



First reports of *Grania* (Clitellata: Enchytraeidae) from Africa and South America: molecular phylogeny and descriptions of nine new species

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In this article, our knowledge of the geographic distribution of *Grania* species is expanded by describing seven new species, *Grania bekkouchei* sp. nov., *Grania brasiliensis* sp. nov., *Grania capensis* sp. nov., *Grania chilensis* sp. nov., *Grania cryptica* sp. nov., *Grania hinojosai* sp. nov., and *Grania simonae* sp. nov., from poorly investigated regions of the Southern Hemisphere, plus two new species, *Grania carolinensis* sp. nov. and *Grania unitheca* sp. nov., from off the east coast of the USA. An immature achaetous specimen that we call *Grania* cf. *levis* was also included. The newly generated data were combined with a previously published data set in order to update the hypothesis of phylogenetic relationships among *Grania* species. All new species except *G. cryptica* sp. nov. are supported by both morphological and molecular data. In addition, we find that *G. chilensis* sp. nov. is structured in at least four distinct populations along the Chilean coast. The species described from South Africa form a monophyletic clade where two are morphologically indistinguishable but diverging in both mitochondrial and nuclear ribosomal genes, and thus we describe them as different species, *G. bekkouchei* sp. nov. and *G. cryptica* sp. nov. Among the North Carolinian species, *G. unitheca* sp. nov. is indicated as a close relative of *Grania monospermatheca* Erséus & Lasserre, 1976, and *G. carolinensis* sp. nov. is indicated as a close relative of *G. cf. levis*. The updated phylogeny is strongly concordant with geographical species distributions, thus supporting a low level of dispersal within this genus, as has previously been hypothesized.

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INTRODUCTION

Grania Southern, 1913 is one of the largest enchytraeid genera (Erséus *et al.*, 2010), today comprising 72 nominal species (De Wit, Rota & Erséus, 2011b). Exclusively marine, they are found in a wide range of habitats, from the intertidal zone to the deep sea (Coates, 1984;

Erséus & Rota, 2003; Rota & Erséus, 2003; De Wit & Erséus, 2010). Most *Grania* species inhabit the interstitial space of shelly and coarse sediments, and they are generally small and always slender, between 10 and 20 mm long but only about 0.1 mm wide (Erséus & Lasserre, 1976; Rota & Erséus, 2003; De Wit & Erséus, 2010).

The genus is morphologically well separated from other enchytraeid genera. For instance, in *Grania* the cephalization, which is a common feature in the family

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Enchytraeidae, is unusually complex, with a well-developed nervous system that includes, in some species, the so-called 'head organ', which is thought to be a compound georeceptor (Rota *et al.*, 1999; Rota & Erséus, 2003). Moreover, in this genus the chaetal bundles are each represented by only one stout chaeta (Erséus & Lasserre, 1976; De Wit *et al.*, 2011b), and the circular body musculature is reduced, resulting in a nematode-like, coiling, form of locomotion (Rota, 2001; De Wit, Erséus & Gustavsson, 2011a). Yet species distinction in *Grania* is complex, and a unique combination of multiple morphological features must be used (e.g. chaetal distribution and shape, and details of the penial apparatus and spermatheca) for an accurate and unambiguous identification of morphospecies (Erséus & Lasserre, 1976; De Wit & Erséus, 2010).

In addition, a cryptic species, *Grania occulta* De Wit & Erséus, 2010, which is sympatric with, yet morphologically indistinguishable from, *Grania ovipecta* Erséus, 1977, was recently described from Scandinavian waters using molecular data. This provides a first hint that the species diversity of the genus may be greater than previously thought (De Wit & Erséus, 2010), which highlights the need for including genetic data in species descriptions.

An initial phylogenetic analysis of 20 species of *Grania* was recently performed using a combination of molecular and morphological data (De Wit *et al.*, 2011b). According to that study, the genus is well supported as monophyletic and contains three main lineages (referred to as clades A, B, and C by De Wit *et al.*, 2011b). Clade A consists of all North Atlantic species, and is further subdivided into one European and one North American subclade. Clade B contains some peculiarly coloured (yellowish green) species from the Indo-Pacific region, as well as Chilean species and *Grania Americana* Kennedy, 1966, whereas clade C comprises species from Australia and Hong Kong (De Wit *et al.*, 2011b).

In an ancestral state reconstruction of the total evidence phylogeny (De Wit *et al.*, 2011b), most morphological characters, such as chaetal shape, midventral glands, and shape of penial apparatus and spermathecae, were found to be highly homoplasious. In addition, a morphology-based phylogeny was found to be incongruent with one based on genetic data from six loci; however, the geographic distribution of the species in the analysis showed great congruence with the phylogeny, suggesting limited dispersal capabilities (De Wit *et al.*, 2011b). One caveat, though, was that the geographic sampling was skewed to locations from which recent sampling efforts had been conducted, and it was concluded that including data from other parts of the world, such as Africa and South America, would be crucial to updating the phylogeny of this genus.

This is not a simple task, however, as the biogeographical knowledge of *Grania* species is largely restricted to the North Atlantic (Southern, 1913; Knöllner, 1935; Lasserre, 1966, 1967; Erséus, 1974, 1977; Erséus & Lasserre, 1976; Kossmagk-Stephan, 1983; Locke & Coates, 1999; Rota & Erséus, 2003; De Wit & Erséus, 2010) and some Indo-Pacific regions (Jamieson, 1977; Coates, 1990; Erséus, 1990; Coates & Stacey, 1993, 1997; Rota & Erséus, 2000; Rota, Erséus & Wang, 2003; De Wit & Erséus, 2007; Rota, Wang & Erséus, 2007; De Wit, Rota & Erséus, 2009). Except for some Antarctic (Rota & Erséus, 1996) and sub-Antarctic (Erséus & Lasserre, 1977; Rota & Erséus, 1997) areas, in most parts of the Southern Hemisphere, particularly along the Atlantic and Pacific coasts of South America, and both sides of southern Africa, the genus has remained almost unexplored (De Wit *et al.*, 2011b; Prantoni, Di Domenico & Lana, 2013).

Herein we describe nine new species, seven from coastal areas of Brazil, South Africa, and Chile, and two from eastern USA, combining molecular and morphological information. We also present an updated hypothesis of the phylogenetic relationships among *Grania* species.

MATERIAL AND METHODS

COLLECTION AND IDENTIFICATION

The Chilean specimens of *Grania* were collected by the second author, in February 2009. South African *Grania* were collected by Nicholas Bekkouche, during a workshop on annelid systematics arranged in South Africa by Dr Carol Simon (Stellenbosch University) in December 2011, and the North American species were collected by the third author during a cruise with R/V *Cape Hatteras*, off North Carolina, in May 2011. In Chile and South Africa, sediment samples were collected by hand, stirred with seawater, followed by the decantation of suspension into a 0.25-mm mesh. The North American material was obtained from sieved sediments collected with a box corer from the research vessel.

The Brazilian specimens from Ponta do Baleeiro beach, Sao Paulo State, were sampled in October 2012 during the workshop *Taxonomy and Diversity of Marine Meiofauna – Brazil*. These, as well as the specimens from Paranaguá Bay collected in August 2013, were sampled by taking sediment cores with PVC pipes of 10 cm in length and 10 cm in diameter, stored in a plastic bucket with seawater, relaxed with isotonic MgCl₂, and with salinity up to 36 ppt. The supernatant containing numbered animals was washed with fresh seawater in a 0.063-mm sieve and transferred into a Petri dish.

All worms were sorted alive using a stereomicroscope and, with few exceptions, preserved in 80%

ethanol. Some specimens were collected incomplete, but most had a complete body with pygidium. Many of these were intentionally cut and a few (sometimes counted) 'amputated segments' (posterior or middle) were removed and stored in 95% ethanol for subsequent DNA extraction and sequencing. The holotype of *Grania brasiliensis* sp. nov. was killed and stored in 99% ethanol, and then its posterior end was cut for DNA analysis; the paratypes of the same species were fixed in 4% formaldehyde and left uncut. The anterior end of the cut specimens from Chile, South Africa, and Brazil, as well as all complete worms, were stained in alcoholic paracarmine solution and mounted whole in Canada balsam on slides for the purpose of identification. The worms were morphologically examined using light and interference contrast microscopy, and drawings were made with the aid of a camera lucida.

In the descriptions, the chaetal length means the straight line from the distal tip to the furthest proximal point of the chaeta. The chaetal foot length is understood as the maximal breadth of the L-shaped chaetae at their ental end, and the chaetal index was calculated as the average ratio of the chaetal length by the chaetal foot length, \pm standard deviation (Rota & Erséus, 2003). Penial bulb lengths were measured as their extension parallel with the long body axis, and widths were taken as perpendicular to the longitudinal body axis.

Type specimens are deposited in the Museu de Zoologia da Universidade Estadual de Campinas 'Adão José Cardoso' (ZUEC), Campinas, Brazil, Iziko South African Museum (SAMC), Cape Town, South Africa, Swedish Museum of Natural History (SMNH), Stockholm, Sweden, and, US National Museum of Natural History (USNM), Washington, DC, USA.

ABBREVIATIONS USED IN THE FIGURES

amp, spermathecal ampulla; cl, coelomocyte; ed, ectal duct of spermatheca; ggc, granular gland cell of clitellum; hgc, hyaline gland cell of clitellum; mp, male pore; pb, penial bulb; sb, sperm bundle; sf, sperm funnel; sr, sperm ring; spp, spermathecal pore.

GENETIC ANALYSES

The DNA of 38 individuals (Table 1) was extracted using the DNeasy Blood & Tissue kit (Qiagen), or the QuickExtract DNA Extraction Solution 1.0 (Epicentre), after which polymerase chain reactions (PCRs) were performed for the mitochondrial cytochrome *c* oxidase subunit I (*COI*) locus, using the universal 'barcoding primers' LCO1490/HCO2198 (Folmer *et al.*, 1994). One evolutionary lineage represented by an immature specimen from off North Carolina lacks chaetae completely; we refer to it as *Grania* cf. *levis* Coates

& Erséus, 1985, also from the north-west Atlantic Ocean and the only formally described *Grania* with this striking feature. In addition, one specimen (CE20818) of *G. brasiliensis* sp. nov., one specimen (CE21024) of *Grania hinojosai* sp. nov., and nine specimens of *Grania chilensis* sp. nov. (CE21036, CE21037, CE21038, CE21039, CE21040, PDW189, PDW197, PDW200, and PDW202) were *COI* barcoded but not found suitable for closer morphological study. To compare the mitochondrial data with nuclear DNA patterns, 23 individuals were also chosen to be amplified at the *ITS* region (Table 1), using primers ITS5/ITS4 (White *et al.*, 1990) and 5.8 mussF and 5.8 mussR (Källersjö *et al.*, 2005). Finally, one individual from each putative species was chosen for phylogeny reconstruction (Table 1). For each of these ten individuals, four additional loci were amplified (18S rDNA and the D1 region of 28S rDNA of the nuclear genome; and 12S and 16S rDNA of the mitochondrial genome) using primers TimA/TimB for 18S (Norén & Jondelius, 1999), 28SC1/28SC2 (Dayrat *et al.*, 2001) and 12SE1/12SH for 12S (Jamieson *et al.*, 2002), and 16SarL/16SbrH for 16S (Palumbi *et al.*, 1991).

The amplicons were sequenced either by Macrogen Inc. (Seoul, South Korea) or by Eurofins mwg operon (Ebersberg, Germany) using additional sequencing primers for 18S (600F, 1806R, 4FBK, 4FB, 5f and 7fk; Norén & Jondelius, 1999), assembled using GENEIOUS PRO 4.8.5 (Rozen & Skaletzky, 2000) from Biomatters Ltd (Auckland, New Zealand), and aligned using MAFFT 6 (Kato & Toh, 2008), applying the L-INS-i setting (slow-accurate). Alignments were tested for models of best fit using the BIC criterion in jModeltest (with the *COI* alignment partitioned by codon position; Guindon & Gascuel, 2003; Darriba *et al.*, 2012).

The *COI* and the *ITS* loci were first analyzed individually using Bayesian inference implemented in the parallel version of MrBayes 3.2 (Ronquist & Huelsenbeck, 2003), with two parallel runs of 20 million generations, sampling trees every 1000 generations (*COI* partitions unlinked in all parameters except topology). After examining the output files for convergence using the online software AWTY (Wilgenbusch, Warren & Swofford, 2004), majority-rule consensus trees were created by summarizing all trees after a burn-in period of 5 million generations. Bayesian support values were then plotted on the consensus trees. For *COI*, a distance matrix was plotted (Table S1), which was then used to plot a histogram to examine the prevalence of the so-called 'barcoding gap'. In addition, a haplotype network was created using TCS 1.21 (Clement, Posada & Crandall, 2000) of one of the putative Chilean species, in which there was significant genetic variation.

For the phylogeny reconstruction, the newly generated data were combined with the data set of De Wit *et al.* (2011b), and realigned and tested for models of

Table 1. Specimens of the new species used for genetic analysis, with places of origin, voucher numbers, and GenBank accession numbers

Taxon	Spm	Locality	Voucher	COI	ITS	16S	12S	18S	28S
<i>Grania chilensis</i>	CE21036	Chile, Valdivia	SMNH 147308	KT428079					
<i>Grania chilensis</i>	PDW193	Chile, Valdivia	ZUEC-CLI 13	GU902190	GU902182	GU902141	GU902127	GU902154	GU902168
<i>Grania chilensis</i>	PDW194	Chile, Valdivia	ZUEC-CLI 16	KT428080	KT428058				
<i>Grania chilensis</i>	CE21037	Chile, Valdivia	SMNH 147309	KT428081					
<i>Grania chilensis</i>	CE21038	Chile, Valdivia	SMNH 147310	KT428082					
<i>Grania chilensis</i>	CE21039	Chile, Valdivia	SMNH 147311	KT428083					
<i>Grania chilensis</i>	CE21040	Chile, Valdivia	SMNH 147312	KT428084					
<i>Grania chilensis</i>	PDW190	Chile, Valdivia	ZUEC-CLI 14	KT428085	KT428059				
<i>Grania chilensis</i>	PDW191	Chile, Valdivia	ZUEC-CLI 15	KT428086	KT428060				
<i>Grania chilensis</i>	PDW185	Chile, Coquimbo	ZUEC-CLI 17	KT428087	KT428061				
<i>Grania chilensis</i>	PDW189	Chile, Coquimbo	SMNH 147313	KT428088	KT428062				
<i>Grania chilensis</i>	PDW197	Chile, Concepcion	SMNH 147314	KT428092	KT428066				
<i>Grania chilensis</i>	PDW198	Chile, Concepcion	ZUEC-CLI 18	KT428089	KT428063				
<i>Grania chilensis</i>	PDW199	Chile, Concepcion	ZUEC-CLI 19	KT428090	KT428064				
<i>Grania chilensis</i>	PDW200	Chile, Concepcion	SMNH 147315	KT428091	KT428065				
<i>Grania chilensis</i>	PDW202	Chile, Concepcion	SMNH 147316	KT428093					
<i>Grania hinojosai</i>	PDW177	Chile, Coquimbo	ZUEC-CLI 10	KT428094	KT428067				
<i>Grania hinojosai</i>	PDW182	Chile, Coquimbo	ZUEC-CLI 12	KT428095	KT428068				
<i>Grania hinojosai</i>	PDW186	Chile, Coquimbo	ZUEC-CLI 08	GU902189	GU902181	GU902140	GU902126	GU902153	GU902167
<i>Grania hinojosai</i>	PDW181	Chile, Coquimbo	ZUEC-CLI 11	KT428096	KT428069				
<i>Grania hinojosai</i>	PDW187	Chile, Coquimbo	ZUEC-CLI 09	KT428097	KT428070				
<i>Grania hinojosai</i>	CE21024	Chile, Coquimbo	SMNH 147317	KT428098					
<i>Grania simonae</i>	CE14058	S Africa, Van Dyks Bay	SAMC A82479	KT428101	KT428071	KT375040	KT375048	KT375055	KT375063
					(ITS2 only)				
<i>Grania simonae</i>	CE14060	S Africa, Van Dyks Bay	SAMC A82480	KT428099					
<i>Grania simonae</i>	CE14110	S Africa, Van Dyks Bay	SAMC A82477	KT428102					
<i>Grania simonae</i>	CE14111	S Africa, Van Dyks Bay	SAMC A82476	KT428100					
<i>Grania simonae</i>	CE14093	S Africa, Van Dyks Bay	SAMC A82481	KT428103					
<i>Grania capensis</i>	CE14015	S Africa, False Bay	SAMC A82474	KT428104	KT428072	KT375041	KT375049	KT375056	KT375064
<i>Grania capensis</i>	CE14016	S Africa, False Bay	SAMC A82475	KT428105					
<i>Grania bekkouchei</i>	CE13975	S Africa, Saldanha Bay	SAMC A82467	KT428106	KT428073	KT375042	KT375050	KT375057	KT375065
<i>Grania bekkouchei</i>	CE13996	S Africa, Saldanha Bay	SAMC A82466	KT428107					
<i>Grania bekkouchei</i>	CE14059	S Africa, Van Dyks Bay	SAMC A82472	KT428108					
<i>Grania cryptica</i>	CE14031	S Africa, False Bay	SAMC A82473	KT428109	KT428074	KT375043	KT375051	KT375058	KT375066
<i>Grania brasiliensis</i>	CE20734	Brazil, Paranagua	ZUEC-CLI 04	KT428110	KT428075	KT375044	KT375052	KT375059	KT375067
<i>Grania brasiliensis</i>	CE20818	Brazil, Baleiro	–	KT428111					
<i>Grania carolinensis</i>	CE11569	USA, N Carolina, 492 m	USNM1283174	KT428112	KT428076	KT375045	KT375053	KT375060	
<i>Grania carolinensis</i>	CE11659	USA, N Carolina, 17 m	USNM1283175	KT428113	KT428077	KT375046		KT375061	
<i>Grania cf. leviss</i>	CE11570	USA, N Carolina, 492 m	USNM1283176	KT428114	KT428078	KT375047	KT375054	KT375062	

Note that for some of the species not all specimens were suitable for closer morphological study.

best fit as described above. The alignments were first concatenated into two loci (mitochondrial and nuclear ribosomal DNA) and run in MrBayes as described above, in order to test whether the two loci share the same phylogeny (Figs S1, S2). After determining that no statistically supported incongruences exist between the gene trees, the two loci were combined into one matrix and analysed again as described above.

RESULTS

TAXONOMY

GRANIA BRASILIENSIS SP. NOV.

FIGURE 1

Holotype

ZUEC CLI 04, individual CE20734, whole-mounted, sexually mature specimen, with some segments amputated, from Ponta do Poço, Paranaguá Bay, Paraná State, southern coast of Brazil, 25°32'54"S, 48°23'18"W, estuarine subtidal, 7 m depth, medium to coarse sand with some mud, and lots of shell and cirriped fragments. Collected by A. L. Prantoni, 19 August 2013. COI barcode sequence, GenBank acc. no KT428110; for other sequence data, see Table 1.

Etymology

Named for Brazil.

Paratypes

ZUEC CLI 05–CLI 07, three whole-mounted adult mature specimens, one from the type locality and two from the rocky promontory of the Ponta do Baleiro

beach, São Sebastião, São Paulo State, south-east coast of Brazil, 23°49'41"S, 45°25'23"W, lower intertidal pond, gravel and coarse sand with shell fragments, A. L. Prantoni, 28 October 2012. Paratypes not barcoded.

Description

Holotype > 5.71 mm long, > 38 segments (posterior end used for genetic analyses), 0.18 mm wide at segment III, 0.15 mm wide at segment XII, and 0.12 mm wide at segment XXI. Paratypes (complete adults) 5.28–13.7 mm ($n = 3$), 40–61 segments ($n = 3$), 0.12–0.14 mm wide at segment III, 0.13–0.15 mm wide at segment XII, 0.13–0.17 mm wide at segment XXI ($n = 2$); one paratype not suitable for width measurements). Prostomium small, rounded, 45–65 μm long, 39–81 μm wide ($n = 4$); epidermis not reduced at front tip, 7–16 μm thick. Ventral chaetae from segment IV, lateral chaetae from segment XVII ($n = 2$) or segment XVIII ($n = 2$). Chaetae (Fig. 1A) 35–60 μm long, shaft straight, 3.5–5.0 μm thick at midpoint, L-shaped, proximally bent into a short oblique foot, with low instep and receding heel. Chaetal index (Rota & Erséus, 2003) 3.61 ± 0.56 ($n = 5$). Epidermal gland cells inconspicuous. Clitellum (not well developed in the holotype) maximally 18 μm thick, extending over segment XII and anterior half of segment XIII, comprising more or less regular transverse rows of hyaline cells alternating with granular cells; hyaline cells more abundant than granular cells; both types of cell absent midventrally between male pores (Fig. 1C). Spermathecal pores paired, lateral, somewhat posterior to 4/5. Male pores ventrolateral in middle of segment XII. Female pores ventrolateral, just posterior to 12/13. Anus directed ventrad (paratypes).

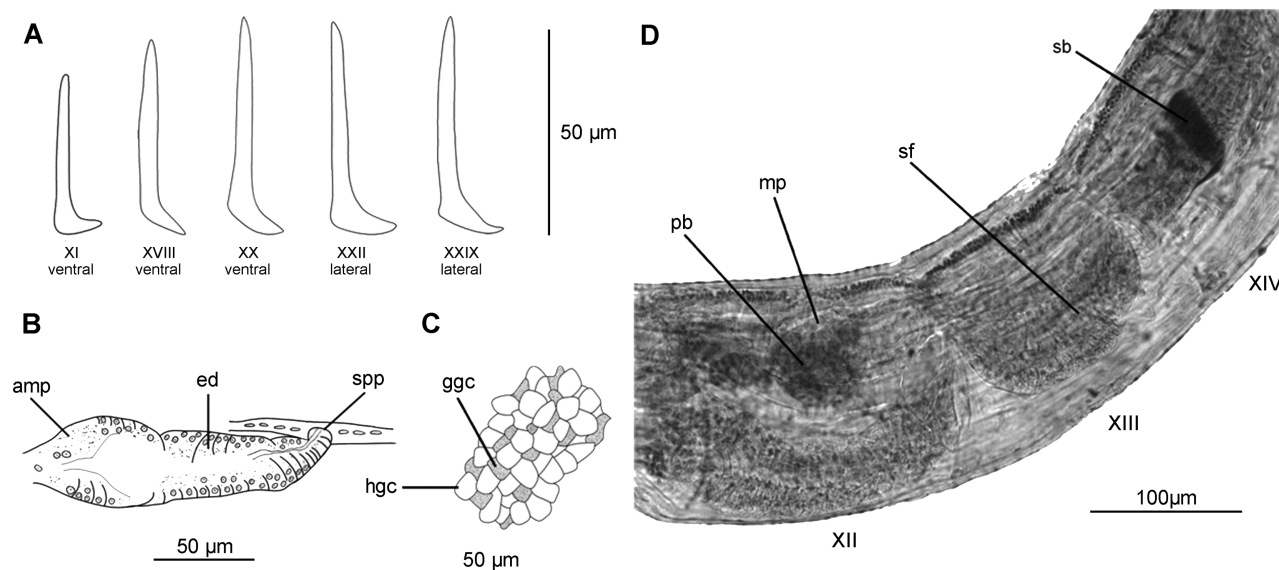


Figure 1. *Grania brasiliensis* sp. nov. A, chaetae. B, spermatheca. C, clitellar cell pattern, oriented diagonally. D, penial bulb and sperm funnel. See text for abbreviations.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI; dorsal lobes present in segment IV (one pair), segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells inconspicuous. Dorsal blood vessel arising in segment XIX or XX. Coelomocytes sparse, small, up to 11.5 µm long, irregularly oval or circular; cytoplasm with fine granulation around cell nucleus. Sperm sac extending into segments XIV–XVI. Egg sac extending into segments XVII–XXII. Sperm funnels very long, in segments XI–XII (paratype) or segments XI–XIV (holotype), 10–12 times longer than wide, directed posteriorly ($n = 2$) (Fig. 1D). Vasa deferentia 7 µm wide, coiled and reaching at least into segment XV. Penial apparatus type 1 (*sensu* Coates, 1984), with small, round bulb, 59 µm long, 62 µm wide ($n = 1$), or oval bulb, 69 µm long, 38 µm wide ($n = 1$); bulb glandular, surrounding a simple invaginated male pore; stylet absent (Fig. 1D). Midventral copulatory gland (in segment XIV) present. Spermathecae communicating with the oesophagus close to septum 5/6 through narrow ental ducts. Ectal ducts spindle-shaped, thick-walled, muscular, 67–79 µm long, 31–40 µm thick at midcourse, joining ampullar cavity through conspicuous deep conical intrusion. Ampullae 41–66 µm long, 36–59 µm wide; oval, ectally granulated (Fig. 1B). No glands at ectal pores of spermathecae. Sperm rings not observed in ampullae.

Remarks

The combination of the long sperm funnel, presence of a midventral copulatory gland in segment XIV, and the unusual clitellum, with hyaline cells more abundant than granular cells, may differentiate this new species from all other described species of *Grania*.

Grania brasiliensis sp. nov. appears to be similar to the Caribbean species *G. americana*, by the shape of the spermathecae, total length, and number of segments. In addition, the genetic analyses indicate that these two species are closely related (see Phylogenetic analyses). There are at least two obvious morphological differences, however: *G. americana* is distinguished from *G. brasiliensis* sp. nov. by its large glands at the spermathecal pores and the presence of a head organ.

Two species from the South Atlantic, sub-Antarctic island of South Georgia, i.e. *Grania monochaeta* (Michaelsen, 1888) and *Grania lasserrei* Rota & Erséus, 1997, share the presence of a midventral copulatory gland (in segment XIV) with *G. brasiliensis* sp. nov. A third species from the same island, *Grania stephensoniana* Rota & Erséus, 1997, also seems to have this gland, but it was not clearly seen because of the

poor quality of the specimens available for the original description (see Rota & Erséus, 1997); *G. stephensoniana* is also larger than all the other species just mentioned. *Grania monochaeta* is similar to *G. brasiliensis* sp. nov. in the location of the spermathecal pores (at some distance from 4/5), but it has glands at its spermathecal pores and stylets in its penial bulbs; *G. brasiliensis* sp. nov. lacks these characters. Both *G. lasserrei* and *G. stephensoniana* possess a head organ (Rota & Erséus, 1996), a structure observed neither in *G. brasiliensis* sp. nov. nor in *G. monochaeta*.

The presence of a copulatory gland in segment XIV is also characteristic of the Tasmanian species *Grania tasmaniae* Rota & Erséus, 2000, but like *G. lasserrei* and *G. stephensoniana*, this species also has a head organ, and furthermore, an unusual type of coelomocytes; according to Rota & Erséus (2000), these cells resemble the spindle-shaped coelomocytes found in *Cernovitoviella* and some species of *Chamaedrillus* (formerly *Cognettia*; see Martinsson, Rota & Erséus, 2014).

Despite sharing some characters with *G. brasiliensis* sp. nov., all of the species from South Africa described below (see their respective remarks), have sperm funnels that are short, not long as in the *G. brasiliensis* sp. nov., and a clitellar epidermis that seems to contain only granular cells (no rows of hyaline cells as in the Brazilian species).

The length of the sperm funnels and the absence of stylets also distinguish *G. brasiliensis* sp. nov. from the Caribbean *Grania bermudensis* Erséus & Lasserre, 1976, *Grania laxartus* Locke & Coates, 1999, and *Grania hylae* Locke & Coates, 1999. Moreover, these species differ from *G. brasiliensis* sp. nov. by their complex penial apparatus, and the presence of a head organ in both *G. laxartus* and *G. hylae*.

Among the *Grania* species with elongate sperm funnels, a pattern of clitellar cells similar to that in *G. brasiliensis* sp. nov., i.e. alternating rows of granular and hyaline cells, has also been described for the Western Australian *Grania sperantia* Rota, Wang & Erséus, 2007; however, this species lacks lateral chaetae and has an unusually long clitellum (extending over segments XI–XIII and sometimes over a part of segment XIV), with a thicker and more complex ‘multi-band’ pattern, i.e. the hyaline cells are more numerous than the granular cells only at the two ends (‘borders of clitellum’; Rota *et al.*, 2007).

The sperm funnels are very long (between nine and 18 times longer than wide), in *G. brasiliensis* sp. nov. and *G. sperantia*, as well as in the other Australian species *Grania conjuncta* Coates & Stacey, 1993, *Grania vacivasa* Coates & Stacey, 1993, *Grania eurystila* Coates & Stacey, 1997, and *Grania integra* Coates & Stacey, 1997; however, all these species are totally devoid

of lateral chaetae, which is not the case in *G. brasiliensis* sp. nov.

GRANIA BEKKOUCHEI SP. NOV.

FIGURE 2

Holotype

SAMC A82466, CE13996, whole-mounted, sexually mature specimen, ten posterior segments amputated, from Saldanha Bay, West Coast district, Province of the Western Cape, South Africa, 33°00'25"S, 17°56'45"E, intertidal coarse sand in rock crevice. Collected by N. Bekkouche, 13 December 2011. *COI* barcode sequence, GenBank acc. no. KT428107; for other sequence data, see Table 1.

Etymology

Named for Nicolas Bekkouche, the collector of the type material.

Paratypes

Six whole-mounted, sexually mature specimens, all collected by N. Bekkouche. SAMC A82467, CE13975, with 13 posterior segments amputated; SAMC A82468, CE13995, with some segments amputated, SAMC A82469, CE13997, with 11 middle body segments amputated; SAMC A82470, CE13998, with nine middle body segments amputated; all from the type locality and the type date. SAMC A82471, CE14035, with some segments amputated, from Glencairn Heights, False Bay, City of Cape Town, Province of the Western Cape, South Africa, 34°09'29"S, 18°26'01"E, lower

intertidal rocky pool, 15 December 2011. SAMC A82472, CE14059, with seven middle body segments amputated from Van Dyks Bay, Overberg District, Overstrand Local Municipality, Province of the Western Cape, South Africa, 34°37'00"S, 19°21'21"E, rocky beach, shallow subtidal, 16 December 2011. For *COI* barcodes of paratypes, see Table 1.

Description

Body > 6.6–12.5 mm long ($n = 5$), comprising 55–72 segments ($n = 5$) (including the segments used for DNA analysis), 0.16–0.17 mm wide at segment III, and 0.15–0.23 mm wide at segment XII ($n = 7$). Prostomium rounded, 50–77 μm long, 55–80 μm wide, occasionally epidermis slightly reduced at front tip, 7–12 μm thick ($n = 7$). Ventral chaetae from segment IV, lateral chaetae from segment XIV ($n = 4$) or segment XV ($n = 3$), sometimes present in preclitellar segments VII–VIII ($n = 1$), VII–IX ($n = 1$), or VII–X ($n = 1$). Chaetae (Fig. 2A) 54–74 μm long, shaft straight, 3.7–6.2 μm thick at midpoint, L-shaped, proximally bent into a foot with broad instep and indistinct heel. Chaetal index (Rota & Erséus, 2003) 4.92 ± 0.68 ($n = 5$). Free chaetae (partly resorbed?) scattered in coelomic cavity, mostly in preclitellar segments. Epidermal gland cells inconspicuous. Clitellum 12–19 μm thick, extending from segment XII to middle of segment XIII, formed by more or less regular transverse rows of granular cells, absent between male pores, hyaline cells not observed. Spermathecal pores in lateral lines, at short distance from 4/5. Male pores ventrolateral in mid segment XII. Female pores lateral (?) in 12/13.

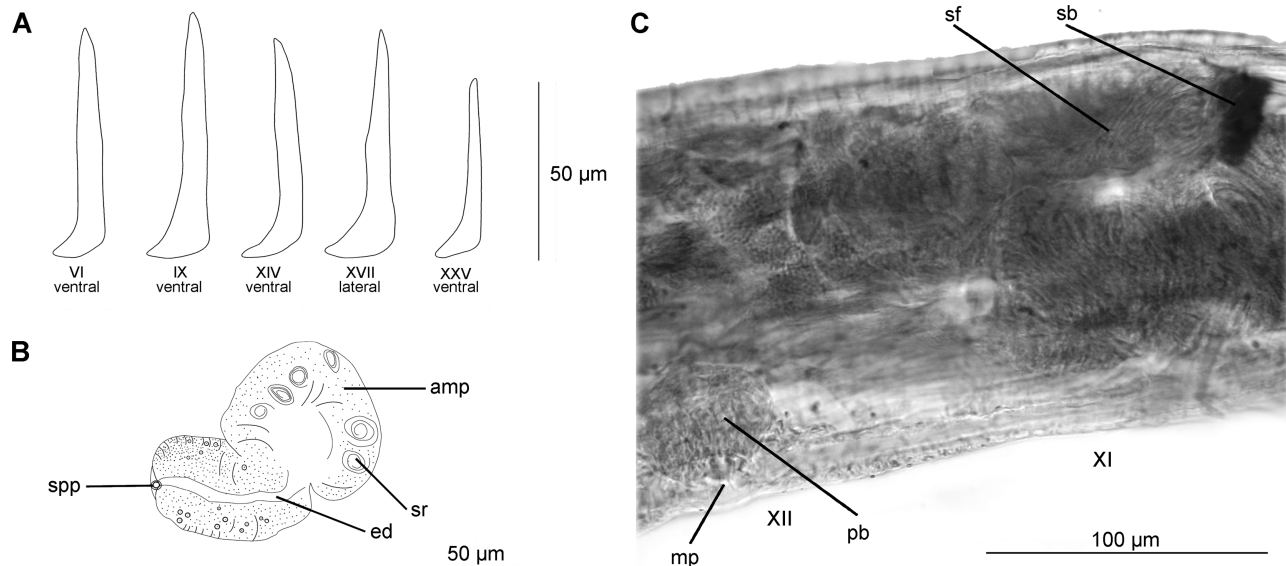


Figure 2. *Grania bekkouchei* sp. nov. A, chaetae. B, spermatheca. C, penial bulb and sperm funnel. See text for abbreviations.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI; dorsal lobes present in segments IV (one pair), V (one pair), and VI (one pair), ventral lobes present in segments IV (one pair), V (two pairs), and VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells inconspicuous. Dorsal blood vessel commencing in segments XVIII–XX. Coelomocytes not observed. Sperm sac reaching XVII. Egg sac extending into segments XVII–XXII. Sperm funnels about 2.5 times longer than wide ($n = 5$; Fig. 2C). Vasa deferentia 7.5 μm wide, internally ciliated, coiled, extending into segments XII–XV. Penial apparatus type 1 (*sensu* Coates, 1984), with oval or round bulb, 55–77 μm long, 45–87 μm wide ($n = 7$); bulb glandular, surrounding a simple invaginated male pore; stylet absent (Fig. 2C). Midventral copulatory gland (in segment XIV) present. Spermathecae communicating with oesophagus at posterior end of segment V. Ectal duct of spermatheca 75–110 μm long, 27–43 μm wide, muscular, maintaining uniform width over its entire length, proximally curved to enter ampulla, devoid of glands at pore. Spermathecal ampulla 60–75 μm long, 60–82 μm wide, dome-shaped with granular walls, containing sperm rings, each maximally 15 μm wide (Fig. 2B).

Remarks

Although noted for some unidentified specimens from the Marion and Crozet islands in the Southern Indian Ocean (see Rota & Erséus, 1997), the presence of lateral chaetae in preclitellar segments is rather unusual in this genus, and is only formally reported for *G. lasserrei* and one specimen of *G. monochaeta*, both from South Georgia Island (Rota & Erséus, 1997). According to Erséus & Lasserre (1976) and Rota & Erséus (1997), the beginning of the chaetal distribution, particularly that of the lateral chaetae, is subject to considerable intraspecific variation. Even so, *G. lasserrei* differs from *G. bekkouchei* sp. nov. by its spermatheca, which has a dorsal, thin-walled, and sacciform diverticulum on the spermathecal ampulla. In *G. monochaeta* the ectal duct of the spermatheca narrows at both ends. The ampulla of *G. bekkouchei* sp. nov. is dome-shaped and the ectal duct maintains the same width along its entire length. In addition, *G. lasserrei* possesses a head organ, which is absent in *G. bekkouchei* sp. nov.

Although seldom noted in descriptions of Enchytraeidae, we observed a lateral ring of histologically distinct cells indicating a small pore-like structure in the intersegmental furrows 12/13 of the holotype; we interpret these structures as the female pores.

Among the new South African species described here, *G. bekkouchei* sp. nov., and its cryptic sister species *Grania cryptica* sp. nov., differ from *Grania capensis* sp. nov. in the morphology of the spermathecae.

Unlike the former two species, *G. capensis* sp. nov. has a pear-shaped ampulla and an ectal duct that narrows near the pores. The species are also separated by the chaetal distribution, although some anterior lateral chaetae are irregularly distributed in all three (see the preceding paragraph). The postclitellar lateral chaetae start in segments XIV–XV in *G. bekkouchei* sp. nov., in segment XVIII in *G. cryptica* sp. nov., and in segments XXI–XXII in *G. capensis* sp. nov. In addition, the chaetae of *G. bekkouchei* sp. nov. have a broad instep and no prominent heel, which make them morphologically different from those of *G. capensis* sp. nov. (see Figs 3, 5).

Grania simonae sp. nov., also from South Africa, is easily distinguished from *G. bekkouchei* sp. nov. by the absence of lateral chaetae, the absence of a midventral copulatory gland (in segment XIV), the morphology of the spermathecae, and the start of the dorsal blood vessel (see remarks for *G. simonae* sp. nov. below).

A cryptic form, morphologically similar to *G. bekkouchei* sp. nov., is described as a separate species below (*G. cryptica* sp. nov.).

GRANIA CRYPTICA SP. NOV.

FIGURE 3

Holotype

SAMC A82473, CE14031, whole-mounted, sexually mature specimen, with 11 midbody segments amputated, from Glencairn Heights, False Bay, City of Cape Town, Province of the Western Cape, South Africa, 34°09'29"S, 18°26'01"E, lower intertidal rocky pool, 15 December 2011. Collected by N. Bekkouche, 15 December 2011. COI barcode sequence, GenBank acc. No. KT428109; for other sequence data, see Table 1.

Etymology

Named *cryptica* because it is morphologically 'hidden' (cryptic) vis-à-vis *G. bekkouchei* sp. nov.

Description

Body > 11.6 mm long, 64 segments (including 11 segments used for DNA analysis), 0.17 mm wide at segment III, and 0.18 mm wide at segment XII. Prostomium rounded, 72 μm long, 62 μm wide, epidermis 15 μm thick, reduced to 10 μm at front tip. Ventral chaetae from segment IV, lateral chaetae in preclitellar segments in segments VI–IX; in postclitellar segments from segment XVIII. Chaetae (Fig. 3A) 66–75 μm long, shaft straight, 5–6 μm thick at midpoint, entally hook-shaped. Free chaetae (partly resorbed?) scattered in the coelomic cavity, present in both preclitellar and postclitellar segments. Epidermal gland cells inconspicuous. Clitellum 10 μm thick, extending from segment XII to middle of segment XIII, formed by more or less regular transverse rows of granular

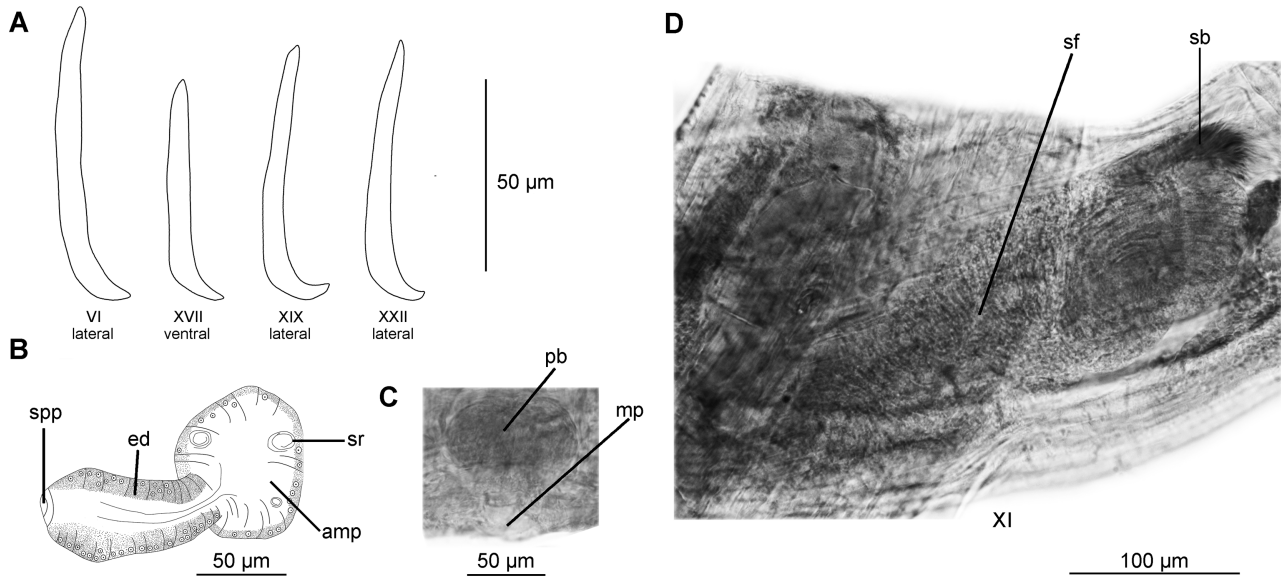


Figure 3. *Grania cryptica* sp. nov. A, chaetae. B, spermatheca. C, penial bulb. D, sperm funnel. See text for abbreviations.

cells, absent between male pores, hyaline cells not observed. Spermathecal pores in lateral lines, located at short distance from 4/5. Male pores ventrolateral in mid segment XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI; dorsal lobes present in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells inconspicuous. Dorsal blood vessel commencing in segment XVIII. Coelomocytes not observed. Sperm sac reaching segment XVII. Egg sac not developed. Sperm funnels about 2.5 times longer than wide (Fig. 3D). Vasa deferentia 10 µm wide, internally ciliated, coiled, extending into segments XII–XVI. Penial apparatus type 1 (*sensu* Coates, 1984), with round bulb, 88 µm long, 88 µm wide; bulb glandular, surrounding a simple invaginated male pore; stylet absent (Fig. 3C). Midventral copulatory gland (in segment XIV) present. Spermathecae communicating with oesophagus at posterior end of segment V. Ectal duct of spermatheca 94 µm long, 40 µm wide, muscular, curved, and slightly narrowing at distal end, devoid of glands at pore. Spermathecal ampulla 77 µm long, 80 µm wide, dome-shaped with granular walls, enclosing sperm rings, each maximally 16 µm wide (Fig. 3B).

Remarks

The only distinguishing morphological feature observed between *G. cryptica* sp. nov. and *G. bekkouchei* sp. nov. is the shape of the chaetae. The

latter has L-shaped chaetae with a broad instep (Fig. 4A, B, C), whereas in *G. cryptica* sp. nov. the chaetae are hook-shaped (Fig. 4D, E, F). The shape of the spermatheca is virtually identical in *G. bekkouchei* sp. nov. and *G. cryptica* sp. nov., with a possible difference, suggested by the single specimen of *G. cryptica* sp. nov., that the ectal duct gently narrows to its junction with the ampulla in the latter species. This, however, may be an artifact from slide mounting.

Although *G. bekkouchei* sp. nov. and *G. cryptica* sp. nov. are difficult to distinguish morphologically, they genetically differ from each other in all loci investigated, clearly indicating that they are separately evolving lineages. This, in combination with their sympatric distribution, strongly supports them to be two different species.

GRANIA CAPENSIS SP. NOV.

FIGURE 5

Holotype

SAMC A82474, CE14015, whole-mounted, sexually mature specimen, with 11 midbody segments amputated, from Glencairn Heights, False Bay, City of Cape Town, Province of the Western Cape, South Africa, 34°09'29"S, 18°26'01"E, lower intertidal rocky pool. Collected by N. Bekkouche, 15 December 2011. *COI* barcode sequence, GenBank acc. no. KT428104; for other sequence data, see Table 1.

Etymology

Named *capensis* for the Cape Town area.

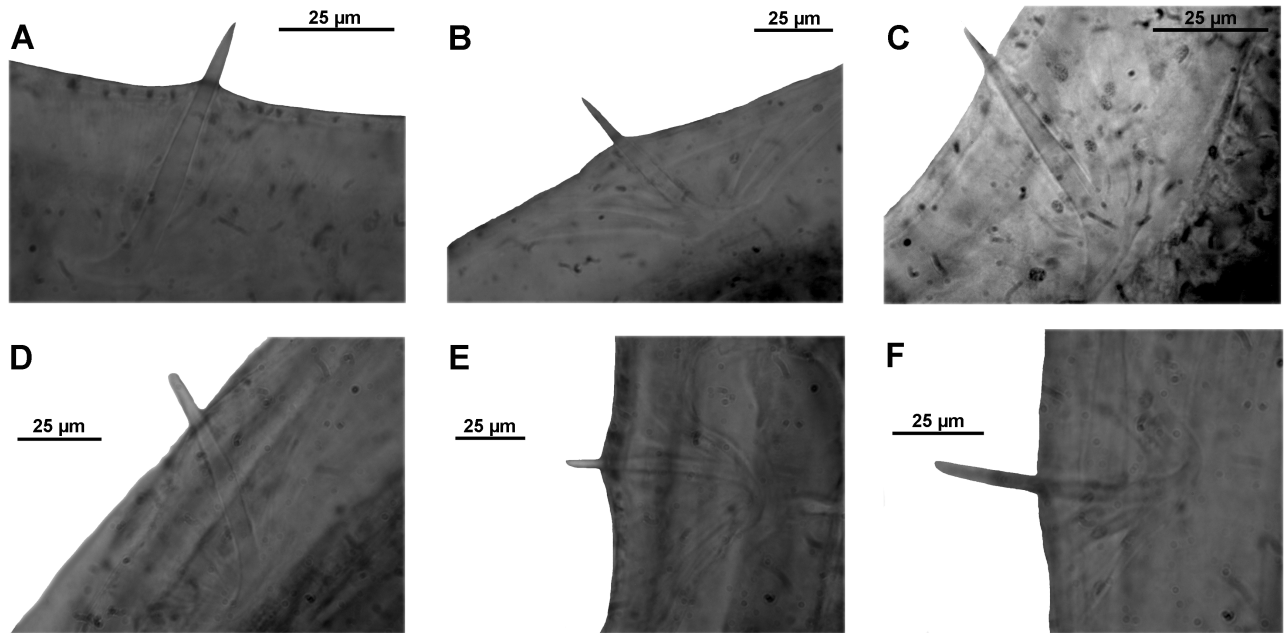


Figure 4. Chaetae of *Grania bekkouchei* sp. nov. A, ventral, from segment VII. B, ventral, from segment X. C, ventral, from segment XXXI. Chaetae of *Grania cryptica* sp. nov. D, lateral, from segment XXI. E, ventral, from segment XXVII. F, ventral, from segment XVIII.

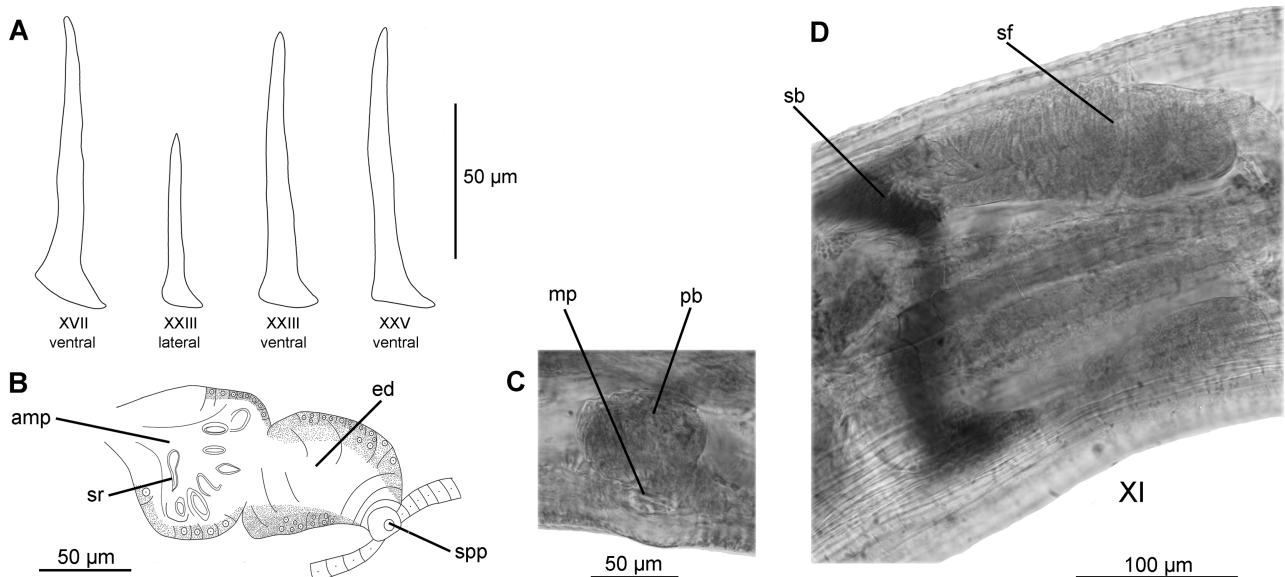


Figure 5. *Grania capensis* sp. nov. A, chaetae. B, spermatheca. C, penial bulb. D, sperm funnel. See text for abbreviations.

Paratype

SAMC A82475, CE14016, one whole-mounted, sexually mature specimen, ten middle body segments amputated, from type locality, N. Bekkouche, 15 December 2011. For *COI* barcode of paratype, see Table 1.

Description

Body > 9.2 and 9.9 mm long, 49 and 63 segments ($n = 2$) (including segments used for DNA analysis), 0.22 and 0.24 mm wide at segment III, 0.20 and 0.26 mm wide at segment XII ($n = 2$). Prostomium rounded, 83 and 120 µm long, 100 and 110 µm wide; epidermis 17 and

12 µm thick ($n = 2$), not notably reduced at front tip. Ventral chaetae from segment IV, lateral chaetae occasionally present in segment VII ($n = 1$), but otherwise from segments XXI or XXII. Chaetae (Fig. 5A) 45–90 µm long, shaft straight, 5.0–7.5 µm thick at midpoint, L-shaped, proximally curving into an 11–25 µm long foot with indistinct heel. Chaetal index (Rota & Erséus, 2003) 4.42 ± 0.88 ($n = 4$). Free chaetae sometimes present, scattered in coelomic cavity. Epidermal gland cells inconspicuous. Clitellum not well developed, 12.5 µm thick, extending from segment XII to two-thirds of segment XIII, formed by more or less regular transverse rows of granular cells, absent between male pores, hyaline cells not observed. Spermathecal pores in lateral lines, slightly posterior to 4/5. Male pores ventrolateral in mid-XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI; dorsal lobes present in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells not observed. Dorsal blood vessel originating in segments XIX (paratype) or XX (holotype). Coelomocytes not observed in available specimens. Sperm sac extending into segments XVI–XVII. Egg sac not developed. Sperm funnels cylindrical, about three times longer than wide (Fig. 5D). Vasa deferentia 15 µm wide, internally ciliated, coiled, reaching at least segment XV. Penial apparatus type 1 (*sensu* Coates, 1984), consisting of a glandular bulb, 75–100 µm long, 75–87 µm wide ($n = 2$) next to epidermal invaginations; stylet absent (Fig. 5C). Midventral copulatory gland (in segment XIV) present. Ectal duct of spermatheca short, thick, 53–75 µm long, 39–55 µm wide, muscular, slightly curved, and narrowing towards pore. No gland at pore. Spermathecal ampulla 57–75 µm long, 75–87 µm wide, pear-shaped, attached to oesophagus in posterior half of segment V. Sperm rings few, maximally 15 µm wide (Fig. 5B).

Remarks

Grania capensis sp. nov. differs from its South African congeners by the shape of the spermathecae, the distribution of the lateral chaetae (see remarks for *G. bekkouchei* sp. nov. and *G. cryptica* sp. nov. above, and *G. simonae* sp. nov. below), and wide vasa deferentia; the latter are 15.0 µm wide in *G. capensis* sp. nov., but only 7.5–10.0 µm wide in the others. The spermathecae of *G. capensis* sp. nov. are pear- or heart-shaped, resembling those of the South Atlantic species *G. stephensoniana* (see Rota & Erséus, 1997), but these two species are distinguished by the length of the sperm funnel (about three times longer than wide in

G. capensis sp. nov., and six or seven times longer than wide in *G. stephensoniana*).

Although geographically distant, two species from the north-east Atlantic, *Grania roscoffensis* Lasserre, 1967 and *Grania pusilla* Erséus, 1974, also share some characteristics with *G. capensis* sp. nov., such as the spermathecal morphology, the length of the sperm funnel, and the distribution of the lateral chaetae. This South African species is distinct from all of these, however, by the simple penial bulb (the other species have a stylet), and the presence of a midventral copulatory gland in segment XIV (absent in the others).

GRANIA SIMONAE SP. NOV.

FIGURE 6

Holotype

SAMC A82476, CE14111, whole-mounted, sexually mature specimen, 11 posterior segments amputated, from Van Dyks Bay, Overberg District, Overstrand Local Municipality, Province of the Western Cape, South Africa, 34°37'00"S, 19°21'21"E, intertidal crevice between rocks. Collected by N. Bekkouche, 16 December 2011. COI barcode sequence, GenBank acc. no. KT428100; for other sequence data, see Table 1.

Etymology

Named for the South African annelid specialist, Dr Carol Simon, who arranged and generously assisted with the workshop/fieldwork that led to the collection of this species.

Paratypes

Six whole-mounted, sexually mature specimens, all from the type locality and date, and collected by N. Bekkouche. SAMC A82482, complete, not DNA barcoded specimen. SAMC A82477, CE14110, with 14 midbody segments amputated. SAMC A82478, CE14112, with 14 middle body segments amputated. SAMC A82479, CE14058, with some segments amputated. SAMC A82480, CE14060, with eight posterior segments amputated. SAMC A82481, CE14093, with some segments amputated, from the type locality too, but in shallow subtidal. For COI barcodes of paratypes, see Table 1.

Description

Body of only complete specimen 16 mm long, 79 segments (paratype SAMC A82482), 0.16–0.20 mm wide at segment III, 0.15–0.22 mm wide at segment XII ($n = 7$). Prostomium rounded, 55–87 µm long, 62–80 µm wide, epidermis 7–10 µm thick ($n = 7$), not reduced at front tip. Ventral chaetae present from segment IV, lateral chaetae absent. Chaetae (Fig. 6A) L-shaped, 45–90 µm long, 5–6 µm thick at midpoint, shaft straight and broad at the base, foot 16–20 µm

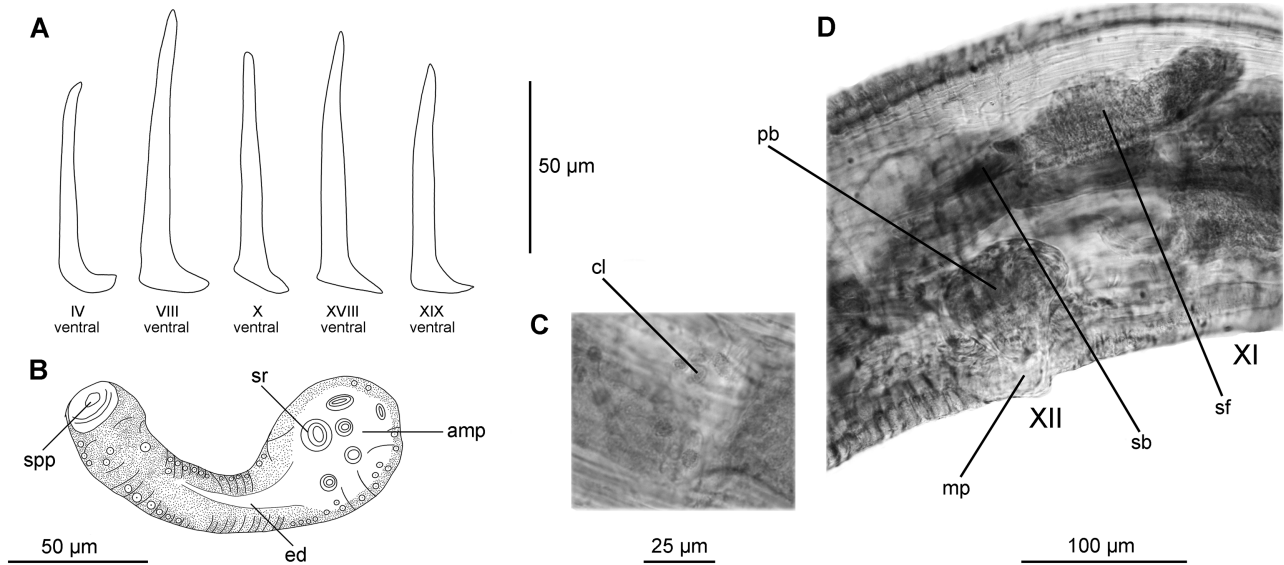


Figure 6. *Grania simonae* sp. nov. A, chaetae. B, spermatheca. C, coelomocytes. D, penial bulb and sperm funnel. See text for abbreviations.

long, with slight heel, and sometimes with slight toe. Chaetal index (Rota & Erséus, 2003) 4.70 ± 0.39 ($n = 5$). Free chaetae scattered in the coelomic cavity, sometimes numerous. Epidermal gland cells inconspicuous. Clitellum maximally 22 μm thick, extending from segments XII to XIII, comprising rows of large granular cells, absent between male pores. Spermathecal pores in lateral lines, immediately posterior to 4/5. Male pores located ventrolaterally in mid-segment XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI, dorsal lobes in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells from segment VII distinctive, containing dark granules, dense in posterior segments. Dorsal blood vessel from segments XXI or XXIV ($n = 3$). Coelomocytes irregular, at least 10 μm wide, most abundant posteriorly (in complete specimen) (Fig. 6C). Sperm sac extending into segment XVI, egg sac reaching segment XX. Sperm funnels in segment XI about two times longer than wide, folded, occasionally directed posteriorly (holotype) (Fig. 6D). Vasa deferentia reaching at least segment XIV, coiled, internally ciliated, 15 μm wide near sperm funnel, narrowing to about 10 μm . Glandular penial bulb type 1 (*sensu* Coates, 1984), 75–80 μm long, 50–68 μm wide ($n = 6$); stylet absent (Fig. 6D). Copulatory gland (in segment XIV) absent. Spermathecal ampulla small, more or less spherical, 37–55 μm long, 37–52 μm wide

(Fig. 6B). Sperm rings up to 15 μm wide. Ectal duct slender, 90–115 μm long, 17–25 μm wide, not narrowing at ends. No gland at spermathecal pore.

Remarks

Grania simonae sp. nov. is unique among the South African species described here and all other members of the genus by the combination of the following characters: lack of lateral chaetae, spermathecae with small and spherical ampulla, dense chloragogen cells, dorsal blood vessel commencing in segment XXIV, and absence of head organ and a midventral copulatory gland in segment XIV. This is the only South African species with numerous and conspicuous coelomocytes scattered throughout the body, but as stated by Rota *et al.* (2007) and De Wit & Erséus (2007), the apparent lack of coelomocytes in the other species described herein may be an artifact, caused by the fixation and staining procedures.

The absence of lateral chaetae has been recorded for at least 13 different species of *Grania* to date. Among them, *G. simonae* sp. nov. is reminiscent of the Antarctic species *Grania carchinii* Rota & Erséus, 1996, by the absence of penial stylets and head organ, and the possession of dense chloragogen cells; however, the adult specimens of *G. simonae* sp. nov. are at least twice as large as those of *G. carchinii* known so far. These two species are also distinguished from each other by the morphology of the spermatheca. *Grania carchinii* has a larger (pear-shaped) spermathecal ampulla and a shorter spermathecal duct than *G. simonae* sp. nov.; the latter has a spherical spermathecal ampulla.

Moreover, *G. carchini* has a midventral copulatory gland in segment XIV, which is lacking in *G. simonae* sp. nov.

The Western Australian *G. conjuncta* and *G. vacivasa* also resemble *G. simonae* sp. nov. by the absence of lateral chaetae, stylets, and head organ, but they both differ from the latter by possessing a midventral copulatory gland in segment XIV and by extremely long sperm funnels; the funnels are only about twice longer than wide in *G. simonae* sp. nov., but 19 times longer than wide in *G. conjuncta*, and 11–15 times longer than wide in *G. vacivasa*.

GRANIA HINOJOSAI SP. NOV.

FIGURE 7

GRANIA SP. CHILE 1; DE WIT ET AL., 2011B

Holotype

ZUEC CLI 08, PDW186, whole-mounted, sexually mature specimen, with some segments amputated, from Puerto Aldea, Coquimbo, Elqui, Chile, 30°18'19"S, 71°39'33"W. Intertidal, sand among rocks, P. De Wit and I. Hinojosa, 6 February 2009. *COI* barcode sequence, GenBank acc. no. GU902189; for other sequence data, see Table 1.

Etymology

Named for Ivan Hinojosa, who was instrumental to all of the Chilean fieldwork.

Paratypes

Four whole-mounted, sexually mature specimens, and all collected by P. De Wit. ZUEC CLI 09, PDW187, with

some segments amputated, from type locality. ZUEC CLI 10–CLI 12, PDW177, PDW181, PDW182, with some segments amputated, from Pampilla Point, Coquimbo, Elqui, Chile, 29°57'23"S, 71°21'39"W, heterogeneous sand with organic material, 6 February 2009. For *COI* barcodes of paratypes, see Table 1.

Description

Body > 4.15–5.85 mm long, > 18–28 segments ($n = 5$) (posterior ends used for DNA extractions), 0.17–0.20 mm wide at segment V, 0.16–0.20 mm at segment XII ($n = 5$). Prostomium conical or rounded, 67–87 μm long, 100–110 μm wide, epidermis not reduced at front tip, 10–12 μm thick ($n = 5$). Ventral chaetae from segment IV, lateral chaetae from segments XVII–XIX. Chaetae (Fig. 7A) increasing in size towards the posterior, 30–65 μm long, shaft straight, 3.7–6.2 μm thick at midpoint, L-shaped, proximally bent into a foot, with low instep and indistinct heel. Chaetal index (Rota & Erséus, 2003) 3.99 ± 0.42 ($n = 5$). Epidermal gland cells inconspicuous. Clitellum maximally 7–10 μm thick, extending from posterior half of segment XI to anterior half of segment XIII, formed by more or less regular transverse rows of granular cells, absent between male pores. Spermathecal pores in lateral lines, somewhat posterior to 4/5. Male pores ventrolateral in mid-segment XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI, dorsal lobes in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in

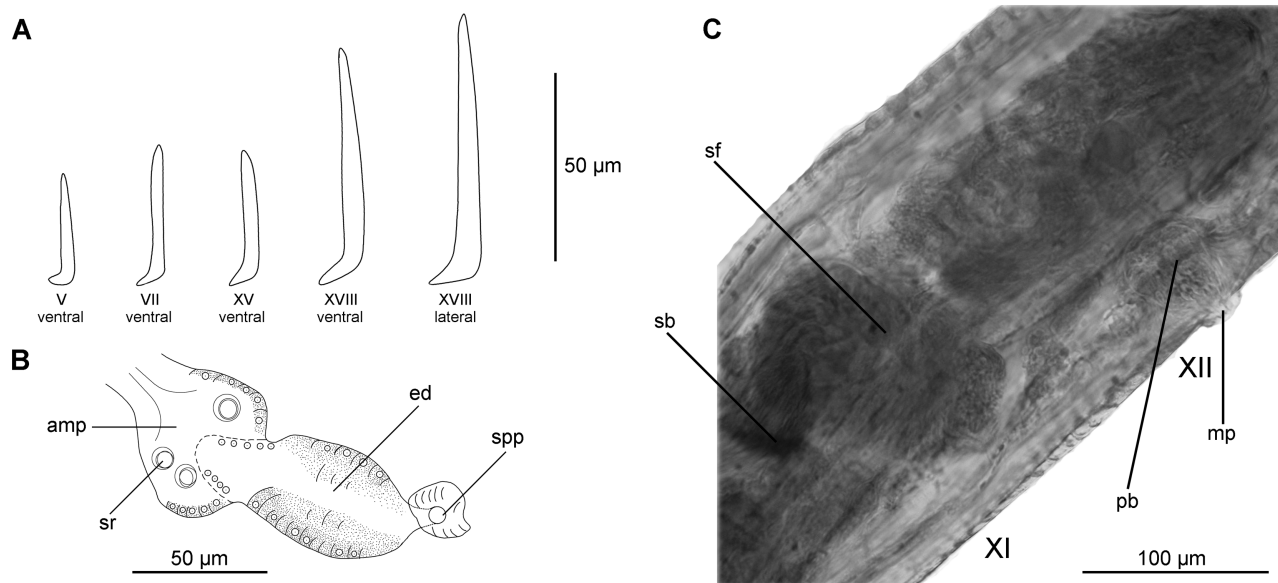


Figure 7. *Grania hinojosai* sp. nov. A, chaetae. B, spermatheca. C, penial bulb and sperm funnel. See text for abbreviations.

segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells not observed. Dorsal blood vessel commencing in segments XV or XVI. Coelomocytes not observed. Sperm sac extending into segment XIV, egg sac extending into segment XVI (holotype). Sperm funnels about 1.5 times longer than wide (Fig. 7C). Vasa deferentia observed in segments XI–XII, internally ciliated, tightly coiled near sperm funnel, 10 µm wide. Penial apparatus type 1 (*sensu* Coates, 1984), small, compact glandular bulb, 47–50 µm long, 55–67 µm wide ($n = 5$); stylet absent. Midventral copulatory gland (in segment XIV) present. Each spermatheca attached to oesophagus in posterior half of segment V through narrow ental duct. Ampulla ‘heart-shaped’, 37–55 µm long, 37–52 µm wide (Fig. 7B). Sperm rings maximally 17 µm wide, but few ($n = 5$). Ectal duct narrowing at both ends, 80–92 µm long, 37–40 µm wide, joining ampulla through a conical intrusion, with a prominent gland attached near pore (Fig. 7B).

Remarks

The diagnostic characters for *G. hinojosai* sp. nov. are the unique combination of the glands at the spermathecal pores, the location of these pores, at some distance from 4/5, the short sperm funnels, and the small and compact penial bulbs.

Among the group of *Grania* species possessing glands at the spermathecal pores, *Grania novacaledonia* De Wit & Erséus, 2007 resembles this Chilean species by sharing characters such as the distribution of lateral chaetae, the chaetal size distribution (increasing in size posteriorly), presence of a midventral copulatory gland (in segment XIV), and the absence of copulatory stylets; however, *G. hinojosai* sp. nov. is distinguished by its shorter sperm funnel and its spermathecal ducts narrowing ectally. Spermathecal glands have also been reported for some species in the West Indian Ocean [*Grania ersei* Coates, 1990 and *Grania darwinensis* (Coates & Stacey, 1997)], North Pacific waters [*Grania paucispina* (Eisen, 1904)], and the North (*G. americana*) as well as the South Atlantic Ocean (*G. monochaeta*). The heart-shaped spermathecal ampulla, the somewhat spindle-shaped spermathecal duct, and the more posterior distribution of the lateral chaetae appear to distinguish *G. hinojosai* sp. nov. from *G. ersei* and *G. paucispina*; the lateral chaetae begin in segments XVII–XIX in *G. hinojosai* sp. nov., but in segments XIV or XV in the other two taxa. *Grania ersei* has a top-shaped spermathecal ampulla, and a long, coiled spermathecal duct, whereas *G. paucispina* has a spermatheca with an ovoid ampulla. Moreover, *G. ersei* possesses very long copulatory stylets, structures not even present in *G. hinojosai* sp. nov.

The Western Australian *G. darwinensis* has a more complex penial apparatus than *G. hinojosai* sp. nov., i.e.

it has accessory glands, covered by muscle layers, at both sides of the bulb, whereas in *G. hinojosai* sp. nov., the whole apparatus is smaller and compact.

Grania americana is easily differentiated from *G. hinojosai* sp. nov. by the length of the sperm funnel (eight times longer than wide in the former, about 1.5 times longer than wide in the latter) and the presence of a head organ, absent in *G. hinojosai* sp. nov.

Although *G. monochaeta* and *G. hinojosai* sp. nov. share the somewhat unusual, more posterior, position of the spermathecal pores, and the presence of a midventral copulatory gland in segment XIV (Rota & Erséus, 1997), the lateral chaetae start in segment XIII in *G. monochaeta*, but start in segments XVII–XIX in *G. hinojosai* sp. nov. Finally, *G. hinojosai* sp. nov. differs from all species mentioned above by its short sperm funnel.

GRANIA CHILENSIS SP. NOV.

FIGURE 8

GRANIA SP. CHILE 2; DE WIT *ET AL.*, 2011B

Holotype

ZUEC CLI 13, PDW193, whole-mounted, sexually mature specimen, with some segments amputated, from Punta Loncoyen, Valdivia, Chile, 39°49'27"S, 73°24'25"W. Lower intertidal, sand among rocks. Collected by P. De Wit, 9 February 2009. COI barcode sequence, GenBank acc. no. GU902190; for other sequences, see Table 1.

Etymology

Named for Chile.

Paratypes

Six whole-mounted, sexually mature specimens, collected by P. De Wit. ZUEC CLI 14–CLI 16, PDW190, PDW191, PDW194, with some segments amputated, from type locality. ZUEC CLI 17, PDW185, with some segments amputated, from Puerto Aldea, Coquimbo, Elqui, Chile, 30°18'19"S, 71°39'33"W, intertidal, sand among rocks, 6 February 2009. ZUEC CLI 18–CLI 19, PDW198, PDW199, with some segments amputated, from Caleta Tumbes, Talcahuano, Concepción, Chile, 36°38'00"S, 73°05'27"W, lower intertidal, heterogeneous sand with organic material between boulders, 16 February 2009. For COI barcodes of paratypes, see Table 1.

Description

Body > 3.85–7.45 mm long, > 26–40 segments ($n = 6$) (posterior ends used for DNA extractions), 0.13–0.17 mm wide at segment V, 0.11–0.19 mm at segment XII ($n = 6$). Prostomium conical, 55–80 µm long, 60–80 µm wide, epidermis not reduced at front tip, 7 µm thick ($n = 6$). Ventral chaetae from segment IV, lateral chaetae beginning in segment XVI ($n = 1$), in

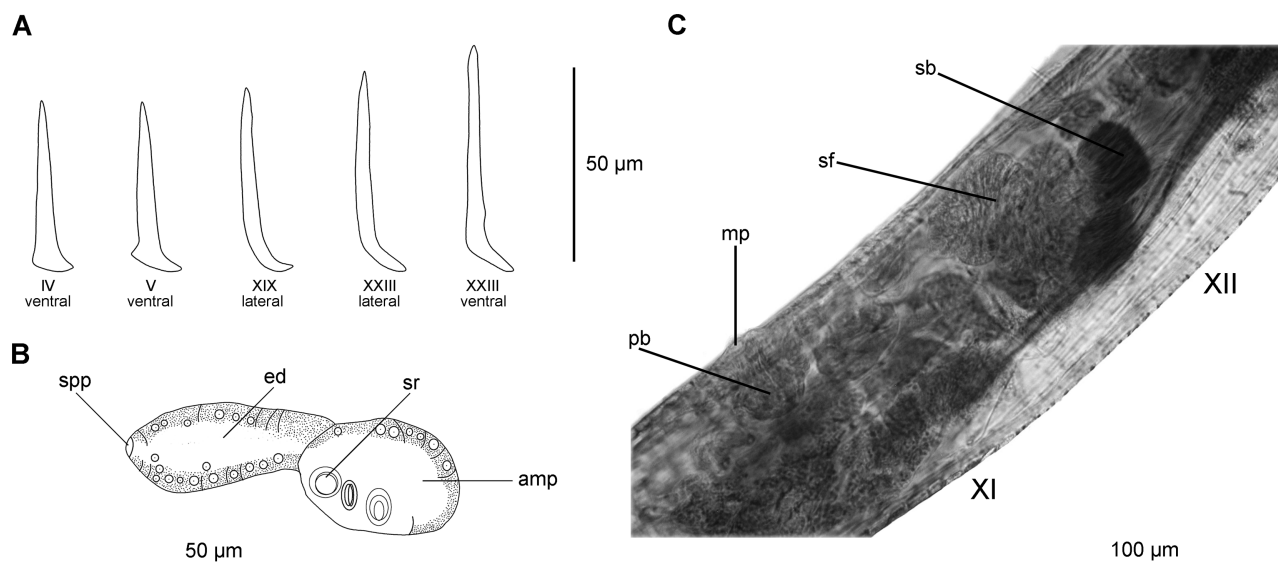


Figure 8. *Grania chilensis* sp. nov. A, chaetae. B, spermatheca. C, penial bulb and sperm funnel. See text for abbreviations.

segment XVII ($n = 3$), in segment XVIII ($n = 1$), or segment XIX ($n = 1$). Chaetae (Fig. 8A) of uniform size, 44–59 μm long, shaft straight, 3.7–5.0 μm thick at midpoint, L-shaped, proximally bent into a foot with low instep, and with distinct heel only in preclitellar segments. Chaetal index (Rota & Erséus, 2003) 4.03 ± 0.52 ($n = 5$). Epidermal gland cells inconspicuous. Clitellum maximally 10–12 μm thick ($n = 4$), extending from segment XII to anterior half of segment XIII, consisting of transverse rows of granular gland cells interspersed with hyaline cells; with the latter, however, absent ventrally. Spermathecal pores in lateral lines, just posterior to 4/5. Male pores ventrolateral in middle of segment XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI, dorsal lobes in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells not observed. Dorsal blood vessel commencing in segments XVI–XVIII. Coelomocytes not observed. Sperm sac extending to segments XIII–XV, egg sac extending to segments XV–XVII. Sperm funnels about 1.5–2.5 times longer than wide (Fig. 8C). Vasa deferentia observed in segments XI–XIII, internally ciliated, coiled, 10 μm wide. Penial apparatus type 1 (*sensu* Coates, 1984), small, compact glandular bulb, 35–55 μm long, 37–50 μm wide ($n = 6$); stylet absent. Midventral copulatory gland (in segment XIV) present. Spermatheca attached to oesophagus in posterior half of segment V by narrow ental duct.

Ampulla oval, 42–65 μm long, 30–42 μm wide (Fig. 8B). Sperm rings maximally 17 μm wide, but few. Ectal duct, 54–95 μm long, 13–30 μm wide at midcourse. No gland at spermathecal pore.

Remarks

The compact and small penial bulb, the sperm funnels being 1.5–2.5 times longer than wide, the absence of penial stylets, the presence of a midventral copulatory gland in segment XIV, and the distribution of the lateral chaetae, starting from segments XVI–XIX, make *G. chilensis* sp. nov. reminiscent of *G. hinojosai* sp. nov.; however, *G. hinojosai* sp. nov. has a spermathecal ampulla that is heart-shaped, a distinct chaetal size distribution (chaetae becoming larger towards the posterior end), and possesses a gland at the spermathecal pore, whereas in *G. chilensis* sp. nov. the ampulla is ovoid, the chaetal size is uniform, and there are no glands at the spermathecal pore.

Three species from South Pacific, *Grania galbina* De Wit & Erséus, 2007, *Grania breviductus* De Wit, Rota & Erséus, 2009, and *G. novacaledonia* share a similar distribution of the lateral chaetae to that in *G. chilensis* sp. nov. Moreover, as in *G. chilensis* sp. nov., a midventral copulatory gland is present in *G. breviductus* and *G. novacaledonia* too. *Grania chilensis* sp. nov., however, differs from all the species mentioned above by the short length of the sperm funnel; in the other three species these funnels are about four or five times longer than wide. Furthermore, *G. galbina* has its male pore surrounded by a large granular mass, *G. breviductus* has the communication between the spermatheca and the oesophagus in the middle of segment V, and

G. novacaledonia possesses glands at the spermathecal pores, none of which is the case for *G. chilensis* sp. nov.

The ovoid spermathecal ampulla, the length of the sperm funnel, the distribution of the lateral chaetae, and the absence of a penial stylet make *G. chilensis* sp. nov. (see discussion above) similar to the Irish *Grania mira* Locke & Coates, 1998, but in addition to being geographically distant from this species, *G. chilensis* sp. nov. differs from *G. mira* by the presence of a midventral copulatory gland in segment XIV, and the lack of thickened muscles along the male ducts.

GRANIA UNITHECA SP. NOV.

FIGURE 9

Holotype

USNM1283175, CE11659, whole-mounted, sexually mature specimen, eight posterior segments amputated, from North Carolina, USA, 34°47'22"N, 76°13'43"W. Sublittoral, 17 m in depth, sand. Collected by C. Erséus, 24 May 2011. *COI* barcode sequence, GenBank acc. no. KT428113; for other sequences, see Table 1.

Etymology

Named *unitheca* for its single spermatheca.

Description

Body > 8.56 mm long, 61 segments (including segments used for DNA analysis), 0.09 mm wide at segment V, 0.09 mm at segment XII. Prostomium rounded, 40 µm long, 60 µm wide, epidermis not reduced at front tip, 6 µm thick. Ventral chaetae from segment XV, lateral chaetae absent. Chaetae (Fig. 9A)

50–62 µm long, shaft straight, 3.7–5.0 µm thick at midpoint, hook-shaped. Epidermal gland cells inconspicuous. Clitellum not well developed. Spermathecal pore unpaired, mid dorsal, just posterior to 4/5. Male pores ventrolateral in middle of segment XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI, dorsal lobes in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells not observed. Dorsal blood vessel commencing in segment XV. Coelomocytes not observed. Sperm sac extending to segment XIV, egg sac in segment XVII. Sperm funnels as long as wide (Fig. 9D). Vasa deferentia observed in segments XII–XIII, internally ciliated, coiled, 8 µm wide. Penial apparatus type 1 (*sensu* Coates, 1984), small, compact glandular bulb, 44 µm long, 39 µm wide; stylet absent (Fig. 9C). Midventral copulatory gland (in segment XIV) present. Spermatheca attached to oesophagus near 5/6. Ampulla nearly spherical, 52 µm long, 54 µm wide. Sperm rings maximally 9 µm wide (Fig. 9B). Ectal duct, 61 µm long, 35 µm wide at midcourse. No gland at spermathecal pore.

Remarks

An unpaired spermatheca is unusual in enchytraeids and, among *Grania* species, it has been only reported for *Grania monospermatheca* Erséus & Lasserre, 1976, also known from the continental shelf off the

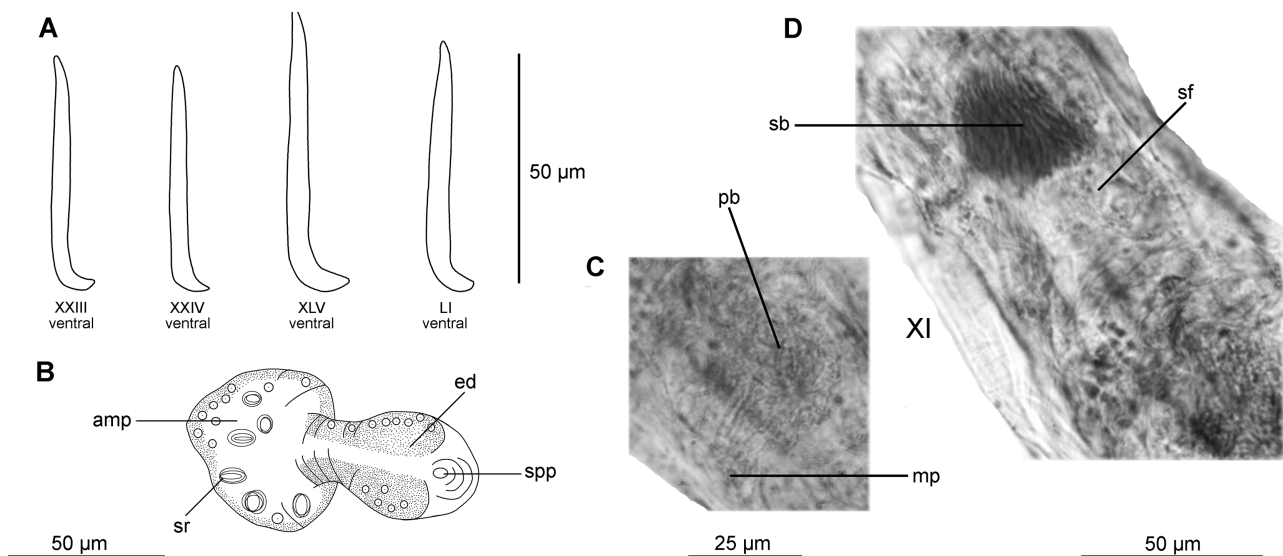


Figure 9. *Grania unitheca* sp. nov. A, chaetae. B, spermatheca. C, penial bulb. D, sperm funnel. See text for abbreviations.

eastern USA. In fact, *G. unitheca* sp. nov. is morphologically similar to *G. monospermatheca* in almost all characters observed; however, it may be distinguished from *G. monospermatheca* by its small and compact penial bulb, with no stylet. *Grania monospermatheca* has a more complex penial apparatus, with a large glandular structure attached to the penial bulb, and the presence of a stylet. Furthermore, *G. unitheca* sp. nov. possesses a midventral copulatory gland in segment XIV. This gland was not observed in *G. monospermatheca* (see Erséus & Lasserre, 1976 and Coates & Erséus, 1985).

Genetically, these two species are well distinguished from each other; however, the phylogenetic analysis, as well as the fact that the two species are from the same geographical area, suggest that *G. unitheca* sp. nov. and *G. monospermatheca* are closely related (see 'Phylogenetic analysis' below).

GRANIA CAROLINENSIS SP. NOV.

FIGURE 10

Holotype

USNM 1283174, CE11569, whole-mounted, sexually mature specimen, with some segments amputated, from off North Carolina, USA, 33°10'23"N, 76°45'23"W. Continental shelf slope, 492 m in depth, sand. Collected by C. Erséus, 20 May 2011. *COI* barcode sequence, GenBank acc. no. KT428112; for other sequences, see Table 1.

Etymology

Named for North Carolina.

Description

Body > 5.10 mm long, > 30 segments (posterior end used for DNA extractions), 0.08 mm wide at segment V, 0.09 mm wide at segment XII. Prostomium rounded, 51 µm long, 66 µm wide, epidermis 10 µm thick, reduced to 4 µm at front tip. Ventral chaetae from segment XIII, lateral chaetae absent. Chaetae (Fig. 10A) 42–50 µm long, shaft straight, 3.7 µm thick at midpoint, L-shaped, proximally bent into a foot with low instep and slight heel. Chaetal index (Rota & Erséus, 2003) 3.60 ± 0.33 ($n = 4$). Epidermal gland cells inconspicuous. Clitellum not well developed. Spermathecal pores in lateral lines, immediately posterior to 4/5. Male pores ventrolateral in mid-segment XII. Female pores not observed.

Brain posteriorly indented. Head organ (*sensu* Rota & Erséus, 1996) absent. Pharyngeal glands in segments IV–VI, dorsal lobes in segment IV (one pair), in segment V (one pair), and in segment VI (one pair), ventral lobes present in segment IV (one pair), in segment V (two pairs), and in segment VI (two pairs); glands not connected dorsally. Nephridia not observed. Chloragogen cells not observed. Dorsal blood vessel commencing in segment XVII. Coelomocytes not observed. Sperm sac extending into half of segment XV, egg sac extending into segment XVII. Sperm funnels about six times longer than wide (Fig. 10C). Vasa deferentia observed in segments XII–XIII, internally ciliated, coiled, 7 µm wide. Penial apparatus type 1 (*sensu* Coates, 1984), small, glandular bulb, 40 µm long, 31 µm wide; stylet absent. Midventral copulatory gland (in segment XIV) not observed. Each spermatheca attached to oesophagus in posterior half of segment V. Ampulla large, oval, 100 µm long, 44 µm wide. Sperm

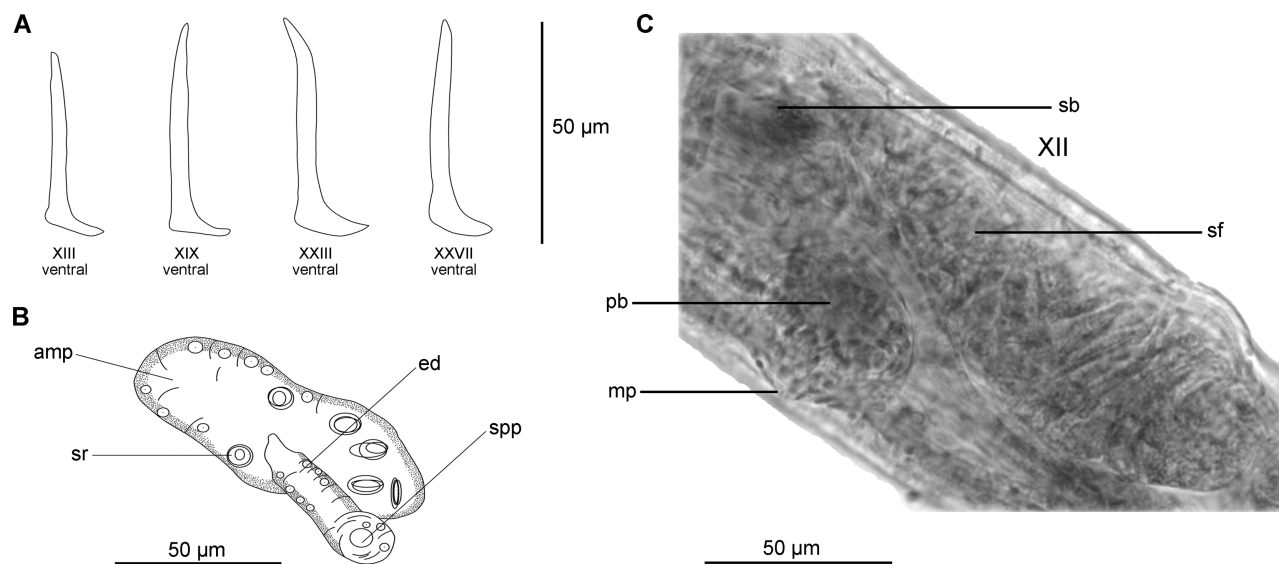


Figure 10. *Grania carolinensis* sp. nov. A, chaetae. B, spermatheca. C, penial bulb and sperm funnel. See text for abbreviations.

rings maximally 11 µm wide, but few. Ectal duct slender, 53 µm long, 14 µm wide at midcourse, narrowing at distal end (Figure 10B). No gland at spermathecal pore.

Remarks

Grania carolinensis sp. nov. is similar to the north-east Atlantic, *Grania postclitellochaeta* (Knöllner, 1935), *Grania ovitheca* Erséus, 1977, and the cryptic congener to *G. ovitheca*, *Grania occulta* De Wit & Erséus, 2010, a species constellation also supported by the genetic data (see 'Phylogenetic analyses' below). All of these four species have the same distribution of ventral chaetae (beginning in segment XIII), and they lack lateral chaetae and the midventral copulatory gland in segment XIV. *Grania carolinensis* sp. nov., *G. ovitheca*, and *G. occulta* also share the shape of the spermatheca, which has a short and slender ectal duct and a large oval ampulla. In *G. ovitheca* and *G. occulta*, however, the sperm funnels are longer (between eight and 15 times longer than wide, as opposed to six times longer than wide in *G. carolinensis* sp. nov.) and they lack the ventral lobes of the pharyngeal glands in segment IV that are present in *G. carolinensis* sp. nov. and also in *G. postclitellochaeta*. *Grania postclitellochaeta* differs from *G. carolinensis* sp. nov. by possessing a racket-shaped diverticulum in the spermathecal ampulla, which is absent in *G. carolinensis* sp. nov. In addition, *G. carolinensis* sp. nov. differs from all the species mentioned above by its penial apparatus, with a simple invagination surrounded by a small and glandular bulb. In *G. postclitellochaeta* the penial bulbs are covered by a large supplementary glandular body, whereas in *G. ovitheca* the bulbs instead have lateral aglandular sacs directed posteriorly (Rota & Erséus, 2003). The aglandular sacs were not mentioned in the original description of *G. occulta* (see De Wit & Erséus, 2010), but the authors noted that the single available specimen possessed oval structures next to the epidermal invaginations. No accessory glands or sacs could be observed near the male pores of *G. carolinensis* sp. nov.

The deep-sea Atlantic *Grania atlantica* Coates & Erséus, 1985 also resembles *G. carolinensis* sp. nov. by the proportions of the sperm funnel and the morphology of the penial apparatus, but the former differs from the latter by its spermathecal ampulla, which has a large sacciform diverticulum. Moreover, *G. atlantica* possesses lateral chaetae, a head organ, as well as a midventral copulatory gland in segment XIV, all of which are absent in the new species.

GRANIA CF. LEVIS COATES & ERSÉUS, 1985

PROBABLY *GRANIA LEVIS* COATES & ERSÉUS, 1985:
111–112, FIG. 6

Material examined

USNM 1283176, CE11570, whole-mounted, sexually immature specimen, with some segments amputated, from

off North Carolina, USA, 33°10'23"N, 76°45'23"W. Continental shelf slope, 492 m in depth, sand. Collected by C. Erséus, 20 May 2011. *COI* barcode KT428114; for other genes, see Table 1.

Remarks

This barcoded, but immature specimen, and thus unsuitable for complete morphological description, was included in the phylogenetic analysis, to enlarge the taxonomic sampling from the north-western Atlantic region. Phylogenetically, this specimen came out as closely related to *G. carolinensis* sp. nov. (Fig. 15), but it is morphologically distinct by its complete lack of chaetae. The latter trait suggests that this specimen could belong to *Grania levis* Coates & Erséus, 1985, originally described from somewhat further north, from Georges Bank, south-east of Massachusetts, USA.

GENETIC ANALYSES

COI clustering

The Bayesian inference of the *COI* sequences divide the 38 individuals into ten well-supported clades (Fig. 11), four of which are found in South Africa, two in Chile, one in Brazil, and three in the North Atlantic. Within-clade variation is generally low, but in one clade, i.e. all specimens referred to the new taxon *G. chilensis* sp. nov., there is a notable subclustering pattern, dividing this clade into four subclades. A haplotype network (Fig. 12) indicates that *G. chilensis* sp. nov. is structured geographically, with two subclades found in the southernmost site (Valdivia), one subclade in the northernmost site (Coquimbo), and an intermediate subclade in the intermediately located site (Concepcion). Pairwise genetic distances indicate that in general there is a strong barcoding gap present between lineages within this group. In the *G. chilensis* sp. nov. clade, however, there is higher than average within-species divergence, although not nearly as great as the lowest between-species differences (Fig. 13).

ITS clustering

The Bayesian inference analysis of 23 *ITS* sequences supports all ten clusters found in the mitochondrial data (Fig. 14); however, although there is also variation within the *G. chilensis* sp. nov. cluster in the *ITS* region, the geographic substructuring is not seen here. Instead, the variation seems to be randomly distributed with respect to geography.

Phylogenetic placement of new species

The updated phylogeny is completely congruent with that described in De Wit *et al.* (2011b), containing three main clades (A, B, C in Fig. 15). All of the South African species form one strongly supported clade within clade A,

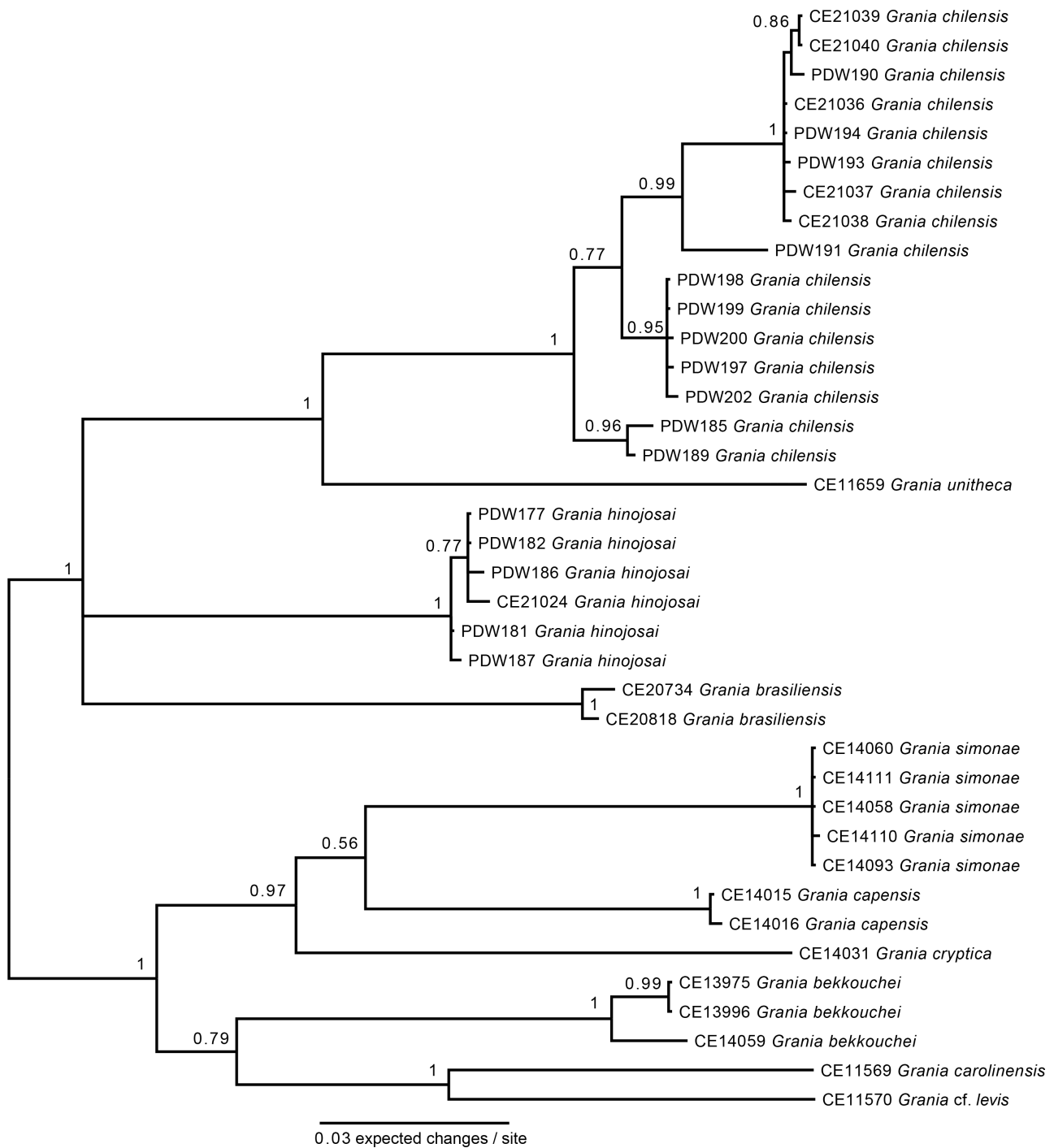


Figure 11. Majority-rule consensus gene tree of cytochrome *c* oxidase subunit I (*COI*) locus from all species described in this study. Posterior probabilities calculated from Bayesian Markov chain Monte Carlo (MCMC) inference are noted above the branches. Note the four clades within *Grania chilensis* sp. nov.

together with all the North Atlantic species. By contrast, the Chilean and the Brazilian species are placed in clade B, together with *Grania curta* De Wit & Erséus, 2007 and *G. americana*. *Grania unitheca* sp. nov. from shallow water in North Carolina (North Atlantic) is

placed together with the other North American species *G. monospermatheca* (its sister taxon) and *G. laxartus*; however, *G. carolinensis* sp. nov. and the closely related immature specimen of *Grania cf. levis*, both found in deep water off the North Carolinian coast, are placed

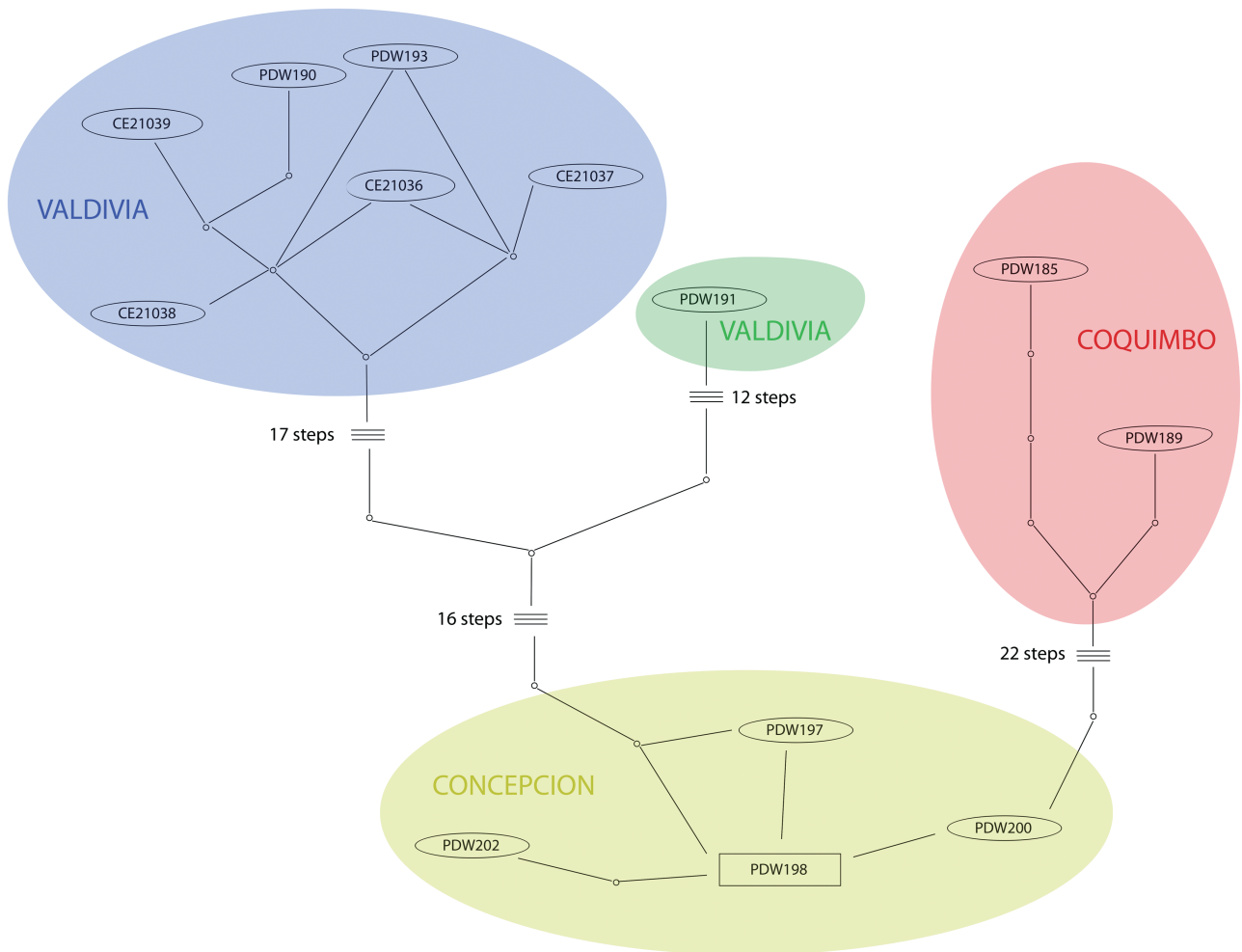


Figure 12. Haplotype network of individuals of *Grania chilensis* sp. nov. Note the long distance (35 steps) between the two groups found in Valdivia.

as the sister clade of *G. postclitellochaeta* and the cryptic *G. occulta*, whereas *G. ovithecra*, which is morphologically identical to *G. occulta*, is strongly supported as the sister to this four-taxon group (Fig. 15).

DISCUSSION

Based on molecular and morphological data, species of *Grania* from Brazil, South Africa, and Chile are described for the first time and, together with the two new species described for north-western Atlantic, the number of nominal species belonging to this genus is increased from 72 to 81. The phylogeny of the group was updated by the addition of these representatives from poorly known areas in the southern hemisphere, plus the species from the North Atlantic Ocean. This study clearly shows that within *Grania* commonly used morphological characters are unsuitable for phylogenetic reconstruction, supporting the previous

conclusion (De Wit *et al.*, 2011b) that homoplasy is abundant within the genus.

The finding of a new cryptic species in South African waters again illustrates the importance of an integrative taxonomic approach to the delimitation of closely related ciliates; for other similar cases see Gustafsson, Price & Erséus (2009), De Wit & Erséus (2010), and Martinsson *et al.* (2013). As *G. cryptica* sp. nov. and *G. bekkouchei* sp. nov. are sympatric and hard to distinguish without genetic analyses, they probably would otherwise be identified as the same species, leading to an underestimation of the diversity of the group. Interestingly, *G. cryptica* sp. nov. and *G. bekkouchei* sp. nov. do differ in their chaetal shape (L- versus hook-shaped), which constitutes the only observed distinguishing difference between these two species (Fig. 4A, B, C, D, E, F). Chaetal shape has previously been indicated as one of the few morphological characters showing some degree of congruence with

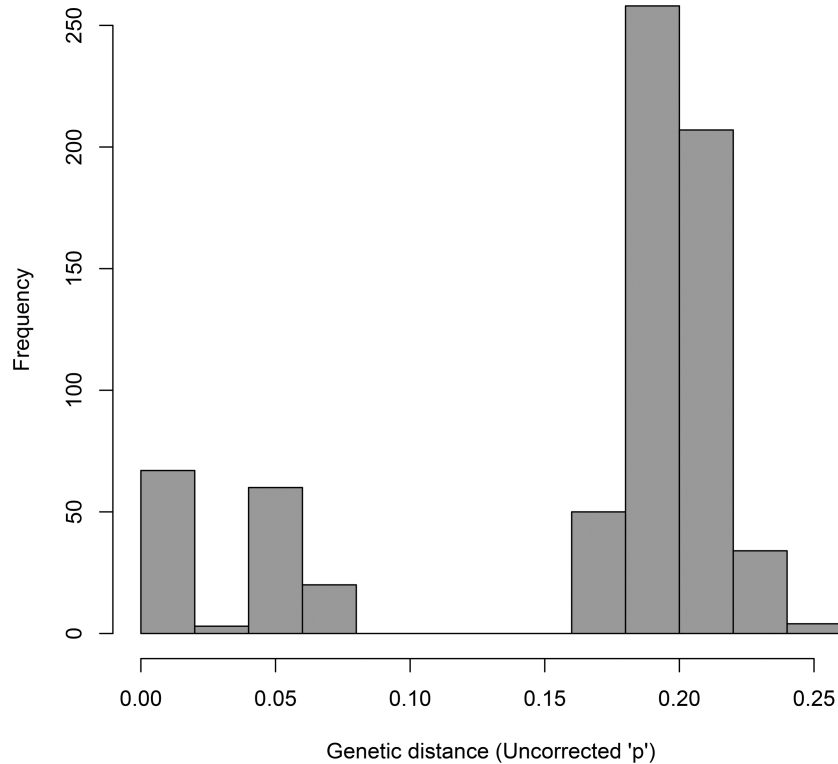


Figure 13. Distance histogram of pairwise uncorrected p distances between all individuals analysed genetically in this study. Within-species distances range from 0 to 8% (0–3% if not counting *Grania chilensis* sp. nov.); between-species distances range from 16 to 25%.

molecular data (De Wit *et al.*, 2011b), and could thus be an interesting avenue for future research.

The high differences of 19.5% in *COI* between *G. cryptica* sp. nov. and *G. bekkouchei* sp. nov. strongly support that these taxa represent two separately evolving lineages. A similar situation was found in Scandinavian species of *Grania*, where a molecular analysis suggested that no gene flow occurs between the cryptic *G. ovitheca* and *G. occulta* populations (De Wit & Erséus, 2010). In these two cases, the genetic differences suggest that the process of speciation is complete, despite their morphological similarities. Most of the species concepts (i.e. biological, ecological, evolutionary, and phylogenetic) share the common point that species are separately evolving metapopulation lineages (De Queiroz, 2007). According to De Queiroz, the species will acquire different properties over the speciation process, for instance, reciprocal monophyly, phenetic differences, and reproductive incompatibility, although not necessarily in the same order or on the same time scale. Thus, for a complete speciation event, the criteria mentioned above should be fulfilled (De Queiroz, 2007; Nygren, 2014). Although not tested, in this study we assume that *G. cryptica* sp. nov. and *G. bekkouchei* sp. nov. are not reproductively compatible, because of the great difference in both

mitochondrial and nuclear markers between these two species. In a study that combined genetic data with reproductive crosses, *Ophryotrocha japonica* Paxton & Åkesson, 2010 and *Ophryotrocha notoglandulata* Pfannenstiel, 1972, two sympatric species of Polychaeta, only a 5% difference in *COI* was shown to be associated with full reproductive isolation (Wiklund *et al.*, 2009; Nygren, 2014).

Unfortunately, only one specimen each of *G. brasiliensis* sp. nov., *G. cryptica* sp. nov., *G. unitheca* sp. nov., *G. carolinensis* sp. nov., and *G. cf. levis* was available for genetic analysis, and thus it is not possible to draw any conclusions regarding intraspecific variation in these taxa. Even so, it is clear that the morphological features (and differences) observed suggest that these individuals represent different taxa, of which at least four are new to science. Moreover, the combined DNA analysis (Fig. 15) shows that all these four specimens are genetically distinct from at least all other species of *Grania* genetically analysed to date.

On the other hand, *G. chilensis* sp. nov. is structured in four different maternal lineages (as shown by *COI*; Fig. 12): two at Valdivia and one each at Coquimbo and Concepción. At a first glance, the *G. chilensis* sp. nov. populations match an isolation-by-distance (IBD) pattern (Wright, 1943; Malécot, 1948), indicating a decrease

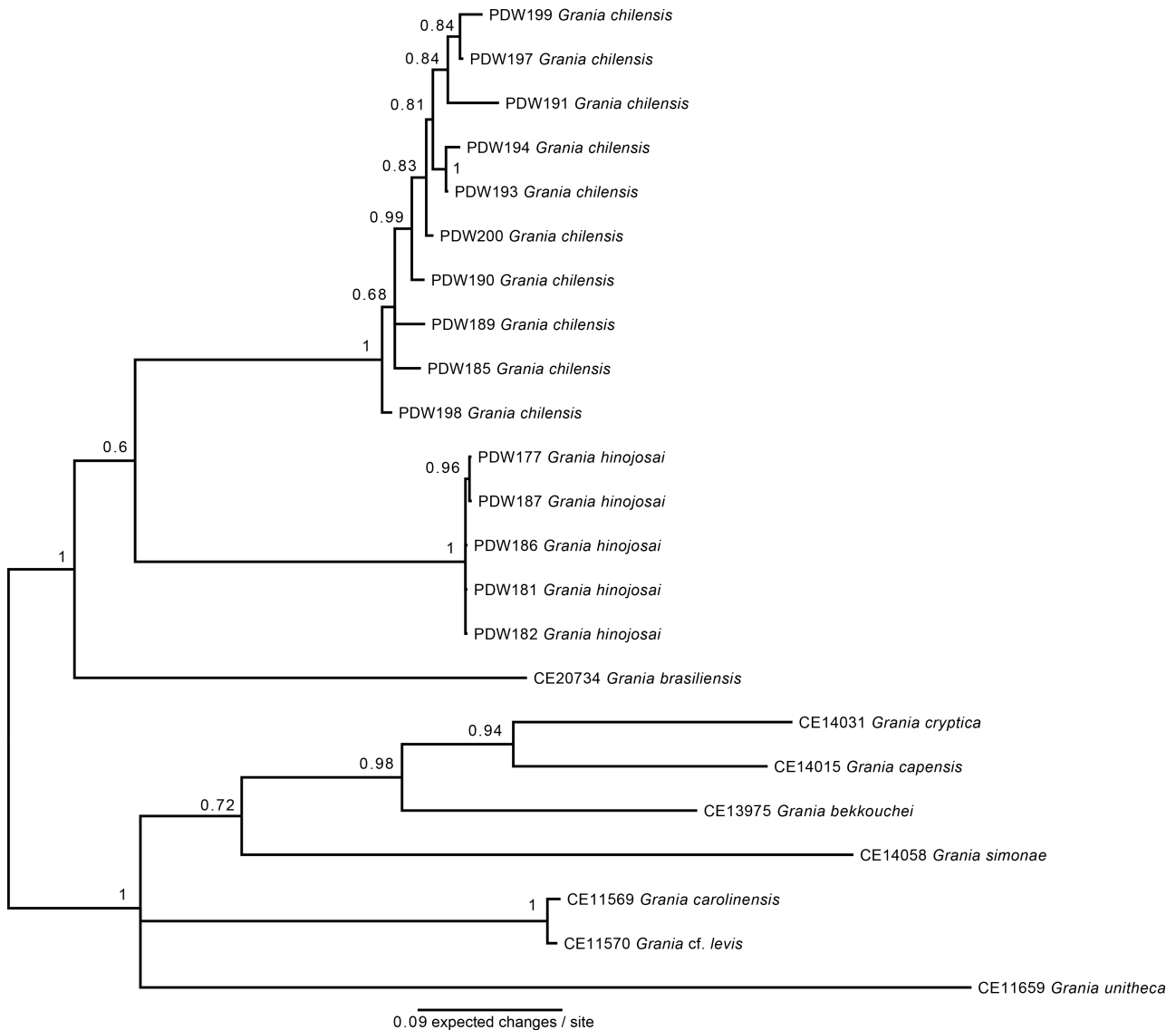


Figure 14. Majority-rule consensus gene tree of the *ITS* region (*ITS1-5.8S* rDNA-*ITS2*) from all species described in this study. Posterior probabilities calculated from Bayesian Markov chain Monte Carlo (MCMC) inference are noted above the branches. Note that the four *Grania chilensis* sp. nov. clades seen in the *COI* locus are absent here.

in gene flow with increasing distance, and migration probabilities depend only on distances between birth-places of offspring and their parents (Ishida, 2009). A likely cause for this population structure is the limited mobility of these organisms, which is associated with their infaunal/interstitial lifestyle and the lack of dispersal phases in their life cycle (Costa *et al.*, 2013). Interestingly, however, two genetically distinct populations of *G. chilensis* sp. nov. are observed in sympatry at the southernmost site, Valdivia. There are several potential explanations for this pattern: it could result from an ancient separation with secondary contact, ecological niche separation with respect to local environmental variables that were not assessed herein, or two

separate colonization events from a more northern site. In any case, the four population clusters are not supported by the more slow-evolving, recombining nuclear *ITS* locus, suggesting that the separation is fairly recent or alternatively that there is a limited rate of gene flow at present.

The updated phylogenetic tree (Fig. 15) maintained the three main clades (A, B, and C) found already by De Wit *et al.* (2011b), although some differences are seen in clades A and B. All the South African species come out as a strongly supported monophyletic group, which (with low support: posterior probability, PP 0.81) clusters together with the north-west Atlantic *G. monospermatheca*, *G. laxartus*, and

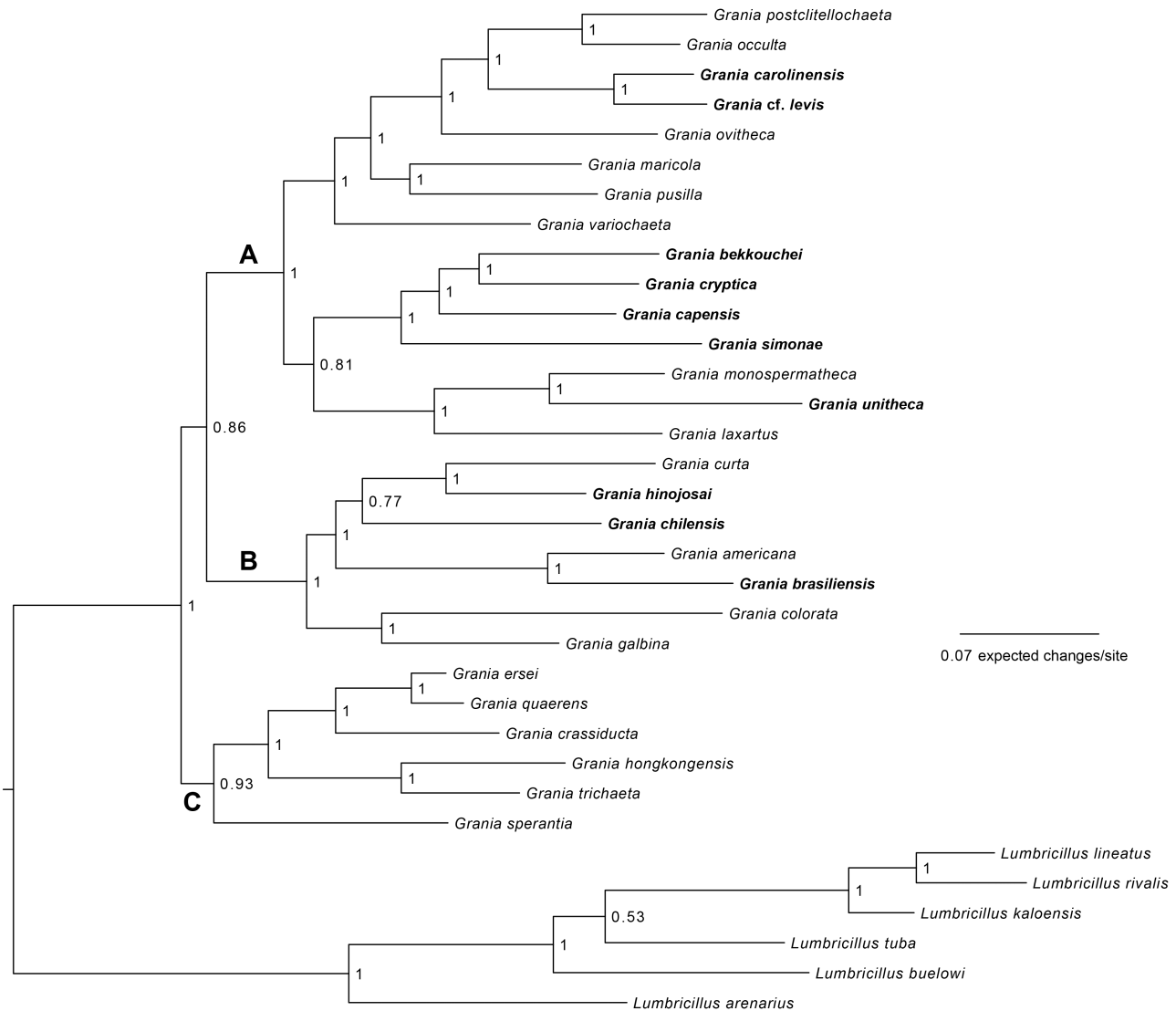


Figure 15. Multi-locus phylogeny of all available genetic data in *Grania* to date, using three mitochondrial and three nuclear loci. New species from this study are marked in bold. Majority-rule consensus tree from Bayesian Markov chain Monte Carlo (MCMC) inference; clade posterior probabilities are given next to the nodes.

G. carolinensis sp. nov. Consequently, the former western and eastern Atlantic subclades within clade A (see De Wit *et al.*, 2011b) now appear as one unique pan-Atlantic clade. In this clade, *G. monospermatheca* and *G. unitheca* sp. nov. are sister taxa. The morphology and the geographic distribution (north-west Atlantic) of these two species are strongly concordant with phylogeny. The main diagnostic character (the unpaired spermatheca) is found only in these species, supporting that they share a common ancestor.

The inclusion of the seven additional species in the phylogeny (*G. hinojosai* sp. nov. and *G. chilensis* sp. nov. have already been included as *G. sp. Chile 1* and *G. sp. Chile 2*, respectively, in the analy-

sis described in De Wit *et al.*, 2011b) raises another interesting aspect. The two Chilean species as well as the Western Atlantic *G. brasiliensis* sp. nov. and *G. americana* are placed within clade B, which largely is an Indo-West-Pacific clade. As suggested by De Wit *et al.* (2011b), the ancestor of *G. americana* probably migrated from the eastern Pacific to North Atlantic through Central America before the closing of the Isthmus of Panama (3 Mya). The addition of the new Brazilian species to the phylogenetic analysis suggests that this ancestor is shared by *G. americana* and *G. brasiliensis* sp. nov. The origin of the ancestor of the two Chilean species remains unclear (De Wit *et al.*, 2011b).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Mitochondrial DNA phylogeny of all available genetic data in *Grania* to date, using three loci (*12S* rDNA, *16S* rDNA, and COI).

Figure S2. Nuclear ribosomal DNA phylogeny of all available genetic data in *Grania* to date, using three loci [*18S* rDNA, *28S* rDNA (D1 region), and the *ITS* region (*ITS1-5.8S* rDNA-*ITS2*)].

Table S1. Distance matrix of uncorrected p distances between all individuals analysed genetically in this study.