

## How to deal with cryptic species in Enchytraeidae, with recommendations on taxonomical descriptions

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**Abstract.** During the 12th International Symposium on Enchytraeidae, held in Tihany, Hungary (27–29 June 2016), the participants discussed cryptic species, *i.e.*, species that are morphologically so similar that they are classified as the same species (Bickford *et al.* 2007), and how to deal with them taxonomically. Here we summarise the discussion together with a few additional comments, and we give recommendations for species descriptions in Enchytraeidae.

**Keywords.** Annelids, morphospecies, sequencing.

### INTRODUCTION

Species are basic biological units, and the first step in the exploration of biodiversity. Species are also entities of generalisation: information from different studies of individuals of the same species can be generalised to that species, but not necessarily for a more inclusive taxon, *e.g.*, a genus or a family. However, for this generalisation it is important that the specimens are correctly identified to species, and species are correctly delimited, to avoid, for example, that

several species differing in various properties, *e.g.* ecological or physiological, are included under the same name. Both correct identification of previously named species, and the naming and description of new taxa are crucial steps for describing the biota of the world, and also to ensure that scientists mean the same thing when using a species name. Taxonomic names are also needed to link species to data, produced in different studies, so that they can be related in various analyses. If data (*e.g.* ecological, morphological, and molecular) cannot be linked to formal species

and well-referenced names, these data will lose much of their value. The proper naming and description of species is therefore essential.

Enchytraeids have traditionally been studied alive using light microscopy, and the morphological investigation of whole worms, either alive or fixed, is still the basis for the identification of specimens and descriptions of new species. However, with the introduction of widely accessible molecular methods, notably the sequencing of DNA 'barcode' fragments and refined analytical tools, a new standard set of data has become available to recognize and to delimit species. DNA sequences often confirmed the distinctions drawn between morphologically defined species (Klinth *et al.* 2017, De Wit & Erséus 2010), but in some cases they showed that species described on the basis of morphological differences are in fact synonyms (Dózsa-Farkas *et al.* 2012). More importantly, they also revealed the existence of cryptic species, *i.e.* species that, so far, cannot be differentiated with morphology-based methods (Martinsson & Erséus 2014, Matamoros *et al.* 2012).

It is important to note that cryptic species have been known in enchytraeids for more than half a century, based on karyology (Christensen 1961), protein patterns (Brockmeyer 1991, Christensen *et al.* 1992, Schmelz 2003, Westheide & Graefe 1992) or other techniques, but they were never formally recognized and described, with the notable exception of *Enchytraeus crypticus* Westheide & Graefe, 1992. A list in Collado *et al.* (2012) contains 40 enchytraeid species as candidates for species complexes; they include almost all commonly cited species. Formal recognition of cryptic species has increased with the establishment of DNA sequencing as standard taxonomic method. There are currently four described truly cryptic species-pairs in Enchytraeidae (Table 1) and we expect many more to come. Cryptic species cannot be distinguished using the traditional and widely-used method of studying the morphology using light microscopy. Therefore, a discussion on how cryptic species should be treated was held during the symposium.

During the discussion, the participants agreed that cryptic species are distinct evolutionary lineages, which deserve recognition in a classificatory system. There is a growing body of evidence that cryptic species may differ in ecological and physiological properties, and therefore the separation of cryptic lineages within morphospecies can be important when such species are used as models in ecology, ecotoxicology and physiology (see Feckler *et al.* 2013, Römbke *et al.* 2016). ('Morphospecies' is used here to denote mainly *named* species, described and identified in the traditional way, using morphological characters. Our use differs from the one in ecology, where 'morphospecies' often means morphologically distinguishable but *unnamed* species of unknown identity.) It was also agreed that a morphospecies that comprises an assemblage of cryptic species still deserves recognition even though it cannot be considered, due to reproductive barriers within, as one biological species any more. The reasons are not only practical but also biological: The assemblage of cryptic species (*i.e.* the morphospecies) may form a monophyletic group and may have common ecological properties that are different from the rest of the species in the genus. Morphospecies that turn out to be polyphyletic assemblages, however, should be abandoned. It should be noted that this consensual opinion differs from previous practice, where either the cryptic species or the morphospecies was discarded (Christensen 1961, Sturmbauer *et al.* 1999, Gustafsson *et al.* 2009, James *et al.* 2010).

However, opinions differed as to how cryptic lineages should be recognised. Two options included the use of informal categories:

- Maintain the species name of the morphospecies and denote the cryptic species appending a series of alphanumerical codes to the name of the morphospecies;
- Give full species rank to the cryptic species and denote the morphospecies with the old name plus an epitheton like "*sensu lato*", or "species group" or "species complex".

**Table 1.** Cryptic species pairs in Enchytraeidae. Included are also species pairs with morphological differences inconclusive or difficult to access.

	Habitat	Type of difference	Morphological differences
<i>Enchytraeus variatus</i> Bouguenec & Giani, 1987 <i>Enchytraeus crypticus</i> Westheide & Graefe, 1992	compost, soil	Isozyme patterns, total protein patterns (Brockmeyer 1991) DNA-RFLPs (Schlegel et al. 2009) CIE, crossed immuno-electrophoresis (Gabrich et al. 1991) RAPD-PCR (Schirmacher et al. 1998)	Ultrastructure of spermatozoa (Westheide et al. 1991)
<i>Grania bekkouchei</i> Prantoni, De Wit & Erséus, 2016 <i>Grania cryptica</i> Prantoni, De Wit & Erséus, 2016	marine sediment	DNA sequences (Prantoni et al. 2016)	none
<i>Chamaedrillus/Cognettia</i> * <i>sphagnetorum</i> (Vejdovský, 1878) <i>Chamaedrillus pseudosphagnetorum</i> Martinsson, Rota & Erséus, 2015a	soil	DNA sequences (Martinsson and Erséus 2014, Martinsson et al. 2015b)	none
<i>Grania oviheca</i> Erséus, 1977 <i>Grania occulta</i> De Wit & Erséus, 2010	marine sediment	DNA sequences (De Wit and Erséus 2010)	none
<i>Enchytraeus bigeminus</i> Nielsen & Christensen, 1963 <i>Enchytraeus japonensis</i> Nakamura, 1993	compost, soil	Isozyme patterns, Total protein patterns (Schmelz et al. 2000)	Male sexual glands (species with predominantly asexual reproduction) (Schmelz et al. 2000)
<i>Chamaedrillus/Cognettia glandulosus</i> (Michaelsen, 1888) <i>Chamaedrillus varisetosus</i> Martinsson & Erséus, 2015b	soil, aquatic sediments	DNA sequences (Martinsson and Erséus 2014, Martinsson et al. 2015a)	Body size, chaetal numbers (Martinsson et al. 2015a)

\* Priority of *Cognettia* or *Chamaedrillus* awaits ruling by the International Commission on Zoological Nomenclature, see <http://www.iczn.org>, Case 3689.

Two further options excluded informal categories and promoted the integration of the taxic diversity into the Linnaean system:

- Maintain the species rank for the morphospecies and use the subspecies rank for the cryptic species.
- Give full species rank for the cryptic species and a supraspecific rank (*e.g.*, subgenus), for the morphospecies.

All of these options have their pros and cons. Using the morphospecies with an alphanumerical code to represent the different cryptic lineages will let us continue using the morphospecies as taxonomical units in inventories, species lists etc., but there is the risk that the knowledge about the cryptic lineages is ignored, as they are not formally recognised and described taxa. Whereas if

cryptic lineages were formally described as species, identification of specimens to species based on morphology would become impossible, and specimens could only be identified to species groups or species “*sensu lato*”. However, the cryptic lineages would at least be recognised as species, and could thereby be included in counts of biodiversity and be seen as different units. A drawback of this option is the possible confusion caused by the same name used with two different meanings, either *sensu lato* (morphospecies) and *sensu stricto* (cryptic species).

Informal ranks and categories have the advantage of being flexible but the disadvantage of not being regulated, which may promote confusion in the meaning of names; they should therefore be considered only as an interim solution. The preferable full integration of the diversity into the

Linnaean system of names, however, faces other problems: The use of the subspecies category for cryptic species would contradict the traditional concept of subspecies as morphologically distinguishable populations of a species that replace each other geographically (Mallet 2007). In fact, cryptic species fulfil all criteria of being 'species', regardless which species concept is applied here (Bickford *et al.* 2007). On the other hand, a rank elevation of morphospecies to subgenus level would create considerable classificatory and nomenclatural instability: First, it would necessitate a complete reorganization of the classificatory architecture of a genus, because the subgeneric category cannot be applied selectively and hence affects all species of a genus. Second, each morphospecies elevated to subgeneric rank (the type species of the genus excepted) would need a new subgeneric name. The same problems would arise, *mutatis mutandis*, with the elevation of morphospecies to genus rank. To conclude, solutions to this classificatory problem are not straightforward and may differ from case to case.

Another important question is what evidence at the level of genetic markers is necessary to decide whether specimens belong to the same species or to different species, and whether a species is undescribed or not. Traditionally the characters used are morphological, both external and internal, but molecular data are becoming more and more common as the base for taxonomical decisions. Also ecological and physiological data, if available, can aid in the species delimitation process. The most commonly available genetic marker is the mitochondrial gene cytochrome C subunit I (COI) that is used as the barcode for animals (Hebert *et al.* 2003). However, if used alone, COI will often overestimate the number of species, and it should be used with caution and in combination with other data (Dasmahapatra *et al.* 2010). As a broad rule of thumb, in clitellates, if two clusters differ with more than 10% uncorrected genetic distance, *i.e.*, if more than 10% of the nucleotides differ between the two lineages, they are likely to belong to different species, and if they differ with less than 5% they are likely to belong to one species (Rougerie *et al.* 2009, Römbke *et al.* 2016, but see Giska *et al.* 2015,

Martinsson & Erséus 2017 for exceptions). More support is, however, needed in order to make a robust delimitation. Other commonly used markers are the nuclear Histone H3 (H3) and the ribosomal internally transcribed spacer region (ITS) consisting of ITS1, 5.8S and ITS2. H3 has been recommended as a secondary barcode for Enchytraeidae (Schmelz *et al.* 2014), and both H3 and ITS have good discriminatory power and will in most cases separate closely related species.

A third problem with cryptic species was discussed at the symposium: When cryptic species are detected within a morphospecies and are described formally according to nomenclatural rules (ICZN 1999), one lineage should bear the name of the morphospecies, notably the one to which the name-bearing type of the morphospecies belongs. However, finding the correct lineage may be difficult because type material is lost or in a state of preservation that does not allow extraction of DNA; both cases are very common in enchytraeids. A possible solution is to get fresh material from the type locality for sequencing, and in that way tie the name to a genetic lineage. However, in many cases the type localities are vague or missing; in these cases it should be sufficient to use material from the wider area where it can be supposed that the original material was collected. As a further complication, however, more than one cryptic lineage may be present at the type locality or in the wider area. To conclude, the choice of the name-bearing lineage is often a decision based on probabilities, and the task is to raise the probability-level as much as possible. For example, in case that small morphological differences exist between the candidate lineages, the one that fits the original description best should be chosen to bear the name of the old morphospecies. If uncertainty is too high, there remains the radical solution of dismissing the old name as "*nomen dubium*".

## RECOMMENDATIONS

Based on the consensus that both morphological characters and molecular markers are important for species-level taxonomy in enchytraeids, we recommend:

1. The erection of new species should include a good morphological description with illustrations of the important taxonomic characters and also a reference to molecular markers that are informative at the species level: at least one, but preferably two markers, one being mitochondrial (*e.g.*, COI), one nuclear (*e.g.*, ITS, H3).

2. DNA sequences should be generated from at least one of the type specimens, preferably the holotype or a syntype specimen, to link the sequence permanently with the name. However, some of the paratypes and other reference specimens should also be sequenced to avoid errors and to allow estimates of variability.

3. When species are erected based on only one set of data (*i.e.*, either morphological characters alone or DNA sequences alone), the retrieval of missing or additional data should be made possible by appropriate fixation and preservation of at least some specimens of the type series. This means, for DNA, at the present state of knowledge, the use of ethanol as preservation liquid at concentrations higher than 70%.

4. Sequencing is also recommended in specimens that form the basis of redescriptions and in those that are elected as neotypes in taxonomic revisions.

5. Of each sequenced specimen, the anterior part of the animal should be retained as a voucher and deposited in a public collection. (In enchytraeids, most of the taxonomically informative structures are located in the anterior body part.)

We understand that it will not always be possible to extract and sequence DNA from the specimens used as the basis for a new species description, *e.g.*, due to fixation methods, old age and bad storage or due to other factors, but whenever possible we recommend that DNA-sequence-data should be included in future descriptions of enchytraeids.

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