

Systematics of *Grania* (Clitellata:  
Enchytraeidae), an interstitial  
annelid taxon

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## **Dissertation abstract**

Pierre De Wit (2010). Systematics of *Grania* (Clitellata: Enchytraeidae), an interstitial annelid taxon.

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In between the grains of sand on the ocean floor, there exists a world which few people are aware of. Representatives of almost all animal phyla can be found here. The clitellate family Enchytraeidae is in the marine interstitial environment represented in large part by species of a genus called *Grania*, which are long slender worms found in marine sands throughout the world. This thesis is a study on the systematics of these worms. The body wall of *Grania* is searched for phylogenetically informative morphological characters. It is found that the cuticular morphological variation seen in naidids is absent, but the collagen fiber thickness varies between *Grania* species. Also, the circular and outer, triangular longitudinal musculature is reduced compared to that of closely related taxa while the inner, ribbon-shaped longitudinal muscle fibers are well-developed, possibly an adaptation to interstitial life. The *Grania*-fauna of the Great Barrier Reef is investigated, with four new species described and *Grania trichaeta* re-described. The phylogenetic position of *Grania* within the family Enchytraeidae is elucidated by molecular means, where *Lumbricillus arenarius* is shown to be a close relative of a monophyletic *Grania*. Within the genus, a molecular phylogeny is inferred of a sample of 19 species, showing considerable morphological homoplasy, while geographical distribution is concordant with the phylogeny. Thus, we combine morphology with geography, while using the DNA-based tree as a backbone constraint, to estimate a phylogeny of all 71 currently described species within the genus. Finally, the genetic variation within Scandinavian species of *Grania* is studied with the resulting find of a cryptic species, and the realization that although intraspecific variation generally is low, deviant individuals exist. Within this study, we also infer a phylogeny of the Scandinavian species of *Grania*, which seems to be a monophyletic group, and discuss their morphological character evolution.

**Keywords:** Clitellata, Oligochaeta, Enchytraeidae, *Grania*, interstitial habitat, systematics, phylogeny, DNA barcoding, cryptic species

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## Svensk sammanfattning

Mellan sandkornen på havsbotten finns en värld som få känner till. Nästan alla djurgrupper har arter som är anpassade till denna miljö, och dessa bidrar mycket till biodiversiteten i havet. Ringmaskfamiljen Enchytraeidae är här främst representerad av ett släkte kallat *Grania*, som består av slanka, mestadels vita eller genomskinliga maskar, som blir en till två centimeter långa och ungefär en tiondels millimeter tjocka. Arter av *Grania* kan hittas i marin sand världen över, från tidvattenzonen ner till djuphavet. Denna avhandling är en studie i *Granias* systematik, d.v.s. hur många arter av *Grania* det finns, hur evolutionära släktskapsförhållanden inom och mellan arter ser ut, samt deras relationer till andra närbesläktade organismer. Avhandlingen består av fem delar. I del ett letar vi efter morfologiska karaktärer som kan vara hjälpsamma när vi studerar hur släktets evolution har gått till. Vi detaljstuderar kroppsväggen hos tre arter, och finner att kutikulans fibertjocklek varierar mellan arter. Vi finner även att deras muskulatur är annorlunda utformad jämfört med närbesläktade släkten. *Grania* har reducerad cirkulär muskulatur och kraftig längsmuskulatur, något som antagligen är en anpassning till en interstitiell levnadsstil. I del två undersöker vi *Grania*-faunan vid Stora Barriärrevet i Australien. Vi beskriver fyra nya arter, samt återbeskriver *Grania trichaeta* Jamieson, 1977. I del tre studerar vi fylogeni (släktskapsträdet) hos familjen Enchytraeidae, och kommer fram till att *Grania* är monofyletiskt och nära släkt med *Lumbricillus arenarius*, som lever i tidvattenzonen på Europas Atlantkust. I del fyra använder vi en kombination av molekylära och morfologiska data för att skapa ett släktskapsträd över alla för tillfället beskrivna arter av *Grania*. Vi kommer här fram till att den geografiska distributionen av arterna är kongruent med den molekylärt baserade fylogeni. Om man kombinerar morfologi, geografi och samtidigt viktar ned homoplastiska karaktärer, får man ett väl upplöst träd som kan ses som en god start-hypotes att testa vidare när fler molekylära data blir tillgängliga. Del fem är en studie i genetisk inomartsvariation hos Skandinaviska arter av *Grania*. Vi finner att inom dessa arter är variationen överlag liten, men i vissa fall dyker individer upp med distinkt annorlunda mitokondriella DNA-sekvenser. Vi hittar även en kryptisk art i vårt material som vi beskriver som ny, och som bara kan avgränsas med hjälp av DNA. Vi estimerar också en fylogeni baserat på arternas nukleära ITS-sekvenser, och vi diskuterar deras morfologiska karaktärsevolution.



## List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-V).

**I.** De Wit, Erséus & Gustavsson (2009). Ultrastructure of the body wall of three species of *Grania* (Annelida: Clitellata: Enchytraeidae). *Acta Zoologica* 00:00-00 (available online).

**II.** De Wit, Rota & Erséus (2009). *Grania* (Annelida: Clitellata: Enchytraeidae) of the Great Barrier Reef, Australia, including four new species and a re-description of *Grania trichaeta* Jamieson, 1977. *Zootaxa* 2165: 16-38.

**III.** Erséus, Rota, Matamoros & De Wit (manuscript). Molecular phylogeny of Enchytraeidae (Annelida, Clitellata).

**IV.** De Wit, Rota & Erséus (manuscript). Congruence between geography and DNA – a “backbone” approach to estimate phylogeny in *Grania* (Annelida: Clitellata: Enchytraeidae).

**V.** De Wit & Erséus (submitted). Genetic variation and phylogeny of Scandinavian species of *Grania* (Annelida: Clitellata: Enchytraeidae), with the discovery of a cryptic species.

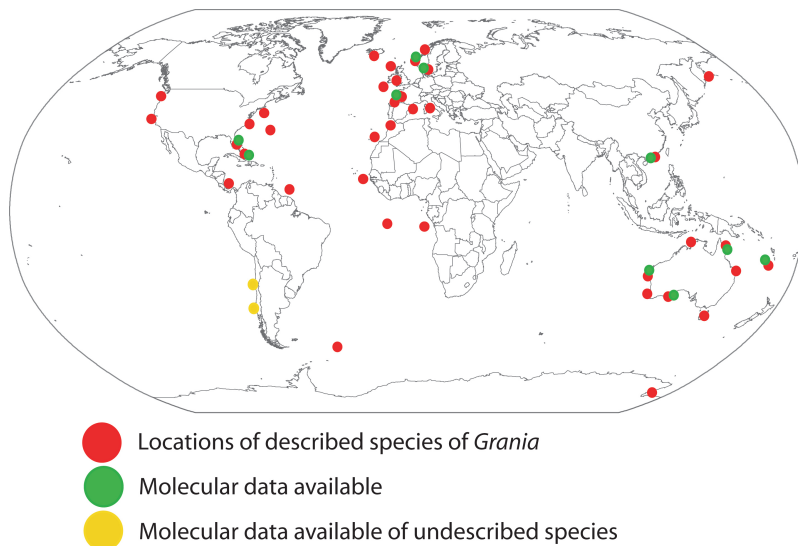
All new taxon names mentioned in this thesis are disclaimed for nomenclatural purposes (ICZN article 8.2).

## Introduction

A world which few people are aware of exists in between the grains of sand on the ocean floor. A multitude of small animals take advantage of the small water-filled space between the uneven sand grains. This space can be of different size depending on a number of factors, such as the size and shape of the individual sand grains and the heterogeneity of grains (i.e. if all grains are the same size or if some are smaller than others). Other physical factors can also differ greatly within this environment, for instance temperature, salinity, nutrient content and oxygen availability (Jansson 1967). Thus, the interstitial space is not homogeneous, but varies from location to location. As different organisms prefer different habitats also within this environment, a great biodiversity usually overlooked (even by many biologists), is to be found here. Representatives of almost all animal phyla can be found in this environment; the ones most commonly found here are crustaceans, annelids, nematodes, flatworms and gastropods (Delamare-Deboutteville 1960). Some animal groups, such as gastrotrichs and kinorhynchans, can be found only in this environment. Many single-celled organisms, especially ciliates, are also known only from interstitial habitats. The animals inhabiting this peculiar environment are adapted to it in a number of ways. Elongated worm-shape is common in most animals groups here, and many animals have hooks, claws or adhesive organs to attach themselves to the sand grains (Ruppert and Barnes 1994). Because of the small size, there is a trend toward reduction of the number of eggs (usually with large amount of yolk) with internal fertilization and direct development (Swedmark 1964) as a common mode of reproduction. Even vivipary (bearing live young) has been reported in some cases (Ruppert and Barnes 1994).

This thesis is a study of the systematics and morphology of an almost exclusively marine interstitial genus of annelid worms named *Grania* Southern, 1913. *Grania* is a member of the family Enchytraeidae within the taxon Clitellata, to which also the earthworms and leeches belong. The traditional name for the animal group containing earthworms and their relatives is “Oligochaeta”, a name which comes from the Latin “oligo” meaning “few” and “chaeta” which translates to “bristles” in English. Thus, the oligochaetes are worms with few bristles, as opposed to the “polychaetes” which have many. The name “Clitellata” refers to the oligochaetes plus the leeches (Hirudinea), a taxon which is discerned by the clitellum, a girdle-like structure associated with the formation of the cocoon. A few years ago, it was shown that leeches actually are derived oligochaetes (Siddall et al. 2001), making Oligochaeta and Clitellata synonymous. As the shared character for all taxa within this group is the clitellum, it is clearly more logical to call the clade Clitellata. Oligochaeta *sensu stricto*, that is the worms with few bristles but not including the leeches, becomes a paraphyletic group. As paraphyletic groups do not necessarily reflect evolutionary history, it is prudent to not use the name Oligochaeta since this discovery. Instead, as an option, one can use the more informal term “oligochaetous Clitellata” to refer to the “few-bristled worms” (Erséus 2005).

Within the family Enchytraeidae there are almost 700 currently described species (Erséus 2005), most of which are living in soil. Some enchytraeid species inhabit the ocean floor, however, and of these, species of *Grania* seem to be the most specialized to this environment as they almost exclusively are found interstitially in environments with full oceanic salinity. As most enchytraeids, species of *Grania* are slender, transparent or whitish worms, and they are usually 1-2 cm long and 0.1-0.2 mm thick. At first sight they resemble nematodes as they are thin and move in a similar way, but at closer inspection observers can see that they are segmented, just like earthworms or polychaetes.



**Figure 1.** World map showing known localities of described spp. of *Grania* and from where genetic material has been available.

### History of *Grania*

Like for many other taxa of small marine animals, we have only just started to realize how much diversity there is within *Grania*. The genus was established in 1913 for *G. maricola* Southern, 1913 (Southern 1913). There was for long confusion regarding the status of the genus, as *G. maricola* was similar to a previously described species, *Enchytraeus macrochaetus* Pierantoni 1901 (which had since its description been moved to the genus *Michaelsena* (Pierantoni 1903)). As a result, *G. maricola* was also transferred to *Michaelsena* (Stephenson 1930), after which both species were moved to *Enchytraeus* (Nielsen and Christensen 1959). In 1966, however, Kennedy (1966) re-described both species as *Grania macrochaeta* and *Grania maricola*, while also adding a third species, *G. americana* Kennedy 1966, to the genus. The same year, Lasserre (1966) transferred *Michaelsena postclitellochaeta* Knöllner, 1935 to *Grania*. One year later, the species *G. macrochaeta* and *G. postclitellochaeta*

were both proposed to consist of several subspecies (Lasserre 1967), and were so considered for about ten years (Erséus and Lasserre 1976; Jamieson 1977). Towards the late 1970s, however, with more and more species being described from many different locations in the world, and increasingly refined studies of morphological variation within and between species and subspecies, it was argued that the differences between the subspecies warranted that they be elevated to separate species (Erséus 1977; Erséus and Lasserre 1977). With better understanding of species-specific characters, the diversity of the genus has steadily increased since then, and today it is clear that *Grania* is composed of a high number of species, most of which with a limited geographical range. In total, 71 species of *Grania* have been described to date (Table 1).

Our knowledge of *Grania*, however, is limited to the locations where sampling has been done. This has in large parts been focused on Europe, the east coast of North America, and Australia (Figure 1). This unequal sampling is the result of history; taxonomic experts of this group have been from Europe and America. Several faunal workshops were organized in Australia, with these taxonomic experts invited as participants, which is why this area also is relatively well-sampled with respect to *Grania*. In large areas of the world, however, the taxonomic expertise on this animal group has not been available to date, for instance in South America, Africa or the Indo-Pacific. In many other animal groups we know that the majority of diversity exists in the tropics (e.g. Jablonski et al. 2006; Stork 2007) so it is clear that a large number of species of *Grania* are still waiting to be found.

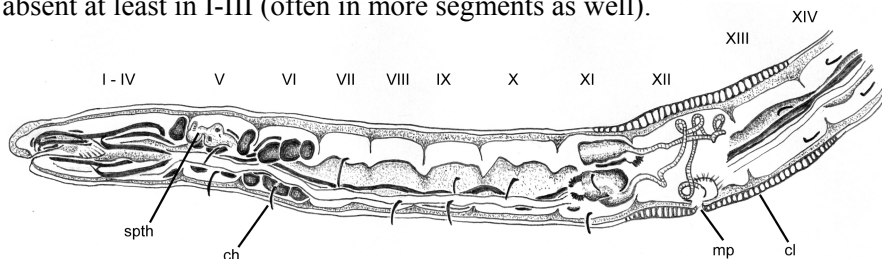
## **Morphology**

As all annelids, oligochaetous clitellates are segmented worms, i.e., they have a coelomic cavity which is compartmentalized into segments by septa (Figure 2). Anterior to the first segment they have a prostomium, and behind the last segment in the posterior end they have a pygidium. These two compartments are already present in the larval stage and are not considered part of the segmentation (Brusca and Brusca 2003). In contrast to most annelids, the clitellate prostomium is in most cases devoid of tentacles or cirri, possibly an adaptation to a burrowing way of life. After the prostomium, segment number is usually denoted with roman numerals (I, II, III etc.) (although sometimes the first segment posterior to the prostomium, the peristomium, is not considered a true segment either). Species within the oligochaetous Clitellata possess a closed circulatory system, with a ventral blood vessel leading hemoglobin-containing blood posteriorly from the head end, and a dorsal blood vessel leading blood forward to complete the circuit. A capillary network stretches in every segment from the ventral vessel to the dorsal one, providing surrounding tissue with oxygen and nutrients. In the posterior part of the worm this network also encompasses the gut, where nutrients are absorbed. Oxygen is generally absorbed through the body wall by diffusion.

Clitellates differ from most other annelids in a number of ways. Perhaps most obvious is the mode of reproduction; clitellates are hermaphrodites. They have both male and female gonads as well as a specialized organ for sperm reception and storage, the spermatheca (Figure 2). When copulation takes place, they arrange themselves so that one worm's male pore is located near another worm's spermathecal pore (which is almost always anterior of the male pore). The other worm's sperm are subsequently stored in the spermatheca. All sexually mature clitellates possess a clitellum, which is a region of the body with thickened body wall due to large numbers of glandular cells in the epidermis. During reproduction, these gland cells secrete a sticky mucus layer in a band around the worm body into which unfertilized eggs are deposited. The worm starts backing out of the ring of mucus with one or more eggs inside, and when the eggs are level with the spermathecal pore, the sperm stored in the spermatheca are squeezed out, fertilizing them externally. The worm then moves out of the mucus ring completely, after which the mucus hardens into a cocoon around the egg (Jamieson 2006). There are notable deviations from this rule, however. Some clitellates are parthenogenetic (Poddubnaya 1984), and some reproduce asexually by fragmentation, such as *Lumbriculus variegatus* (Gustafsson et al. 2009).

Further apomorphies (derived characters) for Clitellata are: a dorsal pharynx, the lack of parapodia and a general trend towards fewer chaetae (still, as a rule, located in four distinct positions per segment, two lateral and two ventrolateral, as in other annelids) (Figure 2).

Oligochaetous Clitellata are divided into a number of taxa, distinguished largely by the number and location of male and female genitalia as well as spermathecae. The family to which *Grania* belongs, Enchytraeidae, has spermathecae in segment V, testes in XI, and ovaries, penial apparatuses and male pores in XII (all are usually paired) (Erséus 2005). Within Enchytraeidae, species of *Grania* are distinct in the level of cephalization, with a well-developed brain and anterior nervous system (Rota et al. 1999), including in some cases the only multicellular sense organ recorded inside the body of clitellates, the head organ, which is thought to be a compound georeceptor (Rota and Erséus 1996; Rota et al. 1999; Locke 2000). They also possess solitary, often stout or proximally enlarged, chaetae (Erséus and Lasserre 1976) which are absent at least in I-III (often in more segments as well).



**Figure 2.** Anterior end of a *Grania* specimen. spth, spermatheca; ch, chaeta; mp, male pore; cl, clitellum. Segment numbers are denoted with roman numerals. (Modified from Locke & Coates, 1999).

**Table 1. Described species of *Grania* Southern 1913, in chronological order.**

<i>Grania monochaeta</i> (Michaelsen, 1888)	<i>Grania laxartus</i> Locke & Coates, 1999
<i>Grania macrochaeta</i> (Pierantoni, 1901)	<i>Grania dolichura</i> Rota & Erséus, 2000
<i>Grania paucispina</i> (Eisen, 1904)	<i>Grania tasmaniae</i> Rota & Erséus, 2000
<i>Grania principissae</i> (Michaelsen, 1907)	<i>Grania aquitana</i> Rota & Erséus, 2003
<i>Grania maricola</i> Southern, 1913	<i>Grania canaria</i> Rota & Erséus, 2003
<i>Grania postclitellochaeta</i> (Knöllner, 1935)	<i>Grania fortunata</i> Rota & Erséus, 2003
<i>Grania americana</i> Kennedy, 1966	<i>Grania mauretanica</i> Rota & Erséus, 2003
<i>Grania roscoffensis</i> Lasserre, 1967	<i>Grania ocarina</i> Rota, Erséus & Wang, 2003
<i>Grania pusilla</i> Erséus, 1974	<i>Grania papillinasus</i> Rota & Erséus, 2003
<i>Grania bermudensis</i> Erséus & Lasserre, 1976	<i>Grania torosa</i> Rota & Erséus, 2003
<i>Grania longiducta</i> Erséus & Lasserre, 1976	<i>Grania vikinga</i> Rota & Erséus, 2003
<i>Grania monospermatheca</i> Erséus & Lasserre, 1976	<i>Grania cinctura</i> De Wit & Erséus, 2007
<i>Grania variochaeta</i> Erséus & Lasserre, 1976	<i>Grania curta</i> De Wit & Erséus, 2007
<i>Grania ovitheca</i> Erséus, 1977	<i>Grania fiscellata</i> De Wit & Erséus, 2007
<i>Grania trichaeta</i> Jamieson, 1977	<i>Grania fustata</i> De Wit & Erséus, 2007
<i>Grania pacifica</i> Shurova, 1979	<i>Grania galbina</i> De Wit & Erséus, 2007
<i>Grania incerta</i> Coates & Erséus, 1980	<i>Grania novacaledonia</i> De Wit & Erséus, 2007
<i>Grania parvitheca</i> Erséus, 1980	<i>Grania papillata</i> De Wit & Erséus, 2007
<i>Grania atlantica</i> Coates & Erséus, 1985	<i>Grania sperantia</i> Rota, Wang & Erséus, 2007
<i>Grania levis</i> Coates & Erséus, 1985	<i>Grania quaerens</i> Rota, Wang & Erséus, 2007
<i>Grania reducta</i> Coates & Erséus, 1985	<i>Grania breviductus</i> De Wit, Rota & Erséus, 2009
<i>Grania ascophora</i> Coates, 1990	<i>Grania colorata</i> De Wit, Rota & Erséus, 2009
<i>Grania bykane</i> Coates, 1990	<i>Grania homochaeta</i> De Wit, Rota & Erséus, 2009
<i>Grania crassiducta</i> Coates, 1990	<i>Grania regina</i> De Wit, Rota & Erséus, 2009
<i>Grania ersei</i> Coates, 1990	
<i>Grania hastula</i> Coates, 1990	
<i>Grania hongkongensis</i> Erséus, 1990	
<i>Grania hyperoadenia</i> Coates, 1990	
<i>Grania inermis</i> Erséus, 1990	
<i>Grania stilifera</i> Erséus, 1990	
<i>Grania alliata</i> Coates & Stacey, 1993	
<i>Grania conjuncta</i> Coates & Stacey, 1993	
<i>Grania longistyla</i> Coates & Stacey, 1993	
<i>Grania vacivasa</i> Coates & Stacey, 1993	
<i>Grania acanthochaeta</i> Rota & Erséus, 1996	
<i>Grania algida</i> Rota & Erséus, 1996	
<i>Grania angustinasus</i> Rota & Erséus, 1996	
<i>Grania antarctica</i> Rota & Erséus, 1996	
<i>Grania carchinii</i> Rota & Erséus, 1996	
<i>Grania hirsuticauda</i> Rota & Erséus, 1996	
<i>Grania darwinensis</i> (Coates & Stacey, 1997)	
<i>Grania eurystyla</i> Coates & Stacey, 1997	
<i>Grania integra</i> Coates & Stacey, 1997	
<i>Grania lasserrei</i> Rota & Erséus, 1997	
<i>Grania stephensoniana</i> Rota & Erséus, 1997	
<i>Grania mira</i> Locke & Coates, 1998	
<i>Grania hylae</i> Locke & Coates, 1999	

## AIMS

The objectives of this thesis are:

- To search for new phylogenetically informative morphological characters which might shed light upon the origin and evolution of the genus and in particular, to gain an understanding of how the body wall of *Grania* is organized and how it differs within the genus.
- To explore the *Grania*-fauna of a poorly known area: the Great Barrier Reef, Australia, both with respect to describing new species, understanding their geographical distributions and facilitating their future identification by non-experts.
- To figure out where, within the family Enchytraeidae, the genus *Grania* is positioned and what taxon is the sister of *Grania*. This is important to be able to root the phylogeny of the genus.
- To estimate the phylogeny of the genus *Grania*, by combining morphological and molecular data, and to evaluate how much information there is in these two different kinds of data.
- To illuminate the relationship between morphological species and separate evolutionary units among closely related taxa of *Grania*.

## Methods of collection and analysis

Collection of individuals of *Grania* species was conducted by a sieving-decantation method, where sediment samples were stirred in seawater to put both small sediment particles and animals in suspension. After this, the water and the suspended material were poured through a sieve with a mesh size of 250  $\mu\text{m}$ , which caught the worms but let smaller things through. After repeating this process about 5 times, most of the worms from the sample had ended up in the sieve. The material in the sieve was then examined under a stereo microscope, and the worms were sorted out. For fixation, formalin was used. As formalin is destructive to DNA, however, I mostly chose to preserve the worms in ethanol.

As mentioned, it is necessary to stain and mount the specimens of *Grania* to identify them to species level. For this, the procedure described by Erséus (1994) was used. This method includes staining the worms with the red dye Paracarmine, dehydration using an alcohol-xylene series and mounting them in Canada balsam on microscope slides.

Worms used for Transmission Electron Microscopy (TEM) were fixed in SPAFG (Ermak and Eakin 1976). Before observation, these specimens were dehydrated using ethanol and propylene oxide, encapsulated in plastic cubes of EPON™, sectioned using an ultramicrotome and stained with lead citrate and uranyl acetate (Daddow 1983).

A tissue sample preserved in ethanol was taken of specimens to be used in DNA analyses before the specimen was mounted. Usually, a piece of the posterior end of the worm was cut for this purpose. As most of the morphological characters used for species identification are located in the

anterior and clitellar regions, the loss of a few segments in the posterior end is normally not an issue. Whole worms were used for DNA extraction on a few occasions, in which case other specimens from the same sample served as identifiers. DNA was extracted from the tissue samples and amplified using PCR reactions. For DNA sequencing, a Beckman Coulter Sequencer was used at first; later samples (2007 and onward) were sent to Macrogen Inc., South Korea for ABI sequencing. The genes used were COI (Paper II, III, IV, V), 12S, 16S, 18S, 28S (Papers III and IV), and also the ITS region (Papers IV and V). Contigs were assembled using the LaserGene software SeqMan at first, then later (from 2008) in Biomatters Geneious Pro. Alignments were done in MUSCLE (Edgar 2004) (Papers III and IV) and MAFFT version 6 (Kato and Toh 2008) (Paper V). Pairwise distance calculations, model testing (through Mr Modeltest 2.2 (Nylander 2004)) and parsimony analyses were done in PAUP\*4.0b (Swofford 2002), and Bayesian analyses were done in the parallel version of MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Character evolution analysis was conducted using the parsimony-based software “ancestres” within the program Mesquite (Maddison and Maddison 2004).

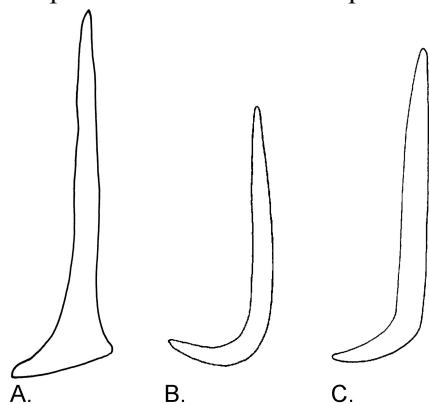
### **The body wall of *Grania***

In all annelids, the body wall is composed of (from the outside to the inside): an extracellular cuticle, an epidermis, a circular muscle layer, a longitudinal muscle layer and finally a coelomic lining (Jamieson 1981). In Paper I, we take a closer look at the ultrastructure of the body wall of *Grania*. Variation has been discovered in the past in other clitellate taxa. The pattern of the collagen matrix constituting the cuticle has been shown to vary considerably in naidids, for example (Gustavsson and Erséus 2000; Gustavsson 2001). The types of gland cells in the epidermis, especially in the clitellum (Burke and Ross 1975; Richards 1977; Fleming and Baron 1982), and the pattern of both kinds of musculature (Giere 1983; Jamieson 1992; Lanzavecchia et al. 1994) also vary between different taxa, and so we wanted to know how the body wall of *Grania* looks, i.e., if it is similar to other enchytraeids and also if there is any variation within the genus. This study was in part also a search for new morphological characters which might give indications about relationships between *Grania* and other enchytraeid taxa. Our results indicate that the amount of cuticular morphological variation seen in naidids does not exist in enchytraeids. The thickness of the cuticular collagen fibers varies between species and might be phylogenetically informative, however. We also find that the circular and triangular longitudinal musculature of *Grania* is reduced, while the ribbon-shaped longitudinal musculature is well-developed. This could be an adaptation to interstitial life, as a similar pattern can also be seen in marine interstitial naidids (Giere 1983). The number of myofibrillar bundles in the muscle fibers also varies between species, another character which might be phylogenetically informative.



### Species distinction and identification

The species concept is still under considerable debate, and there are a number of proposed species definitions, e.g. biological (Wright 1940; Mayr 1942), ecological (Van Valen 1976), evolutionary (Simpson 1951) as well as phylogenetic (Hennig 1966; Rosen 1979) ones. Most of these definitions, however, can be seen as different aspects of the same natural phenomenon, which is the fact that populations of organisms diverge from each other with time in the absence of gene flow. Thus, it has recently been proposed that a single unifying concept of a species could be that each species constitutes a separately evolving metapopulation (De Queiroz 2007). Species hypotheses could then be corroborated by different criteria, e.g. by morphological, reproductive, ecological or genetic distinction (De Queiroz 2007). That a group of populations is found divergent using any of these criteria is evidence for its unique evolutionary history, and thus it could be seen as a distinct species even if it does not meet the criteria of all historically proposed species concepts. This is particularly important for lineages which have only recently been separated, where morphological characters have not yet had the time to diverge to the point where it is possible to use them for species distinction.

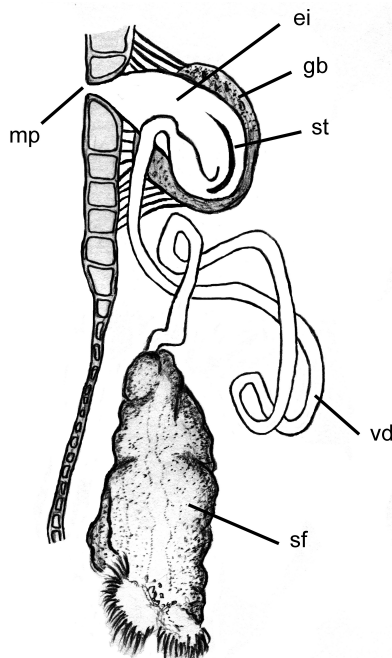


**Figure 3.** Chaetae of *Grania*. **A.** Foot-shaped with a heel. **B.** Hook-shaped. **C.** Entally bent at an angle

### Morphology

Most species of *Grania* have a chaetal distribution either with lateral chaetae absent in all pre-clitellar segments, or with all pre-clitellar segments completely devoid of chaetae, and lacking lateral chaetae also along the remaining length of the body (one species, *Grania levis* Coates and Erséus 1985, completely lacks chaetae). The chaetal distribution can thus be used for species distinction. It is also possible to use the shape of the individual chaetae; chaetae can be entally shaped like a foot with a heel, or it can be hook-shaped, or just bent at an angle (Figure 3). The ratio between the “foot length” and the chaetal length, the “chaetal index (CI)” has been proposed as a diagnostic character as well (Rota and Erséus 2003).

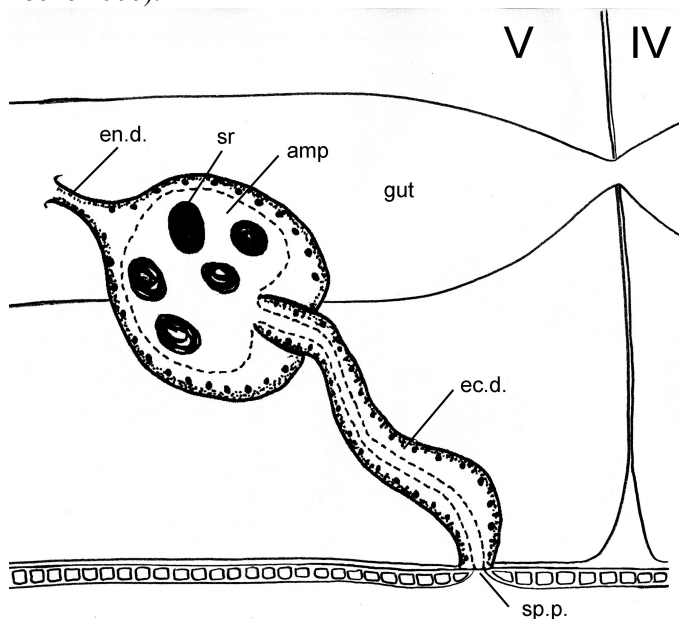
The detailed morphology of the male genitalia (Figure 4) is also useful for species distinction. The sperm, which mature in a sperm sac within the coelomic cavity, are when fully developed collected into a sperm funnel which can be of varying size and shape. The sperm funnel narrows into the vas deferens which may be more or less muscularized. At the ectal end, the vas deferens may open into an (eversible) epidermal invagination or directly into the male pore. At the distal end of the vas deferens, there may be a cuticular stylet present, a narrow copulatory structure assumed to be rather rigid. The stylet can be of varying length in different species and is usually curved. The male pore may also be surrounded by glandular tissue. To organize all of these characters, Coates (1984) recognized a number of “penial bulb types” present in *Grania*.



**Figure 4.** Male genitalia of *Grania*. mp, male pore; ei, epidermal invagination; gb, glandular bulb; st, penial stylet; vd, vas deferens; sf, sperm funnel. (Modified from Locke & Coates, 1999).

The shape of the spermathecae is another character which can be used. A spermatheca (Figure 5) consists of an ectal duct connecting the external pore to the ampulla, where the sperm is stored after copulation. The duct may be long and narrow or short and bulbous, and it may have gland cells or muscle fibers surrounding it. Among the species, the ampullae are of varying size, may have blind-sacs (diverticula), and contain different amounts of sperm (usually, but not always, organized in small rings within the ampullar wall). At the ental end, the spermatheca is connected to the gut through an ental duct which may be narrow or wide.

In Paper II, we describe four new species from the Great Barrier Reef. We also re-describe an already described species, *Grania trichaeta* Jamieson, 1977, because the original description lacks many of the diagnostic characters used today, such as the morphology of the male genitalia and chaetae. One of the new species, *Grania colorata* De Wit, Rota and Erséus, 2009, is colored brightly green, something unusual among enchytraeids; it has only been reported once before, in a *Grania* species described from New Caledonia (De Wit and Erséus 2007). The reason for this coloration is still unknown (see Paper I). Another new species, *Grania regina* De Wit, Rota and Erséus, 2009, possesses a conspicuous head organ, the only multicellular sense organ ever reported in enchytraeids, which is thought to be a geo-receptor (Rota et al. 1999; Locke 2000).



**Figure 5.** Spermatheca of *Grania*. en.d., ental duct; sr, sperm ring; amp, ampulla; ec.d., ectal duct; sp.p., spermathecal pore. Segment numbers are denoted with roman numerals. (Modified from De Wit & Erséus, 2007)

### *Barcoding*

Morphological species identification in *Grania* is complicated. The lack of good diagnostic features necessitates study of a combination of characters for distinction. It is e.g. not enough to examine the penial apparatuses to know what species you have in front of you, it is necessary to look at several other morphological structures as well, to find a species-specific combination of chaetal distribution and shape, penial apparatus structure and spermathecal shape. To complicate things further, we are now realizing that so-called cryptic species are fairly common within these small marine invertebrates. A cryptic

species is an evolutionary lineage which is difficult or impossible to distinguish morphologically from another lineage (Mayr and Ashlock 1991). Larvae and young (pre-copulatory) individuals are also usually impossible to identify to species-level using only morphological features.

In cases where it is difficult or impossible to identify specimens morphologically, the idea of DNA barcoding (Hebert et al. 2003), where a standard sequence of DNA of the specimen to be identified is compared to a sequence database, has gained support within the scientific community. The locus proposed to be used in metazoan animals is mitochondrial, coding for Cytochrome Oxidase subunit I (COI). The application of this tool is still controversial (Will and Rubinoff 2004; Cameron et al. 2006), but it does seem to be useful for the identification of clitellates (Erséus and Kvist 2007; Huang et al. 2007; Chang et al. 2009), providing that sequences first be submitted to a database along with voucher information (Ekrem et al. 2007; Pleijel et al. 2008). As a part of Paper II, we submit COI sequences from specimens of *G. trichaeta* and *G. colorata* through GenBank to the Barcode of Life Database (BoLD), in hope that future identification of these taxa will be easier in the future, as it can now be done by non-experts as long as they have access to a sequencing facility.

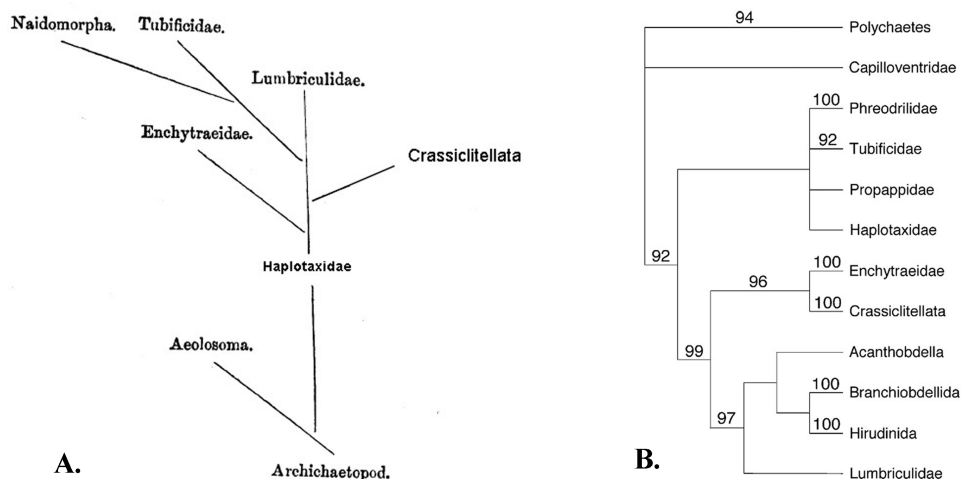
Furthermore, in the last paper of this thesis we study how the morphological species reported from the coast of Sweden are genetically structured. We reach the conclusion that within the populations present in Sweden and Norway there is little variation, and the morphospecies are also well-supported genetically, both by mitochondrial and nuclear DNA. We do show, however, that the morphological species *Grania ovitheca* Erséus, 1977, which inhabits the Atlantic coast of Europe, actually consists of two lineages which are quite different from each other genetically. We choose to describe the lineage inferred to be deviant from the holotype of *G. ovitheca* as *G. occulta* sp.n. This is the first occasion of finding a cryptic species in *Grania*, but probably not the last. Cryptic species might exist in other parts of the world as well, but as in many cases no genetic data of previously described species are available, we will not know until we go out and sample. As in Paper II, we submit sequences from the species under study to GenBank and the Barcode of Life Database as a future identification tool.

### **Position of Clitellata within Annelida**

The phylogeny of Annelida has been investigated in many studies, both using morphology and molecular data, and it is clear today that clitellates are derived polychaetes (Bleidorn et al. 2003; Hall et al. 2004; Rousset et al. 2007; Struck et al. 2007). The family Aelosomatidae (and possibly the taxon *Hrabiella*) have been inferred as being closely related to Clitellata (Jördens et al. 2004; Zrzavý et al. 2009). Unfortunately, no study has provided a good conclusive placement for the Clitellata + Aelosomatidae group within Polychaeta, i.e. the sister taxon has not been found. Several have been suggested though. Dinophilidae and

Lumbrinereidae are polychaete families that in molecular analyses frequently group with Clitellata (Hall et al. 2004; Jördens et al. 2004; Rousset et al. 2007). There are also several other polychaete taxa which morphologically resemble clitellates, such as *Questa* and the terrestrial *Parergodrilus*, but molecular analyses refute a close relationship between these and Clitellata (Jördens et al. 2004; Bleidorn et al. 2009); at least in the case of *Parergodrilus*, the similarity is attributed to evolutionary convergence (Rota 1998; Purschke 1999; Rota et al. 2001). The problems in resolving the basal relationships within Annelida is most probably due to a rapid radiation in the Cambrian (Fauchald 1974; Rota et al. 2001).

The aquatic origin of Clitellata has recently been all but confirmed (Rousset et al. 2008) but it is still not understood if the ancestor of all clitellates lived in freshwater or the ocean. The family Capilloventridae, which has been proposed to be the sister taxon of the remaining Clitellata (Erséus and Källersjö 2004), includes both freshwater and marine species, making it difficult to draw more specific conclusions about the habitat of the common ancestor.



**Figure 6. A.** Tree of life of Clitellata (Oligochaeta) as envisioned by Beddard (1895).

**B.** Tree of life of Clitellata according to 18S rDNA from Erséus & Källersjö, 2004. (Modified from Erséus, 2005).

### Position of Enchytraeidae within Clitellata

Traditional morphological analysis of Clitellata has placed the Enchytraeidae as a rather basal group within the Clitellata (Jamieson 1988; Erséus 2005) (Figure 6A). Lately, molecular studies have provided some new insights, although the results are still not uncontroversial. Siddall et al. (2001) and Erséus and Källersjö (2004) have published well-supported phylogenies in which Enchytraeidae comes out as sister group to Crassicitellata (clitellates with more than one epithelial cell layer in the clitellum, i.e. “earthworms”).

Morphologically, enchytraeids are quite similar to earthworms, with rather simple chaetae, and they also share the terrestrial habitat preference (Erséus and Källersjö 2004). In Paper I, we also see that the cuticular ultrastructure of the two taxa is quite similar. The articles by Siddall et al. and Erséus and Källersjö also both support the hypothesis that Clitellata has an aquatic origin (see e.g. Rousset et al. 2008), with the terrestrial clade Enchytraeidae + Crassiclitellata as a derived group at the tip of the tree (Figure 6B). The family Propappidae has historically been seen as part of, or closely related to Enchytraeidae (Coates 1986), and it is supported as a close relative of Enchytraeidae in some studies (Rousset et al. 2007; Marotta et al. 2008). In particular, Marotta et al. (2008) arrive at the conclusion that Enchytraeidae and Propappidae are sister taxa, and in turn form a sister group to a clade consisting of Crassiclitellata, Lumbiculidae and the leeches, by using a combination of DNA data, morphological details and sperm ultrastructure.

### **Position of *Grania* within Enchytraeidae**

At the beginning of my Ph.D. project in 2005, no conclusive phylogenetic analysis of Enchytraeidae had been conducted. Coates (1989) attempted at a morphological analysis of some enchytraeid taxa with little result. She did, however, reach the conclusion that some genera are not monophyletic, most notably *Marionina*. Just recently, Christensen and Glenner (Christensen and Glenner in press) presented a phylogeny using 17 ingroup enchytraeids and two outgroups, indicating *Enchytraeus* as the sister of other enchytraeids. In Paper III of this thesis, we use a much larger sample of Enchytraeidae as well as more outgroups to infer a DNA-based phylogeny. In our study, which is based on five loci, we get good resolution at the generic level, as well as interesting results above this level. Enchytraeidae is divided into two clades; sister group of all other enchytraeids is a clade containing the largely terrestrial genera *Achaeta* and *Hemienchytraeus*. *Grania* is monophyletic, and is placed as the sister taxon to *Lumbricillus arenarius* (Michaelsen, 1889), a taxon inhabiting the littoral zone of Northern Europe. *Lumbricillus arenarius* is separated from all other studied *Lumbricillus* species, suggesting that it should be removed from the genus.

### **Phylogeny of *Grania***

No phylogenetic analysis of the genus *Grania* has been done before. In Paper IV, we infer the phylogeny of *Grania* using a combination of genetics and morphological analysis. Using DNA-data, it is possible to obtain a well-supported tree, but unfortunately genetic material is only available for a limited number of species of *Grania*. Using this tree, we examine morphological characters of all currently described species of *Grania* and find that almost all characters used in standard species descriptions are highly homoplasious within the genus. Interestingly, however, the pattern of geographical distribution of the species used in the DNA-analysis is congruent with the phylogeny, indicating

that this character also could be used in a phylogenetic analysis. When we add geographical location as a character to the morphological analysis, use the DNA-based tree as a backbone constraint, and successively down-weight highly homoplasious characters (Farris 1969; Goloboff et al. 2008), we get a well-resolved phylogeny of all species of the genus (Figure 7). This tree should be seen as a good starting hypothesis for further testing when more genetic data becomes available.

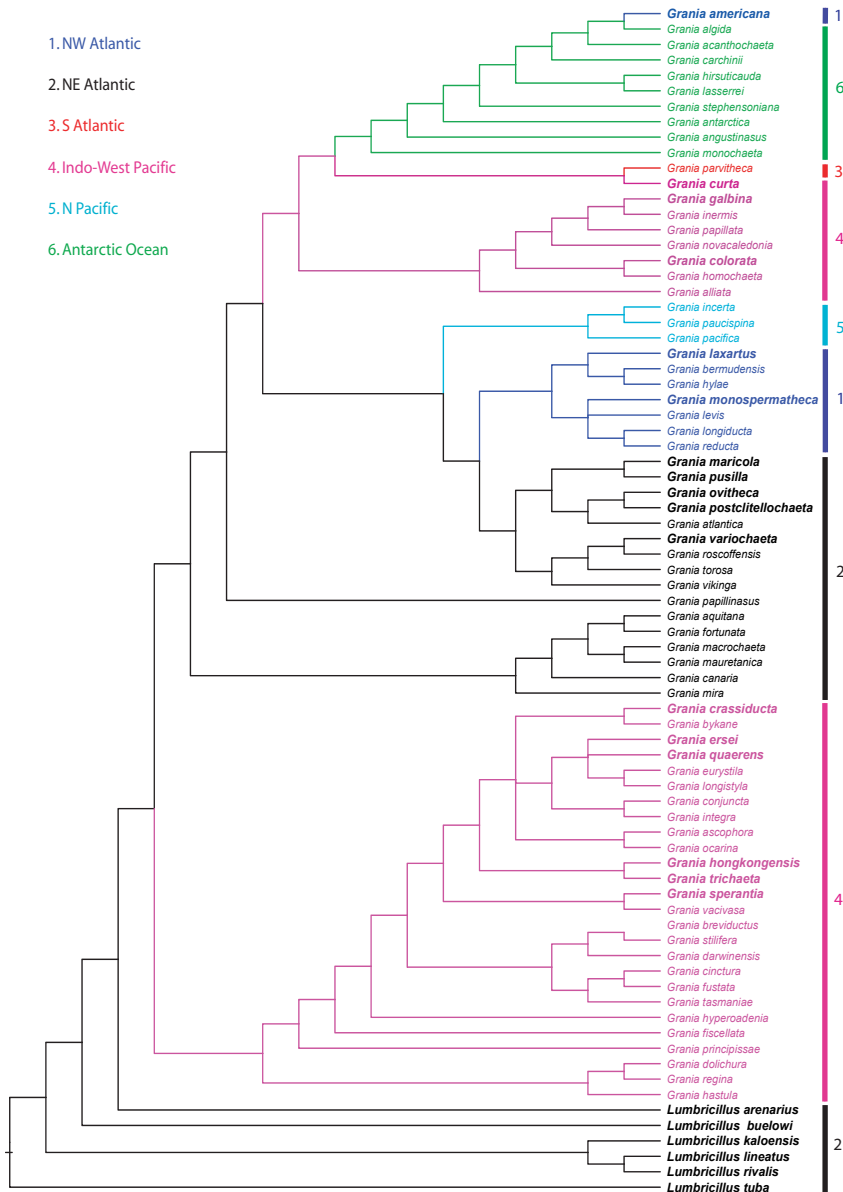


Figure 7. Phylogeny of *Grania* from Paper IV, with geographical region inhabited by the species indicated in colour and with numbers in the right margin. Species from which DNA was available are indicated in bold.

## **Biogeographical patterns**

*Grania* consists of many species with limited geographical distributions. The limited ranges indicate that they cannot disperse easily. Their mode of reproduction might be partly responsible for this; as is common in interstitial animals, the eggs of *Grania* are few and large (each egg is almost as large as an entire segment of the adult worm) and are presumably, as in other clitellates, placed directly in the sand within a cocoon. Thus, *Grania* taxa have no dispersal phase as part of their life cycle. Another factor affecting dispersal ability is selectivity of sediment type and depth. Previously it was thought that the ocean floor was a homogeneous environment, but now we know that this is not the case (Huston 1994). The seafloor is very diverse in type of sediment, organic content, pH, oxygen, temperature etc. etc. and the animals inhabiting it seem to be very specific about where they choose to live (Gage 1996). Species of *Grania* in general prefer sand with particles of varying size and with high organic content. They are most commonly seen in sand made up from mollusk shell fragments; the sand type also favored by the lancelet, *Branchiostoma spp.*

The species that inhabit Swedish waters all seem to have geographical ranges from Gibraltar in the south to Norway in the north, a possible exception being *Grania vikinga* Rota and Erséus, 2003, which so far has only been found on the Swedish west coast (Rota and Erséus 2003). Some have even been reported from the Mediterranean Sea, such as *Grania postclitellochaeta* (Knöllner, 1935) (Lasserre 1971) and *G. ovithec*a (Bonomi and Erséus 1984). The deep waters of the Atlantic seem to be an effective barrier to dispersal, as a different *Grania* fauna can be found on the other side, along the coast of North America. Species which are specialized to live on the deep ocean floor, however, such as *Grania atlantica* Coates and Erséus, 1985, can be found on both sides of the North Atlantic on the continental slopes (*G. atlantica* has even been found south of the equator). Except for this, most of the species found on either side of the North Atlantic seem to be mutually monophyletic, while at the same time they form a monophyletic group together (Paper IV), indicating that the North Atlantic was colonized by an ancestor of the now-living species, which then split up into two lineages on the coasts of Europe and North America, respectively. In Australia, the biogeography is more complex. On the east coast (see Paper II), there seem to be two elements in the fauna, one associated with the tropical Indo-Pacific in the north with close relatives in Hong Kong and New Caledonia, and one associated with the temperate south with relatives in Tasmania and Antarctica.

## **Conclusions and the future**

Since 2005, when I started studying this animal group, we have learned a lot. 14 new species of *Grania* have been described (including the cryptic *Grania occulta*). We now know how the body wall of *Grania* is constructed. We have inferred the phylogeny of the family to which *Grania* belongs, Enchytraeidae, and demonstrated that the genus is monophyletic, with its closest relative in the



genus *Lumbricillus*. We have found some evidence that the common ancestor of all now living enchytraeids was terrestrial and that the ancestors of *Grania* adapted to a life in the marine environment. We have concluded that morphological characters within the genus are highly homoplasious and that the currently recognized species-distinguishing characters cannot estimate a stable phylogeny without the use of genetic data. We have learned that the geographical distributions of species of *Grania* reflect the phylogeny to a great extent, and thus that the large-scale dispersal ability of species of *Grania* is limited. Within the species found in Scandinavia, we have seen that genetic variation is low, whereas it is high between species. In *Grania variochaeta*, we have also found that Scandinavian and French specimens are genetically identical, indicating that there is gene flow between the two populations. We have also found a cryptic species within the relatively few species of *Grania* studied genetically at the population level, suggesting that many more are waiting to be found in other parts of the world.

Thus, we are now starting to realize that the diversity in the interstitial environment is much greater than we could have imagined just a few years ago. We are starting to get pieces of evidence of how some of these animals have evolved, although much work still needs to be done. We still know too little about what the life of an interstitial animal is like or in what ways pollution, eutrophication or global warming affect them, and yet the interstitial fauna contributes greatly to the diversity of our oceans. To be able to separate them to species level and to understand their phylogeny is crucial for biodiversity estimation, which in turn is needed to understand in what ways humans affect the planet, and where conservation efforts should be concentrated.

The search for new species of *Grania* goes on. Large parts of the world need to be surveyed; especially South America, Africa and the tropical Indo-Pacific need to be studied. In the future, it is very important that specimens be preserved in ethanol to allow DNA analysis as well as morphological descriptions of new species. Species that have been described in the past also need to be re-sampled for genetic analysis. Cheaper DNA sequencing techniques and the creation of the Barcode of Life Database provide some hope for a future where we can finally identify and study even the smallest and most inconspicuous organisms on the planet and answer the question “how have they evolved to their current form from a common ancestor many millions of years ago?”

## Summary of Manuscripts

**I. De Wit, Erséus & Gustavsson (2009). Ultrastructure of the body wall of three species of *Grania* (Annelida: Clitellata: Enchytraeidae).** Acta Zoologica 00:00-00 (available online).

The body wall of three species of *Grania*, including the cuticle, epidermis and the musculature, are studied using TEM. The cuticle is similar to previously studied enchytraeids, with an orthogonal grid pattern of collagen fibers. This pattern is also seen in Crassicitellata, which has been suggested as the sister taxon of Enchytraeidae. Variation of epicuticular and fiber zone patterns seen in Naididae (formerly Tubificidae and Naididae) seem to be lacking in Enchytraeidae. The fiber thickness, however, varies between *Grania* species and may be a phylogenetically informative character. The epidermis consists of supporting cells, secretory cells and sensory cells. Basal cells, typical for Crassicitellata, were not observed. The clitellum of *Grania* seems to consist of two types of gland cells, which develop from regular epidermal tissue. It is possible that more cell types exist in different regions of the clitellum, however. The body wall musculature is arranged somewhat differently from that of closely related taxa; this refers to the reduction of circular and outer, triangular longitudinal muscle fibers, while the inner, ribbon-shaped longitudinal muscle fibers are well-developed. A search was conducted for the cause of the peculiar green coloration of *Grania galbina* De Wit and Erséus, 2007, but it was concluded that neither cyanobacteria nor epidermal pigment granules were present in the fixed material.

**II. De Wit, Rota & Erséus (2009). *Grania* (Annelida: Clitellata: Enchytraeidae) of the Great Barrier Reef, Australia, including four new species and a re-description of *Grania trichaeta* Jamieson, 1977.** Zootaxa 2165: 16-38.

This study describes the fauna of the marine enchytraeid genus *Grania* at two locations on the Australian Great Barrier Reef: Lizard and Heron Islands. Collections were made from 1979 to 2006, yielding four new species: *Grania breviductus* sp.n., *Grania regina* sp.n., *Grania homochaeta* sp.n. and *Grania colorata* sp.n.. A re-description of *Grania trichaeta* Jamieson, 1977 based on new material is also included, along with notes and amendments on *G. hyperoadenia* Coates, 1990 and *G. integra* Coates & Stacey, 1997, the two latter being recorded for the first time from eastern Australia. COI barcode sequences were obtained from *G. trichaeta* and *G. colorata* and deposited with information on voucher specimens in the Barcode of Life database and GenBank; the mean intraspecific variation is 1.66 % in both species, while the mean interspecific divergence is 25.54 %. There seem to be two phylogeographic elements represented in the Great Barrier *Grania* fauna; one tropical with phylogenetic affinities to species found in New Caledonia and Hong Kong, and one southern (manifested at the more southerly located Heron

Island) with affinities to species found in Southern Australia, Tasmania and Antarctica.

### **III. Erséus, Rota, Matamoros & De Wit (manuscript). Molecular phylogeny of Enchytraeidae (Annelida, Clitellata).**

Using a multi-locus Bayesian approach with both mitochondrial and nuclear DNA, we infer the phylogeny of the family Enchytraeidae. The family Enchytraeidae is found to be monophyletic and Propappidae is not nested within it. We find statistical support for a majority of the traditionally recognized genera with the family. Enchytraeidae is divided into two groups, one containing *Achaeta* and *Hemienchytraeus*, and one containing all other sampled genera. Within the latter clade, there is support for eight clades. The genus *Marionina* is found to be polyphyletic. The sister taxon to *Grania* is *Lumbricillus arenarius*, which is separated from remaining species of *Lumbricillus*, suggesting that it should be removed from the genus.

### **IV. De Wit, Rota & Erséus (manuscript). Congruence between geography and DNA – a “backbone” approach to estimate phylogeny in *Grania* (Annelida: Clitellata: Enchytraeidae).**

This study is an attempt at inferring a phylogeny of *Grania* using a combination of genetic and morphological analysis. Using DNA-data, it is possible to obtain a well-supported phylogeny, but unfortunately genetic material is only available for a limited number of species of *Grania*. Using the tree given by DNA, we therefore examine morphological characters of all currently described species of *Grania* for homoplasy, and find that all characters used in standard species descriptions are homoplasious to some extent. Interestingly, however, the pattern of geographical distribution of the species of *Grania* used in the DNA-analysis is congruent with the molecular phylogeny, showing that this character can be included in a morphological analysis. When we add geographical location as a character to the morphological analysis, use the DNA-based tree as a backbone constraint, and successively down-weight highly homoplasious characters, we get a well-resolved phylogeny of the genus. This tree should be seen as a good starting hypothesis for further testing when more genetic data becomes available.

### **V. De Wit & Erséus (submitted). Genetic variation and phylogeny of Scandinavian species of *Grania* (Annelida: Clitellata: Enchytraeidae), with the discovery of a cryptic species.**

Individuals of five species of *Grania* (Annelida: Clitellata: Enchytraeidae) were collected from locations in Sweden, Norway and France for studies on the intraspecific variation at the COI locus of mitochondrial DNA and ITS region of nuclear DNA. It was found that the previously described morphospecies in general contain low variation compared to the between-species variation in both loci. In one instance, however, an individual morphologically indistinguishable

from *G. ovitheca* was found to be deviant and instead cluster with *G. postclitellochaeta* both by COI and ITS. We describe this individual as a new species: *G. occulta* sp.n. Furthermore, phylogenetic analyses were conducted, showing a close relationship between *G. variochaeta*, *G. occulta*, *G. ovitheca* and *G. postclitellochaeta*, as well as between *G. pusilla* and *G. maricola*. Using the results from the phylogenetic analyses, we discuss the evolution of morphological characters in Scandinavian species of *Grania*.

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It seems as if it was only yesterday I walked into the Department of Zoology for the first time, yet so much has happened since then. I have had the opportunity to travel the world on more than one occasion, I have lost members of my family, and gained new ones. It is an almost impossible task to mention all the people that have helped me during the past five years, but here I will try to mention some of them. If your name is not here, it is probably because I forgot to mention it, sorry about that. First of all, I wish to thank all fellow zoological systematians who have helped me out: My supervisor Christer for patiently explaining the world of worms to me, Anna and Inger for invaluable help in the lab, Helena for always knowing everything, Judith for speaking her mind at all times, Lisa for being a great roommate, Emma, Jenny and Susanne for teaching me all the practicalities of the institution when I started, Daniel for all funny jokes around the lunch table, and Totte, Arne, Urban, Mattias and Per for support and theoretical help. At the Department of Plant and Environmental Sciences, I thank Henrik for computer help, and Anna, Matz, Martin, Alex, Elisabet, Grazyna, Tine, Bengt and Bente for interesting discussions inside and outside of our journal club. And of course I have to thank old Charlie Darwin for coming up with the theory of evolution in the first place.

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