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Cryptic diversity, intraspecific phenetic plasticity and recent geographic translocations in *Branchiomma* (Sabellidae, Annelida)

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The importance of identifying biological diversity accurately and efficiently is becoming more evident. It is therefore critical to determine the species boundaries between closely related taxa and to establish diagnostic characters that allow us to define species. This is not an easy task when species exhibit high intraspecific phenotypic plasticity or when distinct evolutionary lineages with an unusually large amount of genetic distinctiveness show no apparent morphological diversity (cryptic species). These phenomena appear to be common in the genus of fan worms *Branchiomma* (Sabellidae, Annelida) and consequently taxonomic errors are widespread in the group. Moreover, some *Branchiomma* species have been unintentionally translocated outside the area where natural range extension is expected, increasing the taxonomic problems. We have performed a range of analytical methods including genetic distances, Bayesian Interference, maximum likelihood, maximum parsimony, statistical parsimony analyses and general mixed Yule coalescent model to clarify the taxonomic status and asses the species boundaries of *Branchiomma* in Australia. This study shows that the traditional diagnostic morphological features are greatly homoplastic. Results also indicate that the diversity of *Branchiomma* in Australia is

higher than previously reported and evidence some cases of high phenetic plasticity (in features previously considered as stable within species), high intraspecific genetic variability, cryptic species and several unexpected cases of translocations.

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Introduction

The genus *Branchiomma* is a homogeneous group of fan worms (Sabellidae, Annelida) characterised by the presence of paired compound eyes and stylodes alternating along the radioles (Figs 1A-B, 2). The stylodes are epithelial appendages directed outwards of the radiolar crown, unique amongst members of Sabellidae and their shape and relative length between the radioles (Fig. 1A-G) is considered to be one of the main specific diagnostic features (Knight-Jones, 1994; Tovar-Hernández & Knight-Jones, 2006). There are currently around 30 nominal species of *Branchiomma* (Tovar- Hernández & Dean, 2010) described from a variety of shallow water environments, ranging from fine sediments to hard substrates and from many localities around the world. Some of these nominal species are well defined morphologically with precise data about their reproductive strategies, ecology and geographic distribution being available (e.g. Sordino & Gambi, 1992; Licciano et al., 2002; Tovar-Hernández & Knight-Jones, 2006; Licciano & Giangrande, 2008; Tovar-Hernández et al., 2009 a, b, 2011). But species of *Branchiomma* have been defined by unique combinations of features, rather than unequivocal synapomorphies, and some of these are often quantitative morphological characters (e.g. the relative length of the stylodes, the number of rows of teeth on the uncini, the length of the dorsal lips and radiolar appendages, etc.) that have not been critically analysed. Some of these attributes overlap between putative species and have shown intraspecific variability (not only related to ontogenetic and developmental changes).

In Australia, two species of *Branchiomma* had been reported to date: *B. galei* (Augener, 1914) and *B. punctulatum* (Haswell, 1885), but revision of Australian material housed in museum collections, based on morphological features, suggested these two species were not clearly defined and that a considerably higher diversity could be present. Some of the specimens examined fitted well descriptions of species originally reported from distant geographic areas, suggesting a much broader geographic range than previously encountered, whereas others presented a combination of features that did not match any previously described species. We also found a variety of forms within several samples and

collection sites that could be either attributed to a high intraspecific phenetic plasticity or to the co-existence of several sympatric species. In summary, evidence indicated that morphology-based species delimitation of members of this genus misrepresent its diversity in Australia, and also probably in other locations, and hence specific morphological diagnostic features need to be re-examined and quantified.

Delineation of species within polychaetes from DNA sequences and molecular phylogenies are not scarce, most aiming to resolve the status of species complex or broadly distributed species (Westheide & Hass-Cordes, 2001; Westheide & Schmidt, 2003; Jolly et al., 2006; Iannotta et al., 2007; Barroso et al., 2010; Capa et al., 2010; Nygren & Pleijel, 2010; Canales-Aguirre et al., 2011; Nygren et al., 2011). However, due to the differences between gene trees and species trees (e.g. Pamilo & Nei, 1988; Maddison, 1997) several authors have advocated using multilocus gene trees or an integrative approach, combining or comparing several sources of information (morphological, molecular, ecological, physiological, behavioural, etc.) to define species boundaries (Sites & Marshall, 2004; Pardial & de la Riva, 2010; Schlick-Steiner et al., 2010). An accurate delimitation of species is crucial from a taxonomic and systematic perspective, particularly for their stability, but also from a biodiversity, conservation, policy-making and ecological point of view (e.g. Cracraft, 2002; Agapow et al., 2004; Bickford et al., 2006; Vogler et al., 2008; Richards et al., 2009).

Methodological advances have also occurred rapidly in the last decade accompanying theoretical issues related to species concepts, biological properties of the taxa analyzed and sources of data investigated (summarized in Sites & Marshall, 2003, 2004). Several recent studies used intra- and inter-genetic distances or developed algorithms based on diversification branching patterns and coalescence models to test species boundaries by optimizing particular thresholds of the sampling methods, and hence offering testable hypotheses of species (e.g. Wiens & Penkrot, 2002; Morando et al., 2003; Hendrixson & Bond, 2005; Pons et al., 2006; Knowles & Carstens, 2007; Cummings et al., 2008; O'Meara, 2010; Yang & Rannala, 2010; Fujita et al., 2012). Those approaches despite their

theoretical and practical limitations were generally a fast method to assess biodiversity and to establish preliminary hypotheses of species, especially in poorly known or complex taxonomic groups, which later can be cross-examined in the light of morphological, ecological, behavioral, and other data available.

The aim of this study was to clarify the taxonomic status of *Branchiomma* in Australia by building molecular phylogenies, using genetic divergences and implementing two analytical methodologies for delimiting species boundaries by means of DNA sequences: statistical parsimony analyses (Tempelton et al., 1992) and delineation of branching threshold between coalescence and pure birth diversification models (general mixed Yule coalescent, GMYC; Pons et al., 2006). Evolution of morphological features and evaluation of the traditional specific morphological diagnostic features was also assessed.

Materials and methods

Collecting and preservation of material

Branchiomma specimens were collected from several localities in Australia and from around the world (Table 1, Fig. 3) between 2004 and 2010, trying to include in the analyses at least five representatives of each morphotype and geographic locality (a total of 110 *Branchiomma* specimens). We initially recognised three species from the Mediterranean, one from the Caribbean and we were able to group the Australian material into three morphological entities but with an unclear identity (not assignable to a current nominal species). Moreover, some specimens could not be allocated to any of the morphological groups because of intermediate morphological attributes. The outgroup selected was represented by members of *Pseudobranchiomma* (referred to as sp. A and B) and *Sabellastarte australiensis* (after Capa et al., 2010).

Live specimens were collected by SCUBA diving and were preserved directly in 90% ethanol. Some parapodia were mounted in glycerine and for observation of chaetae under

the microscope. Photographs were taken with a Leica MZ16 microscope and Spot flex 15.2 camera attached. Some specimens were completely dehydrated in ethanol, critical point dried, and covered with 20 nm of gold and examined under a Leo 435VP scanning electron microscope (SEM) in the Australian Museum (AM), using an Everhart-Thornley secondary electron detector. Tissue removed from the posterior end of specimens (without epithelium or gut) was used for DNA extraction, leaving the anterior ends intact as vouchers deposited in the AM collection.

Morphological features

The selected morphological features cover most of the general morphological diversity found within the group. These characters have traditionally been considered as diagnostic and used for grouping species in partial revisions of the genus (Knight-Jones, 1994; Nogueira et al., 2006; Tovar-Hernández & Knight-Jones, 2006). Other features observed to show variation within the sample, such as the overall pigmentation pattern or size of the spots over the body have also been included. A list of 19 characters and states are described in Table 2. The matrix (Sup. Mat.) was constructed in NDE, Nexus Data Editor (Page, 2001). The 'C-method' proposed by Pleijel (1995) was used for character scoring. The codification scheme included absent/present characters and unordered multistate characters. Taxa lacking the feature were scored as inapplicable and indicated as a gap '-' and unknown as a question mark'?'

DNA extraction, PCR amplification and DNA Sequencing

Genomic DNA was extracted from the sample tissue using standard protocols for the DNeasy Blood and Tissues Kit (QIAGEN Pty Ltd). Of the original 110 specimens, successful PCR product was obtained from 69 specimens plus three outgroups after several trials and changing conditions, 46 from Australian localities. Sequences from individuals from Port Philip Bay, Victoria or *Branchiomma nigromaculata* (Baird, 1865) from the Caribbean, among others, were not available and therefore these populations/species are not

represented in the present study. We sequenced 310-432 bp of the mitochondrial gene cytochrome b (*cob*) from 57 individuals, and 232-572 bp of the nuclear gene ribosomal internal transcribed spacer 1 (ITS1) with flanking regions of 18S rDNA and 5.8S rDNA from 58 specimens. We used the oligonucleotides Cytb 424F (RT-1) and *cob*r825 (Burnette et al,. 2005) for *cob*, and ITSF (Chen et al., 2002) and ITSR1 5' GCAATTCACATTAATTCAC 3' (designed for the present study). PCR mixtures contained 1x QIAGEN PCR buffer, 0.5x Q Solution, 3.5mM MgCl2, 0.05mM of each dNTP, 10 pmol of each primer, 1 unit of QIAGEN Taq DNA polymerase, 50-100ng of whole genomic DNA, and up to 25 µl with ddH_2O. Amplifications were performed on a MastercyclerS Gradient (Eppendorf Inc). The PCR thermal cycling profile was 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 50°C for 40 s, 72°C for 1 min and 5 min of final extension at 72°C. Successful amplifications were then purified using the ExoSAP-IT PCR purification system (USB Corporation), and then bi-directionally sequenced, using the original PCR primers, at an external sequencing facility using BigDye v1.1 (Applied Biosystems). Chromatograms were annotated with the program Sequencher v. 5.1 (Gene Codes Corporation). ITS1 sequence chromatograms showed no evidences of doubles peaks suggesting the presence of multiple copies.

DNA Sequence and Phylogenetic Analyses

Nucleotide sequences of *cob* were aligned with MAFFT v. 6.0 (Katoh, 2008) using default parameters, and ITS1 sequences using the Q-INS-i algorithm that takes into account secondary structures. Poorly aligned positions from divergent regions of ITS1 were removed using Gblocks v. 0.91b with relaxed parameters to assess the impact of ambiguously aligned regions on phylogenetic signal (Talavera & Castresana, 2007). The best nucleotide substitution model fitting each marker or partition was estimated in MrAIC (Nylander 2004) based on Bayesian Information Criterion (BIC). Best partitioning scheme was selected based on BIC using the Maximun Likelihood (ML) values estimated in RaxML 7.2.8 (Stamatakis, 2006). Two partitioning schemes were tested for *cob* sequences (as a single unit, and as first plus second codon positions vs third ones) whereas three

divisions were compared in the combined analyses (as unique partition, as two independent marker *cob* vs ITS1, and finally as three partitions, first and second codon sites of *cob* vs third ones vs ITS1). We did not asses *cob* sequences as three codon partitions since substitutions on second codon sites were extremely rare and hence model parameters would be very difficult to estimate accurately.

Maximum Parsimony (MP) heuristic search (with equally weighted characters and gaps as fifth character state) used 5,000 replicates of random taxon addition with tree bisection-reconnection (TBR) branch swapping algorithm, saving 100 trees per replicate, and hitting the most parsimonious trees 20 times using TNT v. 1.1 (Goloboff et al., 2008). Nodal support was estimated by 1,000 jackknife (JK) replicates using TBR (Goloboff et al., 2008). Results of heuristic searches and implementing new technologies alone (ratchet, drift and tree fusing), or combined were compared. Tree metrics are abbreviated as follows: tree length (TL), consistency index excluding parsimony non-informative characters in the data matrix (CI), and retention index (RI). Morphological features were traced in the combined morphological and DNA topology with Winclada v. 1.00.08, (Nixon, 2002) using same settings as for the combined dataset TNT analyses.

Best ML tree was obtained in RAxML v. 7.2.8 (Stamatakis, 2006) by optimizing the best parsimony tree out of 1,000 random searches and bootstrap values by summarizing tree topologies from 1,000 non-parametric replicates. Bayesian phylogenetic analyses were estimated in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001) by combining two independent runs of ten million generations with three heated and one cold chain, starting from default prior values and random trees. All parameters were unlinked and rates were allowed to vary freely over partitions. Burn-in and parameter/run convergence were assessed using Tracer v. 1.5 (Rambaut & Drummond, 2007), aiming at an effective sample size greater than 200. After discarding the initial 25% of trees as burn-in, trees from the stationary phase of both runs were combined to obtain a majority rule tree and *a posteriori* node probabilities (Huelsenbeck & Ronquist, 2001). Conflicting signals across *cob* and ITS1 was assessed implementing Partition Bremer Support (PBS) analyses in Treerot v. 3.0

(Sorenson & Franzosa, 2007) and PAUP v. 4b10 (Swofford, 2002) by performing 200 random parsimony tree searches on the tree topology obtained in the Bayesian analyses of the combined dataset.

Nucleotide divergence (*p*-distance) over sequence pairs within and between lineages was estimated in MEGA v. 5.1 (Tamura et al., 2011). All positions containing gaps and missing data were eliminated.

Two methods of sequence-based species delimitation were applied: identification of independent networks using statistical parsimony (Templeton et al., 1992) and analysis of branch pattern dynamics to detect independently evolving entities (i.e. putative species) using the Generalized Mixed Yule Coalescent model (GMYC; Pons et al., 2006). Statistical parsimony of haplotype networks were performed in TCS v. 1.2.1 (Clement et al., 2000) analysing *cob* and ITS1 aligned sequences independently, calculating 95% connection limit and treating IUPAC ambiguity codes as missing data. The GMYC model (Pons et al,. 2006; Fontaneto et al., 2007 , Monaghan et al,. 2009) optimizes using ML criterion and Likelihood Ratio Test (LRT) the shift in branching patterns of an ultrametric tree from a neutral coalescent model (intraspecific-population branching and null hypothesis) to a simple birth (Yule) model of diversification (interspecific diversification branching). GMYC analyses were performed using the R package SPLITS (SPecies' LImits by Threshold Statistics), which allows single or multiple thresholds (http://r- forge.r-project.org/projects/splits/). Identical haplotypes were removed before making tree clock-like using the perl script uniqHaplo.pl (http://raven.iab.alaska.edu/~ntakebay/teaching/programming/perl-scripts/perl-scripts.html). Ultrametric trees for each marker were estimated in BEAST v. 1.7.2 (Drummond & Rambaut, 2007) using the best model and partition scheme implemented in Mrbayes and by enforcing a relaxed global clock with an uncorrelated log-normal distribution, a coalescent diversification model with constant population size, and arbitrarily setting the basal node of the tree to 100 My. Strict clock was rejected by Bayes Factors estimated on Tracer v. 1.5 (LnBayesfactor -24.901 and -13.365 for *cob* and ITS1, respectively). Two independent

BEAST analyses were run for 100 million generations, sampling every 1,000 generations. Parameter convergence was assessed in Tracer v. 1.5 and clock-like branch lengths annotated in Treeannotator v. 1.7.2 (Drummond & Rambaut, 2007) after discarding the first 10 million generations.

Results

Sequence analyses, phylogeny and species delimitation

The best partition scheme for the mitochondrial *cob* was treating third codon sites as separate partition from first and second codon positions, and for the combined analysis as three divisions, first and second codon sites vs third codon positions vs ITS1. The best evolutionary model for first plus second codon positions of *cob* was HKY+I+Γ, for third ones was GTR+Γ, and for the nuclear ITS1 was HKY+ Γ. The removal of poorly aligned positions from divergent regions of ITS1 from the alignments with Gblocks v. 0.91b did not affect the phylogenetic results, neither tree topology nor node supports (not shown), hence all further analyses were conducted with the complete sequence of ITS1.

Bayesian and ML analyses of combined *cob* and ITS1 fragments provided similar tree topologies, with eleven highly supported lineages (PP=1) separated by long internal branches. Two lineages (BR_004 and BR_026) were composed by a single divergent individual only (Fig. 4). These clades were also outlined after analysing the DNA fragments separately (Sup. Mat.). Although the relationships among these clades varied depending on the data analysed and the methodology used, the topologies were congruent. Topological differences involved low supported internal nodes due to the lack of phylogenetic signal particularly for the mitochondrial *cob*. Partition Bremer support also revealed the absence of conflicting signal at both basal and deep nodes across mitochondrial (*cob*) and nuclear (ITS1) genes (Fig. 4).

The *cob* alignment of 57 sequences had 435 sites, 217 parsimony-informative. The ITS1 alignment included 58 sequences with a total of 661 sites, 352 parsimony-informative. Combined MP analysis of mitochondrial (*cob*) and nuclear (ITS1) sequences (72 taxa) yielded 10,430 equally parsimonious trees with 1652 steps (CI 0.58, RI 0.89). Individual gene analyses produced 68 equally parsimonious trees with 921 steps (CI 0.46, RI 0.86, uninformative characters excluded) for *cob*, and 5 with 811 steps (CI 0.75, RI 0.94, uninformative characters excluded) for ITS1, which topologies although not fully resolved were congruent (Sup. Mat.). Strict consensus was congruent with probabilistic methods topologies and the eleven well supported main clades were also recovered (Fig. 4). The main disparity among all topologies resulted from phylogenetic analyses regarding the relationships between clades. *Branchiomma galei*, for example, is recovered as sister to sp. G in the probabilistic methods and at the base of a clade together with *B. bairdi*, sp. C, sp. D and sp. G. and according to MP analyses (Figs 4, 5).

Statistical parsimony haplotype networks based on a maximum connection limit of 95% separated the 57 *Branchiomma cob* sequences into 13 groups, and the 58 ITS1 sequences into 11 groups. Results of the mitochondrial and nuclear data were mostly congruent except in three cases: i) the mitochondrial fragment split *B. galei* in two clusters whereas these specimens were considered as a single network for the nuclear fragment; ii) the reverse arrangement is found in sp. D since it is a single network after analyses of the *cob* fragment but split into two for ITS1 sequences; iii) finally, a more complex pattern is found in sp. G where the two networks obtained for *cob* fragments, equivalent to the two main clades, were merged in a single network for ITS1 sequences except for individuals BR_030 and BR_28 that belong to separated networks (Fig. 4).

The GMYC analyses using BEAST trees indicated that a branching pattern with single threshold between Yule and coalescent models was statistically better than a simple coalescent model for both makers (LRT 13.203 p=0.004 for *cob*, and LRT 9.758 p=0.044 for ITS1). Multiple thresholds model had lower statistical fitness than a single shift model for both markers and three degrees of freedom (Chi.sq 0.228 p=0.973 for *cob* and Chi.sq

1.228 p=0.746 for ITS1). The exclusion of the outgroups neither affected the number nor composition of the GMYC entities. Those analyses resulted in the identification of 13 putative *Branchiomma* species for *cob* sequences and 11 for ITS1 (Fig. 4). Differences regarding the marker considered are similar to those found after the statistical parsimony analyses, with the exception of the delineation of entities of the nuclear fragment in *Branchiomma* sp. G (Fig. 4).

Intraspecific and interspecific sequence divergence, calculated as uncorrected *p*-distance, was measured for each DNA fragment and for each group of specimens estimated according to three different criteria: morphospecies, haplotype networks and GMYC species (as in Fig. 4). Considering the initial nine morphospecies, the intraspecific sequence divergence was 20.3-0% for *cob* and 15.3-0% for ITS, while the interspecific distances range between 39.3-4.5% for *cob* and 34.3-0.9% for ITS. The networks obtained after statistical parsimony analysis gathered an intraspecific genetic divergence of 1.4-0% for *cob* and 1.1-0% for ITS1, and an interspecific divergence of 40.7-5.6% for *cob* and 35.9-2.1% for ITS. The entities recovered after GMCY were identical to the haplotype networks for *cob* sequences and hence their genetic distances but not for ITS1 data. For the latter, the intraspecific sequence divergence ranges from 3.0-0% and the interspecific from 35.9-0.5%.

Combined analyses, evolution of morphological features and diagnostic characters

Combined maximum parsimony analysis of morphological and molecular datasets yielded 10,430 equally parsimonious trees with 1754 steps (CI 0.56, RI 0.89, after excluding uninformative characters) (Fig. 5). Study of the morphological characters showed that features with the exception of those defining *Branchiomma* (characters 16-19), the ingroup, are homoplasious (Sup. Mat.). Some of the species are defined by a unique combination of them (Fig. 5). For example *B. bombyx* is characterised by long dorsal lips and body covered in spots; *B. luctuosum* has long necked-uncini and thoracic ventral shields separated from tori; sp. A, lacks spots on body surface, has a short thorax with 4-5 segments and

inconspicuous ventral shields; sp. B. presents cylindrical stylodes and red radiolar eyes and *B. galei* has collar dorsal margins widely separated and red radiolar eyes. But there are other species not supported by unambiguous changes and these are *B. lucullanum*, *B. bairdi*, sp. G and sp. C (Fig. 5).

The level of fusion of the dorsal peristomial collar to the faecal groove has an ambiguous origin. It is fused to the faecal groove in *B. lucullanum* and *B. bombyx*, widely separated in sp. F, and slightly separated in the rest of species. Macrostylodes are always present in *B. bairdi*, but this character shows variation within other species (e.g. present in only some members of sp. B and *B.* cf *punctulatum*). The shape of stylodes, was revealed as highly homoplastic and with great intraspecific variability; only consistent within sp. D. *Branchiomma galei* shows the widest phenetic plasticity for this attribute, with specimens presenting only cylindrical stylodes and others presenting both strap-like and palmate ones (Fig. 1C, D). The plesiomorphic condition for the number of basal stylodes is ambiguous and single basal stylodes are present in distant lineages (*B. lucullanum, B. bombyx* and *B. galei*) being paired in the rest of the *Branchiomma* terminals. The length of these basal stylodes and the dorsal lips is short in origin but is highly variable within most species. The uncini evolved, within the ingroup, tending to a reduction in number of the rows of teeth above the main fang, with numbers being more or less consistent within species. Two distant clades, *B. bairdi* and sp. D, are characterised by possessing uncini with long necks but in the rest of species this character is not stable. Even though the body general pigmentation was revealed as highly homoplastic, we observed that a purple body is characteristic of the Mediterranean species *B. bombyx* and *B. lucullanum*, green is typical of the *B. bairdi*, sp. A, sp. C and sp. D. The rest of clades have a combination of whitish or brown bodies with both patterns mixed within each lineage. The presence of spots or flakes all over the body is the general rule and the plesiomorphic condition, but some species have lost this pattern (*B. lucullanum*, sp. A, sp. C, sp. D and sp. H). The size of these spots is also phylogenetic informative and consistent within most species, as sp. B and *B. galei* presents small spots; sp. G presents medium spots; and *B. bombyx* has irregular-shaped large spots. The plesiomorphic condition for the colour of the radiolar eyes is dark and according to the

phylogenetic hypothesis these have changed to a light red in sp. B, sp. D and *B. galei*. Ventral shields are well developed and clearly noticeable in *B. lucullanum, B. bombyx, B. luctuosum*, sp. B and *B. galei* and separated from the thoracic neuropodial tori in *B. lucullanum*, *B. bombyx* and *B. luctuosum,* being in contact in sp. B and *B. galei*. The number of thoracic segments, although being generally eight the common and plesiomorphic condition, shows variation within most species and others, like sp. D that typically have 4-5 thoracic segments.

Discussion

The reconstruction of ancestral states of morphological characters based on molecular phylogenies highlight that traditional diagnostic morphological features are inaccurate in *Branchiomma* and that the accepted putative species based on morphological grounds need revision. Moreover, genetic divergence and molecular entities (putative species) resulting from species delineation based on mitochondrial and nuclear DNA fragments provide evidence of cryptic diversity, large intraspecific phenetic plasticity, genetic introgression, geographic structure and recent geographic anthropogenic translocations; processes reported here for the first time in the genus. Results also reveal a high diversity of species of *Branchiomma* in Australia, with eight species, seven of which are new species or new records for the continent, and *B. galei*, previously reported in Western Australia (Augener, 1914).

Sequence based species delimitation, genetic introgression and geographic structure

Species delineation was in most cases consistently supported by statistical parsimony, GMYC analyses, and clade monophyly by both mitochondrial and nuclear markers. This is the case for *B. lucullanum, B. bombyx, B. bairdi, Branchiomma* sp. A, B and C. But some incongruences between datasets and analytical methods were also found (summarised in Fig. 4). An example of conflicting results between analysed datasets is *B. galei*. Two distinct entities resulted after statistical parsimony and GMYC analysis of *cob*, coinciding

with the two clades recovered after phylogenetic analyses (Figs 4, 5) but only one entity after analyses of the ITS1 region. This result can be explained by geographic structure of populations within the same species, i.e. early stages of speciation, along with the faster evolutionary rate of mitochondrial genes relative to nuclear ones (Zink & Barrowclough, 2008; Kaltenpoth et al., 2012). This hypothesis was also supported by the geographic distribution, Western Australia and New South Wales respectively, of members of these two clades.

Members ascribed to *Branchiomma* sp. D were recovered as two sister clades after phylogenetic analyses, coinciding with the haplotype networks and GMYC entities found for the ITS1 fragment. On the other hand, the mitochondrial gene considered them all as a single group. A plausible explanation for this conflicting pattern is a genetic introgression (see Johnson et al., 2006; Alves et al., 2008; Mallet, 2008; Frade et al., 2010), where interspecific hybridization has been followed by back-crossing, resulting in two distinct ITS1 allele groups (<0.75 within clades; 2.1% between clades) but almost imperceptible in *cob* sequences (identical haplotype for all individuals except for BR_83 and BR_91 with 0.9% and 1.5% divergence, respectively). The homogenization and the fixation of divergent ITS1 variants at the species level in a short period of time have been related to the concerted evolution of variants already present in the parental lineage (Dover, 1982; Chen et al., 2002). It also has to be highlighted that members of those two alleles were found in Hawaii and different localities in Australia.

A more complex case is represented by the species *Branchiomma* sp. G. Phylogenetic analyses recover two main clades, being ML and MP incongruent about the position of the individual BR_30 (Figs 4, 5). The haplotype networks and GMYC analyses of the *cob* fragment group the terminals in two entities, while the analyses of the ITS1 regions recover two networks and three GMYC entities, that are not congruent with any of those found for the *cob* fragment, of phylogenetic hypothesis. The overall genetic divergence in the species *Branchiomma* sp. G. is low (0.7% for *cob* and 1.1% for ITS1). These results could be

explained by a combination of introgression and genetic and geographic structure of populations.

The interspecific genetic distances for ITS1 were similar or higher (11-35%) to those found in other polychaetes groups (Pleijel et al., 2009; Nygren & Pleijel, 2010; Nygren et al., 2010; 2011). Examples of species genetic distance on *cob* in polychaetes have not been published, but the *cob* of *Branchiomma* specimens (17-41%) is greater than that measured between closely related molluscs (<2% de Aranzamendi et al., 2009), nematodes (< 9% de Gruijter et al. 2002; Hu et al. 2002) or insects (<9.4%, Pons et al. 2006). The 'consensus species' based on all evidences gathered in this study are indicated and named (with a Linnaean binomial or sp. X) in figures 3 and 4. The intraspecific variation for these putative species is, in most cases, one order of magnitude smaller than the interspecific variation (Fig. 6 and Sup. Mat).

Diversity in the Australian coasts

The present results increase the total number of species of *Branchiomma* in Australia to eight. One of them, *B. galei*, was previously reported in Western Australia (Augener, 1914). Another agreed with the diagnosis of *B. bairdi*, originally from the Caribbean and now reported in Australia for the first time. But there are also other six species that cannot unequivocally be identified as any of the currently recognised nominal species of *Branchiomma*. We were suspicious about the presence of *B. punctulatum* (e.g. BR-069, Fig. 1A), originally described from Port Jackson, Sydney (Haswell, 1884) and never reported again, no sequences were obtained and therefore was not included in the analyses. Another species expected to be in the analyses is *B. cingulatum* (Grube, 1870), originally from the Philippines but reported in several distant localities along the Indo-Pacific (Grube, 1870; Augener, 1914; Hartman, 1948; Knight-Jones, 1994; Fitzhugh, 2002). *S*ince we collected specimens from several localities in these oceans, including Hawaii, Saipan and Australia, the chances of collecting members of this apparently common species were anticipated to be high. But none of the putative species delineated herein matches the description of this

species. The description of *B. boholense* (Grube, 1878) described from the Philippines and reported as a translocated species (Knight-Jones *et al*., 1991; Zenetos *et al*., 2005, 2010; Román et al., 2009) does not match any of putative species in this study, and the combination of back radiolar eyes, presence of strap-like macrostylodes, and thoracic uncini with two rows of teeth over the main fang (Grube, 1878; Knight-Jones, pers. comm.) is not shared by individuals in our study.

Morphological features, phenetic plasticity and cryptic diversity

We have demonstrated that most of the traditional specific 'diagnostic features' in *Branchiomma* are homoplastic, showing a broad intraspecific plasticity and/or being shared between species, becoming evident they should not be considered for taxonomic purposes. The establishment of new features, non-overlapping between species and showing intraspecific stability, will only be possible after a complete revision of the genus. As summarised by Padial & de la Riva (2010), regardless of the nature of the characters used for describing species (qualitative or quantitative, discrete or continuous, fixed or being polymorphic within species) character states need to be distributed in different frequencies across species. The shape and relative size of stylodes, for example, considered as two of the most important features for distinguishing between species (Knight-Jones, 1994; Nogueira et al., 2006; Tovar-Hernández & Knight-Jones, 2006; Tovar-Hernández et al., 2009a) have shown to be homoplastic, highly variable within *Branchiomma* species, and with states shared by several species. The pigmentation pattern has been shown to give some taxonomic and phylogenetic insights, even though it has not always been included in the descriptions in the literature. The scoring of these features included the general body colour and the presence and size of the spots (Fitzhugh, 2002; Nogueira et al., 2006; Tovar-Hernández & Knight-Jones, 2006; Licciano & Giangrande, 2008) and colour of radiolar eyes. This is not the first time that colour has revealed to be the main difference between polychaete species (Pleijel et al., 2009, Capa et al., 2010) but there are also some examples of intraspecific colour variation (Pleijel et al., 2009; Nygren et al., 2011).

Examples of intraspecific phenetic plasticity are *B. galei* and sp. G, with polymorphism in the length and size of stylodes among other characters. While members of species B and D are only distinguished by minor morphological features including the degree of development of the glandular ventral shields and could be considered as pseudocryptic. These two species that belong to distant lineages (Figs 4, 5) and show a high genetic distance (Table 3) have been shown to have a wide distribution, co-occurring at the same collecting sites in Oahu, Hawaii and at Ningaloo, Western Australia, but all collected in coral rubble and other hard substrates. Detailed studies about the micro-distribution within these environments and also their life histories should be made in order to establish differences between them. Sympatric cryptic species often have characteristic differences in ecology or life history, and can, in retrospect, be identified by elusive differences in morphology or colour pattern (Knowlton, 1993, 2000).

Taxonomy

With the aim of facilitating the identification of species of *Branchiomma*, Knight-Jones (1994) established seven artificial groups according to shared morphological features and these have been used widely by taxonomists (Nogueira et al., 2006; Tovar-Hernández & Knight-Jones, 2006; Licciano & Giangrande, 2008; El Haddad et al., 2008). Group A gathered species with a peristomial collar fused to the sides of the dorsal faecal groove, and group B species with a dorsal collar showing widely separated margins. The latter group was again subdivided into group C with radioles with macrostylodes and group D with even gradations of stylode length distal to the basal ones. Group D was further subdivided into group E, species with numerous teeth on the thoracic uncinal crests and group F with sparse teeth on the uncinal fang.

The relationships within *Branchiomma* species are far from understood and present analyses did not find strong relationships between species. But both probabilistic and MP methods indicate that the groups proposed by Knight-Jones (1994) have no evolutionary foundation and species sharing a wide gap in the collar dorsal margins, presenting

macrostylodes or bearing many rows of teeth in the uncini are not necessarily most closely related to each other. In contrast the ingroup accumulates high levels of homoplasy on morphological features and these barely define basal clades or species groups.

Geographical distribution and species translocation

The species outlined in this study present, in most cases a wide geographic distribution (Figs 3, 5). *Branchiomma* sp. B has the widest distribution range as it has been found in Northern Australian, but also in Saipan, Hawaii and Florida (Figs 3, 5) besides the very little genetic intraspecific variation (0.6 and 0.3% in *cob* and ITS1 fragments respectively), followed by *B. bairdi*, described from the Caribbean, and found in tropical Queensland, and *Branchiomma* sp. D, found in Western Australia, Queensland and Hawaii (again with none or little intraspecific genetic divergence 0-0.5% in both cases).

Branchiomma species show relatively low dispersal capabilities, some being broadcast spawners (Berrill 1977; Licciano et al., 2002, Tovar-Hernandez et al., 2011) with a larval stage that can last between three days in the water column (Licciano et al., 2002) and other reported as brooders (Dragesco-Kernéis 1980; Rouse, 1993; Tovar-Hernández et al., 2011). Recent anthropogenic translocations could therefore explain the wide geographic distribution range of the species included in the present study that shows none or little genetic variation. Several *Branchiomma* species have already being reported as unintentionally moved out their natural expected distribution range. These are *B. bairdi*, originally from the Caribbean and recently reported in the Gulf of California, the Eastern Mediterranean (Tovar-Hernández et al., 2009 a, b, 2011; Çinar, 2009; Zenetos et al., 2010; Arias et al., 2012; Giangrande et al., 2012); *Branchiomma boholense*, described from the Indo-Pacific and reported as introduced to the Mediterranean (Knight-Jones *et al*., 1991; Zenetos *et al*., 2005, 2010; Román *et al*., 2009); *Branchiomma curtum* (Ehlers, 1901) translocated from Chile to the Caribbean (Tovar-Hernández & Knight-Jones, 2006; Tovar-Hernández & Dean, 2010) and *B. luctuosum* originally described from the Red Sea and

translocated to the Mediterranean (Bianchi, 1983; Licciano et al., 2002; Zenetos et al., 2005, 2010; Çinar et al., 2006; El Haddad et al., 2008).

Some *Branchiomma* species are able of reproduce asexually by scissiparity, breaking off the posterior end that will become a new individual (Tovar-Hernández et al., 2009a). This ability is responsible of the large aggregations found in some disturbed environments and artificial surfaces such as buoys, hulls of ships or docks in ports (Díaz-Díaz & Liñero-Arana, 2006; Tovar-Hernández et al., 2009b, 2011; Giangrande et al., 2012; pers. obs. Fig. 2D, E), as some of the specimens collected for this study (Fig. 5). When *Branchiomma* species aggregate in high densities (Fig. 2D, E) they become a structural species, modifying the habitat, with the consequential effects to the rest of the ecosystem, especially if they are invasive, because they can drastically alter the receiving habitat (Coleman & Williams, 2002; Teske et al., 2011). Some of the species delineated in the present study, and with evidence of being translocated, were found in medium-high densities in artificial surfaces in ports of Australia (Fig. 5) highlighting a potential biosecurity threat for endemic ecosystems and species.

Conclusions

Genetic and evolutionary methods for species delimitation have proved to be an effective tool, or at least a capable and useful point of departure, to approach biodiversity studies especially in those cases where morphology fails due to complexity or lack of expertise (e.g. Pons et al., 2006; Barraclough et al., 2009; Camargo et al., 2012; Esselstyn et al., 2012. Harrinton & Near, 2012; Puillandre et al., 2012). Therefore polychaetes seem to be an ideal candidate for applying these methodologies, since it is a group of invertebrates with several taxonomic issues, high level of homoplasy in some groups, species diagnostic features not well delimited and ‘species’ with cosmopolitan or broad geographic distributions. Some recent studies have dealt with the discovery of sibling species based on molecular phylogenies and/or statistical parsimony networks analyses (e.g. Nygren et al.,

2005, 2009, 2010; Bleidorn et al., 2006; Mahon et al., 2009, Pleijel et al., 2009; Nygren & Pleijel, 2010) but no study has used coalesce methodologies to date.

The genus *Branchiomma* has shown to be more diverse that previously reported and the traditional morphological features inaccurate for taxonomic purposes. A revision of the genus is needed to determine the overall diversity in the genus and establish valid attributes for identifying species. The total number of species reported from Australia after this study is eight . A number that could increase after a more comprehensive study including specimens from other localities. It would also be interesting to compare results after a more intensive sampling within lineages and populations although it has already been demonstrated in other studies, that accurate species delimitation can be achieved despite widespread incomplete lineage sorting and discordance among loci (Pons et al., 2006; Knowles & Castern, 2007; Papadopoulou et al., 2009) and even with the presence of singletons (Lim et al., 2012).

We recommend a comprehensive study within the genus in order to assess the status of certain species with broad distributions to determine their origin so a careful follow up of the size of their populations can be achieved to determine their potential threat as pests. Since the identification of the *Branchiomma* species from morphological features has shown to be elusive, we recommend the use of molecular markers to corroborate the identification and status of these species (e.g. Bastrop et al., 1998).

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FIGURES

Fig. 1. Scanning electron micrographs of morphological features in *Branchiomma* species. – A. Detail of paired cylindrical stylodes and paired radiolar compound eyes (white arrow). – B. Flat and varying in size stylodes.– C. Palmate stylodes. D. Base of radiolar crown, peristomial collar and first segments, dorsal view. — E. Base of crown, with short unpaired basal stylodes. – F. Base of crown with long, unpaired basal stylodes. – G. Radiolar crown with flattened and bifurcated stylodes (white arrow). – H. Thoracic uncini with 2-3 rows of teeth over the main fang. – I. Thoracic uncini with 2-3 compact rows of teeth over the main fang. - J. Paired basal stylodes, longer than rachis; Thoracic ventral shields in contact with tori. K.- short, unpaired basal stylodes. Scales: A, B, D, E-G, J, K: 100 μm; C: 10 μm; H, I: 3 μm.

Fig. 2. Photographs of live specimens where paired dark dots on radioles are the compound radiolar eyes and appendages directed outwards the stylodes. – A. *Branchiomma* cf. *punctulatum* (BR-079); –B. *Branchiomma* sp. G. (BR-078); – C. *Branchiomma* sp. D (BR-091); – D. Soft bottom covered by members of *Branchiomma* sp. in Suruga Bay, Japan. Photographs A and B, taken by Roger Springthorpe; D and E by Kenji Nin.

Fig. 3. Map with collecting sites and list of species sampled from each geographical area surveyed.

Fig. 4. Phylogenetic relationships in members of the genus *Branchiomma* based on a Bayesian analysis of combined *cob* and ITS1 datasets. Boxes below nodes show the following support values (from left to right and too to bottom): PP, Bayesian Interference posterior probabilities; BS, Maximum likelihood bootstrap values; B*cob*, partitioned Bremer support for *cob*; Bits, partitioned Bremer support for ITS; BS*cob*, Maximum likelihood bootstrap values for *cob*; BSits, Maximum likelihood bootstrap values for ITS. X stands for a clade not found after analyses of one partition. Maximum parsimony Jackknife

support values over 0.50 are indicated above nodes. The longitudinal coloured columns on the right side show the clustering of terminals according different methodologies and datasets: purple bars correspond to the preliminary groups based on morphological features; the red column represents the statistical parsimony haplotype networks recovered for *cob*; the green column the haplotype networks for ITS1 (considering gaps as missing data); the blue columns are the species recovered after GMYC analyses for *cob*; and the orange columns are the species recovered after GMYC analyses for ITS1. Finally, the black columns represents our final hypothesis of species after considering all results.

Fig. 5. Strict consensus of 10,430 most parsimonious trees after analyses of combined morphological, *cob* and ITS datasets. Values at left of branches indicate the Jackknife support value of clades, back dots represent the synapomorphies and white dots the homoplasies. The coloured bars on the right side represent the geographic region where the specimens were collected. The stars indicate the specimens collected in the docks or pylons of ports.

Fig. 6. Boxplot for uncorrected p-distances for each DNA marker at the intra- and interspecific level for species and molecular entities delimited by two methods, parsimony networks and GMYC. Values within lower and upper quartiles are represented with a box and median as thicker line. Largest values are indicated with dotted lines and outliers as circles.

Table 1. List of specimens included in the study, ordered by project specimen number, Australian Museum (AM) voucher numbers and GenBank accession numbers.

Species	Specimen	Voucher	*cob*	ITS1	Locality	Country
Sabellastarte australiensis	BR_076	AM W.35608	XXX	XXX	NSW	Australia
Pseudobranchiomma sp. 1	BR_018	AM W.35576	XXX	XXX	Oahu, Hawaii.	USA
Pseudobranchiomma sp. 1	BR_019	AM W.35577	XXX		Oahu, Hawaii.	USA
Pseudobranchiomma sp. 1	BR_020	AM W.35578	XXX	XXX	Oahu, Hawaii.	USA
Pseudobranchiomma sp. 2	BR_056	AM W.35590		XXX	Kalbarri NP, WA	Australia
Branchiomma lucullanum	BR_001	AM W.35598	XXX		Catalonia	Spain
B. lucullanum	BR_002	AM W.35599	XXX	XXX	Catalonia	Spain
B. lucullanum	BR_003	AM W.35600	XXX		Catalonia	Spain
B. bombyx	BR_004	AM W.35601	XXX		Catalonia	Spain
B. bairdi	BR_005	AM W.30508		XXX	Fort Pierce, Florida	USA
Branchiomma sp. B	BR_006	AM W.31816	XXX	XXX	Fort Pierce, Florida	USA
B. bairdi	BR_007	AM W.35562	XXX	XXX	Fort Pierce, Florida	USA
Branchiomma sp. B	BR_008	AM W.35563	XXX		Fort Pierce, Florida	USA
B. bairdi	BR_011	AM W.35564		XXX	Fort Pierce, Florida	USA
B. bairdi	BR_012	AM W.31822	XXX	XXX	Fort Pierce, Florida	USA
Branchiomma sp. D	BR_015	AM W.35573	XXX	XXX	Oahu, Hawaii	USA
Branchiomma sp. B	BR_016	AM W.35574	XXX	XXX	Oahu, Hawaii	USA
Branchiomma sp. B	BR_017	AM W.35575		XXX	Oahu, Hawaii	USA
Branchiomma sp. D	BR_022	AM W.35580	XXX	XXX	Oahu, Hawaii	USA
Branchiomma sp. D	BR_023	AM W.35581		XXX	Oahu, Hawaii	USA
Branchiomma sp. D	BR_024	AM W.35582	XXX	XXX	Oahu, Hawaii	USA
B. luctuosum	BR_026	AM W.32604	XXX		Bulleji, Karachi	Pakistan
Branchiomma sp. G	BR_028	AM W.32594		XXX	Solitary IS., NSW	Australia
Branchiomma sp. G	BR_030	AM W.29050	XXX	XXX	Forster, NSW	Australia
Branchiomma sp. G	BR_039	AM W.32600	XXX	XXX	Port Stephens, NSW	Australia
Branchiomma sp. B	BR_043	AM W.35568		XXX	Darwin, NT	Australia
Branchiomma sp. B	BR_044	AM W.35569		XXX	Darwin, NT	Australia
Branchiomma sp. B	BR_045	AM W.35570		XXX	Darwin, NT	Australia
Branchiomma sp. B	BR_046	AM W.35571		XXX	Darwin, NT	Australia
Branchiomma sp. B	BR_047	AM W.35572		XXX	Darwin, NT	Australia
Branchiomma sp. C	BR_048	AM W.35587	XXX		Darwin, NT	Australia
B. bairdi	BR_052	AM W.35566	XXX	XXX	Cairns, QLD	Australia
Branchiomma sp. F	BR_054	AM W.35594	XXX	XXX	Abrolhos Group, WA	Australia
Branchiomma sp. A	BR_055	AM W.35593	XXX	XXX	Abrolhos Group, WA	Australia
Branchiomma sp. A	BR_057	AM W.35591	XXX	XXX	Kalbarri NP, WA	Australia
Branchiomma sp. F	BR_059	AM W.35597	XXX	XXX	Shark Bay, WA	Australia
Branchiomma sp. G	BR_060	AM W.35583	XXX	XXX	Green Head, WA	Australia

Branchiomma sp. G	BR_061	AM W.35584	XXX		Green Head, WA	Australia
Branchiomma sp. C	BR_062	AM W.35592	XXX	XXX	Port Denison, WA	Australia
Branchiomma sp. F	BR_064	AM W.35585		XXX	Shark Bay, WA	Australia
Branchiomma sp. F	BR_065	AM W.35586	XXX	XXX	Shark Bay, WA	Australia
Branchiomma sp. G	BR_066	AM W.35312	XXX	XXX	Umina, NSW	Australia
Branchiomma sp. G	BR_067	AM W.35313		XXX	Tathra, NSW	Australia
Branchiomma sp. G	BR_068	AM W.35277	XXX	XXX	Tathra, NSW	Australia
Branchiomma sp. G	BR_069	AM W.35276	XXX	XXX	Tathra, NSW	Australia
Branchiomma sp. G	BR_070	AM W.35602	XXX	XXX	Botany Bay, NSW	Australia
Branchiomma sp. G	BR_071	AM W.35603	XXX	XXX	Kurnell, NSW	Australia
Branchiomma sp. G	BR_072	AM W.35604	XXX	XXX	Jervis Bay, NSW,	Australia
Branchiomma sp. F	BR_074	AM W.35606	XXX	XXX	Sydney Harbour, NSW	Australia
Branchiomma sp. F	BR_075	AM W.35607	XXX	XXX	Botany Bay, NSW	Australia
Branchiomma sp. G	BR_077	AM W.35611	XXX	XXX	Jolong Reef, NSW	Australia
Branchiomma sp. G	BR_078	AM W.35614	XXX	XXX	Jolong Reef, NSW	Australia
Branchiomma sp. C	BR_080	AM W.35616	XXX	XXX	Ningaloo Reef, WA	Australia
Branchiomma sp. C	BR_082	AM W.35618	XXX		Ningaloo Reef, WA	Australia
Branchiomma sp. D	BR_083	AM W.35619	XXX	XXX	Ningaloo Reef, WA	Australia
Branchiomma sp. C	BR_084	AM W.35620	XXX	XXX	Ningaloo Reef, WA	Australia
Branchiomma sp. B	BR_088	AM W.35632	XXX	XXX	North Invasion Beach	Saipan
Branchiomma sp. D	BR_090	AM W.36342		XXX	Heron Island, QLD	Australia
Branchiomma sp. D	BR_091	AM W.36343	XXX	XXX	Heron Island, QLD	Australia
Branchiomma sp. G	BR_095	AM W.36432		XXX	Jolong Reef, NSW	Australia
Branchiomma sp. G	BR_096	AM W.36433		XXX	Jolong Reef, NSW	Australia
Branchiomma sp. G	BR_097	AM W.36434		XXX	Jolong Reef, NSW	Australia
Branchiomma sp. G	BR_099	AM W.36436	XXX		LakeMacquarie, NSW	Australia
Branchiomma sp. G	BR_100	AM W.36443		XXX	Cape Banks, NSW	Australia
Branchiomma sp. D	BR_102	AM W.36438	XXX		Heron Island, QLD	Australia
Branchiomma sp. D	BR_103	AM W.36439	XXX	XXX	Heron Island, QLD	Australia
Branchiomma sp. D	BR_104	AM W.36440	XXX	XXX	Heron Island, QLD	Australia
Branchiomma sp. D	BR_105	AM W.36441	XXX	XXX	Heron Island, QLD	Australia
Branchiomma sp. G	BR_106	AM W.36442	XXX	XXX	Tathra, NSW	Australia
Branchiomma sp. B	BR_107	AM W.36444	XXX	XXX	Darwin, NT	Australia
B. lucullanum	BR_110	AM W.36446		XXX	Catalonia	Spain

Table 2. Character description

0. Dorsal margins of peristomial collar: 0, fused to faecal groove; 1, separated (Fig.1D); 2, widely separated.

1. Macrostylodes (stylodes with more than twice the length of contiguous: 0 absent (Fig. 1A); 1, present (Fig. 1B).
2. Shape of stylodes: 0, cylindrical or digitiform (Fig. 1A); 1, flattened, tongue-like or strap-like (Fig. 1F); 2, dendritic or palmate (Fig.1C); 3, bifurcated.
3. Basal stylodes: 0, paired (Fig. 1G, J); 1, single (Fig. 1F, K).
4. Length of basal stylodes: 0, shorter or equal to rachis (Fig. 1K); 1, longer than rachis (Fig. 1F, G).
5. Length of dorsal lips (related to number of thoracic segments): 0, four; 1, three; 2, five; 3, six; 4, seven; 5, eight.
6. Rows of teeth over main fang in thoracic uncini: 0, one; 1, two (Fig 1I); 2, three (Fig. 1H); 3, four or more.
7. Length of neck of thoracic uncini, compared to the distance of tip of main fang to breast: 0, short (<0.6); 1, long (>0.6); 2, medium (0.6).
8. General body pigment: 0, whitish; 1, greenish; 2, brownish; 3, purple.
9. Spots or flakes: 0, absent; 1, present.
10. Size of spots: 0, small; 1, medium; 2, large.
11. Radiolar eyes: 0, light red; 1, dark red (almost black).
12. Thoracic ventral shields: 0, inconspicuous, not raised; 2, raised, noticeable.
13. Ventral shields and neuropodial tori: 0, in contact (fig. 1J); 1, separated.
14. Thorax: 0, 8 segments; 1, 4-5; 2, 7.
15. Stylodes: 0, absent; 1, present.
16. Branchial compound eyes: 0, absent; 1, present.
17. Supporting cells (skeleton) in dorsal radiolar appendages: 0, one cell; 1, many rows of cells.
18. Ventral sacs: 0, inside branchial crown; 1 outside branchial crown.

SUPPLEMENTARY MATERIAL

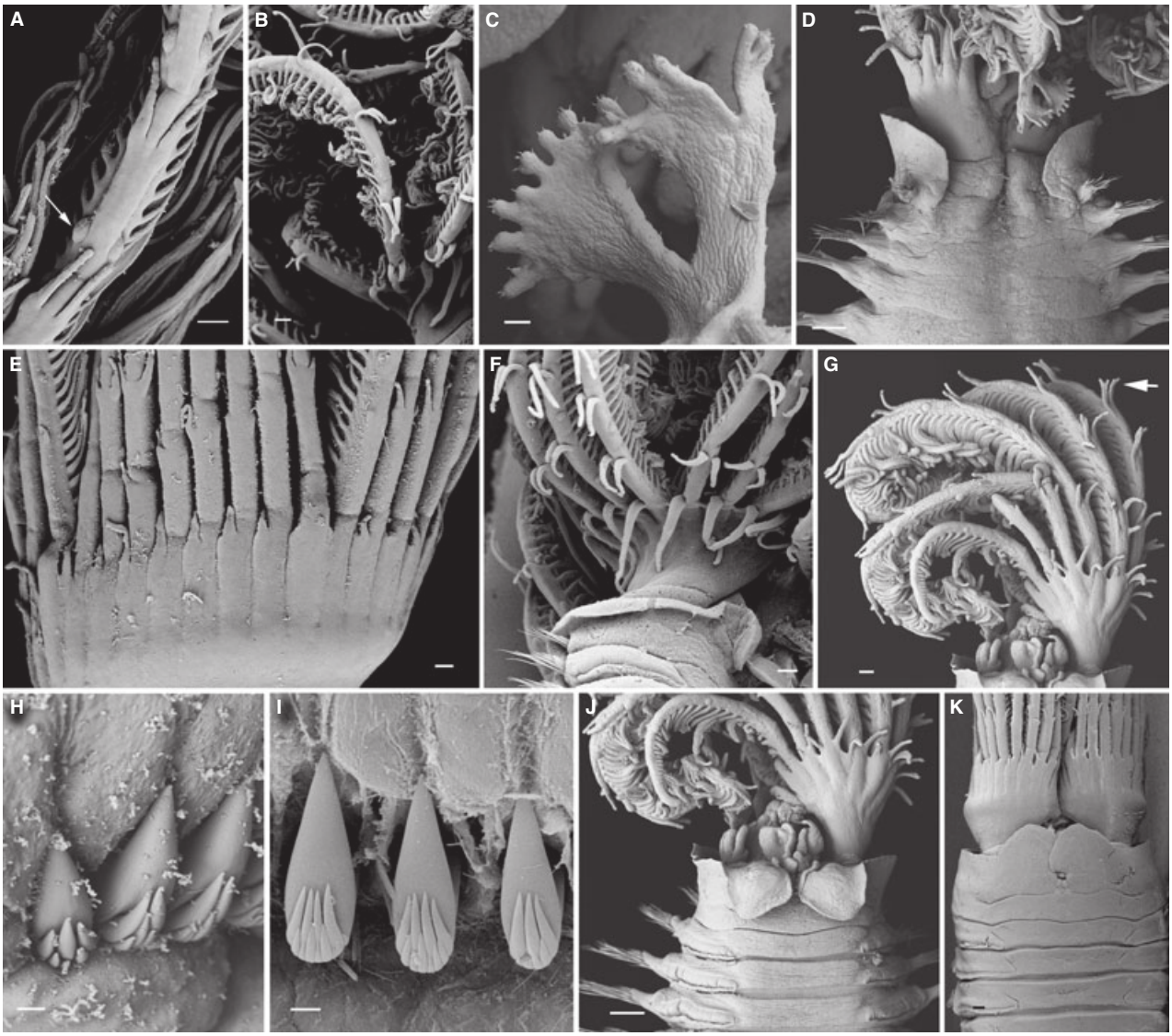
Table 1. Matrix with scored morphological features.

Fig. 1. Maximum likelihood trees. ML bootstrap values indicated on branches. – A. Inferred from the mitochondrial Cytochrome Oxidase b (*cob*) sequences. – B. Inferred from the internal transcribed spacer1 (ITS1) sequences.

Fig. 2. Strict consensus of most parsimonious trees after Values at left of branches indicate the Jackknife support value of clades. – A. Inferred from the mitochondrial Cytochrome Oxidase b (*cob*) sequences. – B. Inferred from the internal transcribed spacer1 (ITS1) sequences.

Fig. 3. Strict consensus of most parsimonious trees of combined datasets (morphological, *cob* and ITS1) showing some of the character transformations. – A. Macrostylodes (0) absent; (1) present. – B. Shape of stylodes: (0) cylindrical or digitiform; (1) flattened, tongue-like or strap-like; (2) dendritic or palmate; (3) bifurcated. – C. Length of basal stylodes: (0) shorter or equal to rachis; (1) longer than rachis. – D. Length of dorsal lips (related to number of thoracic segments): (0) four; (1) three; (2) five; (3) six; (4) seven; (5) eight. – E. Rows of teeth over main fang in thoracic uncini: (0) one; (1) two; (2) three; (3) four or more. – F. General body pigment: (0) whitish; (1) greenish; (2) brownish; (3) purple. – G. Spots or flakes: (0) absent; (1) present. H. Size of spots: (0) small; (1) medium; (2) large. – I. Radiolar eyes: (0) light red; (1) dark red (almost black). – J. Thorax: (0) 8 segments; (1) 4-5; (2) 7.

Fig. 4. Photographs of specimens of different species showing main morphological features scored for the present study. – A-C. *Branchiomma luctuosum* (BR-026). – D-F. *Branchiomma* sp. B (D: BR-43; E: BR-008, F: BR-017). – G-I. *Branchiomma bairdi* (G, H: BR-007, I: BR-052). – J-K. *Branchiomma* sp. D (J, K: BR-90). – L, M. *Branchiomma galei* (BR-075). – N-S *Branchiomma* sp. G (N, O: BR-030; P, Q: BR-066; R, S. BR-068).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B
C
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K


A
B
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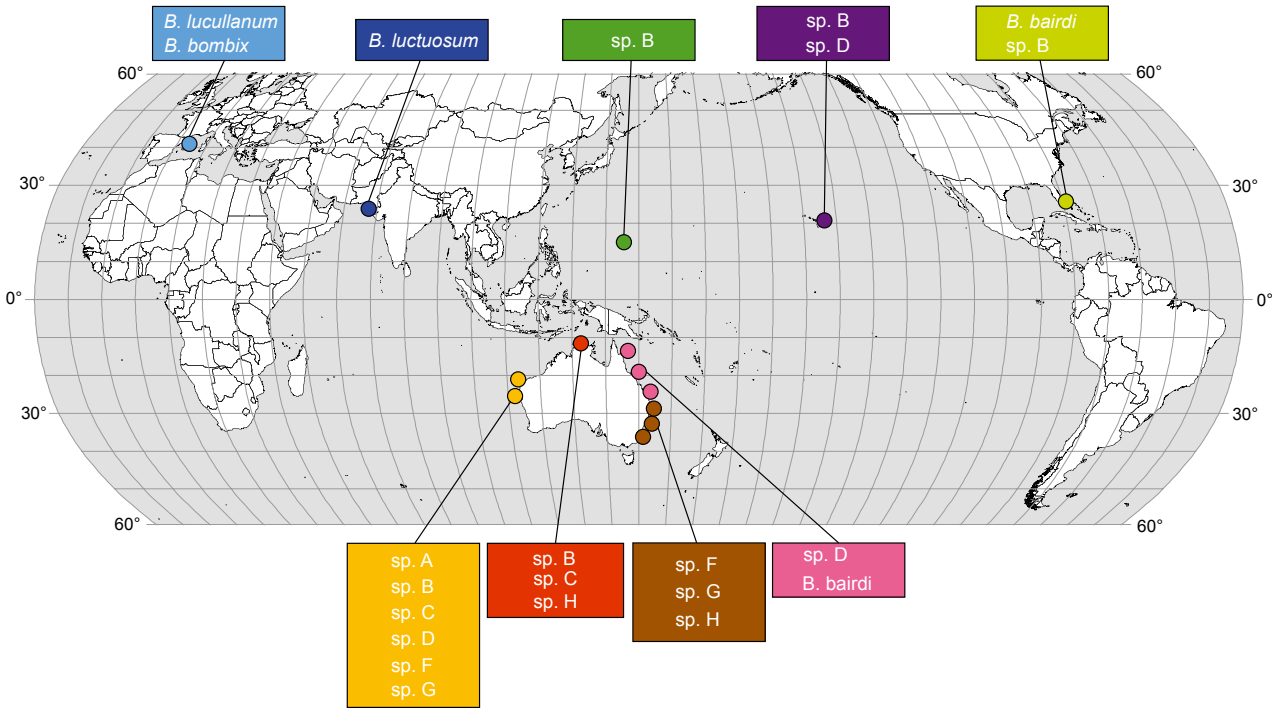
D
E


B. lucullanum
B. bombix
B. luctuosum
sp. B
sp. B
sp. D
B. bairdi
sp. B
60°
30°
0°
30°
60°
sp. A
sp. B
sp. C
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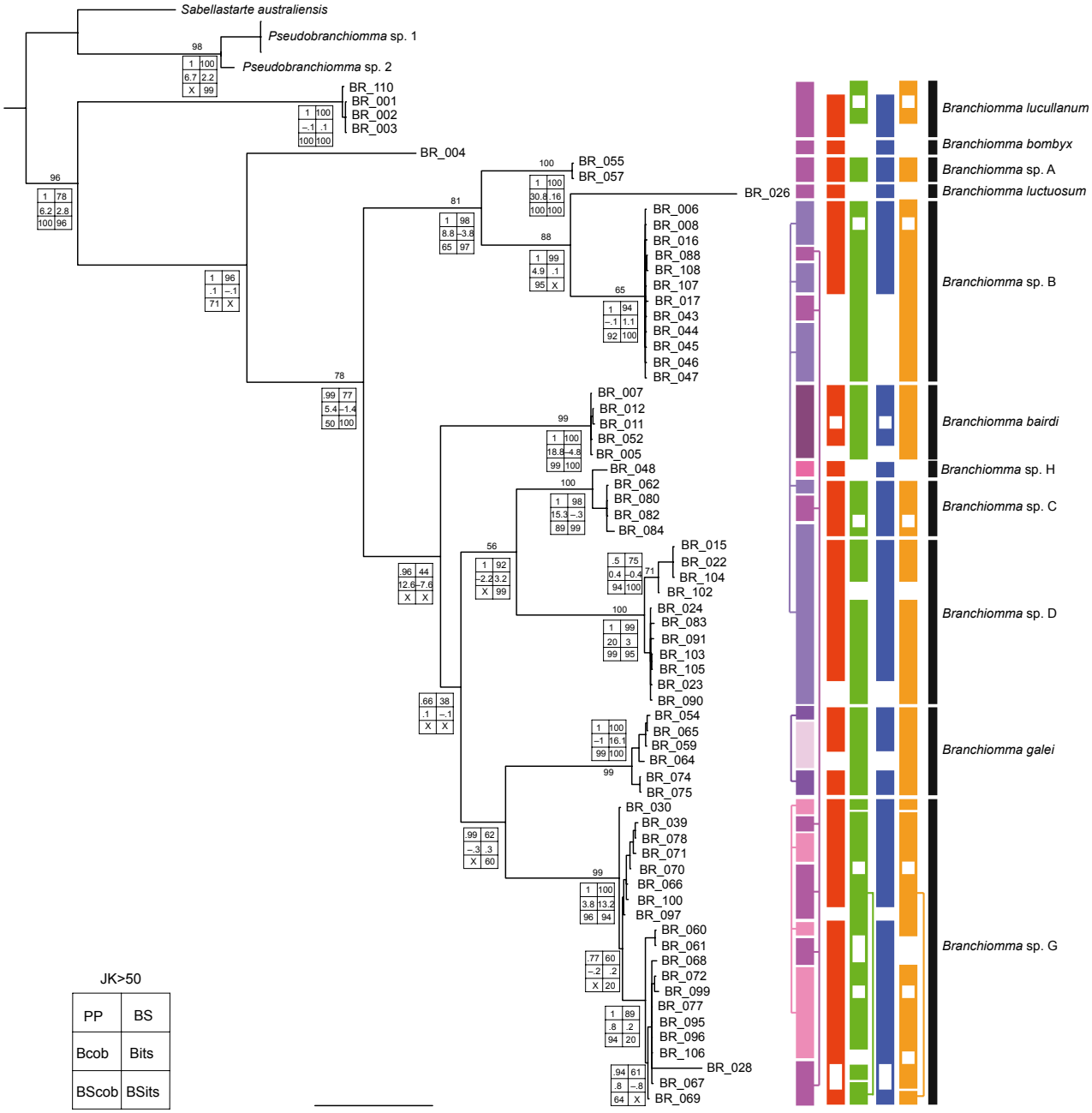
sp. H
sp. F
sp. G
sp. H
sp. D
B. bairdi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Pseudobranchiomma sp. 2
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BR_003
BR_004
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BR_026
BR_006
BR_008
BR_016
BR_088
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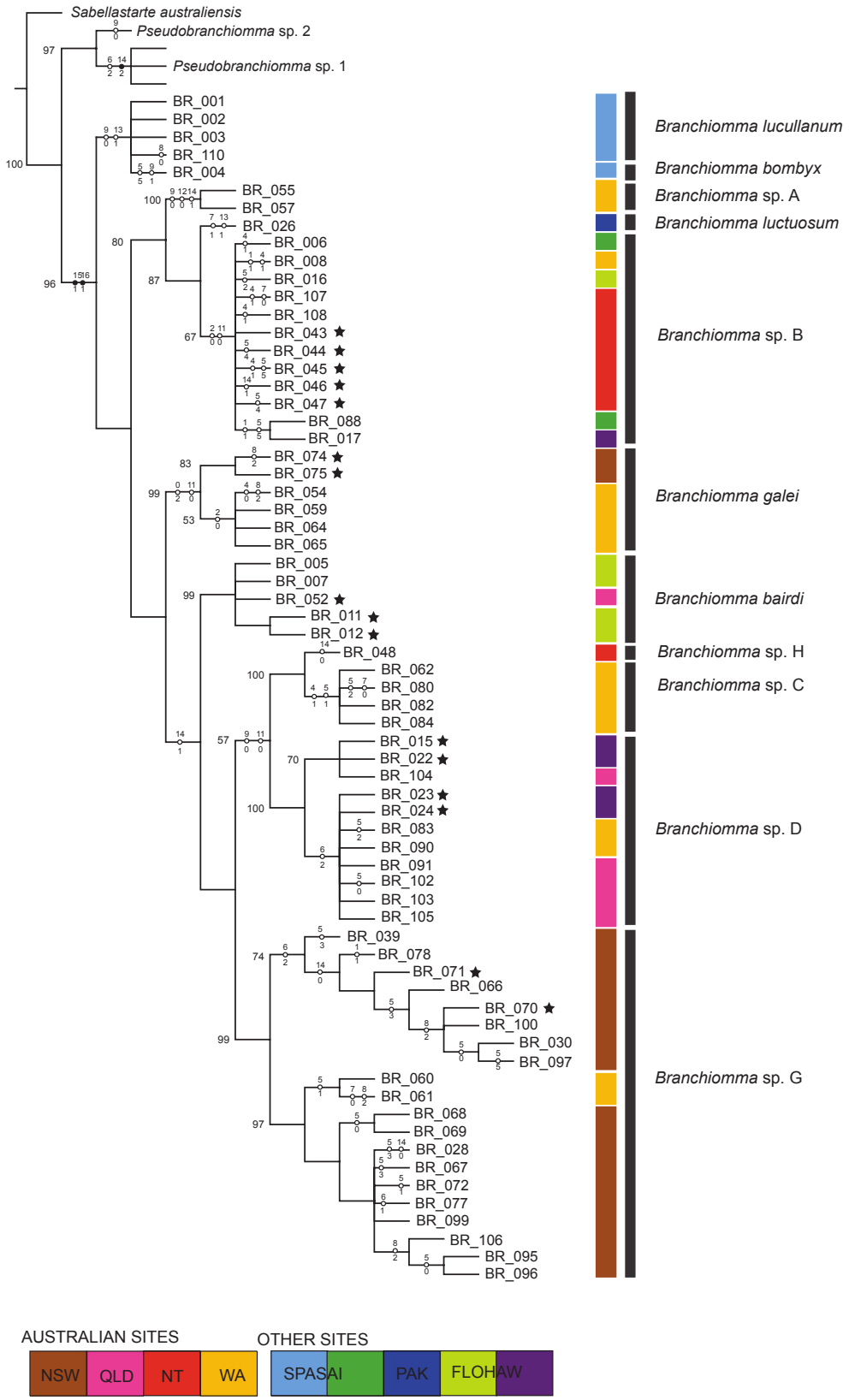
BR_064
BR_074
BR_075
BR_030
BR_039
BR_078
BR_071
BR_070
BR_066
BR_100
BR_097
BR_060
BR_061
BR_068
BR_072
BR_099
BR_077
BR_095
BR_096
BR_106
BR_028
BR_067
BR_069
Branchiomma lucullanum
Branchiomma bombyx
Branchiomma sp. A
Branchiomma luctuosum
Branchiomma sp. B
Branchiomma bairdi
Branchiomma sp. H
Branchiomma sp. C
Branchiomma sp. D
Branchiomma galei
Branchiomma sp. G
JK>50
PP
BS
Bcob
Bits
BScob
BSits
0.2


Sabellastarte australiensis
Pseudobranchiomma sp. 2
Pseudobranchiomma sp. 1
BR_001
BR_002
BR_003
BR_110
BR_004
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BR_057
BR_026
BR_006
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BR_072
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BR_099
BR_106
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BR_096
Branchiomma lucullanum
Branchiomma bombyx
Branchiomma sp. A
Branchiomma luctuosum
Branchiomma sp. B
Branchiomma galei
Branchiomma bairdi
Branchiomma sp. H
Branchiomma sp. C
Branchiomma sp. D
Branchiomma sp. G
AUSTRALIAN SITES
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NT
WA
OTHER SITES
SPAS
AI
PAK
FLOH
AW