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Taxonomic status of *Tambja abdere* and *Tambja fusca* based on morphological and molecular evidence, with comments on the phylogeny of the subfamily Nembrothinae (Nudibranchia, Polyceridae)

Marta Pola[1], Yvonne Vallès[2,3], J. Lucas Cervera[1], Mónica Medina[4,5] & Terrence M. Gosliner[4]

[1)] *Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro, s/n. Ap. 40., 11510 Puerto Real, Cádiz, Spain (e-mail: marta.pola@uca.es)*
[2)] *Department of Integrative Biology, 3060 Valley Life Sciences Bulding, University of California, Berkeley CA 94720, USA*
[3)] *current address: DOE Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek CA 94598, USA*
[4)] *Department of Invertebrate Zoology and Geology, California Academy of Sciences, 875 Howard Street, San Francisco CA 94103-3009, USA*
[5)] *current address: School of Natural Sciences, University of California, Merced, P.O. Box 2039, Merced CA 95344, USA*

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The use of morphological characters as the basis for species recognition and identification has permitted the development of a consistent taxonomy. However, limitations are evident when dealing with cryptic speciation or when intra-specific variability matches the total inter-species variation. Molecular techniques complement or enhance morphological inference by providing sets of data directly applicable to the taxonomic problem. Cases in which molecular techniques are particularly relevant are those involving larval or juvenile identification for which taxonomic characters are based on adult organisms and also those in which the original taxon description leads to uncertainty over the applicability of the species name. In this paper we report the use of mitochondrial DNA sequence data in a group of nudibranchs to exemplify the two cases mentioned above. The first issue is the longstanding debate on the taxonomic status of *Tambja abdere* and *Tambja fusca*, and the second issue is the identification of two juvenile specimens previously considered to represent two different undescribed species of the genus *Tambja* from the scarcely explored waters of Costa Rica. We also present a preliminary molecular phylogeny of the subfamily Nembrothinae.

Introduction

The use of morphological characters as the basis for species recognition and identification has permitted not only the development of a consistent taxonomy but also the generation of keys that allow for taxon identification (Wägele & Willan 2000, Schröld *et al*. 2001, Valdés 2002, Wiens & Penkrot 2002, Lydeard & Lindberg 2003, Fahey & Gosliner 2004, Dayrat 2005, Wägele 2005). However, the exclusive use of morphology-based taxonomy might present problems since it presents some limits (Medina & Walsh 2000, Wiens & Penkrot 2002, Fall *et al*. 2003, Dayrat 2005). The choice of characters is the most critical step for morphological studies (Ghiselin 1987), but it is also a drawback for morphological phylogenetic inference since there is not a set of well-defined standard characters, and the choice of them and their states depends on the criterion of the taxonomist (Mikkelsen 1998). Moreover, morphological characters might vary within the same species as the result of selective pressures and adaptation to the variation of environmental parameters (Wägele 2005). Thus, the individual variation in morphology should be thoroughly addressed before a "morphospecies" (Cain 1954) can be proposed (Dayrat 2005). All these limitations are evident when dealing with cases of cryptic speciation or when intra-specific variability matches the total inter-species variation (Avise & Ball 1990, Avise 2000, García-París *et al*. 2003, Sites & Marshall 2003, Hebert *et al*. 2004).

Molecular techniques complement or enhance this criterion by providing sets of data directly applicable to the taxonomic problem (Grande *et al*. 2002, 2004, Lydeard & Lindberg 2003, Medina & Collins 2003, Wägele *et al*. 2003). In many cases, when the selection of the marker is adequate, molecular data might help making a more objective taxonomic decision (Wägele 2005). Thus, the recent quarrels over "DNA barcoding" have argued that one should use DNA sequences of one (or a few) particular gene(s) to identify species, based on the idea that every species has its own "diagnostic" sequence (Hebert *et al*. 2003, 2004, Blaxter 2004, Dayrat 2005). However the use of DNA as a simple identity tag cannot replace taxonomy since applying a given name to the sequence is not a trivial matter and requires the same expertise that providing a name for the specimen from where the sequence was obtained (Will & Rubinoff 2004, Dayrat 2005). Moreover, recent studies have demonstrated that evolutionary rates for a given gene differ independently across clades thus rendering the use of a single gene as a barcoding device impossible (Vences *et al*. 2005). Besides the triviality of the barcoding issue, the use of adequate markers can in some cases provide faster and more reliable identifications than can morphological features.

The cases in which molecular techniques are particularly relevant are firstly those involving larval or juvenile identification for which taxonomic characters are based on adult organisms (Sánchez-Tocino *et al*. 2000, Chow *et al*. 2003), and secondly those in which the original taxon description leads to uncertainty over the applicability of the species name (Collin 2000, Martínez *et al*. 2002, Dayrat 2005). In this paper we report the use of molecular characters (mitochondrial DNA sequence data) in a group of sea slugs to exemplify the two cases mentioned above. The first issue is the longstanding debate on the taxonomic status of *Tambja abdere* (Farmer, 1978) and *Tambja fusca* (Farmer, 1978), and the second issue is the identification of two juvenile specimens previously considered to represent two different undescribed species of *Tambja*.

Both *T. abdere* and *T. fusca* from Baja California, Mexico were described in the same paper by Farmer in 1978. Both of the original descriptions of the external coloration, the radular formulae, and the features of the reproductive systems were almost identical. Thus, *T. adbere* and *T. fusca* were only differentiated by slightly dissimilar coloration, by the presence of three to four outer lateral plates in *T. fusca* and only four outer lateral plates in *T. abdere*, and by a first lateral tooth apparently more robust in *T. fusca* than in *T. abdere*. These minor differences, together with the presence of a high number of very similar pictures of both species in different book guides (Kerstich 1989, Behrens 1991, Debelius 1996) and web pages, considerably increased the confusion between them.

In the past few years, *T. abdere* and *T. fusca* were considered to be color variants of a single species (Behrens 2004) but no morphological

or molecular studies were performed to demonstrate this empirically. Recently, we had the opportunity of collecting two specimens morphologically identical to those forms described by Farmer and two juvenile specimens believed to represent two new species from the scarcely explored waters of Costa Rica (*Tambja* sp1 and *T.* sp2 in Table 1).

Solving taxonomic problems is not a trivial matter since an adequate taxon sampling is necessary to develop consistent phylogenetic hypothesis. Phylogenetic relationships of the subfamily Nembrothinae (Polyceridae, Nudibranchia) are poorly understood (Medina *et al.* 2001, Vallés 2002, Pola *et al.* 2005a). In a previous paper we reviewed the taxonomy and proposed a phylogenetic hypothesis for the genus *Roboastra* (Pola *et al.* 2005a) while taxonomic reviews of the genera *Tambja* and *Nembrotha* based on morphological characters are in progress. Here we present a preliminary phylogeny of the subfamily Nembrothinae based on molecular data with the purpose of reconciling the current morphology-generated classification and the mitochondrial DNA (mtDNA) data.

The aims of this paper are (1) to confirm the taxonomic status of *Tambja abdere* and *Tambja fusca* based on detailed morphological and molecular data, (2) to identify two juvenile specimens based on mtDNA sequences, and (3) to discuss the congruence and conflict between our preliminary molecular phylogeny of the Nembrothinae and the current classification of the subfamily based on morphological data.

Material and methods

In this study we include 16 taxa ascribed to the subfamily Nembrothinae and four outgroup species (Table 1). Fourteen of the ingroup species included in the analysis are currently considered valid in the literature and the other two are potentially new species of the genus *Tambja*.

We dissected specimens, previously assigned to *T. abdere* and *T. fusca* based on color, by dorsal incision. We examined and sketched their internal features under a dissecting microscope with a camera lucida. Interesting soft parts were critical point dried for scanning electron microscopy (SEM). We paid special attention to the morphology of the reproductive system. We removed the buccal mass and dissolved in 10% sodium hydroxide until the radula was isolated from the surrounding tissue. The radula and soft parts were then rinsed in water, dried, and

Table 1. Samples used in this study for molecular systematics and GenBank accession numbers.

Sample	Species	Locality	GenBank accesion No. (COI)	Reference
01	*Tambja abdere*	Costa Rica	DQ230995	This study
02	*Tambja abdere* [*T. fusca*]	Baja California	DQ230996	This study
03	*Tambja morosa*	Philippines	DQ230997	This study
04	*Tambja eliora*	Baja California	DQ230998	This study
05	*Tambja* sp1 (juvenile)	Costa Rica	DQ230999	This study
06	*Tambja* sp2 (juvenile)	Costa Rica	DQ231000	This study
07	*Tambja ceutae*	Spain	AY345038	Grande *et al.* 2004
08	*Roboastra luteolineata*	Australia	DQ231001	This study
09	*Roboastra tigris*	Baja California	DQ231002	This study
10	*Roboastra europaea*	Spain	AY083457	Grande *et al.* 2002
11	*Nembrotha cristata*	Philippines	DQ231003	This study
12	*Nembrotha mullineri*	Philippines	DQ231004	This study
13	*Nembrotha lineolata*	Philippines	DQ231005	This study
14	*Nembrotha chamberlaini*	Philippines	DQ231006	This study
15	*Nembrotha kubaryana*	Philippines	DQ231007	This study
16	*Nembrotha* cf. *rutilans*	Western Australia	DQ231008	This study
17	*Limacia jansi*	Costa Rica	DQ231009	This study
18	*Notodoris citrina*	Western Australia	DQ231010	This study
19	*Thecacera pennigera*	Spain	AJ223277	Thollesson 2000
20	*Polycera quadrilineata*	Spain	AJ223275	Thollesson 2000

mounted for examination by scanning electron microscopy. We deposited the examined material in the California Academy of Sciences, San Francisco (CASIZ), the Natural History Museum of Los Angeles County, Los Angeles (LACM) and the Museo Nacional de Ciencias Naturales, Madrid (MNCN).

We isolated Total DNA by standard SDS/ Proteinase K digestion (Sambrook *et al.* 1989), and resuspended in TE buffer (10 mM Tris-HCl [ph 8.0], 1 mM EDTA). Amplifications were performed in 100 μM of a solution containing approximately 50 ng of DNA, 1X PCR buffer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 2.5 units of Taq polymerase (Bioline). After an initial denaturation step of 7 min at 94 °C, 30 cycles of 30 sec at 94 °C, 30 sec at 46 °C and 30 sec at 72 °C were performed, followed by a final extension step of 10 min at 72 °C. A fragment of the cytochrome *c* oxidase subunit I gene (COI) was amplified with the universal primers LCO1490 (5´-GGT CAA CAA ATC ATA AAG ATA TTG G-3´) and HCO2198: (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'), developed by Folmer *et al.* (1994). PCR products (658 bp) were directly sequenced in both directions with an ABI 3100 capillary DNA Sequencer (PerkinElmer Instruments).

We compiled and aligned manually COI sequences using Sequence Navigator™ version 1.0.1 (Applied Biosystems). We obtained observed proportional sequence divergence (*p*-distance) and corrected sequence divergence (Kimura 2-parameter — K2p; Kimura 1980) in pairwise comparisons using the computer program PAUP*4.0b10 (Swofford 2002). *P*-distance versus corrected (K2p) estimates of proportional sequence divergence were plotted to test saturated of nucleotide substitutions (Fig. 1).

We performed the analyses using 14 new Nembrothinae sequences and two new outgroup sequences. We obtained additional sequences of two species of Nembrothinae and two outgroups from GenBank (Table 1). The outgroup species are members of the family Polyceridae (1 Triophinae + 2 Polycerinae) and another closely related phanerobranch slug. Table 1 shows the studied taxa, along with their locations of collection and the GenBank accession numbers for their sequences.

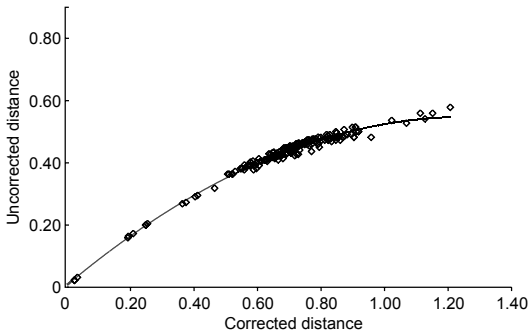

Fig. 1. Scatter plot of corrected *vs.* uncorrected genetic distances in the COI dataset.

We used Model Test 3.06 (Posada & Crandall 1998) to find the best model of evolution that fit the data for subsequent Maximum Likelihood analyses (ML: Felsenstein 1981, 1993) selected under the Akaike information criterion (AIC). The General Time Reversible (GRT) model of evolution with corrections for a gamma distribution and proportion of invariable sites was used for ML analyses (Yang 1994, Gu *et al.* 1995, Swofford *et al.* 1996). ML analyses with empirical base frequencies were performed using PAUP* 4.0b10 (Swofford 2002) via a heuristic search of 100 replicates of random addition sequence followed by tree-bisection-reconnection (TBR) swapping. We used nonparametric bootstrapping (100 pseudoreplicates) (bs) to assess the support of internal branches (Felsenstein 1985, Felsenstein & Kishino 1993). Bayesian phylogenetic analyses were conducted with MrBayes 3.0b (Huelsenbeck & Ronquist 2001). The same parameters used for the ML analysis were used for this analysis. Analyses were initiated with random starting trees and run for 2 000 000 generations. The Markov chains were sampled each 100 generations. Of the resulting 20 000 trees, 2500 were discarded as "burn-in".

We estimated parsimony (MP) phylogenies using the heuristic search algorithm for each tree-building methodology. We used 20 randomized input orders of taxa for all MP analyses in order to minimize the effect of entry sequence on the topology of the resulting cladograms. Parsimony analyses were conducted without the steepest descent option, and with accelerated character transformation (ACCTRAN) optimization, tree bisection-reconnection (TBR) branch swapping,

and zero-length branches collapsed to yield polytomies. We used nonparametric bootstrapping (1000 pseudoreplicates) to assess the support of internal branches in the resulting topologies (Felsenstein 1985, Felsenstein & Kishino 1993). Each base position was treated as an unordered character with four alternative states.

Results

Phylogenetic analyses

We analyzed eighteen sequences of 658 bp (plus two outgroup sequences from GenBank of 603 bp) of the cytochrome oxidase I (COI) gene, 227 characters were phylogenetically parsimony-informative. Sequence divergence (p) within the ingroup was as high as 20.7% (*Roboastra tigris* compared to *Nembrotha* cf. *rutilans*). The smallest divergence between two nominal species was from *Tambja abdere* to *T. fusca* (0.8%). The saturation plots of uncorrected sequence divergence against corrected sequence divergence divided by codon position indicated slight saturation at third position (first and second not shown, Fig. 1).

COI sequences used for this analysis have a typical "mitochondrial" behavior (Zhang & Hewitt 1996) in that most variable sites are in the third codon position as is typical for protein coding. Accordingly, molecular evolutionists have generally assumed that third codon positions are often regarded as less reliable than first and second positions as indicators of phylogeny. However, extensive evidence indicates that third positions are of great value precisely because of their high and differential rate (Yoder *et al*. 1996, Lewis *et al*. 1997, Björklund 1999, Källersjö *et al*. 1999, Baker *et al*. 2001).

The maximum likelihood analysis resulted in a tree ($\ln L = -4404.963$) where all samples of Nembrothinae form a monophyletic group with low bootstrap support (59 bs) (Fig. 2). Deeper nodes in the topology are poorly or not supported at all, basically yielding on a basal polytomy, which contains six well-supported clades. The genus *Roboastra* appears as a monophyletic group with moderated bootstrap support (bs 72). In this clade *R. tigris* and *R. europaea* are sister taxa (bs 68) with *R. luteolineata* basal to them. Even though the three clades containing all *Nembrotha* species are grouped together as a monophyletic clade, no bs support was obtained. *Nembrotha lineolata*, *N. chamberlaini* and *N.* cf. *rutilans* form a clade (bs 88) sister to *N. kubaryana*. *Nembrotha cristata* and *N. mullineri* are sister taxa with high bs value (bs 92). The monophyly of *Tambja* is not recovered. There are two clades including the species of *Tambja* that are not sister to each other. One clade consists of *T. abdere*, *T. fusca* and *T.* sp2 (bs 100), and the other of *T. eliora*, *T.* sp1, *T. ceutae* (bs 87) and *T. morosa* (bs 100).

Bayesian analysis resulted in a consensus tree (50% majority rule) with a topology in which the genus *Roboastra* (pp 100) and *Nembrotha* (pp 97) are monophyletic. In the Bayesian topology *Roboastra* is sister to the *T. morosa* clade of *Tambja* (pp 60), and the *Nembrotha* clade is sister to them (pp 53). Basal to all of them is the *Tambja abdere* clade (Fig. 3). The monophyly of Nembrothinae shows a high posterior clade probability (pp 99) although most of the basal clades have low posterior probability values.

The parsimony analysis including all positions and using equal weighting yielded a single tree (L = 898 steps; 227 characters were parsimony informative; CI = 0.463, RI = 0.566). The topology of the MP tree differs from the ML tree in that *Nembrotha* does not appear as a monophyletic group. However support for the basal nodes is very small, and the bs consensus tree shows a basal polytomy of five clades. The existence of a clade, although weakly supported (bs 61), including *N. kubaryana* as sister of the *N. cristata-mullineri* clade, is the only difference with the ML tree. The placement of the outgroups is identical to the ML tree. Analyses performed excluding third positions produced 35 equally parsimonious trees (L = 134 steps; 38 characters were parsimony informative; CI = 0.493, RI = 0.580). The strict consensus tree (not shown) is mostly unresolved.

Taxonomy and identification of problematic samples

The phylogenetic analyses show that there are two distinct clades within *Tambja* each including one of the juvenile specimens belonging to

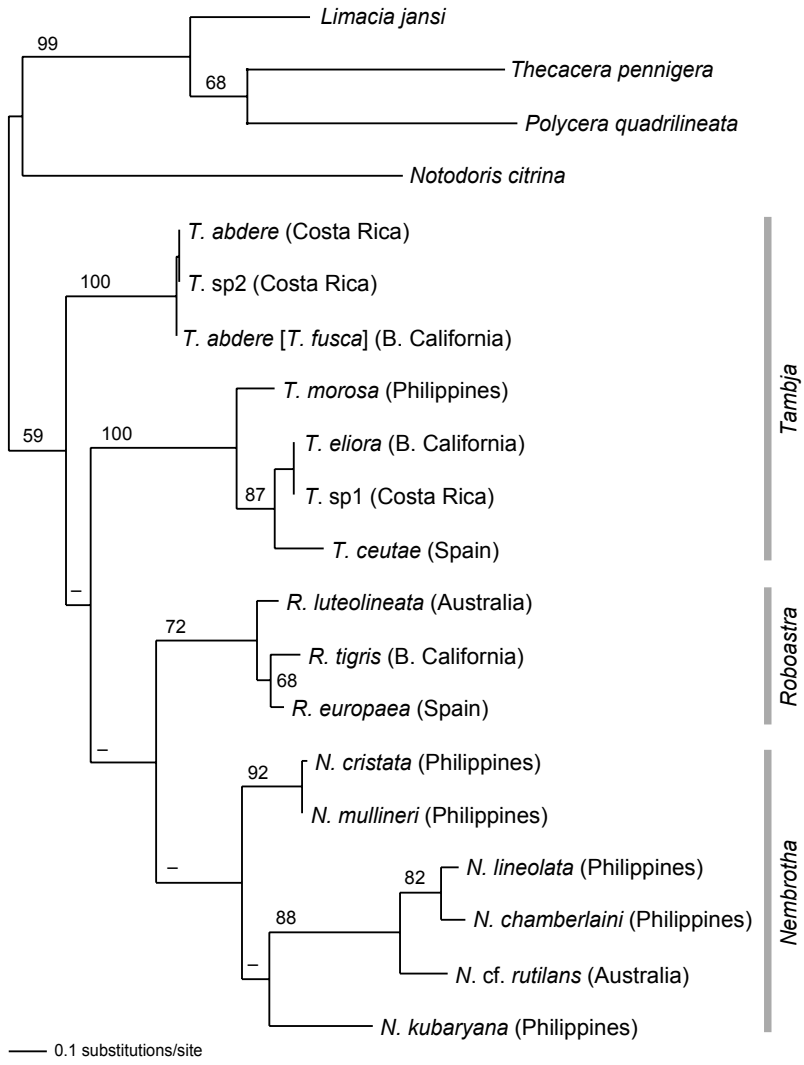

Fig. 2. Maximum likelihood analysis single tree ln*L* = –4404.963, obtained using the GTR + G + I model. Bootstrap probabilities are shown on internodes.

the potential new species. Our data show that *Tambja* sp2 from the Pacific in Costa Rica and our sample of *T. abdere* from Baja California have identical sequences despite their large geographic distance, suggesting that both might be members of the same species. *Tambja* sp2 could be perfectly assigned to the morphotype shown in Fig. 4C, since the ground color was turquoise blue, with yellow-ochre longitudinal lines but without yellowish patches between them. *Tambja* sp1 and *T. eliora* differ in seven nucleotides, which together with their sister relationship in the phylogenetic analyses suggests that *T.* sp1 is not a new species. Without further studies on the geographic variation of *T. eliora*, however, this problem is still unresolved.

Farmer (1978) described *T. abdere* and *T. fusca* as distinct species based on a relatively darker coloration of *T. fusca* as compared with the lighter color of *T. abdere*, and the first lateral tooth being more robust in *T. fusca* than in *T. abdere*. However, analysis of the radulae of the holotypes as well as other specimens shows that the robustness of the teeth is variable within species and thus is not an adequate diagnostic character.

These two named species differ from other species in the genus in showing an inner lateral tooth with a simple, wide, elongate and sharp cusp. The reproductive system is also very different from that of other species in the genus, with a bilobed bursa copulatrix and a very long

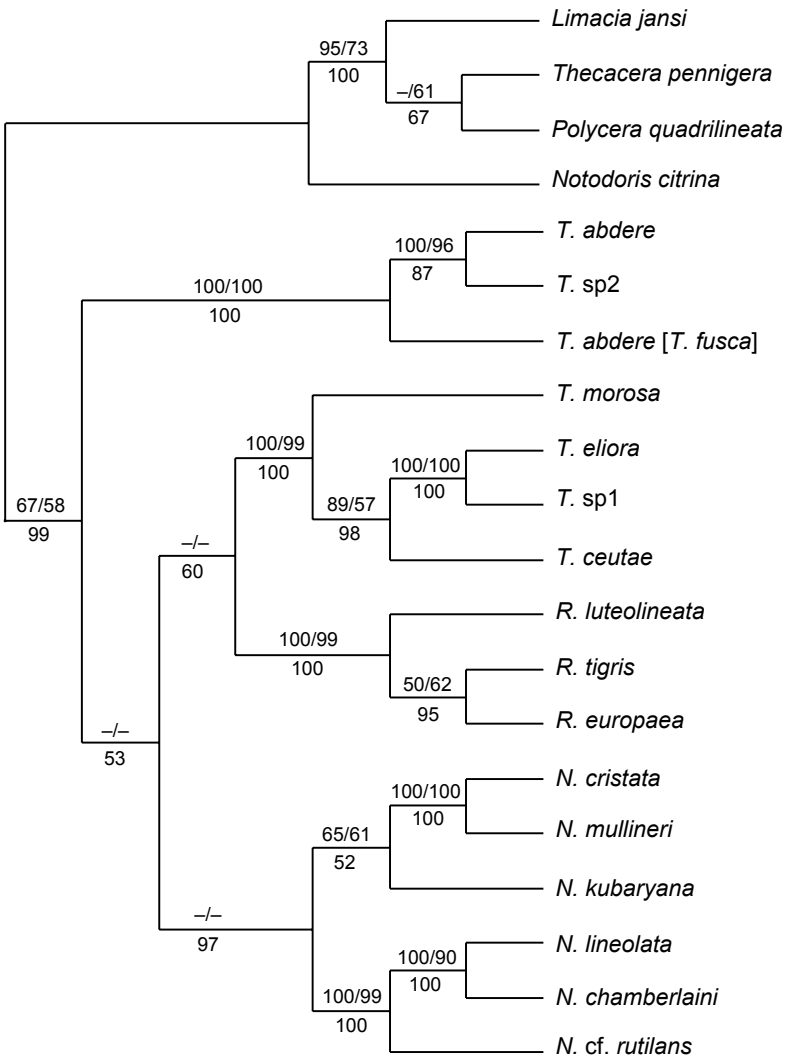

Fig. 3. Bayesian 50% majority rule consensus tree inferred from the partial nucleotide sequence of the mitochondrial COI. The numbers below branches represent Bayesian posterior probabilities (only values above 95% are considered statistically significant). The numbers above branches are bootstrap values corresponding to the NJ and MP analyses, respectively.

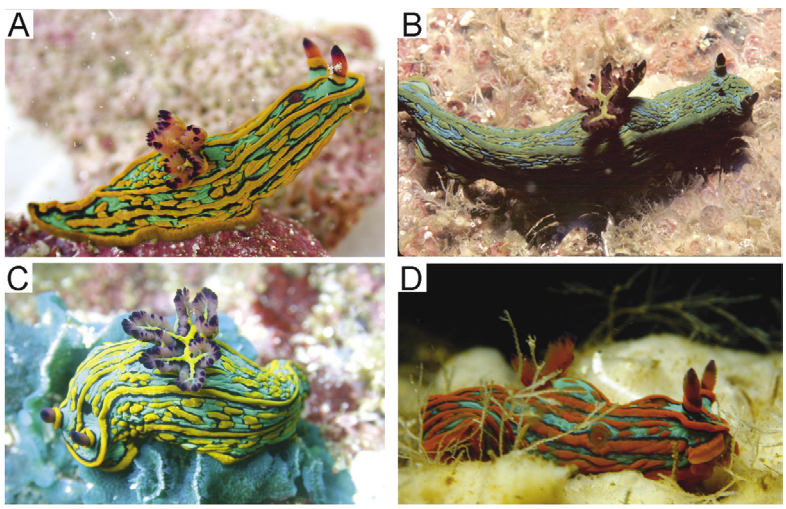

Fig. 4. *Tambja abdere*. Photographs of the living animals: **A**, **C**, **D** (pictures taken by Alicia Hermosillo): Specimens from Jalisco, Bahía Banderas, Baja California, México; **B** (picture taken by Mike Miller): Specimen from Bahía de Los Angeles, Baja California, México.

and coiled vagina. These two features seem to be autapomorphic for this taxon. There are three features that *T. abdere* and *T. fusca* share with two other species of the genus (*T. limaciformis* and *T. amakusana*): (1) the absence of a vaginal gland, (2) a prostate morphologically well differentiated from the rest of the vas deferens, and (3) the presence of a very long and coiled duct joining the bursa copulatrix and the seminal receptacle.

Tambja abdere and *T. fusca* differ in five nucleotid changes in third positions; all mutations resulted in synonymous substitutions. This variation is generally encountered among populations of a single species (Renard *et al*. 2000, Duran *et al*. 2004, Obst *et al*. 2005, Vörös *et al*. 2006). Given the morphological and molecular similarity between the studied samples of *T. abdere* and *T. fusca* we thought that they belong to a single taxonomic entity and therefore we are in agreement with the synonymy of *Tambja fusca* Farmer, 1978: 377, with *Tambja abdere* Farmer 1978: 378 (Behrens 2004). Nevertheless, further morphological and molecular studies of populations should be provided to support this synonymy.

Redescription of *Tambja abdere*

Species redescription follow the standard format for *Tambja* and include the same basic characters and measurements (Cervera *et al*. 2000).

Tambja abdere Farmer, 1978

Tambja abdere Farmer, 1978. The Veliger 20(4): 377, figs. 4–6.

Tambja fusca Farmer, 1978. The Veliger 20(4): 378, figs. 7–9.

CASIZ 687. Holotype (*Tambja abdere*), La Paz, Baja California, México, Leg. Edwin Janss. Microscope slide of radula.

CASIZ 688. Holotype (*Tambja fusca*), Isla Monserrate, Baja California, México, Leg. Edwin Janss, April, 1974. Microscope slide of radula.

CASIZ 074281. Gulf of California, Baja California, México, 3 specimens, 18 May 1985, 40–60 feet depth, collected by Lynn Dunne.

LACM 34800. Gulf of California, Mexico, 1 specimen, February 1978, 20 mm in lengths preserved, collected by Edwin Janss.

CASIZ 071669. Isla Coronados, Gulf of California, Baja California Sur, Mexico, 1 specimen, 30 June 1987, 45 mm in length preserved, 55 feet depth, collected by T. M. Gosliner, H. Bertsch and S. Millen.

LACM 34799. Isla de San Francisco, Gulf of California, Mexico, 3 specimens, February 1974, 20–30 mm in length preserved, collected by Edwin Janss.

INB0003118100. Bahía de Tamarindo, Costa Rica, 1 juvenile specimen, 2001, identifed by Terrence Gosliner. Used for DNA taxonomy.

MNCN 15.05/46742. Barco hundido, Gulf of California, Baja California, México, 1 specimen, March 2000, collected by H. Bertsch. Identifed as *Tambja fusca*. Used for DNA taxonomy.

MNCN 15.05/46658. Islas Marietas, Bahía Banderas, Baja California, México, 11 specimens, 9 June 2003, collected by A. Hermosillo.

MNCN 15.05/46659. Chimo, Bahía Banderas, Baja California, México, 2 specimens, 10 June 2003, collected by A. Hermosillo.

DISTRIBUTION. Pacific coast of Central America. To date, this species has been reported from Mexico (Farmer 1978, Kerstich 1989, Behrens 1991, Debelius 1996, Hermosillo-González 2003) and Costa Rica (INBIO 2003, Behrens 2004).

EXTERNAL MORPHOLOGY (Fig. 4). The body is elongate and limaciform, but robust and widest closer to the circle gill with a long and pointed posterior end of the foot. The head is rounded. The living animals reach 30 to 80 mm in total length with the body surface noticeably wrinkled. The pattern of longitudinal lines and patches is variable. The general pattern consists in a ridge that anteriorly extends around the head suggesting a mantle margin. This same ridge converges behind the gills to form a single line until the end of the tail. A wide longitudinal line circumscribes both sides of the body, surrounding the basal part of the oral tentacles and the mouth and ending in the posterior end of the foot and another band borders the foot. There is another wide mediodorsal stripe running from the rhinophores to the base of the gill. A variable number of irregular patches extend between these areas. There is a pair of large and conical, completely retractile, perfoliate rhinophores with approximately 20 to 25 tightly packed lamellae. The rhinophoral sheaths are well developed. The oral tentacles are short, dorso-ventrally flattened and horizontally grooved. There is a very distinctive translucent dark patch behind each rhinophore above where the eye is situated on the nerve ring. There are five non-retractile bipinnate gill branches. These form a semicircle surrounding the tubular anal papilla. The genital pore opens on the right

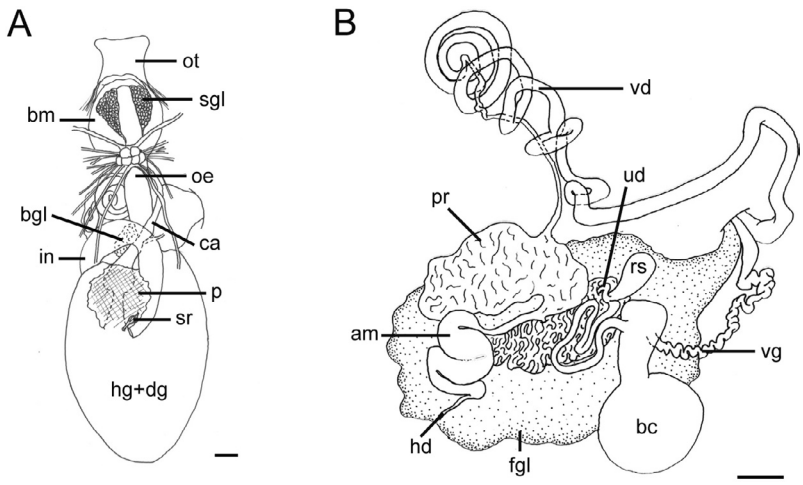

Fig. 5. *Tambja abdere*. — **A**: General arrangement of the internal organs. — **B**: Reproductive system. Scale bar: 1 mm. Abbreviations: am, ampulla; bc, bursa copulatrix; bm, buccal mass; bgl, blood gland; ca, cephalic artery; fgl, female gland; hd, hermaphroditic duct; hg + dg, hermaphrodite gland + digestive gland; in, intestine; oe, oesophagus; ot, oral tube; p, pericardium; pr, prostate; rs, renal syrinx; sgl, salivary glands; sr, seminal receptacle; ud, uterine duct; vd, vas deferens; vg, vagina.

side, midway between the gill and rhinophores. The lateral slots located between the rhinophores and the oral tentacles present in other species of *Tambja* (Yonow 1994, Pola *et al*. 2005b, 2005c) appear to be absent in this species.

The coloration of this species is very variable (Fig. 4). The basic body coloration consists of turquoise blue-green, with mottled patches of yellowish and bluish ochre splattered throughout and separated by black lines but this color pattern is not quite orderly in some specimens. The rhinophoral sheaths, the oral tentacles, the inner and outer rachis of the gills and the foot are yellow-ochre. The rhinophores and the gill branches are translucent pinkish purple or blue-black with darker tips. The eye-spots have a similar color to the latter. In some specimens reported from Baja California, the longitudinal lines and patches, the rhinophores, the oral tentacles, the gill and the foot were orange-red in color (Fig. 4D).

INTERNAL MORPHOLOGY (Fig. 5A). The anterior digestive tract begins with a short muscular oral tube that continues into the buccal mass. The buccal mass is bigger than the oral tube. There is a pair of large, wide salivary glands on the buccal mass, flanking the oesophagus. The elongated pouches at the junction of the buccal mass and the oral tube present in other species of *Roboastra* (Pola *et al*. 2003, 2005a) are not visible in this species. The chitinous labial cuticle is brown, thick, and smooth (Fig. 6A). The radular formula is 16 × 4.1.1.1.4 in two 30 mm long specimens (preserved) (MNCN 15.05/46658), 16 × 4.1.1.1.4 in one 45 mm long specimen (preserved) (CASIZ 071669) and 14 × 4.1.1.1.4 in three 20–30 mm long specimens (preserved) (LACM 34800) (Fig. 6B). The rachidian tooth is quadrangular, without denticles and very slightly notched at the anterior edge. The inner lateral tooth is elongate with two well-developed wide cusps. The inner cusp is simple, with a wide, elongate and sharp denticle. The inner edge of this tooth is smooth. The outer cusp is also well developed and quadrangular in shape. The outer lateral teeth are roughly rectangular, without denticles and decrease in size towards the outer margin. There is a blood gland located near the reproductive system, below the intestinal loop. The renal syrinx is visible under the pericardium, close to the anal papilla.

The reproductive system is triaulic (Fig. 5B). The hermaphroditic duct is short and narrow. It expands into a narrow highly convoluted ampulla, which divides into the oviduct and the vas deferens. At this point the oviduct enters the massive female gland. The branch of the vas deferens widens into a large, curved prostatic

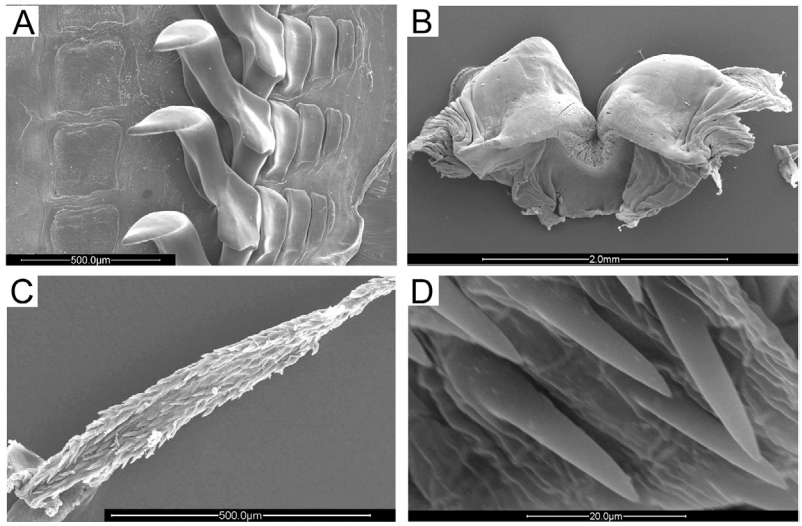

Fig. 6. Scanning electron micrographs of *Tambja abdere* (MNCN 15.05/46659). — **A**: Right half of the radula. — **B**: Labial cuticle. — **C**: Penis. — **D**: Detail of penial spines.

portion, which has a dense network of interconnecting tubules over its surface. The vas deferens narrows into a very thin duct, which descends through the center of the large and highly convoluted final portion of the vas deferens. The penis is located in this latter portion. The penial spines are numerous and seem to be arranged in longitudinal lines. They are relatively small, elongated and sharp (Fig. 6C and D). There is no prominent penial bulb. The bursa copulatrix has two well-differentiated portions, a large and oval distal portion and a small bulbous proximal portion. The smaller pyriform seminal receptacle joins the small bulbous portion of the bursa copulatrix via a long and coiled duct. The elongate uterine duct emerges from the female gland mass and joins the seminal receptacle. An elongate, wide and coiled vagina is also connected to the proximal portion of the bursa copulatrix at the opposite side of the seminal receptacle and joins the vas deferens near the genital aperture. There is no vaginal gland. The female gland is well developed. *In situ*, the bursa is partially surrounded by the prostate.

Discussion

Since the original description of *T. abdere* and *T. fusca* (Farmer 1978) the taxonomic status of both species has been controversial. Some authors have treated them as color variants of a single species (W. B. Rudman unpubl. data) and now they are considered synonyms (Behrens 2004), but this hypothesis has never been tested. There is no significant measure of the genetic differences that separate species (Renard *et al.* 2000), however, the good agreement between our morphological and molecular analyses seems to point toward a clear taxonomic identity of these two forms. *Tambja abdere* is thus a polymorphic species.

Taxonomic identification of juvenile specimens is problematic in Nembrothinae (Sánchez-Tocino *et al.* 2000). Species diagnosis in this group is based mainly on anatomical features including the morphology of the reproductive system and the radula. Coloration is also frequently used as a distinctive trait (Schick & Cervera 1998, Cervera *et al.* 2000), however, coloration is not a reliable character, not only because of intraspecific polymorphisms (*see* above) but also because patterns change during the development of the species (Sánchez-Tocino *et al.* 2000). Our molecular data allowed us to identify with precision one of the two juvenile specimens from Costa Rica, previously treated as putative new species (Medina *et al.* 2001), as *Tambja abdere*. In the results of the different analyses Nembrothinae appears as a monophyletic taxon although with low support (Bayesian posterior clade probabilities excepted) (Figs. 2 and 3). Given our limited sampling these results should be treated as preliminary.

Within Nembrothinae, the monophyly of *Roboastra* is generally well supported, which is

congruent with previous morphological analyses (Pola *et al*. 2005a). *Nembrotha* is monophyletic in most of the analyses. *Tambja* seems not to be a monophyletic group. Relationships among *Roboastra*, *Nembrotha* and the two clades of *Tambja* are basically unresolved. This could be either a consequence of the limited sampling, saturation of the COI sequences for this level of analysis (Fig. 1) or the biological effect of a rapid radiation within Polyceridae. The effect of taxon sampling or rapid radiation cannot be tested in this preliminary paper.

We have shown that a thorough re-analysis of types combined with a detailed redescription of morphological characters, the examination of additional specimens (to gain a better appreciation of character variation), and the addition of molecular characters is a fruitful endeavor that yields stronger results for both phylogenetic inference and taxonomic assignment. The examples used here from the Nembrothinae illustrate the importance of an integrative approach to taxonomy.

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