

Among the 91 *Branchiodrilus* specimens included in this study, 83 were available for morphological scrutiny, while eight had already been used for DNA sequencing prior to this

study and, hence only the anterior part of the latter worms remained for morphology (vouchers CE16868, CE16869, SMNH-153643, SMNH-153644, SMNH-153646, CE1822, SMNH-153645, CE2213; Supporting Information Table S1). Most specimens were stained in alcoholic carmine, dehydrated, cleared in xylol and mounted in Canada balsam; some were mounted in polyvinyl lactophenol and sealed with "DPX Mountant." All specimens were subsequently examined under a compound microscope with differential interference contrast (DIC). All material studied, including the vouchers of the sequenced specimens, is deposited at the Swedish Museum of Natural History (SMNH), Stockholm (Sweden) and the Royal Belgian Institute of Natural Sciences (IRSNB), Brussels (Belgium).

2.2.2 | Characters and identification of morphotypes

An exhaustive list of morphological characters was first drawn up, to tentatively divide specimens into morphotypes. Characters were mainly related to chaetae (size—length and thickness, distribution, number per bundle; size of teeth in bifid chaetae—ventral chaetae and needles; ectal shape of chaetae—ventral chaetae, needles and hair chaetae), gills (size, distribution) and body (diameter, number of segments).

It soon appeared, from a superficial overview, that most characters were too variable, both intra-individual and/or intrapopulation, to have potential value to discriminate species. They were discarded from the analysis without any further consideration, to avoid the daunting task to document a character matrix of 2075 cells (25 characters × 83 specimens). Characters retained are listed below. Segment numbers are denoted by Roman numerals.

C1. Maximal length of gills in relation to body diameter at insertion point (anterior part of the body);
C2. Percentage of body length with non-gilled segments (anterior non-gilled segments omitted);
C3. Number of ventral chaetae in anterior segments devoid of gills and dorsal chaetae (number on one side of the body);
C4. Length of teeth in needles (median value—percentile 50, of 10 measurements made on needles counted from the posterior end—10 will not be reached when specimens have fewer than 10 observable needles);
C5. Thickness of ventral chaetae (from segments VI-XV; maximal thickness above the nodulus, evaluated by the percentile 90—that is, maximal value after removing the 10% highest values).

Characters were expressed as quantitative variables prior to analyses. Multivariate morphometrics were used as a tool for assessment of patterns of variation at the specific level (see Marhold, 2011). PCAs (Principal Component Analyses) were carried out following a twofold approach: (a) identification of the most differentiating characters and of potential morphotypes, and (b) search for morphological differences among groups identified through the genetic approach. Data were normalized prior to PCA analyses. Missing data were replaced by extrapolated values (mean value of observations of other individuals in the same group for the missing character). Data were also weighted so that the weight of a specimen is proportional to the number of characters for which an observation is available. Data processing and PCAs were carried out and illustrated with XLStat v.19.7 (Addinsoft) for Microsoft Excel.

3 | RESULTS

3.1 | Molecular analyses

We successfully sequenced 44 specimens (including the outgroups) for 16S, 42 specimens for COI, 36 specimens for H3 and 39 for ITS1. The length of the alignments after trimming was 333 base-pairs (bp) for 16S, 658 bp for COI, 320 bp for H3 and 514 bp for ITS1.

3.1.1 | ML phylogeny and MOTUs

The ML analysis resulted in a well-resolved tree, which enabled the identification of ten, in most cases highly supported, clades within *Branchiodrilus* (Figure 2a), and they are from now on referred to as numbered MOTUs 1–10 (= M1–M10; see Supporting Information Table S1, Figure 2a). The genus *Branchiodrilus* as a whole was found to be a monophyletic group with maximum support. M4, a clade consisting only of Cambodian specimens, is the sister group to all remaining *Branchiodrilus* MOTUs, but the latter with moderate support ($p = 0.83$, BV = 70%). The remaining *Branchiodrilus* specimens are separated into groups that are consistent with biogeographic regions (Balian, Segers, Lévêque, & Martens, 2008). One group brings together specimens from the Oriental biogeographic region (M3 and M9, Indonesia; M6 and M8, Cambodia; M7, Thailand; M10 India), with maximum support. This group is sister to a group, also maximally supported, divided between M5 on the one hand, including Palaearctic specimens (South Korea, China, France and the Netherlands), which are moderately supported ($p = 0.91$, and the Afrotropical *Branchiodrilus* on the other hand, maximally supported and consisting of M1 (Ghana and Senegal) and M2 (Cameroon) as sister groups; M1 and M2 are well to maximally supported.

3.1.2 | Multilocus species delimitation

The BPP analysis strongly supported all MOTUs. Ten of the input groups, including the two *Dero* species, had a mean

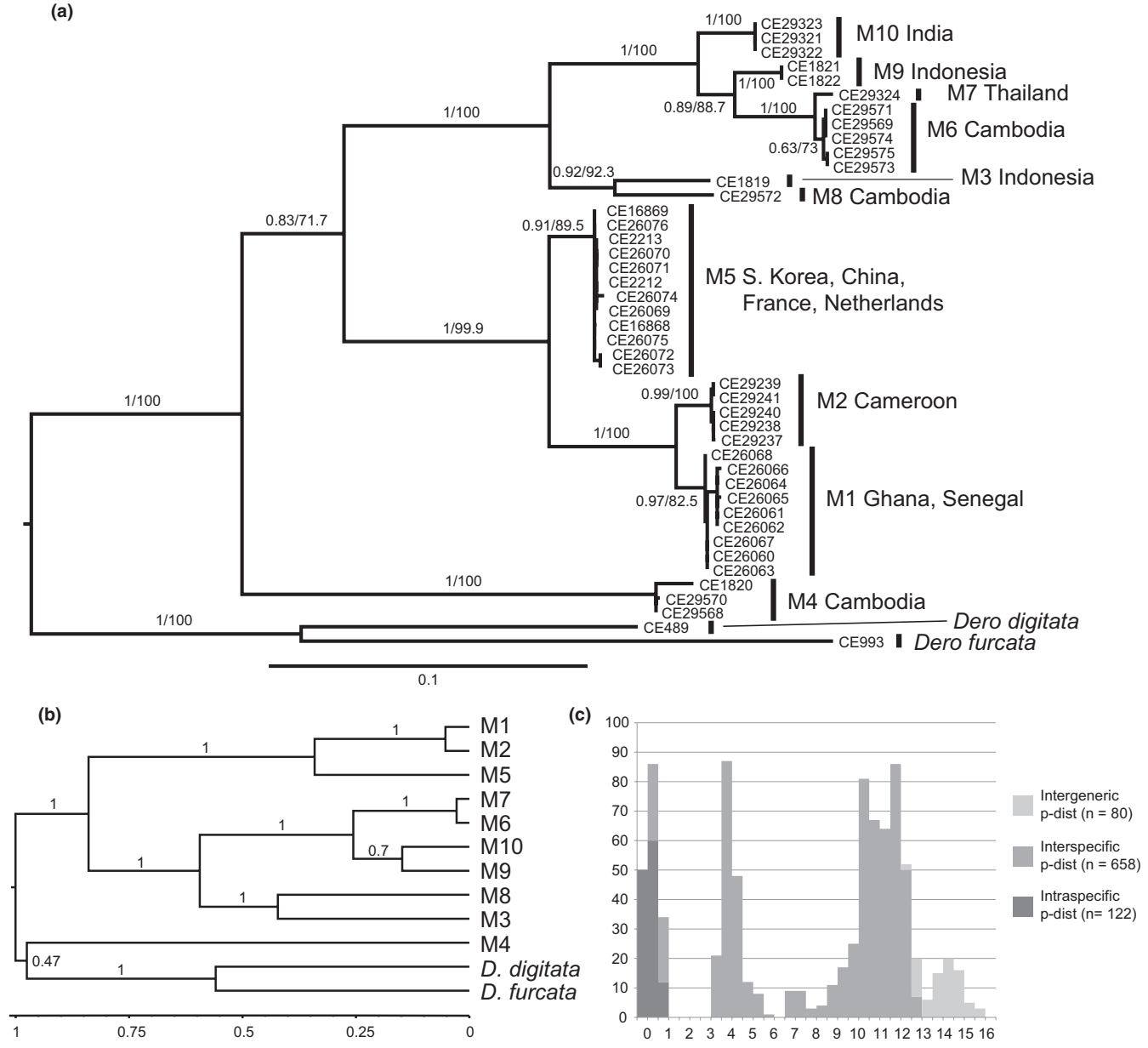

FIGURE 2 (a) Tree from maximum likelihood analysis performed in PhyML; concatenated dataset (COI, 16S, ITS1, H3); the MOTUs (Molecular Operational Taxonomic Units) of the *Branchiodrilus* part of the tree are denoted as M1-M10; values at branches are SH-like aLRT nodal support; scale bar represents expected number of substitutions per site. (b) Species tree from multispecies coalescence analysis performed in *BEAST and based on all four loci (COI, 16S, ITS1 and H3); the MOTUs (Molecular Operational Taxonomic Units) of the *Branchiodrilus* part of the tree are denoted as M1-M10; scale represents proportion of total tree height. (c) Histogram showing the distributions of uncorrected pairwise genetic distances in COI; note that intraspecific variation and interspecific variation are defined in accordance with the outcome of the BPP analysis

posterior probability (PP) >0.99, whereas the support for the last two input species, M6 and M7, was 0.987.

3.1.3 | Single-locus species delimitation

The ABGD analyses resulted in, including outgroups, 10 clusters in COI, five in 16S, seven in H3 and 10 in ITS1 (Supporting Information Table S3). In the four markers, the two outgroups form separate clusters, and M6 and M7 are found in the same cluster, as well as M1 and M2 in COI. In 16S, there is one large cluster containing M1-M2 and M5-M10, but M3 and M4 form separate clusters. In H3, M1 and M5 form a single cluster, as well as M3 and M8, and M6, M7 and M9. In ITS1, M4 is split into two clusters, and M9 and M10 form a single cluster. Of the two statistical tests, $P_{(RD)}$ could not be calculated for the majority of clusters and was not significant ($p < 0.05$) for any cluster whereas P_{AB} could be calculated for the majority of clusters and was significant in much higher degree (Supporting Information Table S3).

TABLE 1 Genetic distances, in per cent, given for within-group comparisons as maximum pairwise distance, between-groups comparisons are given as minimum pairwise distances. All distances are calculated as uncorrected pairwise distances

	M2	M1	M3	M4	M5	M10	M6	M7	M8	M9	*Dero digitata*	*Dero furcata*
M2	0.0											
M1	0.3	0.5										
M3	10.1	10.6	*n*/a									
M4	9.9	9.6	10.9	0.0								
M5	3.3	3.6	9.2	9.9	0.8							
M10	11.5	11.9	7.9	11.7	10.6	0.0						
M6	11.2	11.3	8.5	11.9	11.2	6.8	0.0					
M7	11.7	11.7	9.3	12.3	11.7	7.3	0.5	n/a				
M8	9.4	9.9	5.7	11.4	8.4	9.4	10.2	10.9	n/a			
M9	9.9	10.0	9.3	11.4	10.9	7.3	5.0	5.2	11.1	0.0		
Dero digitata	13.4	13.9	12.3	12.6	12.6	13.7	12.3	13.1	13.5	14.1	n/a	
Dero furcata	14.4	14.7	14.4	13.9	14.0	15.8	14.7	15.1	14.1	15.3	13.1	n/a

3.1.4 | Species tree estimation

The *BEAST analysis resulted in a well-supported maximum clade credibility tree (Figure 2b), with a similar topology as the ML analysis and with only two nodes not having maximum support. However, *Branchiodrilus* was not recovered as monophyletic, instead M4 was found as sister group of the two *Dero* species, although without support (PP = 0.47). The two *Dero* species form a clade with maximum support. The remaining *Branchiodrilus* MOTUs form a clade with maximum support. This clade is divided into two groups both with PP = 1, the first one consists of the two African MOTUs M1 and M2 as sisters, and the Palaearctic M5. In the second group, which only contains specimens with Oriental biogeographic distribution, there is a split between a clade consisting of M3 and M8, and the remaining MOTUs (M6-M7, M9-M10). M6 and M7 are sisters with maximum support, and M10 and M9 are sisters, but with low support (PP = 0.70).

3.1.5 | Distance analysis of COI dataset

When calculating genetic distances, species were defined according to the outcome of the BPP analysis (see above).

The maximal intraspecific distances varied between 0.0% in M2 (Cameroon), M4 (Cambodia), M10 (India), and M9 (Indonesia) and 0.8% in M5 (South Korea, France, the Netherlands and China). The minimal interspecific distances within *Branchiodrilus* varied between 0.3% between M2 (Cameroon) and M1 (Ghana, Senegal) and 12.3% between M4 (Cambodia) and M7 (Thailand; Table 1). There is thus no barcoding-gap between intra- and interspecific distances, but there is a gap in the distances between 0.8% and 3.3% (Figure 2c), and the distances within *Branchiodrilus* are generally smaller than those between species of *Dero* and *Branchiodrilus* (Table 1; Figure 2c).

3.1.6 | Haplotype networks

The haplotype networks (Figure 3a–d) show that the specimens of most MOTUs have unique haplotypes in all markers, not shared with other clusters. The only exceptions are, in the 16S network (Figure 3b), M2 (Cameroon) and M1 (Ghana, Senegal), and in the H3 network (Figure 3d), M6 (Cambodia) and M7 (Thailand). In the first case (16S), they differ in one substitution, which is masked as that site includes gaps in some other specimens. In the second case (H3), the only specimen of M7, CE29324, has two ambiguous sites, which could differentiate it from M6 (Cambodia), but more specimens are needed to resolve this.

3.2 | Morphology

A total of 280 morphological observations were done on a total of theoretically 415 observations (5 characters × 83 specimens), hence an overall percentage of 67%, with large disparities according to the character observed (see Supporting Information Table S4). A few specimens were only available as short fragments; as a result, character 1 (C1, maximal length of gills in relation to body diameter at insertion point in anterior part of the body) was observed on 94% of the specimens, and C2 (the percentage of body length with non-gilled segments) was only visible on <48% of specimens.

Of the five morphological characters selected, the principal component analysis suggested that C2 (% of body length without gilled segments), C4 (length of teeth in needles) and possibly C5 (thickness of ventral chaetae in VI–XV) may be the most

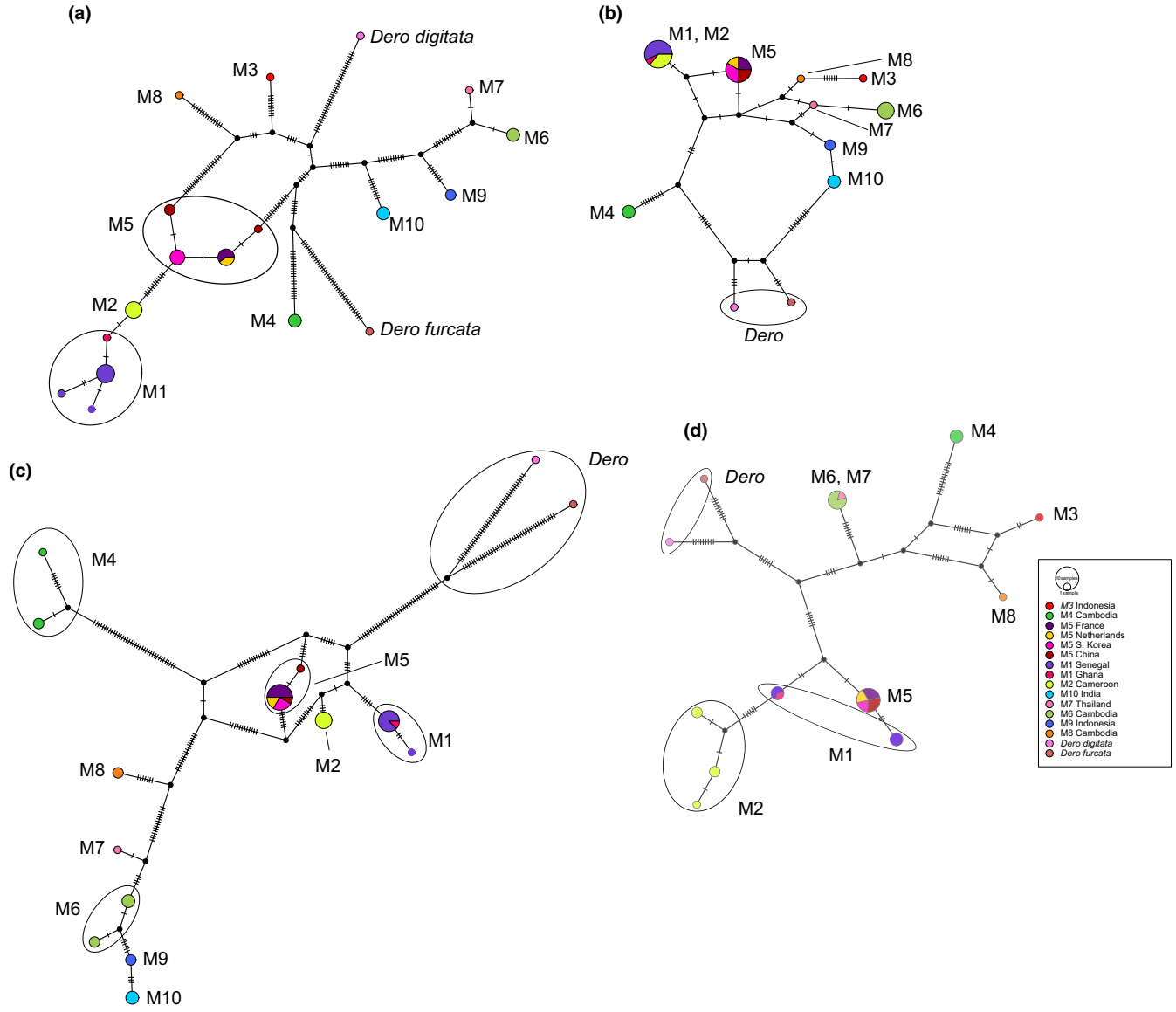

FIGURE 3 Statistical parsimony haplotype networks. The size of the circles is relative to the number of sequences sharing that haplotype, the colours correspond to the input species used in the species delimitation analysis, and the hatch marks correspond to the number of substitutions between haplotypes. (a) COI network; (b) 16S network; (c) ITS1 network; (d) H3 network

discriminant and, hence, have more taxonomical value (Figure 4). The cumulative variance of the two principal components (77%: F1—59%, F2—18%) clearly isolated two clusters along F1-axis: cluster A of individuals with high percentage of body length without gills (C2), and cluster B of specimens with bifid needles (C4) and with thick ventral chaetae in anterior segments (C5) (Figure 4). There is a clear gap of values in C2 between both clusters with minimal value of 21% in cluster A and maximal value of 14.7% in cluster B. As for C4, cluster A can be distinguished by the absence of teeth in needles (=simple-pointed), and cluster B has bifid needles, although there are some exceptions in both cases. Clusters A and B overlap between 3.5 and 4.5 μm for values of thickness of ventral chaetae (C5).

It should be noted that specimens, and the MOTUs M4, M6, M7, M8, M9 and M10 constituting cluster A come exclusively from the Oriental biogeographic region. All specimens and MOTUs of cluster B are of Palaearctic (M5) and Afrotropical (M1, M2) distribution with exceptions from Indonesia (JW33, JW34) and Cambodia (JW31, M4 = JW32, JW55), JW34 and M4 being even divided between the two clusters. Interestingly, M5 and all specimens of the Palaearctic region not available for molecular study are grouped in a consistent subcluster, B1 in Figure 4, fully discriminated when variables are combined, with the highest contribution of the F1-axis to this discrimination (Figure 4B2).

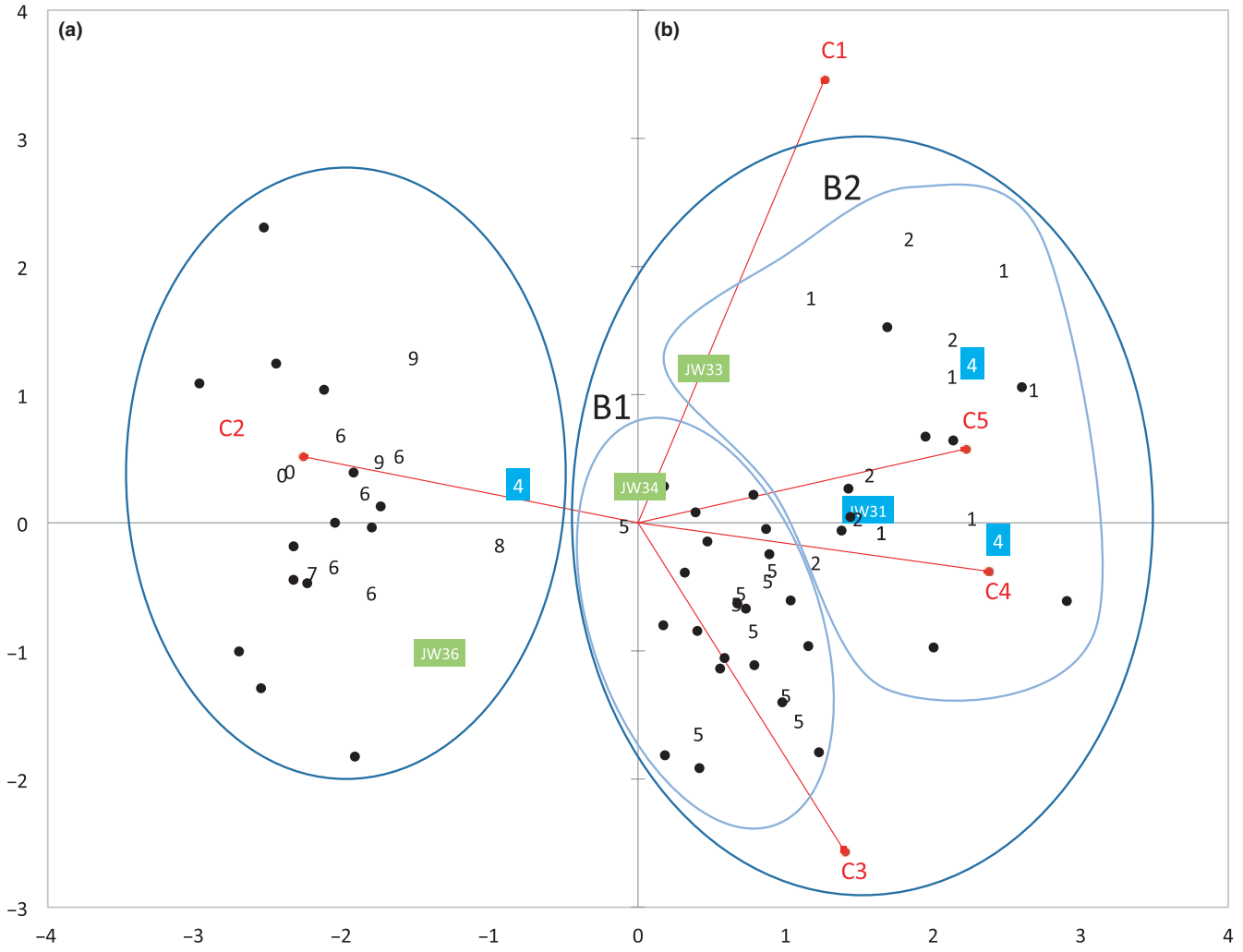

FIGURE 4 PCA-scatter diagram of the *Branchiodrilus* dataset based on five quantitative features. A and B (including B1 and B2) are morphological clusters identified as a result of the PCA (see text). Specimen groups are showed as black dots, with some exceptions when relevant, from Indonesia (green labels) and Cambodia (blue labels) discussed in the text. MOTUs identified via the ML analysis (Figure 2a) are abbreviated as 0 (M10) to 9 (M1–M9) (see Supporting Information Table S1). JW numbers refer to identifiers used for PCA (see Supporting Information, Table S2)

4 | DISCUSSION

4.1 | MOTUs as potentially separate *Branchiodrilus* species

According to the results of the multispecies coalescence species delimitation analysis in BPP, the ten MOTUs represent ten different species of *Branchiodrilus*. However, distance based on the single-locus delimitations gave between three and eight clusters depending on marker. One of the strengths of the multispecies coalescent model is that it is based on a well-defined species concept: that is, species are lineages that no longer exchange genes following divergence from a common ancestor (Toprak et al., 2016), a species concept also in line with the unified species concept by de Queiroz (2007). BPP analyses have been used for other clitellate groups too (e.g., Martinsson & Erséus, 2017a; Martinsson & Erséus, 2017b), but in this case we deal with a group that mainly reproduces asexually, and the samples are largely small and from geographically isolated populations. This increases the risk of splitting the dataset into too many species; it has been showed that BPP can be sensitive to population subdivision (McKay et al., 2013; O'Meara, 2010), and that it may be difficult to distinguish between population structure and species boundaries when the multispecies coalescent model is used (Sukumaran & Knowles, 2017). Most of the delimited MOTUs are genetically well separated (Figure 2a). M6 from Cambodia and M7 from Thailand, however, are very close together in all markers (Figure 3). Since they have been found in the same cluster in all single-locus species delimitations, we are not able to delimit these two MOTUs as separate species without more data. For the other MOTUs, the genetic differences are larger, the separation between them is clearer, and they are found as separate clusters in at least some of the

single-locus delimitations, with the possible exception of the genetic distances in COI between the two African groups M1 and M2 discussed below.

When using COI as a barcoding gene for species separation, a distinct global gap in the pairwise distances between individuals is expected to occur, and further, this gap is ideally reflecting a threshold value between intra- and interspecific variation (Hebert, Ratnasingham, & deWaard, 2003) In the COI dataset of *Branchiodrilus*, there is such a gap between 0.8% and 3.3% (Figure 2c). Two species pairs (each pair a sister group), however, that is, M6/M7 mentioned above, as well as the African species M1 and M2, both show COI distances ≤0.8% within the pair. Each of these two species pairs also show very limited haplotype variation in 16S (Figure 3b). M1 and M2 are separated with a single substitution, but in the haplotype network this is not seen as there are gaps in that position in other species, and the position is therefore masked and not included in the construction of the network. In the M1/M2 case, the two species are well separated by the nuclear markers: in ITS1, they form separate clusters in the single-locus delimitation analysis, and, in H3, M1 is found in the same cluster as M5 whereas M2 form a separate cluster. This strengthens the support for them being different species, and it is possible that the small distances in the mitochondrial markers are caused be introgression, followed by subsequent divergence. It is also worth noting that, in H3, there are two haplotypes, separated by M5.

4.2 | Morphology and nominal species of *Branchiodrilus*

Our morphological scrutiny identified only three non-overlapping clusters of specimens, whereas molecular analyses recognized several clades within the genus *Branchiodrilus*, supporting a much larger species diversity, although, as discussed above, the exact number of species may still be uncertain. Moreover, if some or all of the lineages here regarded as separately evolved are obligatorily asexual, the application of any kind of species concepts based on interbreeding and/or gene flow will involve subjectivity (Fitzhugh, 2009; Lin, Edwards, Kondo, Semple, & Cook, 2017).

Traditionally, the three morphospecies of *Branchiodrilus* are differentiated by the distribution and size of the gills, and the location of hair chaetae with respect to gills (Brinkhurst & Jamieson, 1971: 358). The low number of taxonomic utility criteria is probably due to the fact that sexually mature animals are only exceptionally found; in the literature, there is no mention of the sexual organs of *Branchiodrilus*, except by Mehra (1920) and Chen (1940), who gave a detailed description for specimens identified as *B. hortensis*, collected in Agra (northern India) and in China, respectively.

The main morphological criterion for distinguishing the two main clusters identified in the PCA analysis is the percentage of segments without gills, which isolates groups according to whether they have more than 20% of segments without gills (cluster "A") or less than 14.7% (cluster "B"; Figure 4). This criterion corresponds fairly well to the character traditionally used to distinguish *B. semperi*, with gills restricted to the anterior half of the body, from the other two nominal species, *B. hortensis* and *B. cleistochaeta*. However, in the present study, this feature is shared by at least five MOTUs, all potentially attributable to *B. semperi*.

The taxonomic relevance of the other two criteria traditionally used to distinguish *B. hortensis* from *B. cleistochaeta* can be invalidated even by rather superficial observations. We commonly found individuals with hair chaetae outside the gills, whatever the population studied in this work, as well as gills significantly differing in length along the body. We conclude that there is no longer any morphological character, mentioned in the literature, which allows the diagnosis of the three current nominal species; hence, it seems inevitable to consider *B. hortensis* and *B. semperi*, for which there is no new material coming from the type locality, as *species inquirendae*. *Branchiodrilus cleistochaeta* escapes this taxonomic decision because one of us has obtained material from the locality type, so that it is possible to provide diagnostic criteria for this species, if only genetic. This point will be discussed below.

Biogeographic distribution of specimens is a criterion that enables to recover some consistency in morphology-based groupings, but there are also exceptions. It appears that all specimens with a low percentage of segments without gills and with single-pointed needles are of Oriental origin, whereas, in contrast, most specimens, but not all, characterized by gills on more than 80% of segments and with bifid needles have a Palaearctic and Afrotropical distribution. Among the latter group, the Palearctic specimens are the only ones to form a distinct subgroup, bringing together all specimens from Japan, and including the single MOTU M5 (South Korea, China and European invasive forms; Figure 4B1). It is therefore reasonable to consider this subgroup as one and the same species. On the other hand, the diagnosis of this species on a morphological basis remains problematic because it uses a particular space of values for a combination of characters, which remains to be modelled and makes this approach inconvenient in practice. Regardless of this, a molecular identification of M5 is now straightforward.

4.3 | Peculiarities of specimens from the Oriental region

While the main morphological characters selected in this study do not allow apprehending the full diversity of species in *Branchiodrilus*, careful observation of some morphological details suggests that this diversity may still be higher than what MOTUs have revealed, especially for the Oriental

region. Indeed, the few exceptions to the biogeographic coherence mentioned above are all due to specimens from this region, whether or not they were sequenced (Figure 4). It is therefore interesting to examine in more detail in what way these specimens deviate from this biogeographic coherence. This implies considering not only the specimens that molecular analyses have grouped together but also the specimens from various museum collections, which it has not been possible to address by the molecular tools.

While the percentage of segments without gills is a quantitative trait easy to grasp, careful examination of the needles in the dorsal bundles reveals a more complex situation than what simply "absence vs. presence of teeth" seems to suggest. In reality, one can observe a succession of states between single-pointed needles and bifid needles (Figure 5).

Although most specimens observed have either distinctly single-pointed or distinctly bifid needles, others have needles of which the "simple-pointed" nature may be difficult to specify. Specimens JW31 (voucher A04, Cambodia) (Figure 5a) and JW33 (voucher A05, Indonesia; Figure 5b) have single-pointed needles with a slight furrow at its ectal end, but this could also be interpreted as bifid needles with closely contiguous teeth.

Similarly, some specimens, though essentially with single-pointed needles, also possess bifid needles. This situation is documented for specimen JW35 (voucher A07, Indonesia; Figure 5c,d) but is also observed on specimens from India (voucher IRSBN-16.287.01; Figure 5e,f). However, some elements are intriguing, which suggests that these bifid needles could be a mounting artefact. On the one hand, when present, these teeth are widely bifurcating and of variable size, in contrast to bifid needles seen on Palaearctic and Afrotropical specimens (Figure 5j–o for the latter). On the other hand, they are mostly seen among the needles of an Indian specimen mounted in polyvinyl lactophenol (JW72, voucher IRSNB 16.287.07; Figure 5g), while they are totally absent from all other specimens from the same locality, mounted in Canada balsam (with one exception, voucher IRSNB 16.287.01, JW66, M10). It would seem, in fact, that under certain conditions, the mounting medium will force the ends of the needles to burst, which could give rise to erroneous morphological interpretations.

Individuals belonging to the M4 clade are remarkable inasmuch as it was not possible to see needles in the dorsal bundles of any observed specimen. Their positioning in the factorial space of the ACP analysis therefore results from other variables, which can explain the distribution of the three individuals of this clade between the two clusters "A" and "B." Since only the anterior part of the animals could be examined, the posterior part having been used for the molecular analyses, the absence of needles must be taken with all the usual reservations. The observation of needles has always been very delicate, because of their smallness, their attachment to hair chaetae and their burial, most of the time, in the gills. It should also be noted that specimens of *Branchiodrilus* have particularly thick anterior bristles, with a 4–5 μm thick shaft. This combination of characters, absence of needles and thick anterior ventral chaetae clearly distinguishes M4 from all other MOTUs and confirms its status as a species from all the other studied here.

Finally, it should be noted that while the hair chaetae of a vast majority of *Branchiodrilus* specimens were smooth, there were three cases where plumose hairs were observed. Two are attributable to Oriental specimens; one is specimen JW34 (A06, Indonesia: G11; Figure 5q), the second involves all specimens from Malaysia (S0734-c, -e, -f; G14). Unfortunately, none of these specimens was genetically investigated. A third case was reported from a Cameroonian specimen (JW16.065.04, M2) where some pilosity was exceptionally observed on the ectal part of some hair chaetae, although most hairs were smooth elsewhere. Interestingly, all other Cameroonian specimens had smooth hair chaetae. As they all belong to M2, this implies that the variability of pilosity on hairs is intrapopulational, at least in this Afrotropical population.

4.4 | The Oriental region as a possible centre of origin for *Branchiodrilus* species

Careful examination of specimens from the Oriental region suggests that this region harbours a particularly large diversity of *Branchiodrilus* species, especially in Cambodia where there are no less than three (all suggested by the molecular study; M4, M6 and M8; see Supporting Information Figure S1), if not four species (if single-pointed needles with a slight ectal furrow observed on specimen A04/JW31 can be considered as a species attribute; Figures 3 and 5a,b). On the other hand, it is interesting to note that the phylogenetic analysis of *Branchiodrilus* places the SE Asian M4 clade at the basis of the tree, as a sister group of an assemblage of specimens from the Oriental, Palaearctic and Afrotropical regions. Based on these observations, high diversity and “ancientness” of these clades, it is tempting to consider the Oriental region as the centre of origin from which species have dispersed and radiated. Following this line of reasoning, this also suggests that the colonization of the Afrotropical region was via the Palearctic region, the Afrotropical clades being closer to the Palearctic clade than the Oriental clades, following a surface water network, rather than by a hypothetical exogenous transport over the ocean.

4.5 | Peculiarity of specimens from the Afrotropical region

Branchiodrilus cleistochaeta and *B. hortensis* are the only two morphospecies reported from the African continent at

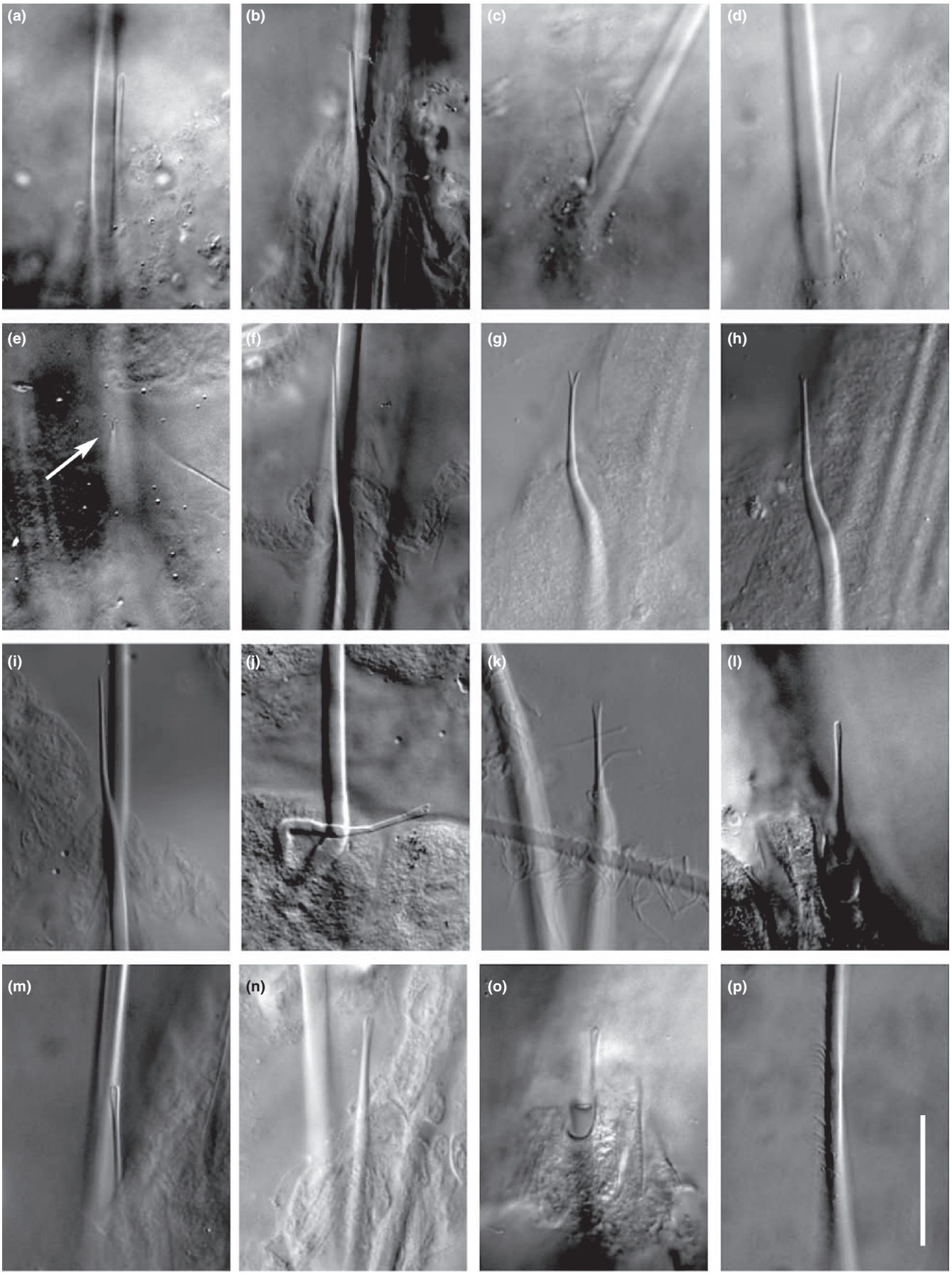
(a)
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FIGURE 5 Diversity of needles among *Branchiodrilus* specimens. (a, b) Simple-pointed with longitudinal furrow in ectal tip (A04, Cambodia); (c, d) bifid and simple-pointed (A07, Indonesia); (e, f) bifid and simple-pointed (IRSBN-16.287.01, India); (g, h) bifid (IRSNB-16.287.07, India); (i) simple-pointed (IRSNB-16.287.02, India); (j–p), bifid needle (j: IRSNB-15.063.06, the Netherlands; k: IRSNB-15.065.04, China; l: JW15.290.07, Japan; m: IRSNB-15.062.07, Ghana; n: *B. cleistochaeta*, holotype ZMUC-OLI-000040; o: JW16.065.03, Cameroon). (p) Plumose hair chaetae (A06, Indonesia). Scale bar = 20 μm

present. However, a brief review of the literature shows that there are questions still pending, upon which this study sheds some new light.

In his revision of the aquatic Oligochaeta of Africa, Brinkhurst (1966) ascribed *Branchiodrilus* specimens from Sudan to *B. hortensis*, apparently on the observation of free hairs in posterior segments, together with a decrease in gill length; although this taxonomic decision was not clearly justified, it can be deduced from characters used in the identification key provided by the author. However, free hairs in the posterior end of the body, as well as a decrease in the gill length, are also illustrated in the original illustration of *B. cleistochaeta* (Dahl, 1957). A few specimens with bifid needles, from Kariba (Zambia—Zimbabwe) and Volta (Ghana) lakes, were also observed and provisionally maintained within *B. hortensis*, noting that "these bifid chaetae may well have been overlooked in earlier work, as needles within the gills are difficult to see" (Brinkhurst, 1966: 137). This remark may be understood as questioning Dahl's observation of straight, simple-pointed needles, which was made on one damaged specimen from French Cameroon. During this study, we had the opportunity to re-examine the holotype of *B. cleistochaeta*, which confirmed that needles are indeed bifid, not simple-pointed (Figure 5), in accordance with observations made on new material sampled from the type locality and identified in the phylogenetic analyses as M2.

Hrabě (1966) gave an emended, detailed description of *B. cleistochaeta*, from specimens collected by a certain T. Petr in the Volta man-made lake. He considered the characters used by Brinkhurst to discriminate African *B. hortensis* from *B. cleistochaeta* as *B. cleistochaeta* specific, namely a decrease in gill length towards the posterior end of the body, and short hair chaetae protruding freely from the body-wall in posterior segments. Needles were also bifid. Interestingly, the material studied by Hrabě was probably the same as the one Brinkhurst saw too, as, in both cases, the Volta material was collected the same year and was acknowledged to the same collector's name, namely T. Petr. When describing *B. cleistochaeta* from Lake Chad, Lauzanne (1968) stated that hairs are enclosed in gills up to segment L (indirectly suggesting that they project freely in the hind end), and that gills are progressively reduced in length posteriorly.

However, our study has shown that it is not possible to distinguish between *B. hortensis* and *B. cleistochaeta* using the traditional taxonomic characters. In addition, phylogenetic analyses do not support a close relationship between Afrotropical and Oriental clades. Taking these observations into account, we believe there are strong reasons for considering the presence of the Pakistani *B. hortensis* in Africa as doubtful and resulting from an identification error.

Although the morphological criteria currently used are mostly unreliable, the Afrotropical origin of specimens should be sufficient to attribute them to *B. cleistochaeta*, in our current state of knowledge. However, molecular data also suggest that at least a second species is present in Ghana and Senegal, which is intriguing given the geographic distance between these two countries, compared to that which separates them from Cameroon.

Theoretically, it is possible to distinguish these two west African forms by the length of the teeth on needles; they are significantly shorter in *B. cleistochaeta* (holotype: 3 μm; M2: 2.9–3.4 μm) than in M1 (4.0–5.2 μm). There is also a clear difference in microhabitat between the Cameroonian, and Senegalese and Ghanaian specimens, the former being systematically found in the bottom litter of the stream, while the latter are only present among the stems of floating plants (e.g., *Ludwigia leptocarpa* [Nutt.] in Senegal). Other environmental differences can also be noted at the station level (Cameroon: small pools of the major bed of a stream of moderate width—100 m, with high slope—0.2%; Senegal and Ghana: side arms of large, gently sloping rivers—the Senegal River is about 350 m wide, with a slope of about 0.002% where sampled, channels or stagnant water), and at the biome level (Cameroon: rainforest without a real dry season; Senegal and Ghana: dry forest and savannah with marked dry season). Although, in the present state of knowledge, *B. cleistochaeta* is distinct from its Afrotropical congener, we cannot exclude that other, morphologically undistinguishable species are present in this biogeographic region, and perhaps even in close areas to the type locality of the species. For this reason, we recommend an integrative taxonomic approach in future biodiversity surveys, in which morphological and molecular data are combined.

5 | CONCLUSIONS

A century ago, Stephenson (1915) noted that the essentially asexual reproduction in "Naididae" (i.e., our subfamily Naidinae today; some species without any known sexual stages altogether) was a major impediment to naidid systematics, which otherwise benefits greatly from being based on the description of genital organs. This statement echoed Piguet's (1906) observation of the usefulness of the genital

organs in separating *Nais communis* Piguet, 1906 from its congener, when the latter author stated: "ce sont surtout ses organes génitaux qui la séparent très nettement des autres *Nais*" (these are mostly its genital organs that neatly separate it from other *Nais*).

A few years ago, Envall et al. (2012) illustrated the relevance of these observations when they showed the existence of an important cryptic diversity within *Nais variabilis/communis*, a species complex with a primarily asexual reproductive mode, leading to their morphology-based identification mostly relying on chaetal characters.

Given this context, our work on the genus *Branchiodrilus* is just another illustration of the relevance of Stephenson' statement. The morphological scrutiny of *Branchiodrilus* species offers few characters operational for their identification. Other characters than the ones examined in this study may be useful on occasion, but we fear that an identification based on morphology will be reduced to the search for small details, whose diagnostic value will be difficult to evaluate. It is also possible that the different species can only be morphologically apprehended in statistical terms, because of the overlap of certain character values, so that their identification could only be expressed in terms of probability.

To document the genital organs of species included in this study remains highly desirable. This requires samples of new material collected during the best period for reproduction, which seems to be when pools dry up and the weather gets colder, for example in northern India (Mehra, 1920). Until this is done, it is likely that the systematics of *Branchiodrilus* will remain as is, and that molecular approaches, including DNA barcoding, will be the main way to document *Branchiodrilus* diversity.

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SUPPORTING INFORMATION

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