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Invertebrate Systematics, 2010, **24**, 616–629

Phylogenetic placement of a new hoplonemertean species commensal on ascidians

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Abstract. The hoplonemertean *Vieitezia luzmurubeae*, gen. et sp. nov. is described from specimens collected in a national park on the north-west Iberian Peninsula, the Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia. The species, previously mistaken as the Mediterranean species *Tetrastemma vittigerum* (Bürger, 1904), is frequently associated with two common species of sea squirt, *Phallusia mamillata* (Cuvier, 1815) and *Ciona intestinalis* (Linnaeus, 1767), inside which the nemertean completes its life cycle. Some of the specimens examined were protandrous hermaphrodites. Data on morphology and anatomy are provided with illustrations. Sequences of the nuclear ribosomal gene 18S rRNA and the mitochondrial gene cytochrome *c* oxidase subunit I were compared with those of other hoplonemertean species and all phylogenetic analyses suggested that *Vieitezia* is sister to the genus *Gononemertes*, which parasitises ascidians, within a clade also containing the genera *Oerstedia* and *Nemertellina*. In contrast, the morphologically similar genus *Tetrastemma* appears in a separate clade. This study stresses the need for combining molecular and morphological data when studying nemertean biodiversity.

Additional keywords: COI, Galicia, 18S rRNA, phylogeny, *Vieitezia luzmurubeae*, gen. et sp. nov.

Introduction

The taxonomy of the members of the phylum Nemertea is among the most difficult of all animal groups. Species have traditionally been described and classified based on a combination of internal anatomy, obtained through histological studies of serially sectioned animals, and external morphological characters (Schwartz and Norenburg 2001; Sundberg *et al*. 2009; Strand and Sundberg 2011). However, the quality of descriptions varies enormously with authors and the important characters are often vaguely described. In addition, old descriptions are often ambiguous and most of the earliest species described lack type material or the types are too poorly preserved for taxonomic purposes. Recently, Sundberg *et al*. (2009) proposed a standardised checklist of characters that could be used for describing species of nemerteans, following modern approaches of establishing a morphological ontology (e.g. Ramírez *et al*. 2007). Modern studies now combine morphological and molecular data for characterising or describing species of nemerteans (e.g. Sundberg *et al*. 2009; Puerta *et al*. 2010), as is commonly done with many other groups of animals. Others have attempted to use external morphology and pigmentation alone, but several groups have shown clear cases of convergence, for example in the genus *Tetrastemma* (see Strand and Sundberg 2005). Even so, the combined use of external morphology and DNA sequence data of selected markers has been viewed as an efficient way to describe new nemertean species (Strand and Sundberg 2011).

During this investigation of the nemertean fauna of the Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia (north-west Spain) a new hoplonemertean species was discovered living among sublittoral algae and sediment, and also inside the atrium of two solitary ascidians, *Phallusia mamillata* (Cuvier, 1815) and *Ciona intestinalis* (Linnaeus, 1767). These specimens were previously identified as *Tetrastemma vittigerum* (Bürger, 1904) by Junoy and Herrera-Bachiller (2010), because their morphology resembles that described by Oxner (1907) for other Atlantic specimens originally assigned to this species. Here we describe these specimens as a new species and combine traditional methods of internal anatomy with the modern approaches suggested by Strand and Sundberg (2011), including DNA sequence data for two targeted genes, with the aim of testing the phylogenetic placement of the species and providing a DNA barcode that could be used for rapid assessment of other specimens.

Materials and methods

Specimens of a new species of nemertean were collected by scuba diving at the Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia and adjacent areas on the north-west Iberian Peninsula, among sublittoral algae and sediment. Additional specimens were collected inside the atrium of two species of solitary ascidians, *Phallusia mamillata* and *Ciona intestinalis.* The nemertean specimens were examined alive both before and after anaesthetisation in 7.5% $MgCl_2$ and then

10.1071/IS10036 1445-5226/10/060616

fixed in Bouin's fluid, embedded in paraffin wax, sectioned at 6 µm and subsequently stained by the Mallory trichrome method for histological examination. All specimens have been deposited in the Department of Invertebrate Zoology of the Museum of Comparative Zoology (MCZ), Harvard University.

Molecular analyses

Total genomic DNA was extracted from two ethanol-fixed specimens (paratype voucher number MCZ DNA104801) using the DNeasy kit (Qiagen, Valencia, CA), following the protocol described in the manual. The nuclear 18S rRNA was amplified by the 1F/5R, 3F/18Sbi and 18Sa2.0/9R primer pairs (Giribet *et al*. 1996; Whiting *et al*. 1997). The mitochondrial protein-encoding gene cytochrome *c* oxidase subunit I (COI) was amplified using the primer pair LCO1490/HCO2198 (Folmer *et al*. 1994). A 25 µL amplification reaction was performed using illustra PuReTaq Ready-to-GoTM PCR beads (GE Healthcare LifeSciences, Piscataway, NJ). Thermal cycling was initiated with 2 min of denaturation at 94°C followed by 35 cycles of 30 s at 94°C, annealing (ranging from 42 to 46°C) for 1 min, and extension at 72°C for 1 min. After cycling, the reaction was completed with an extension phase at 72°C for 10 min and the reaction products were visualised in a 1% agarose gel and purified through enzymatic reaction with ExoSAP-IT (USB Corporation, Cleveland, OH). The purified PCR products were sequenced directly with the same primer pairs used for amplification. Each sequence reaction contained a total volume of 10 µL including 1.5 µL PCR product, 1 µM PCR primer, 0.25 µL ABI BigDye 5× sequencing buffer, and 0.5 µL ABI BigDye Terminator v3.0 (Applied Biosystems, Foster City, CA). The sequencing reactions involved an initial denaturation step for 3 min at 95°C, and 25 cycles (95°C for 10 s, 50°C for 5 s, and 60°C for 4 min). The BigDye-labelled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, MD) and the sequencing reaction products were analysed using an ABI Prism® 3730 Genetic Analyzer (Applied Biosystems).

Sequence analyses

Chromatograms were edited and overlapping sequence fragments were assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Blast searches (Altschul *et al*. 1997), as implemented in the NCBI website (http://www.ncbi.nlm.nih.gov/), were conducted to check for putative contaminations. All new sequences have been deposited in GenBank (see accession numbers in Table 1).

Alignment and phylogenetic analyses

Multiple sequence alignment of the 18S rRNA data for the new species and selected hoplonemerteans (Tetrastemmatidae *sensu* Thollesson and Norenburg 2003) (Table 1) was performed using MAFFT ver. 6 using the strategy G-INS-i (Katoh *et al*. 2005). Direct optimization (Wheeler 1996) was also used as a criterion for assigning homology. The hoplonemerteans *Geonemertes pelaensis* and *Malacobdella grossa* were chosen as putative outgroups in all analyses.

Table 1. List of species included in the phylogenetic analysis with GenBank accession numbers

Accession numbers for the new sequences of *Vieitezia luzmurubeae* in bold

Species	COI	18S rRNA
***Vieitezia luzmurubeae* sp. nov. 1**	–	**HQ443427**
***Vieitezia luzmurubeae* sp. nov. 2**	**HQ443426**	**HQ443428**
Tetrastemma candidum (O. F. Müller, 1774)	AY791973	AY928357
Tetrastemma coronatum (Quatrefages, 1846)	AY791975	AY928358
Tetrastemma flavidum Ehrenberg, 1828	AY791977	AY928359
Tetrastemma laminariae Ushakov, 1928	AY791980	AY928360
Tetrastemma longissimum Bürger, 1895	AY791981	AY928362
Tetrastemma melanocephalum (Johnston, 1837)-1	AY791986	AY928363
Tetrastemma melanocephalum-2	AY791982	AY928364
Tetrastemma peltatum Bürger, 1895	AY791990	AY928371
Tetrastemma robertianae McIntosh, 1874	AY791994	AY928372
Tetrastemma vermiculus (Quatrefages, 1846)	AY791995	AY928377
Tetrastemma sp. 2	–	AY928374
Tetrastemma sp. 3	–	AY928375
Tetrastemma stimpsoni Chernyshev, 1992	–	AY928376
Prostoma graecense (Böhmig, 1892)-1	EF208981	AY039666
Prostoma graecense-2	EU489490	AY928355
Prostoma eilhardi (Montgomery, 1894)	–	U29494
Cyanophthalma obscura (Schultze, 1851)	EF208980	AY039667
Nemertellina yamaokai Kajihara, Gibson & Mawatari, 2000	AJ436907	AB505826
Oerstedia dorsalis (Abildgaard, 1806)-1	AY791971	AY210448
Oerstedia dorsalis-2	FJ855295	AY928353
Oerstedia striata Sundberg, 1988	AY791972	AY928354
Gononemertes parasita Bergendal, 1900	AB505825	AB505824
Outgroups		
Geonemertes pelaensis (Moore, 1975)	EU255602	EU255578
Malacobdella grossa (Müller, 1776)	AJ436905	AY039670

The MODELTEST 3.06 (Posada and Crandall 1998) module in HyPhy (Pond *et al*. 2005) was used to choose the best-fit model of molecular evolution for our dataset under the Akaike information criterion (Akaike 1974). The test suggested the general time reversible model (GTR) as best fitting the data for both nuclear and mitochondrial genes with a discrete Gamma distribution to model rate heterogeneity (GTR + G). Maximum likelihood analyses were performed with Randomized Axelerated Maximum Likelihood (RAxML) v. 7.0.4 (Stamatakis 2006; Stamatakis *et al*. 2008) using the sequences obtained for *V. luzmurubeae*, gen. et sp. nov. and sequences of tetrastemmatids plus outgroups from GenBank (Table 1). Cytochrome *c* oxidase subunit I and 18S rRNA were analysed independently and concatenated. The search for optimal maximum likelihood (ML) trees was performed on the cluster computing facility from the Faculty of Arts and Sciences located at Harvard University. The ML tree search was conducted by performing 200 distinct runs using the default algorithm of the program for random trees (option −d) as a starting point for each run. The final tree was determined by a comparison of likelihood scores under the GTR+G model among suboptimal trees obtained per run. One thousand fast bootstrap replicates were conducted to evaluate nodal support. Bootstrap values ≥70% were considered to indicate strong support, given that bootstrap values appear to be biased but are conservative measures of phylogenetic accuracy (Felsenstein 2004).

A direct optimization analysis was also conducted in the computer program POY v. 4.1.2 (Varón *et al*. 2010) for the independent and simultaneous analysis of both markers, examining alternative parameter sets (for non-linear indel costs; indel opening = 3, indel extension = 1; and two alternative transversion/transition ratios of 2 and 1 (parameter sets 3221 and 3211)). Searches were run in a timed search of one hour performing multiple Wagner additions with subtree pruning and regrafting (SPR) and tree bisection reconnection (TBR) branch swapping, tree fusing and ratchet. Nodal support was evaluated with 100 jackknife replicates (Farris *et al*. 1996) using the command auto_sequence_partition.

Taxonomy

Genus ***Vieitezia***, gen. nov.

Diagnosis

Monostiliferous nemertean with four eyes arranged at the corners of a trapezoid; one cephalic furrow that divides before continuing as the ciliated canal; cerebral organs lying alongside/close to anterior part of brain; dorso–ventral muscles present; rhynchocoel extending to posterior end of body; apical organ present; with a paired structure related to the apical organ; cephalic glands well developed, extending far behind brain; lateral nerve cords without accessory nerves; mid-dorsal blood vessel without vascular plug; intestinal caecum present, with anterior diverticula; protandrous hermaphroditic.

Species included

Vieitezia luzmurubeae, sp. nov., the type species of the genus.

Phylogenetic remarks

The genus is sister to the represented specimens of the genus *Gononemertes* (see results below) and nested within a clade that also includes the genera *Oerstedia* and *Nemertellina*. It is not directly related to the species of the genus *Tetrastemma*, as thought originally based on morphology alone. It is also probably related to other genera lacking a vascular plug, *Antarctonemertes*, *Tetraneuronemertes* and *Vulcanonemertes*.

Etymology

Named after Professor José Manuel Viéitez for his dedication and contribution to the knowledge of marine biology of the Galician coasts and his long-term friendship to J. Junoy.

Vieitezia luzmurubeae, sp. nov.

(Figs 1, 2, Table 2, Appendix 1)

Tetrastemma vittigerum Junoy & Herrera-Bachiller, 2010.

Material examined

Holotype. Specimen in male phase, series of transverse sections (MCZ 99504) Pedras da Praia da Catía (42°31.50′N, 9°0.60′W), Aguiño, Ría de Arousa, 29.viii.2005, collected inside *Phallusia mamillata*, 1–2 m deep.

Paratypes. Specimen in female phase, series of transverse sections (MCZ 99505), same data as holotype; specimen in male phase, series of longitudinal sections (MCZ 99506), same data as holotype; specimen in female phase, series of longitudinal sections (MCZ 99507), same data as holotype. Two specimens in 96% EtOH (MCZ DNA104801) for molecular study, from mussel raft, Ría de Arousa (~42°31′N, 8°59′W), 8 m deep, 6.viii.2009.

Other material examined. Praia de Abra (42°9.30′N, 8°49.80′W), Ría de Vigo, 13.vii.2005, two specimens from algae, 1 m deep; Faro Island (42°17.75′N, 8°54.41′W), Cies Islands, Ría de Vigo, 15.vii.2005, one specimen, from gravel and shell sediment, 5 m deep; Pedra dos Bois (42°28.94′N, 9°00.52′W), Sálvora Island, Ría de Arousa, 26.viii.2005, 17 specimens, from algae and sediment, 5–7 m deep; Pedras da Praia da Catía (42°31.50′N, 9°0.60′W), Aguiño, Ría de Arousa, 29.viii.2005, 70 specimens from 41 *Phallusia mamillata* specimens, 1–2 m deep (sectioned specimens: MCZ 99508 female, MCZ 99509 two males, MCZ 99510 male, MCZ 99511 male, MCZ 99512 male); Pedras da Praia da Catía (42°31.50′N, 9°0.60′W), Aguiño, Ría de Arousa, 13.ii.2009, 18 specimens from 21 *Phallusia mamillata* specimens, 1–2 m deep; mussel raft, Ría de Arousa (~42°31′N, 8°59′W), 6.viii.2009, 17 specimens from six *Ciona intestinalis* specimens, 8 m deep.

Diagnosis

As for the genus; with four brown dorsal longitudinal bands (Fig. 1*A*); the two median bands extending to the anterior of the head before anastomosing; the two lateral bands interrupted between the eyes, not anastomosing at the tip of the head (Fig. 1*B*).

Description

External features. Most specimens examined alive, before or after anaesthetisation, were 5–20 mm long and 0.2–0.5 mm wide. Body of uniform width throughout its length, tapering only near the posterior, ending in a blunt tip (Fig. 1*A*). In histological sections the anterior of the head appears trilobed, with two small shallow furrows that converge at the apical organ. A paired structure related to the apical organ was also observed by Gibson (1974) in the commensal hoplonemertean *Gononemertes australiensis*. The bluntly pointed head is demarcated from the rest of the body by a pair of cephalic furrows, ventrally V-shaped and laterally oblique (Fig. 1*A*, *B*, *E*).

Four dark eyes arranged at the corners of a trapezoid, with the shorter parallel side anterior (Fig. 1*B*). Body generally pale yellow with four dorsal, brown longitudinal bands. Two median bands extend to the anterior of the head before anastomosing; the lateral bands interrupted between the eyes, not anastomosing at the tip of the head. Some variation to this pattern exists; the female phase appears darker than the male phase; in some specimens the brown bands are wider than in others, probably in relation to age and sexual phase. Other specimens present irregular dark spots at each side of the head (Fig. 1*B*), resembling *Tetrastemma quadristriatum* as represented by Langerhans (1880: fig. 67).

Body wall and musculature. Epidermis mostly 25–35 µm thick with typical hoplonemertean arrangement. Dense accumulations of pigment granules located at the base of epidermal cells correspond with the four dorsal brown bands. This pigment is also situated below the body wall longitudinal muscle layer, in the two dorsal central lines (Fig. 2*G*). The difference in the localisation of pigment between the central and lateral bands was also observed by Oxner (1907: 84).

Body wall with outer circular and inner longitudinal muscle layers, respectively 5–7 µm and 25–40 µm thick. Longitudinal muscle fibres reach the tip of the head, intermingling among the

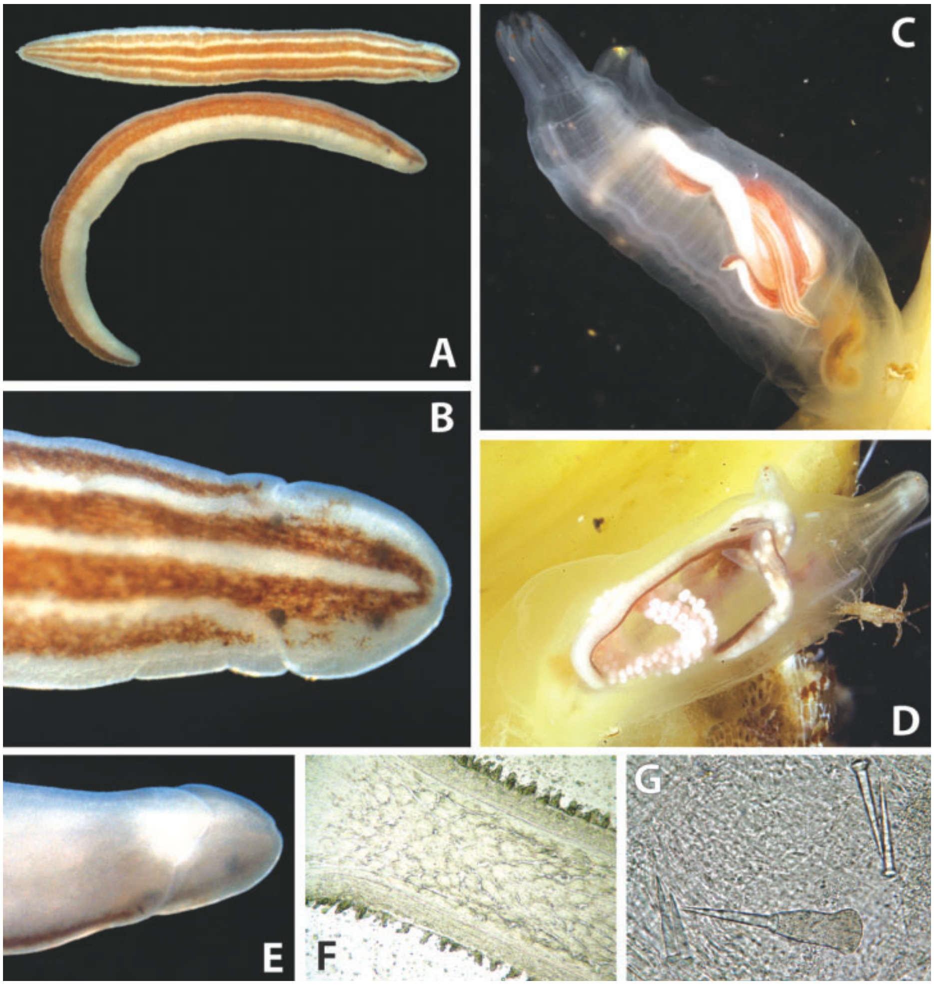

Fig. 1. *Vieitezia luzmurubeae*, gen. et sp. nov. (*A*) Anesthetised specimens showing the body form and colour pattern; (*B*) detail of the cephalic region in dorsal view to show the head of an anesthetised specimen; (*C*) a female (darker specimen) and a male inside the atrium of *Ciona intestinalis* (photo: Jacinto Pérez, Hydronauta); (*D*) two specimens inside the atrium of *Ciona intestinalis*, one female laying eggs and a presumed male (photo: Jacinto Pérez, Hydronauta); (*E*) detail of the cephalic region in ventro-lateral view showing the cephalic furrow in an anesthetised specimen; (*F*) everted proboscis showing the epithelium developed into papillae; (*G*) stylet apparatus showing central stylet and the two accessory pouches containing two stylets each.

lobes of the cephalic glands. In front of the cerebral ganglia bundles of fibres lead inwards from the longitudinal muscle layer to the proboscis insertion; these comprise the pre-cerebral septum, which is the closed type. Dorso–ventral muscles across the body, behind the brain, and between the lateral gut diverticula throughout the intestinal region (Fig. 2*G*).

Proboscis apparatus. Proboscis apparatus similar to that of most other monostiliferous hoplonemerteans. Epithelium of the proboscis developed into papillae (Fig. 1*F*). Ten proboscidial nerves situated between the longitudinal muscle fibres of the proboscis anterior portion, connected by a peripheral neural sheath (Fig. 2*F, H*). Measurements of the stylet apparatus (Fig. 1*G*) of seven specimens shown in Table 2.

Alimentary canal. Stomach apparently emerges directly from the rhynchodaeum, but in some specimens a short oesophagus was observed. As the oesophagus is sometimes difficult to detect, the hoplonemertean genus *Oerstedia* was characterised as lacking an oesophagus (Stiasny-Winjhoff 1930; Kirsteuer 1963; Gibson 1982) until Envall and Sundberg (1993) clarified the situation. A large fraction of the stomach is located in front of the brain (Fig. 2*A, C, D*); this location of the stomach may be due to the maturity of the specimens examined and the fixation process. The pylorus, longer than the stomach, opens into dorsal wall of intestine. Intestinal caecum bears two short anterior pouches.

Circulatory system. Consists of paired lateral and single mid-dorsal vessels, which are transversely joined only by cephalic and caudal loops. The mid-dorsal blood vessel emerges as a branch of one of the lateral vessels, most often the left, in the cerebral region. No vascular plug was observed.

Nervous system. Brain well developed, the dorsal and ventral lobes approximately the same size. A thin outer

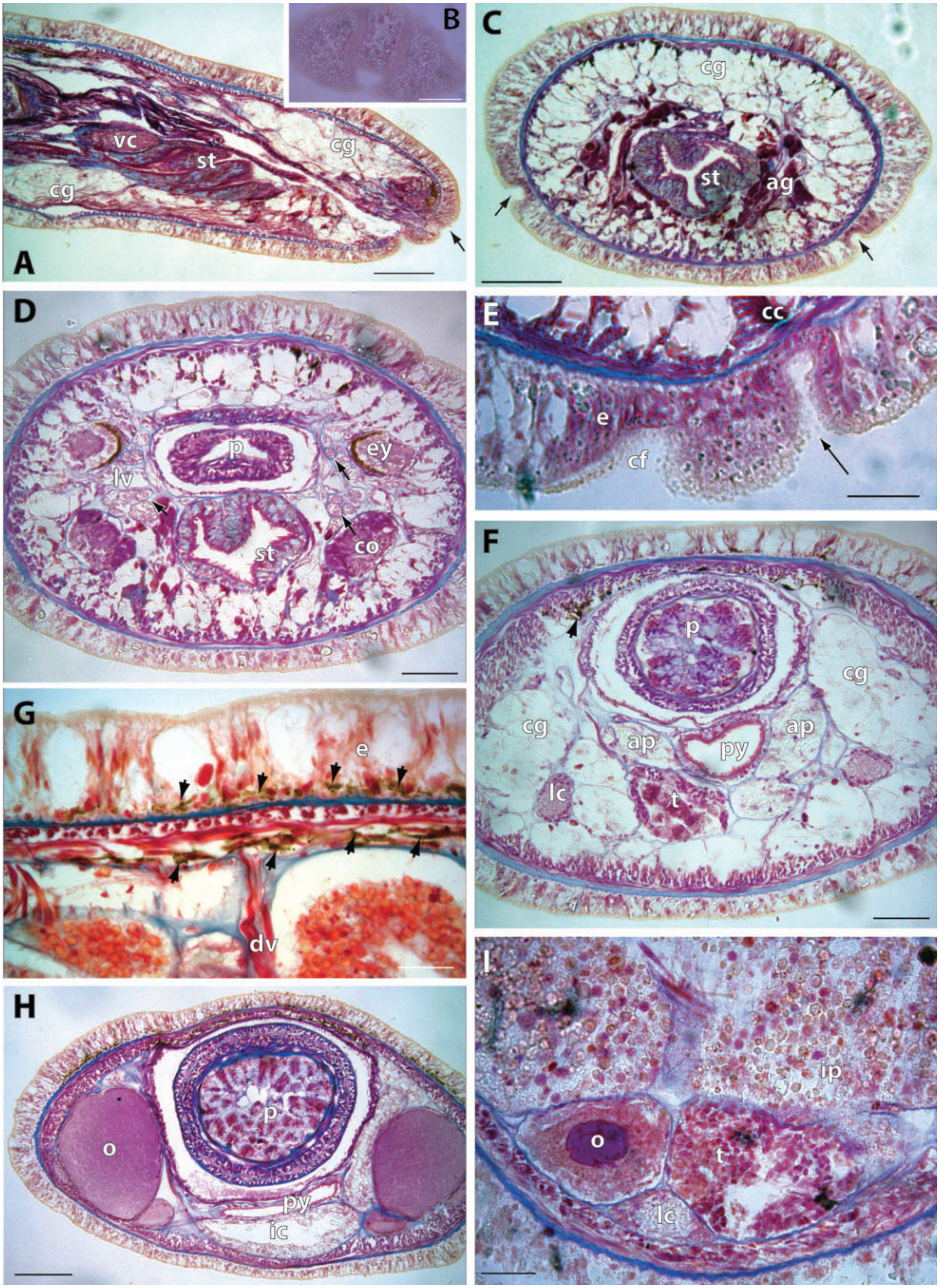
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Table 2. Measurements recorded for the stylet apparatus of seven specimens of *Vieitezia luzmurubeae*, gen. et sp. nov.

	Range	Average
Length of central stylet (μm)	40–96	70.9
Length of basis (μm)	32–88	73.1
Maximum width of basis (μm)	16–36	27.4
Ratio of basis length to central stylet length	0.8–1.3	1.03
Ratio of basis length to basis width	2–3.1	2.6
Number of accessory stylet pouches	2	2
Number of reserve stylets per pouch	2–4	3

neurilemma encloses the brain as a whole, but no inner neurilemma separating the fibrous and ganglionic cerebral components could be distinguished. Dorsal cerebral commissure, 17–30 μm thick, positioned forward of the thicker (30–45 μm) ventral commissure. The lateral nerve cords throughout their length contain only a single neuropile, and there are no accessory lateral nerves. These nerve cords are transversely joined via a supraintestinal commissure. There are neither neurochords nor neurochord cells in the nervous system. Several cephalic nerves lead forward from the anterior borders of the brain lobes (Fig. 2*D*).

Excretory system. Well developed, confined to the post-cerebral region of the body. Consists of two pairs of thick-walled longitudinal collecting tubules, running close to the lateral nerve cords, which open by ventro-lateral nephridiopores in the pyloric region. No flame cells were observed.

Apical organ and cephalic glands. A small single apical organ opens ventro-terminally in front of the proboscis pore (Fig. 2*A*); maximum diameter of ~50 μm; at the very tip of the head, two small lines are confluent to the apical organ, which could represent two rows of bristles observed in live specimens (Fig. 2*B*). Typical basophilic lobules form the most abundant type, occupying much of the cephalic space; posteriorly they extend far behind cerebral ganglia, reaching the gonads (Fig. 2*A, C, D, F*). Acidophilic glands irregularly distributed between the basophilic lobules in the anterior part of head; they concentrate around the foregut, mainly in a lateral position (Fig. 2*C*).

Sensory structures. Two longitudinal rows of sensory cirri are observed at the tip of the head. Four eyes arranged at the corners of a trapezoid. Eyes formed of typical pigmented cup ocellus-type, up to 45 μm in diameter, situated 30–50 μm beneath the epidermal basement layer. The cerebral sensory organs open ventro-laterally from a short bifurcation of the cephalic furrow, via thick-walled ciliated canals. The cerebral organs, 17–50 μm, are located entirely behind the pre-cerebral septum.

Reproductive system. The species is protandrous hermaphroditic; gonads are serially disposed along the body from the back of the cephalic glands backwards, lying dorso-laterally between the diverticula of the intestine and its caecum (Fig. 2*F, H*). The holotype is a mature male, with ~50–60 ripe testes situated in two rows, but the final gonad has also an oocyte, a situation also detected in other sectioned specimen (Fig. 2*I*). The change from testis to ovarium starts at the tail; none of the specimens sectioned had both ripe testes and ripe ovaria. Specimens collected during July and August were sexually mature; frequently several males rode on the back of the female in the ascidian atrium (Fig. 1*C*). Gonoducts open ventro-laterally. Each ovary typically contains a single oocyte, 135–240 μm in diameter, in which the nucleus is 60–75 μm across. Diameter of mature testes is 140–180 μm. A female laying eggs together with a presumable male was observed inside a *Ciona intestinalis* specimen in July 2009 (Fig. 1*D*). A similar observation was recorded by Joubin (1890, 1894) for *Amphiporus vittatus:* '... la trouve en abondance avec ses pontes au mois de juillet dans la branchie de la *Phallusia sanguinolenta*'.

Distribution

Known from multiple localities in the Ría de Vigo and Ría de Arousa, in the Galician provinces of Pontevedra and A Coruña (Spain).

Habitat

Vieitezia luzmurubeae, gen. et sp. nov., lives subtidally both free between algae and sedimentary bottoms, and inside the atrium of the solitary ascidians *Phallusia mamillata* and *Ciona intestinalis.* Of 20 specimens of *Phallusia mamillata* collected at Aguiño (Ría de Arousa, north-west Spain) in September 2005, four specimens (20%) yielded a total of six nemerteans, with up to three specimens occurring in any one host. Of 35 specimens of the same ascidian species collected by trawling (60 m deep) in the CALMEN07 expedition in the north-east of Mallorca Island (Balearic Islands, Mediterranean, July 2007), none contained nemerteans (J. Junoy, pers. obs.). At the Ría de Arousa, specimens have also been collected inside

Fig. 2. *Vieitezia luzmurubeae*, gen. et sp. nov. (*A*) Paratype (MCZ 99506), longitudinal section showing the apical organ (arrow), the foregut and the extension of the cephalic glands; (*B*) holotype (MCZ 99504), transverse section close to the tip of the head showing the trilobed shape and the two rows of bristles; (*C*) holotype (MCZ 99504), transverse section through the pre-cerebral region to show the stomach, the extension of the cephalic glands, and the cephalic furrows (arrows); (*D*) holotype (MCZ 99504), transverse section through the second pair the eyes showing the cerebral sensory organs and cephalic nerves (arrows); (*E*) holotype (MCZ 99504), transverse section, epidermis showing the division of the cephalic furrows, the short branch (arrow) continuing with the ciliated canal; (*F*) holotype (MCZ 99504), transverse section at pyloric region showing the extension of the cephalic glands, the first testis, and the intestinal caecum anterior accessory pouches; note the location of submuscular pigment granules (arrowhead); (*G*) paratype (MCZ 99506), detail of the dorsal body wall in longitudinal section showing the distribution of the pigment granules (arrows); (*H*) paratype (MCZ 99505), transverse section at intestinal region of a mature female; (*I*) oocyte in the intestinal region of a specimen with mature testes (MCZ 99512). Abbreviations: ag, acidophilic cephalic glands; ap, anterior accessory pouches of intestinal caecum; cc, ciliated canal; cf, cephalic furrow; cg, basophilic cephalic glands; co, cerebral sensory organ; dv, dorso-ventral musculature; e, epidermis; ey, eye; ic, intestinal caecum; ip, intestinal pouch; lv, lateral blood vessel; lc, lateral nerve cord; o, oocyte; p, proboscis; py, pylorus; st, stomach; t, testis; vc, ventral cerebral commissure. Scale bars: *A* = 100 μm; *B–D*, *F*, *H* = 50 μm; *E*, *G*, *I* = 25 μm.

Ciona intestinalis, but no specimen was found in *Styela clava*. Of six specimens of *Ciona intestinalis* collected at a mussel raft at the Ría de Arousa (6 August 2009), two ascidians yielded three and fourteen nemertean specimens, the latter being the maximum number of specimens recorded in a single ascidian individual.

Taxonomic remarks

The taxonomic history of *Tetrastemma vittigerum* is complex. Hubrecht (1879) provided a succinct description of the nemertean *Oerstedia vittata* Hubrecht, 1879 for specimens collected in the Mediterranean. Joubin (1890, 1894) merged five colour varieties, including *Oerstedia unicolor* Hubrecht, 1879 and *Tetrastemma quadristriatum* Langerhans, 1880 from Madeira with *Amphiporus vittatus*, but *T. quadristriatum* was recognised by Coe (1905) as a different species and was listed by Gibson (1995) as a valid name. Bürger (1895) listed this species as *Tetrastemma vittatum*, but later Bürger (1904) renamed the species *Prostoma vittigerum*, a name used by Friedrich (1936), and redescribed by Kirsteuer (1963) as *Tetrastemma vittigerum* (Bürger, 1904) because *T. vittata* was pre-occupied by an American species described by Verrill (1874) – currently *T. vittata* is a synonym of *Cyanophthalma cordiceps* (Friedrich, 1933). Anadón and Bitar (1992) studied Moroccan specimens from both the Atlantic and Mediterranean coasts, and referred to them as *Oerstedia vittata*, but these specimens became the basis for the new species *Typhloerstedia anadonae* Chernyshev, 1999.

All these refer to specimens with four longitudinal parallel brown bands running along the dorsal surface of the animals, but there are clear differences between Mediterranean and Atlantic pigmentation patterns, and this pattern has also been found in other oceans (P. Sundberg, pers. comm.). Mediterranean specimens show a coalescence of the bands behind the head in two quadrangular patches with a clear head (Hubrecht 1879; Bürger 1895, 1904; Kirsteuer 1963), whereas the Atlantic ones show the central bands extending to the tip of the head before anastomosing while the lateral bands are interrupted between the eyes (Langerhans 1880; Joubin 1890, 1894; Oxner 1907). There are also differences in habitat, as Atlantic specimens have been found inside solitary ascidians (Joubin 1890; Oxner 1907; Gontcharoff 1955), but this conspicuous habitat has never been reported in the Mediterranean. These differences extend to the internal anatomy of the new species studied here, when compared with *T. vittigerum* or with any other known hoplonemertean. Moreover, DNA sequence data from two molecular markers commonly used in phylogenetic studies of nemerteans, COI and 18S rRNA clearly show that the Galician specimens do not belong to *Tetrastemma*, and that instead they belong within a clade of tetrastemmatid species that includes other genera lacking a vascular plug, such as *Gononemertes*, *Nemertellina* and *Oerstedia.*

Etymology

The species is named in memory of the late Luz Murube (1964–2005), who collected the species during the first expedition of the nemertean project of the Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia.

Results and discussion

The 18S rRNA dataset comprised 26 sequences, which resulted in a multiple sequence alignment of 1828 bp. The resulting likelihood tree ($lnL = -4741.99$; tree not shown) showed monophyly of *Tetrastemma* (86% bootstrap frequency, BF hereafter), while *Vieitezia luzmurubeae*, gen. et sp. nov. was the sister group to *Gononemertes* (53% BF), in a clade including *Oerstedia* and *Nemertellina* (100% BF), not allied with *Tetrastemma* (84% BF). A third clade, containing *Prostoma* and *Cyanophthalma obscura* received low support but was recovered in most analyses. The same result was obtained for the 18S rRNA analyses of the direct optimization data for both parameter sets, 3221 (1054 steps) and 3211 (906 steps), although in these cases *Malacobdella* appeared as sister to *Cyanophthalma*.

The COI fragment was obtained for only one specimen *V. luzmurubeae*, and the MAFFT software yielded a 539 bp alignment. The resulting likelihood tree ($lnL = -4180.11$) produced a similar topology as the 18S rRNA gene tree, however, with low support, especially in the deep nodes (tree not shown). The parsimony and maximum likelihood analyses agreed in the resolution (*Oerstedia* (*Gononemertes*, *Vieitezia*)). Since the genus *Antarctonemertes* also lacks a vascular plug, we did an analysis using the COI sequence available in GenBank (AJ436900), but did not include it in the main analyses because 18S rRNA was not available. These analyses did not place it with the *Vieitezia–Gononemertes*-group.

The maximum likelihood analysis for the combination of all markers gave a tree of $lnL = -9577.60$ (Fig. 3), nearly identical to those of the 18S rRNA analysis, with nodal supports of 86% BF for *Tetrastemma*, 55% for *Prostoma* + *Cyanophthalma obscura*, and 90% BF for the *Nemertellina–Vieitezia–Gononemertes–Oerstedia* clade. Results from the direct optimization analyses were nearly identical (parameter set 3211 yielded a single tree of 2279 weighted steps (Fig. 4); 3221 yielded a single tree of 2905 weighted steps), but 3221 places *Nemertellina* as sister to *Gononemertes* + *Vieitezia* (with low support), as opposed to the 3211 and likelihood trees placing *Nemertellina* as sister to (*Oerstedia* (*Gononemertes*, *Oerstedia*)) (81% jackknife frequency for 3211, 77% BF for ML). The direct optimization analyses placed *Malacobdella* as sister to a clade containing *Prostoma* and *Cyanophthalma*, when rooted with *Geonemertes*.

Phylogenetic studies have consistently shown that many nemertean taxa at different ranks (especially genus and family) are paraphyletic or polyphyletic (e.g. Sundberg *et al*. 2001, 2009; Thollesson and Norenburg 2003; Schwartz 2009; Puerta *et al*. 2010). For this reason, the new genus will not be explicitly placed in any family until a stable classification system for nemerteans is broadly accepted – but it allies with genera lacking a vascular plug, mostly within the traditional Tetrastemmatidae. Gibson (1994) left *Oerstedia* in an uncertain familial designation while other authors have considered it to be within Prosorhochmidae (see Thollesson and Norenburg 2003: table 1), within Tetrastemmatidae (Thollesson and Norenburg 2003: fig. 1), or

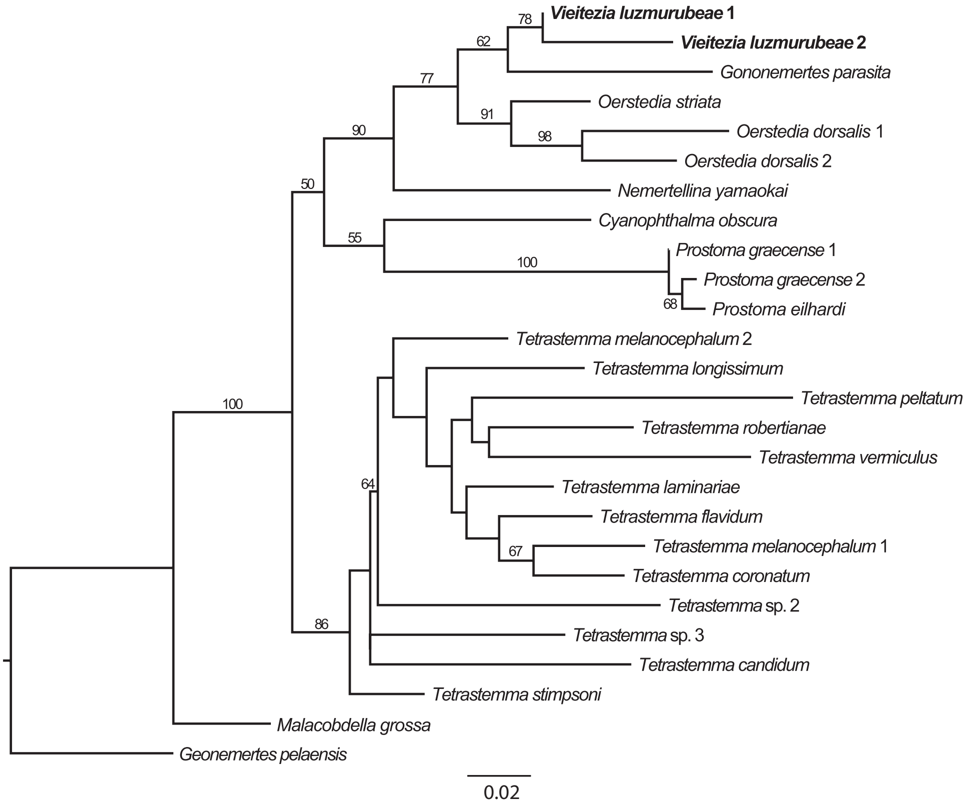

Fig. 3. Phylogeny resulting from the maximum likelihood analysis of combined 18S rRNA and COI ($lnL = -9577.60$). Numbers on nodes indicate bootstrap support values ≥50%. Tree rooted on *Geonemertes pelaensis*.

even in its own family Oerstediidae (see, for example, GenBank). The unequivocal position of *Vieitezia*, gen. nov. in a clade containing *Gononemertes* (Prosorhochmidae), *Oerstedia* (Oerstediidae) and *Nemertellina* (Tetrastemmatidae), but unrelated to *Tetrastemma* may serve as a justification for our decision to leave the family unassigned – although the broader sense of Tetrastemmatidae of Thollesson and Norenburg (2003) would also be broadly compatible with our phylogenetic estimates.

In the genus *Tetrastemma*, different pigmentation patterns have been taken as evidence for differentiating species (Strand and Sundberg 2005), but most European specimens showing four dorsal longitudinal bands have been attributed to *Tetrastemma vittigerum* and synonyms, with the recent exception of *Typhloerstedia anadonae* (see Chernyshev 1999). This colour pattern is so characteristic that differences in pigmentation have been attributed to intraspecific variation, and the specimens from the Mediterranean and the Atlantic have been considered synonymous. Much of this confusion comes from Joubin's work (Joubin 1890, 1894), which describes different variations in colouration from specimens from both French coasts that represent different species in different genera.

Oxner (1907) noted differences in colouration and in the internal morphology between the specimens from Roscoff (France, Atlantic Ocean) and the description of Bürger (1895) from Naples (Italy, Mediterranean Sea). Bürger's specimens have short cephalic glands and the anterior pouches of the intestinal caecum reach the cerebrum, two characteristics confirmed in Kirsteuer's (1963) redescription of the species, based on additional specimens from the Adriatic. Oxner's specimens were collected inside solitary ascidians (*Ciona intestinalis*, *Ascidiella aspersa*) and they belong to the new species here described.

Tetrastemma quadristriatum Langerhans, 1880 was described based on specimens from Madeira as also having four longitudinal dorsal bands and not collected since the original description. However, Langerhans' (1880) succinct description is not sufficient for species identification, and there is no type material that can be compared with our smaller specimens. It seems that the identity of *T. quadristriatum* could not be resolved and it is here considered a *nomen dubium*.

On the basis of morphology, *Vieitezia luzmurubeae*, gen. et sp. nov. resembles the genus *Tetrastemma*, showing the synapomorphies of the genus (four eyes, a flattened body, well demarcated head) (Strand and Sundberg 2005). However, these characters seem insufficient for distinguishing these two genera, and the molecular results clearly place *Vieitezia* with *Gononemertes* (Figs 3, 4). The genus *Gononemertes* was erected by Bergendal (1900) for a nemertean species from the west coast of Sweden, *G. parasita* living in the tunicate genus *Phallusia*, also inhabited by *Vieitezia luzmurubeae*. However, *Gononemertes* shows morphological adaptations related to its

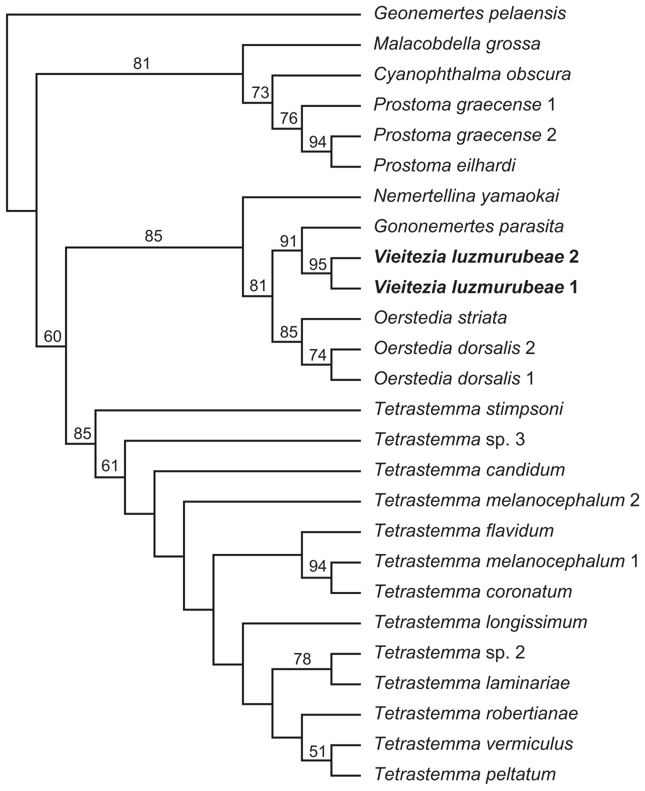

Fig. 4. Single most parsimonious tree for parameter set 3211 (indel opening = 3; transversions = 2; transitions = 1; indel extension = 1) for the combined analysis of 18S rRNA and COI (2279 weighted steps). Numbers on nodes indicate jackknife resampling frequencies ≥50%. Tree rooted on *Geonemertes pelaensis*.

strict parasitic or commensal lifestyle, including the loss or reduction of sensory organs (eyes, cerebral organs) and the extreme multiplicity of the gonads (Bergendal 1900; Brinkmann 1927; Gibson 1974). *Vieitezia* is more similar to other free-living hoplonemerteans – like many *Tetrastemma* species it has four eyes, well developed cerebral organs, and less than a hundred gonads. Differences between *Gononemertes* and *Vieitezia* also relate to rhynchocoel length (short *v.* long), accessory lateral nerve (present *v.* absent), cephalic slits or furrows (absence *v.* well developed), and sex (gonochoristic *v.* hermaphroditic). *Gononemertes parasita*, the type species of the genus, also lacks a stylet-apparatus in its proboscis. There is no doubt that these two putative sister genera have little resemblance other than sharing habitat.

Other members of the clade are *Oerstedia* and *Nemertellina*, two genera with a morphology that differs considerably from that of *Vieitezia*. The analyses also separated *Vieitezia* from the similar genus *Tetrastemma*. As pointed out by other authors (e.g. Strand and Sundberg 2005; Sundberg and Strand, 2010), morphological species and genus delimitation in nemerteans can be questionable and we follow the current trend in description of nemertean species of combining a detailed account of their morphology, with the use of molecular sequence data to include the species in a phylogenetic context (e.g. Envall and Sundberg 1998; Gibson and Sundberg 2002; Sundberg *et al*. 2007, 2009; Maslakova and Norenburg 2008*a*, 2008*b*; Puerta *et al*. 2010). Only in this way will we be able reconcile detailed morphological studies with a sound classification based on phylogenetic evidence.

Vieitezia luzmurubeae, gen. et sp. nov. is hermaphroditic, a characteristic associated with unusual or highly specialised modes of life in nemerteans (Riser 1985, 1988). Hermaphroditic species are found in terrestrial (*Argonemertes*, *Geonemertes*, *Prosadenoporus*) and freshwater habitats (*Apatronemertes*, *Koinoporus*, *Potamonemertes*, *Prostoma*), and many of the marine hermaphrodites are intertidal or supralittoral (*Notogaeanemertes*, *Prosorhochmus)*, or commensal of other invertebrates (*Coenemertes*, *Tetrastemma*). Many specimens of *Vieitezia luzmurubeae*, gen. et sp. nov. were collected inside the ascidians *Phallusia mamillata* and *Ciona intestinalis* but not in other species surveyed. Few reports have mentioned this association between nemerteans and ascidians (reviewed by Harant 1931; Gibson 1974; Monniot 1990). While Gibson (1972) argues that *Tetrastemma* may occur in ascidians occasionally, evidence shows that *Vieitezia luzmurubeae* aggregates in the ascidians for reproduction (Fig. 1*C, D*). All the sectioned specimens collected inside ascidians were sexually mature, with ripe gonads. In the same ascidian individual, several functional males aggregate to one (Fig. 1*C*) or sometimes a few functional females, constituting a true male harem, as early observed by Oxner (1907), suggesting that males are exposed to mating competition. This aggregation ensures high gamete concentrations, a mechanism used by free-spawning species, including nemerteans (Levitan 1995; Thiel and Junoy 2006). Moreover, the eggs are deposited inside the ascidian (Fig. 1*D*), which is interpreted as a system of brood protection. The evolutionary advantage of this strategy seems evident, as the nemertean reduces the mortality resulting from the protection of its eggs inside the ascidian body without the energetic cost of producing a structure to protect the eggs.

In this paper we have illustrated a unique lifestyle of nemerteans, which constitute male harems within the atrium of solitary ascidians. While recorded previously, the specimens constitute a new species that we place in the new genus *Vieitezia*, which is resolved phylogenetically as sister to *Gononemertes* and related to *Oerstedia* and *Nemertellina*. The finding of this new species highlights both the need for new biodiversity studies within nemerteans as well as the urgency of connecting such biodiversity studies with a sound phylogenetic framework that takes into account molecular data in addition to classical morphology.

Acknowledgements

Hiroshi Kajihara, two anonymous reviewers and the associate editor provided comments and advice that helped to improve this article. This research was supported by the Project 79/2003, Spanish *Organismo Autónomo de Parques Nacionales* and Project CGL2004–00709, Spanish *Ministerio de Educación y Ciencia*. The authors wish to thank the staff of the Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia for their collaboration. Juan Pablo Camblor, Alfonso Herrera-Bachiller, Patricia Puerta, Miguel Souto and Juan Souto collaborated in many aspects of the collecting and laboratory work. We are very grateful to Jacinto Pérez (Hydronauta, Ribeira) for allowing us to use his photographs of the new genus and species inside *Ciona intestinalis* (Fig. 2*C*, *D*). Thanks are due to Dr Elsa Vázquez and Dr Jesús S. Troncoso of the Universidad de Vigo for their hospitality. The senior author (J. Junoy) received a yearly research grant (PR2009–1004, *Ministerio de Educación*) for the study of nemertean biodiversity at the Museum of Comparative Zoology, Harvard University.

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Manuscript received 2 November 2010, accepted 11 February 2011

Appendix 1. Character matrix for *Vieitezia luzmurubeae*, gen. et sp. nov.

Character states and code from Sundberg *et al.* (2009). N/A: no applicable character

Character	Character state	Code	Figure
1. Biology	Free-living and commensal	0, 2	1*C*, 1*D*
2. Habitat	Marine	0	
3. Benthic divisions	Sublittoral	2	
4. Pelagic divisions	N/A	N/A	
5. Substrate	Rock/boulders; algae/algal holdfasts/sea grass; other	3, 4, 5	
6. Behaviour when mechanically disturbed	Contracts without coiling into a spiral	0	
7. Cephalic furrows	One pair	1	1*B*
External morphology			
8. Distribution of anterior cephalic furrows	Ventral and lateral	3	1*B*, 1*E*
9. Shape of anterior (dorsal) cephalic furrows	V-shaped or oblique	0	1*B*, 1*E*
10. Shape of posterior (dorsal) cephalic furrows	N/A	N/A	
11. Head demarcated from body	Head not wider than trunk	2	1*A*, 1*B*
12. Position of cephalic furrows	If single pair in front of brain lobes	1	1*B*, 2*C*
13. Shape of head/cephalic lobe	Pointed	2	1*A*, 1*B*
14. Head viewed laterally	Without extensions	0	1*A*
15. Shape of posterior tip	Pointed	0	1*A*
16. Eyes	Four eyes arranged at corners of square or rectangle	3	1*A*, 1*B*, 2*D*
17. Eye morphology	Simple	0	2*D*
18. Relative eye size	All eyes more or less of equal size	0	1*B*
19. Eye distinctiveness	Eyes visible from ventral side	0	1*E*
20. Eye position relative to brain lobes	Confined entirely to pre-cerebral cephalic region but may be located above brain	0	1*B*, 2*D*
21. Colour pattern	Extending full body length on dorsal surface	2	1*A*, 1*B*
22. Primary dorsal body colour	Brown	5	1*A*, 1*B*
23. Body colour hue/tint	Light	2	1*A*, 1*B*, 1*C*, 1*D*, 1*E*
24. Internal organs visible through dorsal epidermis	Yes	1	
25. Lateral margins	No distinction in colour	1	1*A*, 1*B*
26. Distribution of bristles/cirri	Only on head	1	
Internal morphology			
Body wall			
27. Epidermis non-cellular inclusions	Pigment granules	2	2*G*
28. Epidermis of anterior body	N/A	N/A	
29. Ratio thickness of epidermis/lateral body diameter in brain region	N/A	N/A	
30. Dermis	Forming a distinct zone between epidermis and circular muscle layer	0	
31. Thickness of dermis	Approximately the same thickness as circular muscle layer	3	2*E*, 2*G*, 2*I*
32. Muscle processes from dermis into epidermis	N/A	N/A	
33. Muscle layers	Outer circular and inner longitudinal muscle layer	0	2*E*, 2*G*, 2*I*
34. Muscle crosses between body wallcircular muscle layers	N/A	N/A	
35. Body wall longitudinal musclelayer just behind brain	Not anteriorly divided	0	2*F*
36. Pre-cerebral septum	Closed	0	
37. Central (medial) muscle plate	N/A	N/A	
38. Parenchyma	Barely distinguishable other than as a membranes enclosing various body organ systems	0	
39. Muscle fibres in mouth/foregutregion	N/A	N/A	
Proboscis apparatus			
40. Proboscis pore	Subterminal, ventral	1	2*A*
41. Mouth and proboscis pore connection	Open into atrium/rhynchodaeum	1	2*A*
42. Gland cells of rhynchodaeum	Absent	0	
43. Rhynchocoel musculature	Proximal circular and distal longitudinal muscle layer	2	
44. Rhynchocoel musculature in posterior end	N/A	N/A	
45. Rhynchocoel length	Extending to or almost to posterior region of body	2	
46. Rhynchocoelic caeca	Absent	0	
47. Size of posterior third of proboscis region	Small, less than 50% of body diameter in retracted position	0	
48. Musculature of proboscis (everted state)	Outer circular and inner longitudinal muscle layer	0	

(*continued next page*)

Appendix 1. (*continued*)

Character	Character state	Code	Figure
49. Musculature of posterior proboscis region (everted state)	Outer circular and inner longitudinal muscle layers	2	
50. Epithelium of anterior proboscis region when everted	Developed into papillae	1	1*F*
51. Number of proboscis nerves	10	3	2*F*, 2*H*
52. Proboscis nerve arrangement	Peripheral neural sheath distinct	1	2*F*, 2*H*
53. Secondary proboscis nerves	Absent	0	
54. Proboscis armature	With central and accessory stylets	2	1*G*
55. Number of accessory stylet pouches	Two	0	1*G*
56. Number of stylets in each accessory stylet pouch	Three or four	1	1*G*
57. Stylet: basis/stylet ratio	1 : 1	0	1*G*
58. Stylet shaft	Smooth and straight	0	1*G*
59. Shape of stylet basis	Pear-shaped	1	1*G*
60. Median waist of stylet basis	Present	1	1*G*
61. Proboscis used for locomotion	Unknown	0	
Alimentary system			
62. Position of mouth	N/A	N/A	
63. Oesophagus	Present	1	
64. Oesophagus epithelium	Unciliated without glands	1	
65. Stomach	Not regionally differentiated	0	2*C*, 2*D*
66. Stomach connection with intestine	Posterior stomach developing into pyloric canal which opens into dorsal wall of intestine	1	2*F*, 2*H*
67. Length of pyloric canal	Longer than stomach	3	
68. Intestinal caecum	Present ventral	1	2*F*, 2*H*
69. Anterior pouches on intestinal caecum	Short, do not extend forward to reach brain lobes	2	2*F*
70. Lateral diverticula on intestinal caecum	Shallow	1	2*H*
71. Intestinal diverticula	Simple unbranched pouches	1	2*H*
Circulatory system			
72. Cephalic vasculature	Arranged as a simple cephalic loop	0	
73. Vascular plugs	Absent	0	
74. Rhynchocoelic villus	Absent	0	
75. Position of lateral blood vessels	N/A	N/A	
76. Mid-dorsal blood vessel	Divides in brain region to form two vessels	2	
77. Length of mid-dorsal blood vessel	Not observed	–	
78. Extra vascular pouches/valves	Present	1	
79. Pseudometameric transverse connectives linking mid-dorsal and lateral blood vessels in intestinal region	Absent	0	
80. Vascular plexus in foregut region	N/A	N/A	
Nervous system			
81. Location of cerebral ganglia and lateral nerve cords	N/A	N/A	
82. Number of dorsal cerebral commissures	One	1	
83. Distinct outer neurilemma of cerebral ganglion	Present	1	
84. Inner neurilemma of cerebral ganglion	Absent	0	
85. Statocysts in brain tissue	Absent	0	
86. Lateral nerve cords	Without accessory lateral nerve	0	
87. Accessory lateral nerve	N/A	N/A	
88. Four large nerves in head region	N/A	N/A	
89. Number of dorsal nerves	N/A	N/A	
90. Posterior junction of lateral nerve cords	Supraintestinal	1	
91. Neurochord cells in brain	Absent	0	
92. Neurochords in lateral nerve cords	Absent	0	
93. Myofibrillae in lateral nerve cords	Absent	0	
Nervous system			
94. Position of myofibrillae in lateral nerve cords	N/A	N/A	
95. Buccal nerves	Absent	0	
Excretory system			
96. Excretory system	Present	1	
97. Extent of system	Confined to foregut region of body	0	
98. Excretory canal	N/A	N/A	

(*continued next page*)

Appendix 1. (*continued*)

Character	Character state	Code	Figure
99. Nephridial gland	N/A	N/A	
100. Flame cells	No flame cells distinguished	0	
101. Glandular components inexcretory tubules	Absent	0	
102. Number of nephridiopores	Limited to one or two on each side of body	0	
103. Position of nephridiopores	Anterior, close to or alongside brain, in anterior region of excretory system	0	
Reproductive system			
104. Nature of sexes	Protandrous hermaphroditic	2	2*F*, 2*H*, 2*I*
105. Gonad arrangement in heterogamous taxa	N/A	N/A	
106. Gonad arrangement in hermaphrodititctaxa	Testes and ovaries distributed in same regions of body, ovaries and testes separate	2	2*F*, 2*H*, 2*I*
107. Testes	Simple	0	2*F*
108. Sexual colour dimorphism	Present	1	1*C*
109. Gonoduct position	Ventrolateral	1	
110. Nature of reproduction	Oviparous	0	1*D*
111. Apical organ	Present	1	2*A*
Sensory organs			
112. Typical cephalic glands	Extend behind brain	3	2*A*, 2*C*, 2*D*, 2*F*
113. Cephalic gland type	Forming distinct lobules	0	2*A*, 2*C*, 2*D*, 2*F*
114. Opening of cephalic glands	Via apical organ	0	2*A*
115. Position of cerebral sensory organs in relation to brain	Close in front of brain	2	2*D*
116. Position of cerebral sensory organs in relation to epidermis	Separate from blood vessels under body wall muscle layers	2	2*D*
117. Size of cerebral sensory organs	Less than half size of brain lobes/	0	2*D*
118. Ciliated cerebral canal	unforked	0	
119. Side organs	N/A	N/A	
120. Sensory pits in head region	N/A	N/A	