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ORIGINAL ARTICLE

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Cosmopolitan versus cryptic meiofaunal polychaete species: an approach to a molecular taxonomy

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Abstract Polychaete taxonomy is characterised by a high number of apparently cosmopolitan species. Detection of subtle but diagnostic ultrastructural differences and – in recent years – investigations at the molecular level have revealed that many of these "species" are actually complexes of morphologically identical or almost identical cryptic species. To disregard their existence would lead to an underestimation of global meiofauna diversity and undermine the value of many scientific studies. Therefore, we strongly recommend that they be given formal taxonomic recognition, beyond their published presentation as "operational taxonomic units", "types" or by alphabetic or numerical designators. Since there are neither generally accepted practical procedures nor any established consensus regarding the application of genetic data in taxonomy, we here provide examples of, and suggestions for, the treatment of meiofaunal species that are distinguished exclusively by molecular data, e.g. by genetic distance values, cluster analyses, diagnostic (= autapomorphic) DNA fragments from DNA fingerprinting procedures (RAPD) and/or DNA sequence differences (e.g. of ITS 2). Although no holotype material may be available because the molecular procedures require the preparation of entire specimens, practical taxonomic problems can be overcome and the recommendations of the Zoological Code of Nomenclature satisfied, by adopting the following procedures: (1) deposition of band-patterns of an individual obtained with the primers used to find diagnostic markers; (2) deposition of DNA in ethanol of one syntype individual; (3) deposition of fixed specimens (syntypes) from the locus typicus.

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Introduction

For well over 200 years, phenotypic distinctiveness has been the operational basis of polychaete taxonomy, in which light microscopy is generally used to detect species-specific morphological markers. As in many other marine taxa, this has resulted in a large number of species with a wide geographic range (Knowlton 1993) – apparently cosmopolitan species, found on the coasts of more than one continent and in more than one ocean. Now, however, at least since *Capitella capitata* has been found to be not a cosmopolitan but in fact a large complex of cryptic species, the status of other supposed cosmopolitans must also be called into question [see, for example, *Owenia fusiformis* (F.P. Patti, personal communication) and *Marphysa sanguinea* (P. Karageorgopoulos, personal communication)].

The cryptic *Capitella* species were first recognised at the enzyme level (Grassle and Grassle 1976), and subsequently they were found to differ also, or even exclusively, in their ultrastructure, developmental and/or physiological and ecological characteristics and requirements (Eckelbarger and Grassle 1987; Gamenick et al. 1998; Linke-Gamenick et al. 2000). As a result of these investigations, a considerable number of *Capitella* "types" or "species" were distinguished, although they were not formally named (Mendez et al. 2000). Strangely, the non-morphological markers, though distinct and mostly beyond dispute, were never introduced into the taxonomic process proper and the many cryptic *Capitella* species have not yet been accorded formal taxonomic recognition; they have remained nameless units in the sense of the International Commission for Zoological Nomenclature (ICZN). A recent discussion on the Internet has shown that this situation is uncomfortable for many. There is great need for a taxonomic practice that takes account of non-phenotypic, particularly molecular, mark-

ers. In due course, certain taxonomic decisions may be based exclusively on the latter. To ignore these would degrade the quality of taxonomy and considerably underestimate the assessment of biological diversity – with high relevance for practical applications and environmental policies.

So why has molecular taxonomy been used so rarely, and with such reluctance by polychaetologists? There are a number of reasons, of which five are considered here. In the following we try to take issue with these arguments and problems from our own experience and from our own point of view.

Results and discussion

1. Probably the most common reason for rejecting taxonomic decisions based on molecular characters is that the necessary methods are not accessible to many taxonomists and therefore species identification cannot be generally reproduced in the traditional way. This is an understandable, but nevertheless unacceptable, argument, which should ultimately not enter into the discussion. For example, no one would accept inadequate taxonomic treatment of small polychaetes with delicate chaetation on the basis of the lack of a high quality microscope, or, where distinguishing characters can be seen only by scanning electron microscopy, caused by the lack of appropriate laboratory facilities.
2. Another reason may be that polychaetes in general are of slight economic interest, thus removing one of the prime driving forces behind the genetic characterisation of organisms. If taxa are of great medical, veterinary or agricultural importance (see, for example, the high number of molecular diversity studies in ticks and mites: Navajas and Fenton 2000), the impetus for employing molecular techniques is obviously enhanced.
3. Little information seems to be available, but considerable uncertainty propagated among polychaete taxonomists, on whether molecular markers yield results that are in agreement with those obtained with traditional phenotypic markers. This may be due to the fact that very few of the many molecular investigations carried out over the last decade have been concerned with polychaetes. We therefore present here an example of our own research, showing how species that are phenotypically difficult to separate can be easily and congruently distinguished by a DNA fingerprinting technique.

Interstitial meiofaunal polychaetes are excellent examples of the so-called meiofauna paradox (Giere 1993): that species can exhibit world-wide distribution patterns despite lacking pelagic propagation stages and active swimming modes. As a result of long-term research in these animals, we find much evidence that the majority of these cosmopolitan species are actually pairs or complexes of cryptic species (Schmidt and Westheide 1999, 2000).

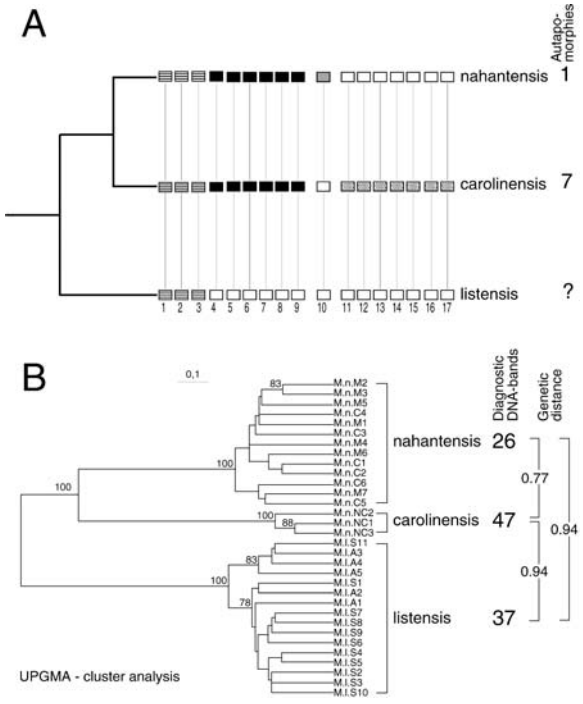

Fig. 1 Phylogenetic relationship within the Micropthalmus–listensis species complex based on A phenotypic characters and B genetic distance values (RAPD). In A, synapomorphic and autapomorphic characters for the three species are indicated by differently marked boxes (after Westheide and Rieger 1987). The phenogram in B is generated by the cluster analysis (UPGMA), bootstrap values of crucial branching points indicated at nodes. Large numerals indicate numbers of diagnostic DNA bands for each species, small numerals show genetic distance values (Nei and Li 1979)

Microphthalmus listensis Westheide, 1967, described and recorded from central European sandy beaches, was first believed to be an amphiatlantic species (see Westheide 1967, 1977; Rieger and Ruppert 1978), also occurring on the western side of the Atlantic Ocean, in North American beaches. This first view, based on fixed material of immature specimens, could not be corroborated when populations from Sylt (North Sea), Emerald Island (North Carolina), Manomet Beach (Massachusetts) and Reid State Park (Maine) were thoroughly compared using a broad spectrum of methodologies, especially electron microscopy (SEM and TEM). These methods uncovered distinct differences between the specimens from the sites in Europe, North Carolina and Massachusetts/Maine (Pietsch and Westheide 1985; Westheide and Rieger 1987; Specht and Westheide 1988), which respectively led to the establishment of three species: *M. listensis* Westheide, 1967, *M. nahantensis* Westheide and Rieger, 1987, and *M. carolinensis* Westheide and Rieger, 1987. Two of these could be characterised by autapomorphies; one, the European *M. listensis*, so far comprises only plesiomorphic characters (Fig. 1A), unless one of their delicate chaetal details (Specht and Westheide 1988) is considered to be autapomorphic. The reconstructed phylogenetic relationship between

these three cryptic species (Fig. 1A) demonstrated a closer relationship between the two American species, supporting a view that they had evolved after their stem species separated from the European *M. listensis*. This was revealed by six synapomorphies for *M. carolinensis* and *M. nahantensis*.

This result was later corroborated in full using the RAPD-PCR (Random(ly) Amplified Polymorphic DNA–Polymerase Chain Reaction) fingerprinting analysis (for details of the methodology, see, for example, Schmidt and Westheide 2000) for specimens from the three sampling sites. With 14 different primers, 335 different DNA fragments were detected and regarded as individual characters, these were used to calculate genetic distances between the species. There is a separation into three units, which are congruent with the phenotypically distinguished species (Fig. 1B). This is evident from three criteria: (1) Genetic distance values according to Nei and Li (1979) are relatively large between specimens from the three sites, although standard interspecific or intraspecific genetic distance values do not exist, not even not within a given taxon. (2) Cluster analyses of pairwise distance values reveal three clusters, i.e. three different gene pools, each containing all specimens investigated from the individual sites. Analyses performed with five different algorithms (UPGMA, WPGMA, Neighbour Joining, Single Linkage, Complete Linkage) show identical tree topologies with bootstrap values at the crucial branch points of 100%. Although numerical algorithms were used, the tree topology is congruent with that of the phylogenetic tree based on morphological data. (3) The most decisive criterion is that each of the three populations is distinguished by a large number of monomorphic diagnostic – i.e. species-specific – bands: *M. listensis* 37, *M. nahantensis* 26, *M. carolinensis* 47. Since markers of this type can be considered as autapomorphies, the RAPD analyses provide an even higher degree of validity for the species status of the three populations than the morphologically based analysis.

Complete congruence between this kind of genotypically based species discrimination and phenotypically based descriptions, also revealed in a number of other taxa by this specific technique (e.g. Schmidt and Westheide 1997/98; André et al. 1999; Lehmann et al. 2000), justifies, in our opinion, the splitting of other species complexes and description of cryptic species-taxa as new to science merely on the basis of RAPD fingerprinting data, even when no morphological discriminating characters are available.

4. There are still serious, even though unjustified, doubts on whether taxa recognised by molecular markers conform with the Code of the International Commission on Zoological Nomenclature. The latest, fourth, edition of the Code (ICZN 1999) acknowledges only morphologically distinguished taxa. However, problems arising from this situation are, in our opinion, concerned mainly with the deposition of type specimens. This can easily be overcome, as demonstrated by the description of the syllid species *Petitia occulta* Westheide and Hass-Cordes, 2001 from the Seychellan Island Mahé (Westheide and Hass-Cordes 2001). The species from Mahé was identified and separated from the cosmopolitan complex (Fig. 2) of the interstitial *Petitia amphophthalma* Siewing, 1956 (Westheide 1977; von Soosten et al. 1998) as a distinct species taxon by (1) the relatively great genetic distance values with respect to other geographically distant populations; (2) complete consistency for phenograms produced by differently generated cluster analyses of the genetic distance values – all revealing a separate clade (bootstrap values 100%) for the Mahé animals; and (3) eight (seven) diagnostic DNA fragments (Fig. 3).

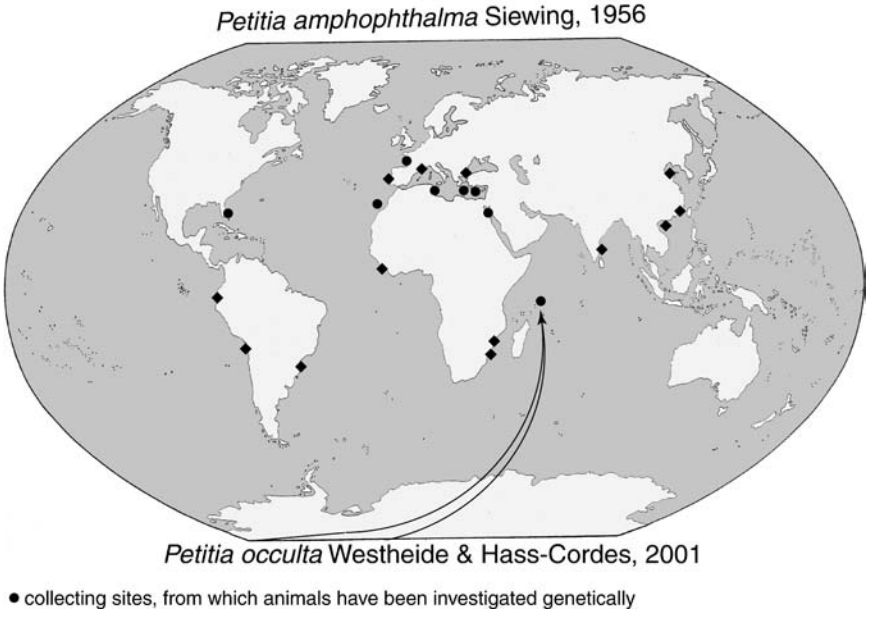

Fig. 2 Worldwide records of the two Petitia species

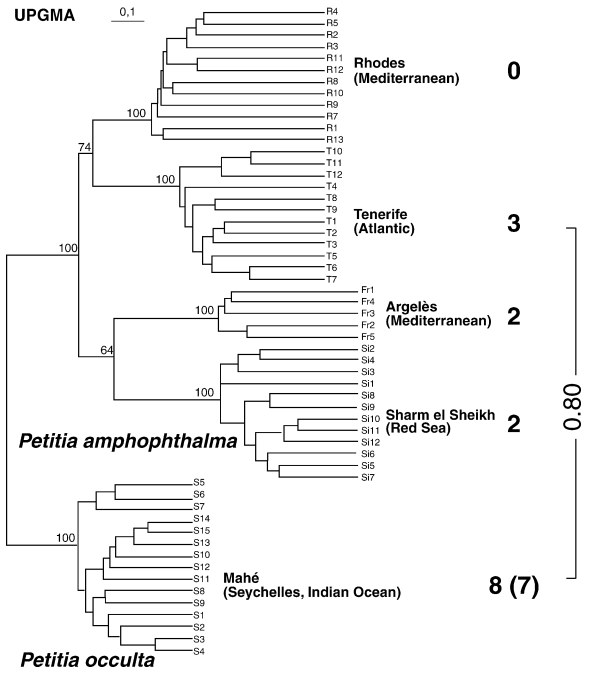

Fig. 3 Hierarchial cluster analysis (UPGMA) of distance values based on RAPD investigations. Bold numbers indicate diagnostic DNA markers for each site. 0.80 is the genetic distance value (Nei and Li 1979) between Petitia occulta and P. amphophthalma. Specimens of Petitia occulta from the Seychellan island Mahé (S) and specimens of P. amphophthalma from the Rea Sea (Si), the Canarian island Tenerife (T) and two sites of the Mediterranean: Rhodes, Greece (R) and Argelès, France (Fr). Bootstrap values of crucial branching points indicated at nodes

Since the RAPD procedure requires the preparation of the total DNA of each tiny specimen (length 1.1 mm) investigated, no holotype could be deposited. In compensation, the band-pattern of an individual with six different primers, in which the eight diagnostic markers are indicated (Fig. 3), has been published (Westheide and Hass-Cordes 2001, p 103). Isolated DNA in ethanol of one specimen from Mahé was deposited in the Senckenberg Museum, Frankfurt, so that the new species can be compared and identified on the molecular level at any time. In addition, a series of complete fixed specimens from the locality (syntypes) was deposited and is accessible for further studies of the phenotype.

5. The most evident uncertainty concerns the question of which methods should be used, whether different methods may give different results, or whether results may differ in reliability. We selected the RAPD-PCR fingerprinting method (Williams et al. 1990), because it has been shown to have a high power of resolution at population and species level analyses – that is, between relatively closely related species. Another major advantage of the method is that it requires only a very small amount of genomic template DNA, so it is possible to work with single specimens of even very small meiofaunal species (Schmidt and Westheide 1999; Leutbecher 2000); and no prior knowledge about sequences of the DNA probes or the PCR target is required (Adamson et al. 1993). An unlimited number of markers can be produced; the analysis is relatively quick, simple and inexpensive, which makes it especially appropriate for taxonomic purposes. An obvious drawback for intraspecific studies is that RAPD markers are dominant, not allowing heterozygotes to be detected (without additional crossbreeding experiments). Though RAPD analysis is often said to be sensitive to experimental conditions (Black 1993; Jones et al. 1998), we have never had any problem in reproducing our RAPD results, as long as we followed the protocol meticulously and used a polymerase obtained from the same manufacturer (see also Penner et al. 1993; van Belkum et al. 1995; Inglis et al. 1996). The other frequently raised criticism is that RAPD fragments of the same length may not represent the same sequence, i.e. are amplified from non-homologous loci; however, this has been shown to be unlikely enough not to represent an important problem (Schierwater 1995). Regardless, there is no question that several other available molecular methods can achieve the same results, or better. Often microsatellites are considered to be the marker of choice, especially for population genetics (e.g., Delaye et al. 1998; Schlötterer and Pemberton 1998; Huang et al. 2000), although they are expensive and sometimes difficult to isolate. Also ITS regions (internal transcribed spacers of the nuclear rDNA) are useful for distinguishing between closely related species, as they evolve more rapidly than the coding regions (Hillis and Dixon 1991). In the case of *Petitia occulta* we confirmed the RAPD results in showing that ITS 2 sequences of specimens from the Seychelles were very different from those of the Mediterranean (Westheide and Hass-Cordes 2001); this is part of a still ongoing investigation of the *P. amphophthalma* complex. Moritz et al. (2000) use ITS-RFLP band patterns for identification of economically important Thysanoptera species.

Conclusions

Cryptic – or sibling – species pose neither operational nor theoretical problems for taxonomy. A now large number of investigations has convincingly demonstrated that even complexes of morphologically identical species can be easily separated and their cryptic species identified with a high degree of validity by methods entirely based on molecular markers. The RAPD procedure is particularly advantageous when animals of small dimensions are concerned. Meiofaunal polychaetes, for example, have body lengths of about 1 mm and body widths of around 100 µm or so, yet even single specimens can yield enough template DNA. The method is appropriate for taxonomic practice also because it is quick, relatively inexpensive, and does not need sophisticated laboratory equipment.

It should be emphasised, however, that not all widely distributed meiofauna species will be cleared up as com-

plexes of cryptic species: individuals of the polychaete *Hesionides arenaria* Friedrich, 1937, from eight European sites between Skagerak and the Mediterranean (including the Canary Islands), and the US Pacific coast did not form separate genetic clades in a RAPD investigation (Schmidt and Westheide 2000). The same is true for the polychaete *Ctenodrilus serratus* (O. Schmidt, 1857) from European and American coastal sites of the Atlantic; this could be corroborated by almost identical ITS 2 sequences (Westheide et al. 2002).

DNA analyses such as the RAPD-PCR provide character states that can be treated in just the same way as morphological ones are in traditionally based descriptions. They provide DNA fragments that are specific for taxonomic units and can be used to discriminate between and identify species as specifically formed chaetae would do, for instance. Any idea that inclusion of genetic data in a species description would contradict one of the competing theoretical species concepts rests on the widespread misunderstanding that an operational concept of taxonomy must simultaneously be its theoretical concept. In fact, the practising taxonomist always thinks and works in terms of a hierarchical system (see also Mayden 1997) – with, for instance, morphological or genetical data as the operational basis and the biological or evolutionary species concept as a theoretical roof. That is, from the constantly different markers used in practice to describe their species, taxonomists draw conclusions about the species' reproductive isolation – and these markers may be morphological as well as molecular markers. Accordingly, the rationale in erecting species on the basis of molecular markers is more or less the same as in the case of phenotypically based species, namely the decision whether differences that are found on the two levels are large enough to establish their carriers as different species or not. How many chaetae should be different or how many diagnostic DNA fragments should be present to decide in favour of a different species? In the case of *Petitia occulta*, Westheide and Hass-Cordes (2001) decided to regard the differences of the Mahé animals as sufficiently great to separate them from the nominate species. However, animals such as those from the Red Sea, with only two diagnostic markers (Fig. 3), still make us hesitate to identify them as a separate species; instead, we regard them as a subpopulation of the cosmopolitan *Petitia amphophthalma*. Thus, molecular markers help us to take a much closer look at species and their distinctions, but they do not automatically make taxonomic decisions easier.

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