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Distinguishing cryptic species in Antarctic Asellota (Crustacea: Isopoda) - a preliminary study of mitochondrial DNA in *Acanthaspidia drygalskii*

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Abstract: Acanthaspid isopods are well known from the deep sea regions of all oceans. Many species have been found also on the continental shelf around Antarctica. Phylogenetic relationships within the Acanthaspidiidae and the genetic differentiation of populations are poorly understood. In this study we analysed 16S rRNA gene sequences of 36 specimens of Acanthaspidiidae, including 17 specimens of *Acanthaspidia drygalskii* Vanhöffen, 1914. This species is known from several locations along the Antarctic shelf, supporting the idea of a circum-Antarctic distribution of this taxon. Our molecular data support the monophyly of all six species analysed, but there is only limited evidence for the interspecific relationships between the species. However, we were able to identify three distinct groups of haplotypes within *Acanthaspidia drygaskii*. Our results indicate the evidence of cryptic, reproductively isolated species. Further data are needed to understand mechanisms underlying speciation in deep sea isopods.

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Key words: Acanthaspidiidae, Antarctica, haplotypes, phylogeny, 16S rDNA, Weddell Sea

Introduction

Isopods belong to the most conspicuous crustaceans in Antarctic waters (Brandt 1991a, 1992). Species of the families Serolidae, Chaetiliidae and Antarcturidae are of large size, many of them over 5 cm long. These families are abundant and are typical components of the marine benthic communities of the Antarctic shelf (e.g. Wägele 1986, Brandt 1990). However, species numbers are much higher in asellote isopods. Asellota are easily overlooked during sampling as most species are less than 10 mm long. They represent one of the most numerous and important elements of the benthos in all oceans, especially in the deep sea (e.g. Wolff 1962, Hessler & Sanders 1967, Hessler *et al*. 1979), which they have colonized through several lineages (Wägele 1989, Raupach 2004, Raupach *et al*. 2004). Within Antarctic waters, many blind species of typical deep sea families (e.g. Munnopsididae, Desmosomatidae, Nannoniscidae or Ischnomesidae) can also be found in shallow waters, while on the other hand some eye-bearing shelf taxa (e.g. Stenetriidae, Paramunnidae or Munnidae) invaded deep waters (Brandt 1991a). These emergence and submergence events are facilitated by the isothermic water column, allowing temperature sensitive animals to migrate up or down the Antarctic shelf.

A well known asellote taxon occurring in Antarctic waters is the family Acanthaspidiidae. The distribution is concentrated in the Southern Hemisphere, specifically in the Southern Ocean. Only five species have been reported from the Northern Hemisphere. The Acanthaspidiidae, revized by Brandt (1991b), include the three genera *Mexicope* Hooker, 1985, *Ianthopsis* Beddard, 1886, and *Acanthaspidia* Stebbing, 1893 (Just 2001). While *Mexicope* is currently recorded from depths of less than 50 m, *Ianthopsis* and *Acanthaspidia* range from the mid shelf to abyssal depths (Just 2001). Most species of the Acanthaspidiidae belong to the genus *Acanthaspidia*. Currently 19 different species are known. All have no eyes, and most of them are typically found in depths below 1000 m (Brandt 1991a, Just 2001). Phylogenetic relationships of the Acanthaspidiidae, especially within genera, are almost unknown, because many members of this family have so far been poorly described or illustrated. Morphological studies suggest that the genus *Ianthopsis* contains the most primitive members of the Acanthaspidiidae (Brandt 1991a, Just 2001).

One of the best known species is *Acanthaspidia drygalskii*, first described by Vanhöffen in 1914 and redescribed by Brandt (1991b). This species has its origin in the deep sea regions of the Southern Ocean and represents one of the most widespread acanthaspidiid isopods on the Antarctic shelf, possibly with a circum-Antarctic distribution (Brandt 1991a). However, several specimens assigned to the same species were also found on the Australian shelf (Brandt 1994). Interestingly, *Acanthaspidia*

Table I. Specimens examined, individual codes, Gen Bank accession numbers for gene sequences, haplotype codes and sources of material of this study.

Genus/ Species	Ind. code	Accession no.	Haplotype	Locality
Ianthopsis multispinosa	BAC10	AY691340		ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
Vanhöffen, 1914	BAC11	AY691341		ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC12	AY691342		ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
Ianthopsis ruseri	BAC1	AY691334		ANT XXI-2, St. 19-1: 54°30'06"S/3°14'13"E–54°30'01"S/3°13'97"E; 247–259 m
Vanhöffen, 1914	BAC5	AY691335		ANT XXI-2, St. 90-1: 70°56'14"S/10°31'70"W–70°55'92"S/10°32'37"E; 274–288 m
	BAC37	AY691336		ANT XXI-2, St. 283-1: 72°32'39"S/17°59'34"W–72°32'45"S/17°59'37"E; 554–542 m
	BAC51	AY691337		ANT XXI-2, St. 297-1: 72°48'50"S/19°31'60"W–72°48'65"S/19°31'85"E; 668–630 m
	BAC52	AY691338		ANT XXI-2, St. 297-1: 72°48'50"S/19°31'60"W–72°48'65"S/19°31'85"E; 668–630 m
	BAC54	AY691339		ANT XXI-2, St. 326-1: 72°51'43"S/19°38'67"W–72°51'33"S/19°38'44"E; 616–606 m
Acanthaspidia bifurcatoides	AC4	AY691345		ANT XIX-3, St. 42-2: 59°40'29"S/57°35'43"W–59°40'42"S/57°35'27"W; 3683–3680 m
Vassina & Kussakin, 1982	AC1	AY691343		ANT XIX-3, St. 46-7: 60°38'35"S/53°57'36"W–60°38'12"S/53°57'49"W; 2894–2893 m
	AC3	AY691344		ANT XIX-3, St. 46-7: 60°38'35"S/53°57'36"W–60°38'12"S/53°57'49"W; 2894–2893 m
	AC6	AY691346		ANT XIX-4, St. 132-2: 65°17'75"S/53°22'82"W–65°17'56"S/53°22'83"W; 2086–2086 m
Acanthaspidia drygalskii	AC10	AY691353	B1	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
Vanhöffen, 1914	AC12	AY691354	B2	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC14	AY691356	B1	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC15	AY691355	A1	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC17	AY691357	B3	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC19	AY691358	A2	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC22	AY691359	B3	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC24	AY691360	B1	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC27	AY691361	B4	ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	BAC17	AY691362	C1	ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC18	AY691363	C1	ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC19	AY691364	C1	ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC20	AY691365	C1	ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC21	AY691366	C2	ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC24	AY691367	C2	ANT XXI-2, St. 232-1: 71°18'61"S/13°56'12"W–71°18'73"S/13°56'57"E; 910–900 m
	BAC45	AY691368	C1	ANT XXI-2, St. 297-1: 72°48'50"S/19°31'60"W–72°48'65"S/19°31'85"E; 668–630 m
	BAC46	AY691369	C1	ANT XXI-2, St. 297-1: 72°48'50"S/19°31'60"W–72°48'65"S/19°31'85"E; 668–630 m
Acanthaspidia pleuronotus	AC7	AY691347		ANT XIX-4, St. 134-4: 65°19'20"S/48°03'81"W–65°19'15"S/48°03'34"W; 4066–4067 m
(Menzies & Schultz, 1967)	AC9	AY691348		ANT XIX-4, St. 134-4: 65°19'20"S/48°03'81"W–65°19'15"S/48°03'34"W; 4066–4067 m
Acanthaspidia sp.	AC13	AY691349		ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC18	AY691350		ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC21	AY691351		ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m
	AC28	AY691352		ANT XIX-4, St. 133-3: 65°20'15"S/54°14'35"W–65°20'06"S/54°14'51"W; 1122–1119 m

drygalskii does not possess the typical morphological apomorphies, e.g. long, caudally inserted uropods with long sympods, of the genus *Acanthaspidia* (Brandt 1991b).

Recent molecular studies on 16S rDNA of two large and widespread Antarctic isopods, *Ceratoserolis trilobitoides* (Eights, 1833) (Serolidae) and *Glyptonotus antarcticus* Eights, 1853 (Chaetilidae), indicate that both "species" consist of several cryptic species (Held 2003, Held & Wägele 2005). Is there any evidence that similar patterns exist within other Antarctic isopods? In addition, can 16S rDNA sequences be used to reconstruct the phylogeny within the Acanthaspidiidae? To answer these questions we analysed the 16S rRNA gene sequences of 36 specimens of the Acanthaspidiidae, including 17 specimens of *Acanthaspidia drygalskii*.

Materials and methods

Specimens and DNA isolation

The studied taxa and their sample localities are listed in Table I. All analysed specimens were collected in the Weddell Sea during the expeditions ANDEEP I and II (ANT XIX/3+4) in 2002 and ANT XXI/2 in 2003/2004. The animals were caught using an epibenthic sledge or an Agassiz trawl employed by RV *Polarstern* (for details see Arntz & Brey 2005, Fütterer *et al*. 2003). A fast fixation with precooled ethanol (0°C) was essential because isopods display highly active nucleases, which digest DNA very quickly (Dreyer & Wägele 2001, 2002). The DNA was extracted on board from several dissected legs of the specimens, using the QIAmp© Tissue Kit (Qiagen GmbH) and following the manufacturer's extraction protocol. In addition, the 16S rDNA sequence of the joeropsidid asellote *Joeropsis dubia* Menzies, 1951 (AF260860) was obtained

from GenBank for outgroup comparison.

Polymerase chain reaction

The polymerase chain reaction (PCR, Saiki *et al*. 1988) was used to amplify a homologous region of the 16S mitochondrial rRNA gene, ranging 509 to 518 base pairs (bp) in 36 specimens of the Acanthaspidiidae. Amplifications were performed in 25 µl reactions containing 2.5 µl 10x Qiagen PCR buffer, 2.5 µl dNTPs (2 mmol μl^{-1}), 0.3 µl of each primer (forward primer 5'-CGC CTG TTT ATC AAA AAC AT-3', reverse primer 5'-CCG GTC TGA ACT CAG ATC ACG-3', both 50 pmol μl^{-1} (Palumbi *et al*. 1991)), 1-2 µl of DNA template, 5 µl Q-Solution©, 0.2 µl Qiagen Taq (5 U μl^{-1}), filled up to 25 µl with sterile H_2O, on a Progene Thermocycler (Techne Ltd.). The temperature profile of the PCR consisted of an initial denaturation of 94°C (5 min), followed by 38 cycles of 94°C (45 s), 44°C (45 s) and 72°C (1.2 min). Three µl of amplified product were controlled by electrophoresis in a 1% agarose gel with ethidium bromide using DNA size standards, the remaining PCR product was purified with the QIAquick© PCR Purification Kit (Qiagen GmbH).

DNA sequencing

Chain-termination cycle sequencing (Sanger *et al*. 1977) was performed using a Thermo-Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech) on a Primus96^{plus} Thermocycler (MWG-Biotech AG). Sequencing-primers used were 5'-CGC CTG TTT ATC AAA AAC AT-3' and 5'-CCG GTC TGA ACT CAG ATC ACG-3' (Palumbi *et al*. 1991). Cycle sequencing conditions were: 2 min at 94°C (initial denaturation), followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 56 to 58°C for 25 s, and

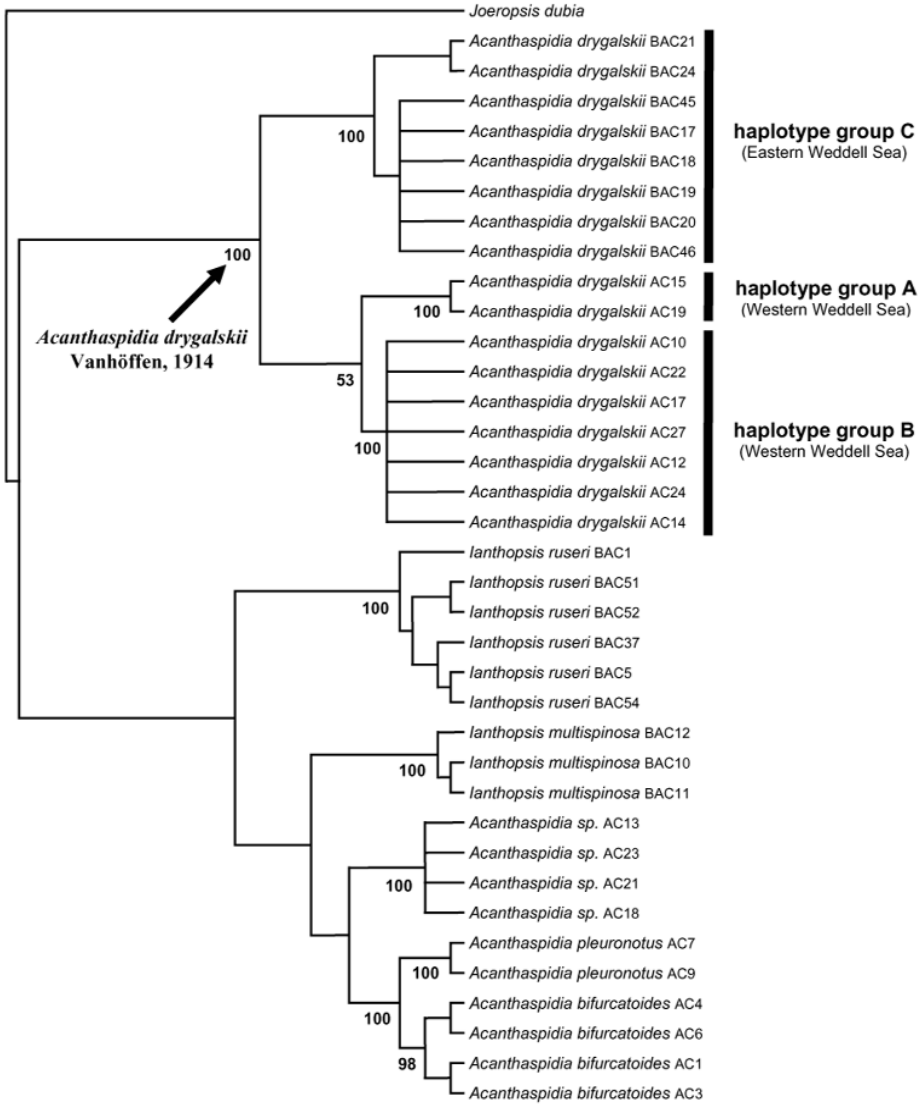

Fig. 1. Strict consensus tree (n = 13 trees) of a maximum parsimony analysis based on 536 nucleotides from the 16S rRNA gene (TL = 602, CI = 0.6944, HI = 3056, RI = 0.9193, RC = 0.6383). Numbers on branches are bootstrap values of 10 000 replicates (only values above 50% are shown).

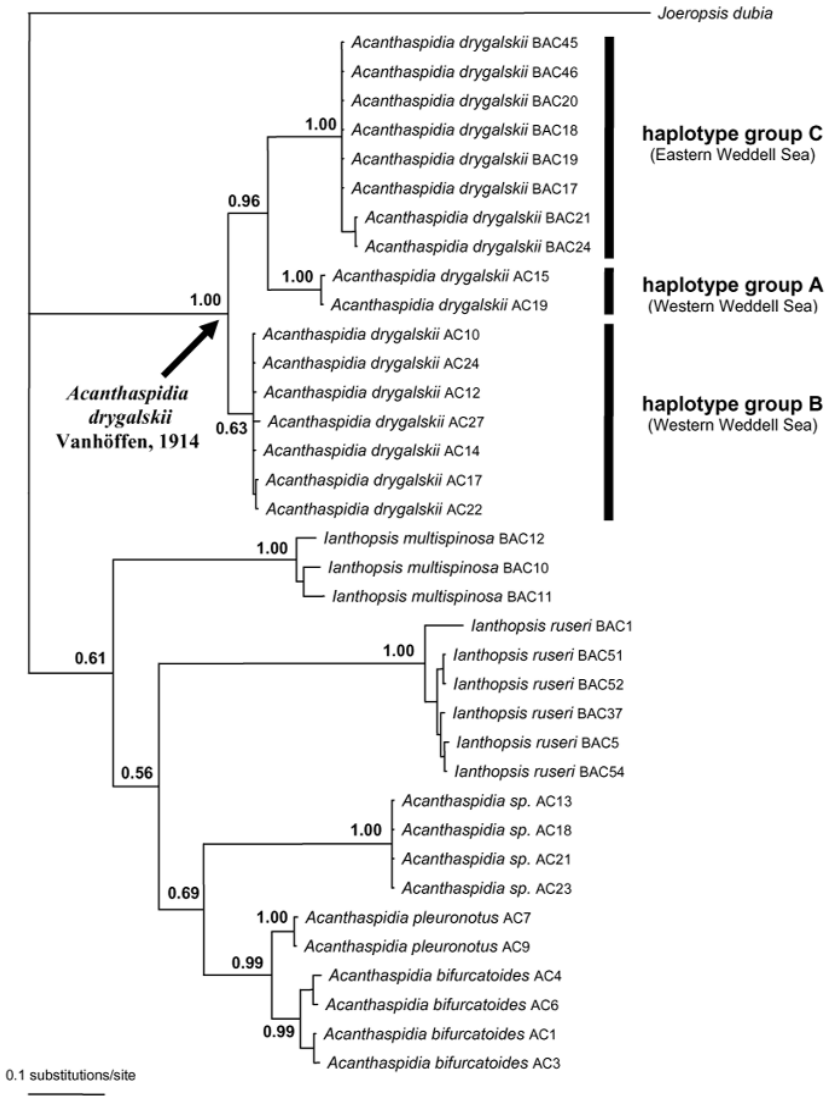

Fig. 2. Bayesian 50% majority rule consensus tree. Model choice based on a hierachical LRT: TVM model with gamma distributed rates (alpha = 0.4192) and no invariant positions (see text). Numbers at the nodes represent posterior probabilities; values below 0.50 are not shown.

extending at 70°C for 35 s. A LI-COR 4000 (LI-COR Inc.) was used for automated sequencing. Gels were proof-read using the image analysis software of the automated sequencer. Double-stranded sequences were assembled with the program AlignIR v1.2. All new 16S rDNA sequences can be retrieved from GenBank (Table I).

Sequence alignment and phylogenetic analyses of sequence data

All 36 partial 16S rDNA sequences were aligned using Clustal X (Thompson *et al*. 1997) with default settings for gap weight and length penalties, generating an alignment of 536 base pairs (bp) with 241 parsimony-informative characters. The alignment was tested for nucleotide bias using a chi-square test of base composition homogeneity across taxa implemented in PAUP*4.0b10 (Swofford 2002). We used PAUP*4.0b10 for performing maximum parsimony analyses. To assess statistical support for hypothesized clades, 10 000 bootstrap replicates were calculated. Node support was also assessed by posterior proabilities with a Bayesian analysis using the program MrBayes v3.0B4 (Huelsenbeck & Ronquist 2001). Markov chain Monte Carlo analyses were run for 1000 000 generations using random starting trees. Trees were sampled every 100 cycles, yielding 9000 samples of the Markov chain after a "burn in" of 100 000 generations. An appropriate likelihood model was determined using the program MODELTEST version 3.6 (Posada & Crandall 1998). This model of nucleotide substitution was used as parameter set for the Bayesian analyses and to calculate maximum-likelihood estimates of pairwise genetic distances in PAUP*4.0b10.

Results

The sequenced fragment of the mitochondrial lsu rRNA gene is AT rich, as it is typically known from this gene (e.g.

Table II. Maximum-likelihood estimates of pairwise genetic distances of haplotypes of mitochondrial 16S rDNA sequences (lower triangle). For more details about the LRT and used model see text. Upper triangle: number of observed genetic distances (transitions versus transversions). *n* = number of specimens.

	A1 *n* = 1	A2 *n* = 1	B1 *n* = 3	B2 *n* = 1	B3 *n* = 2	B4 *n* = 1	C1 *n* = 6	C2 *n* = 2
A1 *n* = 1	-	0/1	31/15	31/15	30/15	32/15	34/21	37/23
A2 *n* = 1	0.0020	-	31/14	31/14	30/14	32/14	34/20	37/22
B1 *n* = 3	0.1186	0.1144	-	1/0	1/0	4/0	32/20	37/22
B2 *n* = 1	0.1198	0.1155	0.0019	-	2/0	3/0	32/20	37/22
B3 *n* = 2	0.1157	0.1115	0.0019	0.0039	-	3/0	33/20	36/22
B4 *n* = 1	0.1230	0.1187	0.0078	0.0058	0.0058	-	35/20	38/22
C1 *n* = 6	0.1450	0.1403	0.1385	0.1394	0.1418	0.1494	-	5/2
C2 *n* = 2	0.1641	0.1590	0.1642	0.1652	0.1609	0.1689	0.0139	-

France & Kocher 1996, Held 2000a, Wetzer 2001, Wetzer *et al*. 2003). Average base frequencies are pi(A) = 36%, pi(C) = 13%, pi(G) = 18% and pi(T) = 33%. However, there are no significant differences in base composition (Chi-square test: df = 108, P = 0.99). The likelihood-ratio test suggests the use of the TVM model with gamma-distributed rates and no invariant sites for the dataset (alpha = 0.4192, $R_{(AC)}$ = 0.56, $R_{(AG)}$ = 6.77, $R_{(AT)}$ = 1.57, $R_{(CG)}$ = 0.26, $R_{(CT)}$ = 6.77, $R_{(GT)}$ = 1.00).

The tree resulting from the maximum parsimony analysis is provided in Fig. 1 whereas Fig. 2 shows the consensus tree recovered using the Bayesian approach. Both topologies strongly support the monophyly of all analysed acanthaspiid groups classified at species level according to morphology (in parenthesis: maximum parsimony bootstrap support and Bayesian support): *Ianthopsis ruseri* (100/1.00), *I. multispinosa* (100/1.00), *Acanthaspidia bifurcatoides* (98/0.99), *A. drygalskii* (100/1.00), *A. pleuronotus* (100/1.00) and *A.* sp. (100/1.00). In contrast to this, relationships between the different species are only poorly supported, and there is no evidence for monophyly at genus level for *Ianthopsis* and *Acanthaspidia*. However, two relationships are found in all of our analyses: *Acanthaspidia drygalskii* represents the sister taxon to all other Acanthaspidiidae studied herein, and *Acanthaspidia bifurcatoides* is the sister group of *A. pleuronotus* (100/0.99).

In all our analyses the molecular data reveal three distinct groups within *Acanthaspidia drygalskii*, well supported by bootstrap values and posterior probabilities. Pairwise sequence comparisons show that divergence is low within (less than 0.0139) but high between the three distinct groups (between 0.1115 and 0.1689), and pairwise haplotype differences range from 1 to 60 observed substitutions over the length of the alignment (Table II). However, there are no intermediate values between the three haplotype groups. Within these three groups, eight unique mitochondrial haplotypes are identified: haplotype group A includes two, group B four and group C also two unique haplotypes (Table II). The amount of genetic diversity between these three groups is much higher than the genetic distances between other closely related species, which is about 0.0645–0.0722 for *Acanthaspidia pleuronotus* and

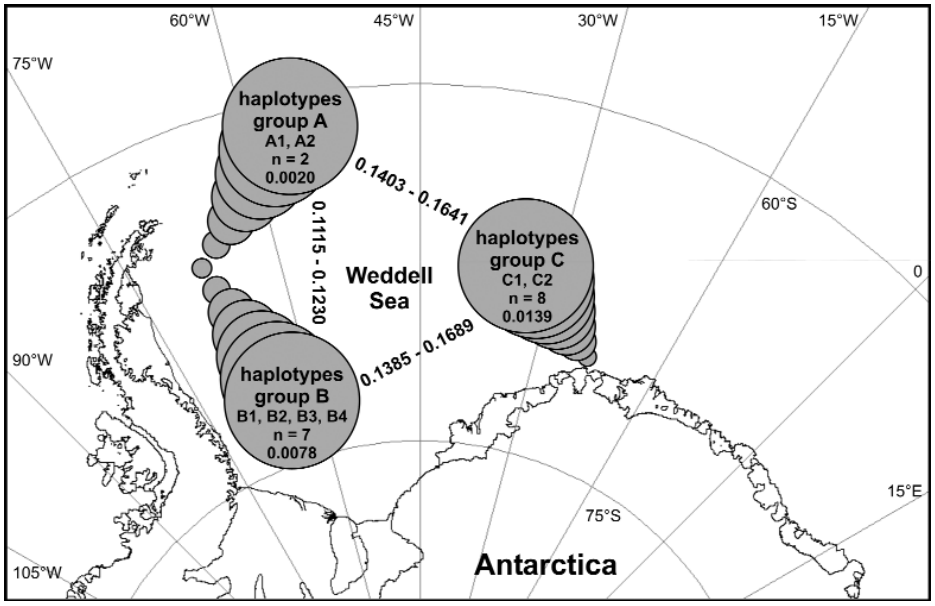

Fig. 3. Pairwise genetic distances (TVM + gamma) within and between three groups (A, B, and C) of eight unique 16S mitochondrial haplotypes (A1, A2, B1–B4, C1, C2) of *Acanthaspidia drygalskii* (*n* = number of specimens).

A. bifurcatoides (not shown). Haplotype groups A and B appear in the western Weddell Sea in sympatry while haplotype group C is only found in the eastern Weddell Sea (Fig. 3).

Discussion

Mitochondrial 16S rDNA sequences have been used before for the identification of cryptic species (e.g. Held 2003, Held & Wägele 2005). Held (2000b, 2003) developed a useful set of criteria to provide evidence for cryptic speciation:

a) bimodal distribution of pairwise distance values without intermediates,

b) differentation at a level known for this gene from undisputed species pairs closely related to the studied species, and

c) persistence of high levels of genetic differentation in sympatry.

The analysed sequences of *Acanthaspidia drygalskii* fulfill all three criteria, but only haplotype group A and B appear in sympatry. However, the observed genetic distances (between 0.1115 and 0.1689) are much higher than values of inter-specific differentiation, which are known from other isopods and crustaceans (e.g. France & Kocher 1996, Held & Wägele 2005, Schubart *et al*. 2000). Since the number of available samples is too small, it is not possible to analyse differences in depth distribution that might indicate an ecological specialization of populations. Nevertheless, the molecular data reveal a high genetic variability within the "species complex" *A. drygalskii*.

Of course, the same phenomena would occur in species with a mitochondrial polymorphism that might be caused by persistent ancestral polymorphisms, by introgression, or by isolation (e.g. Theimer & Keim 1994, Mason *et al*. 1995, Bernatchez *et al*. 1996, Gießler *et al*. 1999, Wares 2001). The fact that two distant localities have different haplotype groups indicates that there is no detectable individual exchange. The high genetic distances suggest that this situation is stable over long periods of time, and where individuals from other sites migrate into a local population, the newly arrived genotype does not spread locally. This would be the result of reproductive isolation of insufficient adaptation to local environmental conditions. Additional morphological studies and molecular analyses (e.g. nuclear genes) will be made in future to investigate the occurrence of cryptic species.

Our study raises important questions about asellote diversity, especially in Antarctic and deep sea taxa. In fact, cryptic speciation may be a common phenomenon within marine Asellota. For example, some morphospecies seem to have a wide distribution (e.g. *Acanthocope galathea*, found in the Caribbean region and in the Angola Basin; Schmid *et al*. 2002) and it is not clear if there are cryptic species detectable using molecular methods.

Cryptic species are well-known from terrestrial habitats (Feldman & Spicer 2002, Bond *et al*. 2003, Hebert *et al*. 2004, Olson *et al*. 2004), and numerous examples for marine and freshwater habitats have been described (e.g. France & Kocher 1996, Bucklin *et al*. 1998, de Vargas *et al*. 1999, Etter *et al*. 1999, Hoarau & Borsa 2000, Müller 2000, Quattro *et al*. 2001), but studies of cryptic Antarctic organisms are few (Beaumont & Wei 1991, Hoelzel *et al*. 1993, Pastene *et al*. 1993, Eastman & DeVries 1997, Gaffney 2000, Pawlowski *et al*. 2002, Held 2003, Held & Wägele 2005). However, Knowlton (1993) argued that marine habitats are filled with cryptic species even though they are rarely recognized as such for two main reasons: firstly, our limited access to marine habitats, and secondly, the fact that speciation processes are less coupled to morphology than to other phenotypic aspects, notably chemical recognition systems. For asellote isopods, some additional aspects may be important and influence cryptic speciation. Asellotes are usually small animals, display reduced mobility (even though some can swim they very rarely appear in the water column), and they have no free-living larvae. This probably reduces gene flow and increases the probability for speciation events.

Although the present dataset includes sequences of only six different species, our analyses show also that 16S rDNA sequences allow only very limited insights into the phylogeny of the Acanthaspidiidae and should not be used to reconstruct the phylogeny of this family. Additional data are needed from other taxa and slower evolving nuclear sequences will probably be more useful for phylogenetic studies of these asellote isopods. Further studies will help us to understand the processes of speciation, phylogeny and generation of diversity in the deep sea.

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