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Systematics of *Marionina* (Annelida: Clitellata: Enchytraeidae)

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Cover picture: *Marionina triplex* Matamoros et al., 2007

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Det är larverna som är papporna och maskarna som är mammorna, vet du väl.

Ylva Matamoros Nilsson

Sammanfattning

Den här avhandlingen handlar om arter av segmenterade maskar som har tillhört släktet *Marionina* Michaelsen, 1890 inom familjen Enchytraeidae, klass Clitellata (gördelmaskar), fylum Annelida (ringmaskar). Arterna ingår i samma klass som daggmaskarna, men är mycket mindre, och de flesta av dem lever mellan sandkornen i havsstränder. Eftersom det inte finns några tydliga gemensamma karaktärer för gruppen, som skiljer ut dem från andra inom familjen Enchytraeidae har *Marionina* länge ansetts vara ett släkte som innehåller artgrupper som inte är nära släkt med varandra. En sådan grupp kallas för icke-monofyletisk, till skillnad mot en monofyletisk sådan, där alla arter härstammar från gruppens senaste gemensamma förfader. Inom modern systematik strävar vi efter att klassificera efter principen om monofyli. Då blir nämligen gruppens alla medlemmar närmare släkt med varandra än med arterna utanför.

Den främsta målsättningen med denna avhandling har varit att reda ut släktskapet mellan arter som tillhör detta icke-monofyletiska släkte. Detta har t.ex. resulterat i att tidigare *Marionina*-arter har överförts till andra släkten för att på så sätt identifiera och avgränsa olika monofyletiska grupper.

Inom min avhandling har jag studerat DNA sekvenser från tre mitokondriella (12S, 16S, COI) och tre nukleära gener (18S, 28S, ITS), från individer av många olika arter av *Marionina*. På grund av att gensekvenserna varierar i förhållande till hur länge arterna har varit reproduktivt isolerade från varandra kan vi få information om hur de evolutionära släktskapssambanden troligen ser ut.

Med DNA analyser har *Marionina* här bekräftats vara en icke-monofyletisk gruppering av arter. Ca 50 arter bildar dock en monofyletisk grupp som även har en unik detalj i blodkärlssystemet. Denna speciella karaktär delar de med typarten för *Michaelsena* Ude, 1896 (den första arten som beskrevs inom släktet *Michaelsena*). *Michaelsena* synonymiserades med *Marionina* 1959, men jag föreslår härmed att detta gamla namn återinförs och att de ca 50 arterna betraktas som medlemmar i släktet *Michaelsena*.

Typarten för ett tredje släkte, *Enchytronia* Nielsen & Christensen, 1959 har ingått i DNA studien och visat sig vara närbesläktad med sju andra arter, som därmed överförs från *Marionina* till *Enchytronia*.

Inom *Michaelsena* har jag beskrivit en ny art, *M. triplex* (Matamoros et al., 2007), från Svarta Havet, och jag har även studerat en grupp av närbesläktade arter (*M. achaeta*-komplexet) som morfologiskt liknar varandra, och gömmer ett stort antal mer eller mindre kryptiska arter. Det innebär att några av dem endast går att identifiera med hjälp av DNA-data. Dessa kryptiska arter tillhör olika evolutionära linjer som har varit reproduktivt isolerade från varandra under lång tid. Jag visar också resultat som tyder på att andra liknande kryptiska art-komplex är vanligt förekommande inom släktet *Michaelsena.*

Abstract

This thesis is about species of segmented worms that previously were placed in the genus *Marionina* Michaelsen, 1890, within the family Enchytraeidae, class Clitellata and phylum Annelida. These species are closely related to earthworms, but are much smaller and many of them are found between the sand grains in marine beaches. Species within *Marionina* have long been suspected to be a non-monophyletic assemblage of only distantly related species, since they lack unique and consistent morphological characters that unify them as a group and distinguish them from other enchytraeids.

The main aim of this thesis has been to revise the systematics of *Marionina*, to obtain a classification that is congruent with the phylogenetic relationships of this assemblage.

To clarify the complex taxonomical history of *Marionina*, a nomenclatural review is conducted, and the type species *Pachydrilus georgianus* Michaelsen, 1888 is re-described. Based on morphological characters it is concluded that a majority of the species bearing the generic name *Marionina* are only distantly related to this type species.

Within my thesis, DNA sequences from three mitochondrial (12S, 16S, COI) and tree nuclear genes (16S, 18S, ITS) were studied, from different specimens. Molecular analyses confirmed that *Marionina* is a non-monophyletic taxon, and revealed, e.g., a monophyletic sub-group of almost 50 species that have a pharyngeal bifurcation of the dorsal blood vessel. This feature is shared with the type species of *Michaelsena* Ude, 1896 and is likely to be an autapomorphy (a derived, unique character) for this group. *Michaelsena*, which was earlier synonymised with *Marionina,* was thereby restored as a genus, and proposed to include these nearly 50 species.

Seven other former *Marionina* species form a monophyletic group together with the type species of another genus, *Enchytronia parva* Nielsen & Christensen, 1959, and they are thus relocated into *Enchytronia* Nielsen & Christensen, 1959, which is the sister group to *Michaelsena.* The majority of species within *Michaelsena* are marine, while *Enchytronia* species are exclusively terrestrial.

Two additional nominal species of *Marionina* appear to be closely related to, respectively, *Bryodrilus* and *Oconnorella*, which are only distantly related to *Michaelsena* and *Enchytronia*. The remaining species of *Marionina* not dealt with in this thesis, may form a non-monophyletic group and their correct phylogenetic position and taxonomy are not yet solved.

In several cases within *Michaelsena*, the molecular variation is large within groups of taxa that are difficult or impossible to separate morphologically. One example is studied in detail: the *Marionina achaeta* complex, which comprises at least nine separate species that all lack chaetae. Some of these species are impossible to distinguish morphologically and are therefore referred to as cryptic species.

A new species, *Michaelsena triplex* (Matamoros et al., 2007) from the Black Sea has been formally described within this thesis.

Keywords: *Marionina*, *Michaelsena*, *Enchytronia*, Enchytraeidae, cryptic species, molecular phylogeny, taxonomy, systematics, genetic diversity

Contents

Papers included in the thesis

I Rota E, **Matamoros L**, Erséus C (2008) In search of *Marionina* (Clitellata, Enchytraeidae): A taxonomic history of the genus and re-description of the type species *Pachydrilus georgianus* Michaelsen, 1888. Italian Journal of Zoology 75: 417 – 436.

II Erséus C, Rota E, **Matamoros L,** De Wit P (2010) Molecular phylogeny of Enchytraeidae (Annelida, Clitellata). Molecular Phylogenetics and Evolution 57: 849 – 858.

III **Matamoros L**, Rota E, Erséus C. Molecular systematics of groups of "*Marionina*" (Annelida, Clitellata, Enchytraeidae). Manuscript.

IV **Matamoros L**, Rota E, Erséus C. Cryptic diversity within the *Marionina achaeta* species complex (Annelida, Clitellata, Enchytraeidae). Manuscript.

V **Matamoros L**, Yildiz S, Erséus C (2007) A new species within the genus *Marionina* (Enchytraeidae: Annelida: Clitellata) from the southern Black Sea. Marine Biology Research 3: 397 – 402.

I LM was responsible for the morphological examination, and contributed with comments on the manuscript.
II LM contributed with fieldwork, and comments on the manuscript.
III LM was responsible for fieldwork, the majority of the laboratory work, all data analyses, the morphological examination, and writing the manuscript.
IV LM was responsible for fieldwork, the majority of the laboratory work, all data analyses, writing the manuscript, and contributed to the morphological examination (that was mostly performed by the 2nd author).
V LM was responsible for the morphological examination, the laboratory work, and writing the manuscript.

This thesis is not to be regarded as a publication in the sense of the International Code of Zoological Nomenclature (ICZN article 8.2), and scientific names mentioned in it should not be cited in any form.

Introduction

Annelids are segmented worms, which traditionally have been divided into three separate groups: (1) the polychaetes that have lateral outgrowths called parapodia and many more chaetae (bristles) than the second group (thereof the Greek name, poly = many); (2) the oligochaetes have a clitellum, which is a thickening of the body wall that produces a cocoon for the eggs and surrounds part of the reproductive organs, oligochaetes also have fewer chaetae than the first group (thereof the Greek name, oligo = few); (3) Hirudinea (leeches) have clitellum as the previous group, they lack chaeta, and have a posterior and anterior sucker. With modern DNA-technique, the division into these three groups has been modified. Leeches have shown to be a derived group within what was traditionally called Oligochaeta, which makes Oligochaeta sensu stricto a paraphyletic group. These two groups are therefore more correctly referred to as Clitellata, and the name Oligochaeta is thus avoided in this thesis. In a similar way Polychaeta is a paraphyletic group that has been shown to include Clitellata (e.g. Rousset 2007; Zrzavý et al. 2009).

Most clitellates are mysterious little hermaphrodite worms that burrow into the ground or live in aquatic sediments. Thus they keep most of their life away from the human eye. They are not an obvious choice for typical study organisms; therefore they are constantly being neglected. Compared to many other animals, little effort has been made to study the class of Clitellata. And within clitellates, most studies have been on larger earthworms, as e.g. *Lumbricus terrestris* Linnaeus, 1758.

This thesis is about systematics of microscopic worms within the genus *Marionina* Michaelsen, 1890 (family Enchytraeidae). For a long time, this genus has been an assembly of unrelated terrestrial, limnic and marine species from all over the world (Coates 1989; Xie & Rota 2001; Schmelz & Collado 2008, and paper I). There has been a number of nomenclatural mistakes in the taxonomical history of *Marionina*, that have contributed to the confusion, and many species have been included in the genus that are only distantly related to the type species *Pachydrilus georgianus* Michaelsen, 1888, because of their similarity to other species that were included in the old (and only) extensive revision of the genus (Nielsen & Christensen 1959). I hope this thesis will help to resolve some of the troublesome issues of the phylogenetic relationships and the taxonomy of the many nominal species of *Marionina*.

Species within the family Enchytraeidae are usually identified by the number of segments, the chaetal distribution, and the form of the reproductive organs. Fig. 1 and 2 give a presentation of the most important morphological characters used in this thesis. A morphological trait shared by many nominal species of *Marionina* is the pattern of the dorsal blood vessel bifurcating in segment III or IV (called the pharyngeal pattern or marionine pattern) instead of in the prostomium or the peristomium (segment I) as in most other Enchytraeidae (Fig. 2). The pharyngeal bifurcation has long been suggested to

be useful for identifying a monophyletic (natural) group of species of the family (Giere 1974; Coates 1980; Rota 1995; Schmelz & Collado 2010), which we show in paper III to be a correct assumption.

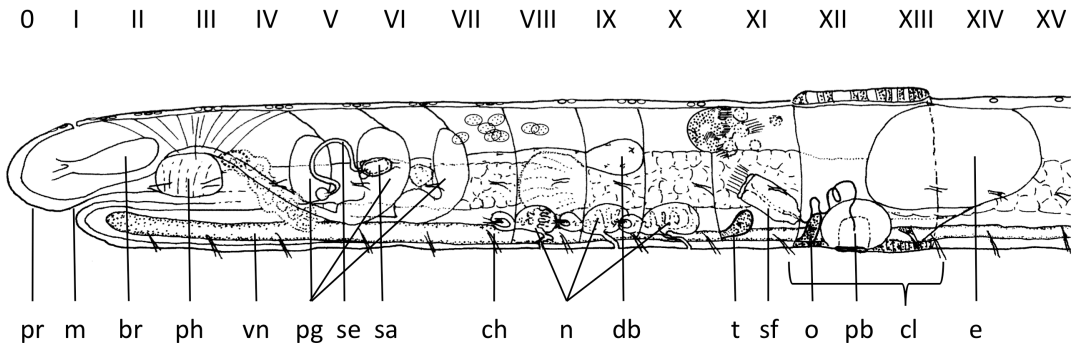

Fig. 1. Enchytraeid morphology. Segments in Roman numbers (I-XV). Abbreviations: pr=prostomium, m=mouth, br=brain, ph=pharynx, vn=ventral nerv cord, pg=pharyngeal glands, se=ectal duct of spermatheca, sa=spermathecal ampulla, ch=chaeta, n=nephridia, db=dorsal blood vessel, t=testis, sf=sperm funnel, o=ovaries, pb=penial bulb, cl=clitellum, e=egg. (Modified from Schmelz & Collado 2010).

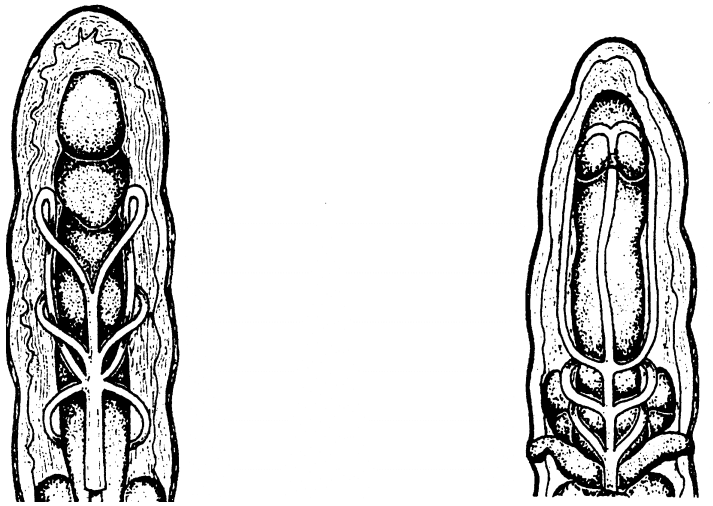

Fig. 2. Anterior bifurcation of the dorsal blood vessel. a) Pharyngeal or marionine pattern. b) Prostomial or peristomial pattern. (From Giere 1974).

Aims

The overall aim of this thesis was to disentangle the taxonomical confusion of *Marionina*. To do this, the problem was divided into the following specific aims:

- Re-description and selection of a lectotype for the type species of *Marionina* (paper I).
- To place groups of "*Marionina"* in a larger phylogeny within the family Enchytraeidae (paper II).
- To resolve the phylogeny of groups of species included in the non-monophyletic genus *Marionina*, and to relocate a large part of the species into other genera to create monophyletic groups (paper III).
- To resolve one example of cryptic diversity within "*Marionina"* (paper IV).
- To formally describe a new species within "*Marionina"* (paper V).

Main methods

The systematic work in papers II, III and IV are mostly based on molecular analyses. Specimens were collected from various places around the world by my co-workers and me, and preserved in ethanol to preserve the DNA. Specimens included in the molecular analyses were cut in two pieces, where the anterior part was used for making whole mounts for microscope observations, and the last few segments that often lacked diagnosable characters were used for DNA extraction. DNA sequences were amplified with PCR technique using gene specific primers, to obtain many copies of the chosen site of the gene. The PCR product was sent to Macrogen Inc. in Seoul, South Korea for sequencing. For most of the included specimens, six genes were sequenced, three mitochondrial (12S, 16S, COI) and tree nuclear (18S, 28S, ITS). All sequences included in a specific analysis were aligned together to detect homologous nucleotides and to enable the estimation of the amount of nucleotide substitutions shared by different specimens. Phylogenetic trees were reconstructed to show the evolutionary relationships of the included gene sequences. This was mainly done with Bayesian analyses using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Maximum Likelihood analyses made with RAxML (Stamatakis et al. 2008), and parsimony analyses with PAUP* 4.0b (Swofford 2003) were made for comparison.

Phylogeny of *Marionina*

In paper I we re-describe the type species of *Marionina, Pachydrilus georgianus*, designate a lectotype, and give a historical overview of the nomenclature and definition of the genus. This is necessary to acquire correct decisions about which species should be morphologically associated with the name *Marionina* and which should not, as done in papers II and III.

The DNA-based assessment of the entire family Enchytraeidae (paper II) is of importance for the understanding of the phylogenetic complexity of the group called *Marionina* and is therefore an important part of my thesis. In our molecular analyses, species earlier regarded as *Marionina* appear in three different places in the phylogenetic tree of Enchytraeidae (Fig. 3). *Pachydrilus georgiana* is a Subantarctic species and we were not able to get hold of fresh material and were therefore limited to studies of museum specimens not suitable for DNA-extraction. For this reason the type species of *Marionina* is not included in the molecular analyses. According to its morphology, it is likely that this type species is close to the genus *Lumbricillus* (paper I); i.e., it does not seem to be closely related to the majority of the *Marionina* species. *Marionina* is thus shown to be a non-monophyletic group (paper II and III), as has been suspected for a long time.

A part of this thesis is about relocating species from the artificial genus *Marionina* into other genera that are likely to be monophyletic groups. The phylogenetic analyses in papers II and III show that two terrestrial nominal species of *Marionina* and five undescribed species (or with uncertain identification) form a monophyletic group together with the type species of *Enchytronia* Nielsen & Christensen, 1959 (*E. parva* Nielsen & Christensen, 1959) (Fig 4). *Enchytronia clavata* (Nielsen & Christensen, 1961) and *E. filiformis* (Nielsen & Christensen, 1959) are accordingly proposed to be included in the terrestrial genus *Enchrytronia* (paper III).

The type species of *Michaelsena* (*M. subtilis* Ude, 1896) has morphological similarities to many of the marine *Marionina* species that appear as a sister group to *Enchytronia* (Fig. 4). The most important feature shared by these species is the pharyngeal bifurcation of the dorsal blood vessel (Fig. 2a). This pattern is neither shared by the type species of *Marionina* nor the species of *Enchytronia,* which have peristomial bifurcation of the dorsal vessel (Fig. 2b). We thereby revalidate the genus *Michaelsena* that has been a junior synonym to *Marionina* since 1959 (Nielsen & Christensen 1959) and include 35 species from the marine clade into *Michaelsena*. Seventeen additional nominal *Marionina* species (with no DNA sequence data) are included in *Michaelsena* on the criterion of having pharyngeal bifurcation of the blood vessel (paper III).

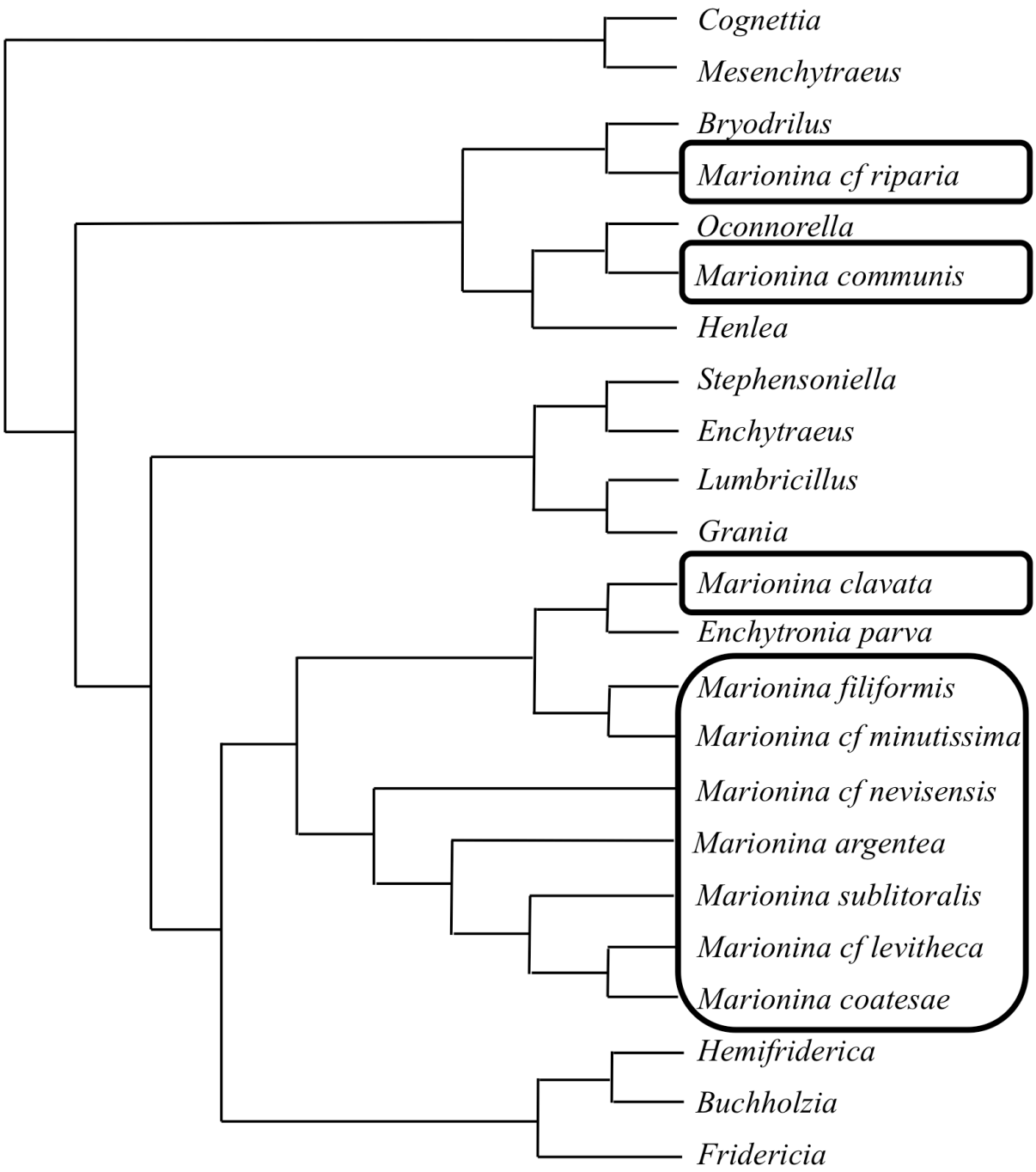

Fig. 3. Enchytraeidae phylogeny. Species of the non-monophyletic genus *Marionina* are marked with frames. Modified from paper II.

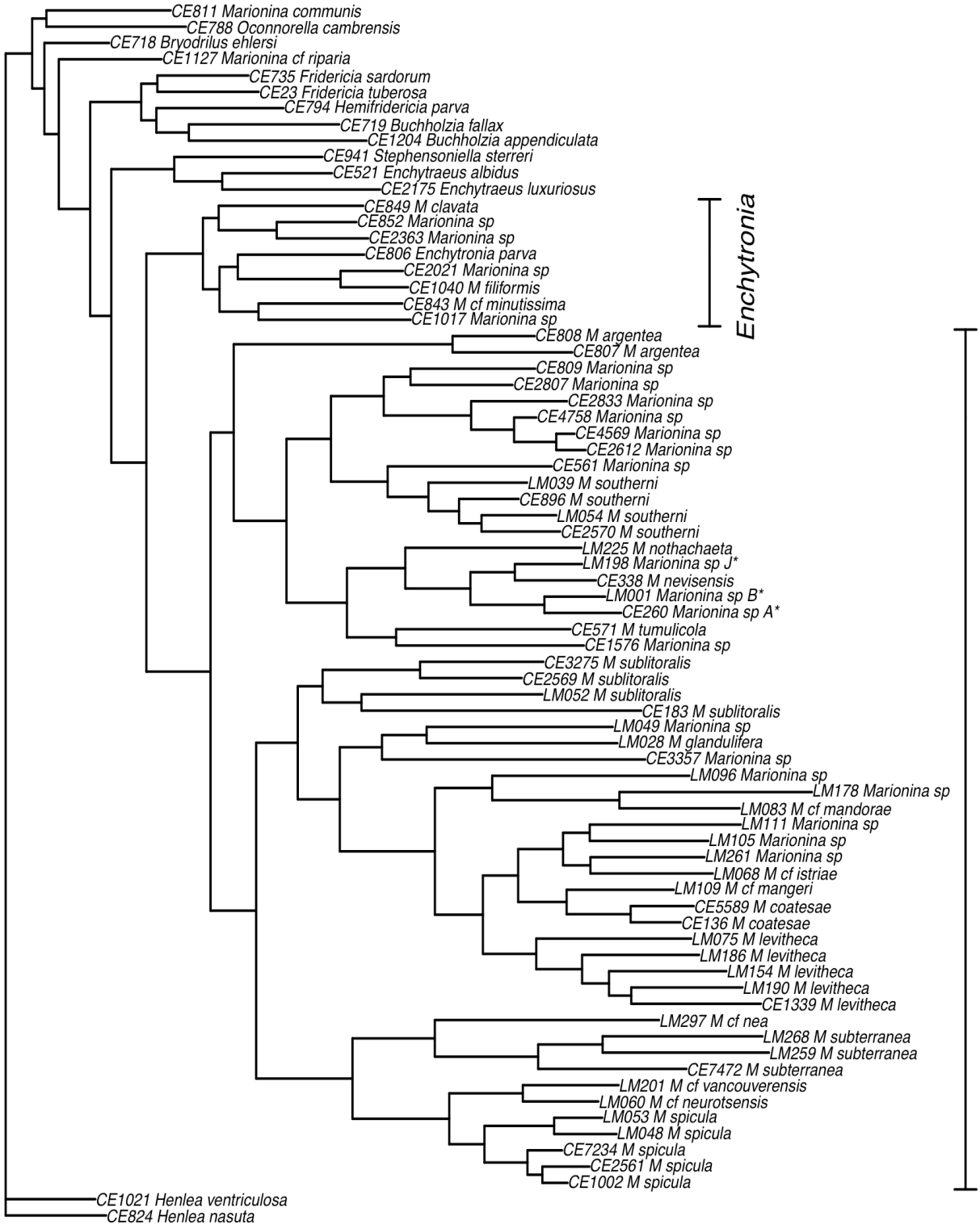

Fig. 4. Phylogeny of groups of *Marionina* species. Modified from paper III. Species transferred to *Enchytronia* and *Michaelsena* marked in margin.

Nominal species included in *Michaelsena* are: *Michaelsena achaeta* Hagen, 1954, *M. appendiculata* (Nielsen & Christensen, 1959), *M. arenaria* (Healy, 1979), *M. argentea* (Michaelsen, 1889), *M. brendae* (Rota, 1995), *M. brevis* (Finogenova, 1972), *M. cana* (Marcus, 1965), *M. coatesae* (Erséus, 1990), *M. eleonorae* (Rota, 1995), *M. gabiae* (Healy & Coates, 1997), *M. glandulifera* Jansson, 1960, *M. istriae* (Giere, 1974), *M. levitheca* (Erséus, 1990), *M. mandorae* (Healy & Coates, 1997), *M. mangeri* Michaelsen, 1914, *M. nea* (Marcus, 1965), *M. neurotsensis* (Coates, 1980), *M. nevisensis* (Righi & Kanner, 1979), *M. nothachaeta* (Matamoros et al., paper IV) (pro *Marionina achaeta* Lasserre, 1964), *M. scintillans* (Boros & Dózsa-Farkas, 2008), *M. seminuda* (Xie & Rota, 2001), *M. sinica* (Xie & Rota, 2001), *M. sjaelandica* (Nielsen & Christensen, 1961), *M. southerni* (Cernosvitov, 1937), *M. spicula* (Frey & Leuckart, 1847), *M. spongicola* (Rota & Manconi, 2004), *M. sublitoralis* (Erséus, 1976), *M. subterranea* Knöllner, 1935, *M. transunita* (Coates, 1990), *M. triplex* (Matamoros et al, 2007), *M. tumulicola* (Healy & Coates, 1997), *M. ulstrupae* (Healy, 1996), *M. vancouverensis* (Coates, 1980), and *M. waltersi* (Healy, 1994).

Two other species currently referred to *Marionina* appear outside of the *Enchytronia* + *Michaelsena* group in the *Enchytraeidae* phylogeny (papers II and III): *Marionina riparia* Bretscher, 1899 is closely related to the genus *Bryodrilus*, and *Marionina communis* Nielsen & Christensen, 1959 to the genus *Oconnorella* (Fig. 3 and 4).

Species concept

Several different species concepts in biology have been proposed and there are never-ending discussions about which concept to use (Mayden 1997). One of the most popular is the biological species concept (Mayr 1942) that states that if two individuals can reproduce and produce fertile offspring, they are members of the same species. This is possible to observe in nature in some cases, but seldom for small worms and other invertebrates. In the lab, it may be possible to do mating experiments with some organisms, but this is costly and cannot be done extensively. Other species concepts are, e.g., based on morphology or ecology alone. Different species concepts give in many cases the same result, but some approaches may fail to identify species that for some reason have not differentiated in morphology or ecology. This could be due to the short time since the populations diverged, or selective pressure for a stable morphology or ecology (e.g. Sites & Marshall 2004). One could claim that "species", to a large extent, is a unit that man has invented for convenience when organizing nature, and that it should be used to facilitate the description and communication of biodiversity. A useful approach however, is the concept of species being separately evolving metapopulation proposed by De Queiroz (2007). He means that if one can show, with whatever tool, that two

metapopulations are separately evolving, this can be used as evidence for them being different species. If you cannot find such proof it does not necessarily mean that speciation has not occurred, it may just not be detectable. For small clitellate species the most convenient way of investigating if populations are separately evolving, is to study the diversity in mitochondrial and nuclear genes that accumulates with time between such metapopulations. As probably most other systematists of today, I have thus used a phylogenetic species approach as the main tool for species delimitation (Baum & Shaw 1995; Mishler & Theriot 2000). You have to bear in mind, however, that speciation is a process with no clear beginning or end, and thereof the difficulty of drawing the line of when two sister lineages have become separate species.

Some differences between mitochondrial and nuclear genes are important for the interpretation of the gene trees in this thesis. First, when the sperm cell penetrates the egg, it is only its nucleus that enters and contributes to the embryo's DNA. Mitochondrial genes are therefore (with few exceptions) inherited from the mother only, while nuclear genes are inherited from both sexes. Second, the two sets of nuclear genes (the maternal and paternal copies) recombine under meiosis and the two copies are therefore continuously mixed in every generation. The mitochondrial genes do not recombine. Third, for most animals, the mitochondrial genes evolve faster than nuclear genes and have therefore more evolutionary information of recent history, while nuclear genes are more stable and are easier to use for studying older splits among lineages (Avise 2000).

Another thing that may be considered when studying species relationships is the effect that hybridization can have on the phylogenetic trees. Hybridization is when individuals from different species reproduce. This leads to mixtures of the two species' DNA. If only a few hybridization events occur in a population (with migrating individuals) the nuclear genome will after many generations have only a small percentage of the introduced DNA, if any. The mitochondria on the other hand could in some cases, by chance, be replaced by the introduced mitochondria, since it is non-recombining (Avise 2000).

Genes do not always have the same phylogenetic history as that of the species from which they are sampled. If e.g. an ancestral population was heterogeneous in respect to a gene (as population "a" in Fig. 5a), having two different gene copies, and this population is split into two different populations ("b" and "c" in Fig. 5a), it is possible for the two new populations to inherit both gene copies. If the new populations are further divided into separate evolving populations before one of the copies has been fixed (a phenomenon called incomplete lineage sorting), it is not sure that sister species ("D" and "E", or "F" and "G" in Fig. 5a) will end up having sister copies of the gene, if different copies are fixed in the recent populations and the heterogeneity in regard to that gene is lost (as in populations D, E, F and G in Fig. 5b). This

incomplete lineage sorting is less likely to occur if ancestral populations were small or if time between species divergences (branching points) was large, since the chance for populations to stay heterogeneous would then decrease. When reconstructing phylogenetic trees based on single genes, the different gene copies could therefore have a common origin that goes further back in time (the point of coalescence) than that of the species divergence, and the gene tree could be incongruent with the species tree (Maddison 1997). To avoid to be misled by this possible disagreement, one should study different unlinked genes and compare the different gene trees.

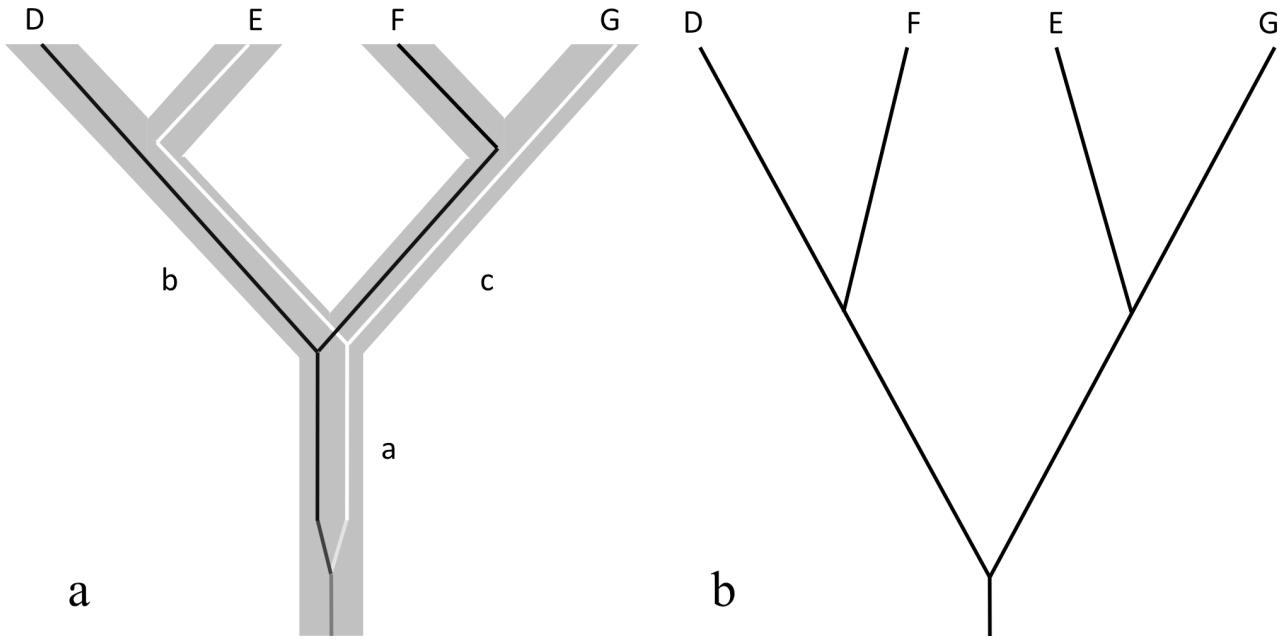

Fig. 5. A hypothetical example where the gene tree disagrees with the species tree due to incomplete lineage sorting a) Species tree is shown as a gray background, with gene lineages in black and white lines respectively, that represent different gene copies in the heterogeneous ancestral populations a, b and c. Recent populations A, B, C and D are homogeneous populations with only one copy of the gene. b) Gene tree that would result if sampling individuals from the same recent populations as in Fig. 5a.

When working with species delimitation one should examine both mitochondrial genes and nuclear genes. If there are contradictions between gene trees this could be interpreted either as a historical introgression of genes (through hybridization), incomplete lineage sorting, or as recent gene flow between populations. In the latter case, one should be cautious in separating species, since it could indicate that the different populations are not separately evolving lineages. An example of this is studied in paper IV, where different

lineages within a species that we now call *Michaelsena sp. E,* show contradictions between the topologies of the nuclear and mitochondrial gene trees. This could be an example of ongoing speciation where reproductive barriers are unstable. In this case we found it reasonable to delimit the species so it includes all lineages with contradicting positions in the different gene trees.

Cryptic species

Geographical boundaries are not as obvious for marine species as for terrestrial or freshwater taxa. However, marine species have been shown to have strong geographical signal resulting from low gene flow between geographically distant populations (Palumbi 1994). Such barriers can result in recent speciation events where the species often accumulate genetic differentiations before morphological differences can be observed. There are many examples of what was earlier thought to be single widespread marine species, which are actually shown to be complexes of cryptic species (Knowlton 1993). Species are said to be cryptic when they cannot be distinguished morphologically and are therefore often misidentified even by experts (Bickford et al. 2007). Such species can vary in, e.g., habitat, ecology and chemical signalling, and are often revealed with molecular phylogenies (Knowlton 1993). Clitellates lack a larval stage, and the geographical barriers could be expected to be even larger for marine clitellates than for most other marine invertabrates that have larval stages, which can be dispersed much longer distances through the water body.

Traditionally for enchytraeids, morphology has naturally been, and is still the main way to distinguish species. The first step of species identification is most likely to be an identification key and for most biodiversity assessments this is the only tool for identification. However, this thesis and many other studies show that species diversity is often much higher than what can be identified with morphology alone (e.g. King et al. 2008; Gustafsson et al. 2009; De Wit & Erséus 2010; Nygren & Pleijel 2011).

In paper IV we studied an example of cryptic diversity within a complex of species lacking chaetae. At that time we called it the *Marionina achaeta* complex, but in paper III we relocated this complex of species into *Michaelsena,* and here, I will refer to it as the *Michaelsena achaeta* complex. Four species have formally been described within this complex: *Michaelsena achaeta* Hagen, 1954, *M. nothachaeta* (Matamoros et al., paper IV), *M. nevisensis* (Righi & Kanner, 1979) and *M. arenaria* (Healy, 1979). The specific name “achaeta” was used for two different species descriptions and there is confusion regarding their taxonomy. In 1954, Hagen used the specific name “achaeta” in her description of a new species, and in 1964, Lasserre described a different species also lacking chaetae, but giving it the specific name “achaeta” since he was only aware of the unpublished version of Hagens species description (Hagen 1951) and thought that his and Hagens specimens

were conspecific. In paper IV, we therefore renamed *Marionina achaeta* Lasserre, 1964 as *Michaelsena nothachaeta* (Matamoros et al., paper IV). With molecular analysis we found nine different species of achaetous worms in this complex. They were: *Michaelsena nothachaeta*, *M. nevisensis* and seven undescribed species, two of which are morphologically identical to *M. nevisensis* and are therefore referred to as cryptic. They can only be distinguished with molecular analyses.

In paper III, we find cryptic species also within each of the nominal taxa: *Michaelsena spicula, M. subterranea*, *M. levitheca*, *M. sublitoralis, M. southerni*, and *M. argentea*. There can be several reasons for this large amount of diversity. It could be that this is a general trend in most animal groups. It is likely however, that one important reason for the large amount of cryptic speciation in *Michaelsena* is that this group is hard to study morphologically. They are small and have only few easily observed characters for identification. Longer terminal branches are observed in the phylogenetic tree within *Michaelsena*, which can be an indication of a faster evolutionary rate in the group compared to other enchytraeids (paper II).

New species

When describing new species it is desirable to publish a genetic sequence, which is associated with a voucher (Pleijel et al. 2008). This sequence can then be used as a barcode for species identification, and DNA from an unidentified specimen can be compared against a database comprising of an amount of sequences barcodes. Different markers have been suggested as barcode genes, but for animals the mitochondrial COI gene (cytochrome c oxidase sudunit I) is the most commonly used so far (e.g. Hebert et al. 2003; 2004; Frezal & Leblois 2008), as it works well both for species identification and delimitation for many animal groups (Ward et al. 2005; Lefébure et al. 2006; Hebert et al. 2004). To use the barcode gene for species delimitation, there needs to be a larger divergence between species than within species, and for some animal groups the COI gene does not work well (King et al. 2008; Chang et al. 2009). For other groups, the COI gene is not suitable for species identification either, as e.g. for cnidarians and plants, where it is a slowly evolving gene giving little evolutionary information (Kress et al. 2005; Erpenbeck et al. 2006). COI is a mitochondrial gene, and therefore inherited only through the maternal lineage and is non-recombining (see above). Analyzing such genes gives information of only a fraction of the historical relationships of the populations. The advantage of working with mitochondrial markers in closely related taxa however, is that they usually evolve at a faster rate than nuclear genes, and therefore have a higher proportion of informative sites.

Michaelsena triplex (Matamoros et al., 2007) is described in paper V. It is a species from the Black Sea and is one of the smallest marine enchytraeids known, only 1.4-1.8 mm long with 20-25 segments. The name “triplex” refers

to that it has three chaetae per bundle. Several attempts were made to extract DNA from *M. triplex*, but they did not succeed. The material used was collected in an ecological study and was not aimed for DNA extraction; it was fixed in 4 % formaldehyde, which degrades the DNA. Hopefully, future samples of this species will be fixed in alcohol to enable the inclusion of its DNA in phylogenetic assessments as well as barcoding.

COI sequences for all nine species within the *Michaelsena achaeta* complex were analyzed (paper IV), which can serve as barcodes for these species, and there are vouchers for all species, except *Michaelsena sp. A*. However, seven of these species have not yet been formally described and named. For the highly variable *Michaelsena sp. E*, the COI sequences show that there is a large variation within this single species. The COI gene can in this case serve for identifying species considering that we have knowledge about the intraspecific variation in the barcodes. However, COI alone is not sufficient for delimitation of *Michaelsena sp. E*, as its intraspecific diversity is higher than some of the interspecific distances of their complex. Nuclear genes are here needed for species delimitation.

Conclusions

The most important conclusion of this thesis is that molecular phylogenetic analyses support that *Marionina* is a non-monophyletic assembly of species. We also provide a revalidation of the mainly marine genus *Michaelsena* Ude, 1896, which now is proposed to include 31 former *Marionina* species. The pharyngeal bifurcation of the anterior dorsal blood vessel is a unique character and appears to be an autapomorphy of this genus. Several other nominal species of *Marionina* are likely to be related to *Michaelsena*, but morphological information of the anterior blood vessels is limited, and DNA sequences for all species are currently lacking.

Two other species of *Marionina* are now included in the terrestrial sister genus *Enchytronia* Nielsen & Christensen, 1959. As for *Michaelsena*, it is likely that yet other *Marionina* species belong to *Enchytronia*, but this suggestion should be evaluated by including more species into future phylogeny reconstructions.

We have described cryptic diversity within the *M. achaeta* complex and showed several examples of great molecular diversity also among other, morphologically similar, species. Many species of *Michaelsena* are still undescribed and if taking into account all the possible cryptic forms, the number of species in this genus will probably increase manifold in the future.

There are still many more nominal *Marionina* species with an uncertain phylogenetic position, and this thesis indicates that studying type material and collecting fresh material for DNA will be needed for a revision of the remaining nominal species of *Marionina*.

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Abstracts of included papers

I. Rota E, **Matamoros L**, Erséus C (2008) In search of *Marionina* (Clitellata, Enchytraeidae): A taxonomic history of the genus and re-description of the type species *Pachydrilus georgianus* Michaelsen, 1888. Italian Journal of Zoology 75: 417 – 436.

An approach towards a systematic revision of *Marionina* Michaelsen, 1890 is made through an historical overview of its nomenclature and definition, and a thorough characterization of its type species, the South Georgian marine littoral *Pachydrilus georgianus* Michaelsen, 1888. Relevant sections of early enchytraeid literature provide the background for appreciating the complex taxonomic history of the genus and giving a final word as to the controversial validity of its name, authority and date. *Marionina* in its current acceptation comprises about 100 nominal species, but the paper documents how, since its establishment, the genus has been an artificial assemblage of unrelated taxa, whose taxonomy cannot be sorted out (1) without finding new morphological characters and improving the standard of descriptions, and (2) without using a total evidence approach (morphology and molecules) within a phylogenetic framework. Confusion about the identity of the type species, originally briefly described and only partially figured, is unravelled upon examination of the syntypes available in Hamburg and Berlin. Since neither series proved to be monospecific, a lectotype is designated to assure correct and consistent application of the name in the future.

II. Erséus E, Rota E, **Matamoros L**, De Wit P (2010) Molecular phylogeny of Enchytraeidae (Annelida, Clitellata). Molecular Phylogenetics and Evolution 57: 849–858.

A multigene data set (12S, 16S, and COI mitochondrial DNA; 18S and 28S nuclear DNA) was analyzed by Bayesian inference to estimate the phylogeny of a sample of the clitellate family Enchytraeidae (86 species representing 14 nominal genera). Monophyly, as well as a basal dichotomy, of the family Enchytraeidae obtained maximum support, with one clade containing *Hemienchytraeus* and *Achaeta*, the other the remaining 12 genera analysed. The latter group is basally resolved in several well-supported clades. *Lumbricillus* and *Grania* are closely related. *Bryodrilus*, *Oconnorella*, *Henlea* and two species of *Marionina* (*M.* cf. *riparia*, and *M. communis*) form a well-supported clade. *Cognettia* is sister to *Stercutus*, and *Cernosvitoviella* sister to *Mesenchytraeus*, and the four together appear to be a monophyletic group. A large part of the taxonomically problematic *Marionina* appears to be a group not closely related to the type species (*M. georgiana*), and this group also includes *Enchytronia*. Further, this *Marionina/Enchytronia* group appears to be sister to a clade comprising the more or less littoral marine genera

Stephensoniella and *Enchytraeus*. *Hemifridericia*, *Buchholzia* and *Fridericia*, the three genera characterized by two types of coelomocytes, also form a well-supported clade. The study corroborates most of the multi-species genera analysed (*Cognettia*, *Cernosvitoviella*, *Mesenchytraeus*, *Oconnorella*, *Henlea*, *Enchytraeus*, *Grania*, *Buchholzia* and *Fridericia*); only *Lumbricillus* and *Marionina* are non-monophyletic as currently defined.

III. Matamoros L, Rota E, Erséus C. Molecular systematics of groups of "*Marionina*" (Annelida, Clitellata, Enchytraeidae). Manuscript.
The enchytraeid genus *Marionina* Michaelsen, 1890 has long been in need of revision. For instance, it was recently concluded that a large majority of the many species historically and currently attributed to this taxon are not closely related to the type species. In the present study we assess the phylogeny of 20 nominal *Marionina* species, and 22 unidentified or undescribed species, using mitochondrial (12S, 16S, COI) and nuclear (18S, 28S, ITS) gene data. As a result, we propose that seven of these species are relocated into the solely terrestrial genus *Enchytronia* Nielsen & Christensen, 1959, and 35 species into the latter's, sister group *Michaelsena* Ude, 1896. *Michaelsena* was synonymised with *Marionina* by Nielsen and Christensen (1959), but is thus hereby resumed as a separate genus. Seventeen additional nominal *Marionina* species, not included in the present phylogeny, are also proposed to be members of *Michaelsena*, on the basis of their morphology. The pharyngeal bifurcation of the anterior blood vessel is argued to be an autapomorphy for *Michaelsena* and is used here as a criterion for including species in *Michaelsena,* when molecular data is unknown. We also reveal a large genetic diversity within several nominal species of *Michaelsena* that we suspect to be complexes of cryptic species.

IV. Matamoros L, Rota E, Erséus C. Cryptic diversity within the *Marionina achaeta* species complex (Annelida, Clitellata, Enchytraeidae). Manuscript.
In this study we define the *Marionina achaeta* complex as a marine group of species within the genus *Marionina* that lack chaetae. It includes the four nominal species: *M. achaeta* (Hagen, 1954), *M. achaeta* sensu Lasserre, 1964, *M. nevisensis* Righi and Kanner, 1979 and *M. arenaria* Healy, 1979. We studied the genetic and morphological diversity of achaetous specimens of *Marionina* collected in Florida, USA; Great Barrier Reef, Queensland, Australia; New Caledonia; South Western Australia; Sweden; England; and the Bahamas. The collection localities are almost all supralittoral and vary from fully marine to brackish environments; some are temporarily in freshwater after rain. Parts of the mitochondrial genes 12S, 16S, COI, and the nuclear genes 18S, 28S, ITS were sequenced and analysed to assess the genetic variation and

estimate the phylogeny of the achaetous *Marionina* species. The molecular data reveal one monophyletic group of at least nine separately evolving lineages: *M. achaeta* sensu Lasserre, 1964*, M. nevisensis s.str.*, *M. sp. A*, *M. sp. B*, *M. sp. D*, *M. sp. E*, *M. sp. I*, *M. sp. J* and *M. sp. K*, most of which are not associated with any described nominal taxon. Variation in COI, shows high variation, both within (up to 13.8 %) and between lineages (15.6-25 %). Morphologically, however, we can discriminate only seven lineages. *Marionina nevisensis* s.str., *M. sp. D*, and *E* match the description of *M. nevisensis* and are therefore considered to be cryptic species. Among the studied species, those from temperate regions of the world appear on two separate branches that diverge from the base of the tree, while the tropical species comprise a single branch, indicating a temperate origin of the whole complex. We also select a neotype for *M. nevisensis* from the Caribbean region*,* and as Hagen's (1954)'*M. achaeta'* appears to be morphologically different from its (then) junior homonym *M. achaeta* Lasserre, 1964, we propose a replacement name for the latter as *M. nothachaeta* nom.nov.

V. Matamoros L, Yildiz S, Erséus C (2007) A new species within the genus *Marionina* (Enchytraeidae : Annelida : Clitellata) from the southern Black Sea. Marine Biology Research 3: 397 – 402.
A new species of Enchytraeidae, *Marionina triplex* sp. n. is described from an intertidal mussel bank in Sinop, southern Black Sea coast of Turkey. *Marionina* Michaelsen, 1889 is a widely distributed and heterogeneous assemblage of species, but while awaiting a revision the new species is placed here, as it appears to belong to a group of small enchytraeids currently associated with this genus. The new species in some aspects resembles other species reported from the Mediterranean Black Sea area [*M. adriaticus* Vejdovsky1877; *M. argentea* (Michaelsen, 1889); *M. brevis* Finogenova, 1972; *M. spicula* (Frey & Leuckhart, 1847)] and some species described from elsewhere (*M. cana* Marcus, 1965; *M. singula* Ude, 1896; *M. sublitoralis* Erséus, 1976; *M. ulstrupae* Healy, 1996), but it can be distinguished by its body length, number of segments, number and form of chaetae, shape and size of pharyngeal glands and spermathecae, etc. The new species is only 1.4–1.8 mm long, with 20–25 segments, and is thus one of the smallest marine enchytraeids known. It has mostly three chaetae per bundle, and spermathecae with the ampulla of about the same size as the ectal duct, and with a few gland cells attached to the base of the ectal duct.