00 Molecular evidence for cryptic speciation within the widespread Antarctic crustacean *Ceratoserolis trilobitoides* (Crustacea, Isopoda)

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ABSTRACT

The controversial taxonomic status of *Ceratoserolis trilobitoides* (Eights 1833) is re-evaluated with sequences from the mitochondrial 16S ribosomal RNA gene. Within *C. trilobitoides* sensu Wägele 1986 two sharply distinct groups of haplotypes are present. The pairwise sequence divergence is small within the groups but large between them with no intermediate distances ($\leq$0.7 vs. $\geq$5.8 percent corrected sequence divergence). The large inter-group distance persists also in areas where both types occur in sympatry. The amount of genetic diversity between the haplotypes is in the lower range of genetic distances between undisputed sister species of the same family for this gene. The molecular data therefore strongly suggest that *Ceratoserolis trilobitoides* sensu lato contains at least one, perhaps more, previously overlooked species. Accounts of *C. trilobitoides* in the literature, especially from the Antarctic Peninsula, should therefore be taken with caution.

Key Words: cryptic speciation, 16S rRNA, biogeography, Antarctica

INTRODUCTION

When James Eights (1833) described *Brongniartia trilobitoides* as one of the first crustaceans from Antarctica, except for whales and seals, very little was known about the marine life in the Southern Ocean. Today this species is contained in the genus *Ceratoserolis* which was first proposed by Cals (1977) and later formally justified by Brandt (1988), who also designated *C. trilobitoides* as its type species.

Ceratoserolis trilobitoides is a locally abundant and unusually large serolid isopod with up to 8cm body length (Wägele 1986). Material that was identified as *C. trilobitoides,* or at least closely related, was subsequently identified almost everywhere in shallower Antarctic waters, around several subantarctic islands, on the high Antarctic shelf and even under the Ross Ice Shelf ice 400 kilometers away from at least seasonally ice-free waters (Stockton 1982). Its size and abundance made this species one of the model organisms for ecological, physiological and biogeographic studies in Antarctica (Brandt 1991;Clarke 1984, Clarke 1982, Clarke & Gore 1992, Luxmoore 1981, Luxmoore 1982, Luxmoore 1984, Wägele 1987).

Despite the fact that this species has one of the longest histories in crustacean research from Antarctic waters, its systematic status has been subject to considerable scientific dispute. At least six recognisable forms with close ties to *C. trilobitoides* were described as different species: *Serolis zoiphila* Stechow 1921, *Serolis cornuta* Studer 1879, *Brongniartia cornuta* Studer 1876, *C. maculatovirgata*, *C. albohirsuta*, *C. griseostrigosa*, *C. lineatocostata* Cals 1977; for more information see Wägele (1986) and references therein. Their distinctness from the species originally described by Eights has been controversial even on the basis of the data available at the time (Hodgson 1910, Sheppard 1933, Wägele 1986). But as more material became available and gaps between earlier sampling sites were closed, many morphological characters that had reliably separated the "species" at the time they were described began to form a continuum and blurred the morphologically outlined species to a degree that forced Wägele (1986) to synonymize them to one, widely distributed and highly plastic species.

The objective of this paper is to use molecular markers as a counterbalance to conflicting evidence from morphological characters in order to assess the validity of *C. trilobitoides* sensu Wägele 1986.

MATERIAL AND METHODS

Sample collection and conservation

The material used in this study was collected during the expeditions ANTXIII/3 (Arntz & Gutt 1997) and ANTXIV/2 (Kattner 1998) with the German research vessel "RV Polarstern" to the Eastern Weddell Sea and the Antarctic Peninsula. Additional material from Potter Cove (King George Island) was collected by scuba diving at approximately

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20 meters and was kindly made available to the author by J. Kowalke. Collection data are given in Table 1.

DNA was either extracted on board using fresh material or specimens were stored in prechilled 70-80 percent ethanol and kept refrigerated until subsequent extraction in the lab. For DNA extraction small amounts of muscle from one peraeopod were dissected with a sterile scalpel and transferred to a sterile microfuge tube. Fragments of the exosceleton were avoided as far as possible to avoid contaminations with DNA from the bycatch. The DNA was extracted as described in Held (2000a).

Amplification and sequencing

DNA amount and purity was estimated by electrophoresis on an ethidium bromide stained 1% agarose gel. Between 0.5 and 1µl of DNA was used in a polymerase chain reaction with primers 16Sar (5'-CGCCTGTTTATCAAAAACAT-3') and either 16Sbr (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.* 1991) or 1472 (5'-AGATAGAAACCAACCTGG-3') (Crandall & Fitzpatrick 1996) in a Perkin Elmer thermal cycler model 2400 or in an MWG Primus cycler, conditions as described in Held (2000a). Some of the amplifications have also been performed using a 5'-tailed version of the above described amplification primers. In these cases the oligomer PFS (5'-CCCAGTCACGACGTTGTAAAAC-3') was added to the 5'-end of the primers 16Sar and the oligomer PRS (5'-AGCGGATAACAATTTCACACAGG-3') was added to the 5'-terminus of the primers 16Sbr or 1472, respectively. The net effect of such oligomer tags on the primers is that the resulting PCR product is flanked by the oligomer sequences which can then be used as priming sites for cycle sequencing. PCR products were purified in spin columns (Qiagen), eluated in 30 to 45µl and run on a 1% agaorse gel to assess purity. Between 0.5 and 2µl of the purified PCR products were sequenced directly in a cycle sequencing reaction. The cycle sequencing reaction was carried out as described in Held (2000a) using fluorescently labeled variants of the amplification primers. For PCR-products carrying tags, fluorescently labeled PFS or PRS sequencing primers were used (see above).

Cycle sequencing reactions were then analysed on a LiCor automatic sequencer (models 4000 and 4200). Both strands were proofread and contigs were assembled using the software of the sequencer. Proofread, double-stranded sequences were aligned using ClustalX v1.64 with default parameters (Thompson *et al.* 1997). In order to test the robustness of the positional homology hypothesis, the gap opening and gap extension penalties were varied in increments of one unit and the resulting alignments were checked for varying positions using the program SOAP (Löytynoja & Milinkovitch 2001).

Phylogenetic analysis of the molecular dataset was carried out with the program Paup 4 beta version 8 (Swofford 1998) using both maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML) as optimality criteria for tree contstruction. The model of nucleotide substitution was chosen on the basis of a likelihood

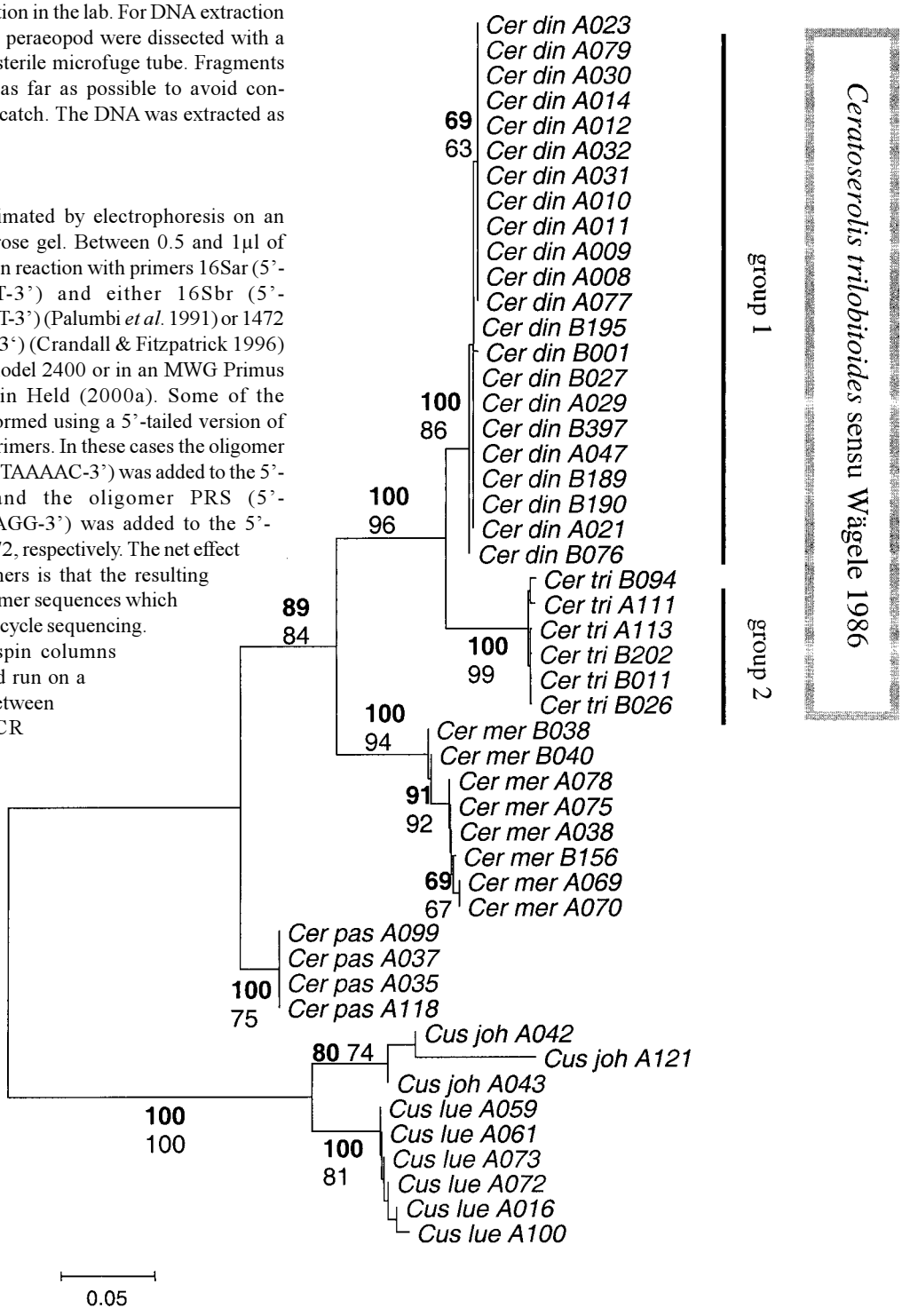

Fig. 1. Neighbour-joining tree of 28 specimens of *Ceratoserolis trilobitoides* sensu Wägele 1986 based on the mitochondrial 16S ribosomal RNA gene excluding the unstably aligned positions 227-265. The two divergent groups of haplotypes are provisionally designated "group 1" and "group 2", respectively. The tree is rooted with *Cuspidoserolis spp.*. *Cer. din* & *Cer. tri* = *Ceratoserolis trilobitoides* sensu Wägele 1986, *Cer. mer.* = *C. meridionalis*, *Cer. pas.* = *C. pasternaki*, *Cus. joh.* = *Cuspidoserolis johnstoni*, *Cus. lue.* = *Cuspidoserolis luethjei*. Numbers on branches are bootstrap support from 100 replicates in the maximum parsimony tree (in bold) and from 1000 replicates, nodes with bootstrap support under 60 are not labeled. Genetic distances were calculated using the Tamura-Nei model of nucleotide substitution gamma distributed rates (alpha=0.41). The model and its parameters were determined in a hierachical likelihood-ratio test (see text for details).

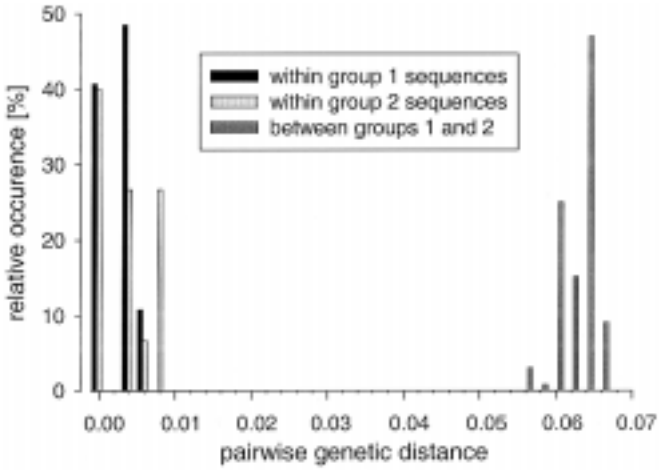

Fig. 2. Frequency spectrum of pairwise genetic distances among *Ceratoserolis trilobitoides* sensu Wägele 1986 (16S rRNA gene, Tamura-Nei model, %invariant=0, gamma distributed rates, alpha=0.41). "Group 1" and "group 2" refer to groups of mitochondrial 16S rRNA haplotypes as illustrated in Figure 1.

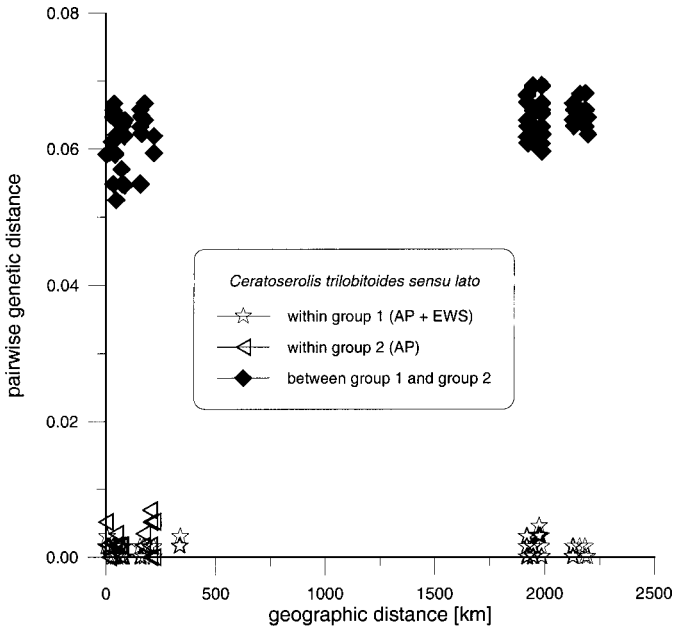

Fig. 3. Geographical distance versus genetic distance (TamNei model, gamma distributed rates) in pairwise 16S rRNA gene sequence comparisons of *Ceratoserolis trilobitoides* sensu Wägele 1986. EWS = Eastern Weddell Sea, AP = Antarctic Peninsula.

ratio test (LRT) as implemented in the program modeltest 3.06 (Posada & Crandall 1998).

RESULTS

The 16S rRNA gene fragment varies between 480 and 488bp in length and shows no significant deviation from homogeneous base frequency distribution (Chi square=13.5174, df=13, p=0.5623). The average AT content (%AT = 66.8) is high as usual for this gene (Simon *et al.* 1994), the alignment is 492bp in length. Thirty-nine aligned bases corresponding to one stem and loop in the secondary structure (Cannone *et al.* 2002) and bases 918-965 in the *Drosophila melanogaster* 16S rRNA sequence (accession number X53506) proved unstable when alignment parameters were varied between 9 and 15 for gap opening and 4 and 9 for gap extension penalties, respectively. They were marked as variable and calculations of genetic distance and inferred tree topologies were carried out with and without these positions. All calculations of base frequency distribution were done with one sequence per species. Of the remaining 453 aligned positions, 133 are variable and 123 parsimony informative.

The likelihood ratio test identified the Tamura-Nei model (Tamura & Nei 1993) with no invariant postions and a gamma shaped rate heterogeneity (alpha=0.41) as the best compromise between fitting the data to a tree and introducing additional paramters. Base frequencies (A=0.3338 C=0.1895 G=0.1459 T=0.3308) and rates for the 6 substitution types were estimated from the dataset (AC=1.0000 AG=2.9398 AT=1.0000 CG=1.0000 CT=11.5236 GT=1.0000).

Maximum parsimony, neighbour joining and maximum likelihood yield very similar trees, which differ only in the placement of nearly identical sequences (nodes with less than 50% bootstrap support in the NJ tree). All nodes with more than 50% bootstrap support are identified regardless of the tree reconstruction method used. Parsimony yields eight equally short trees of 185 steps length each (CI=0.8541; RI=0.9786) for a transition/transversion ratio of 1. This topology remained stable in the strongly supported nodes up to the highest tested ratio (ti/tv=10) and also for trees based on transversions only.

In all methods the sequences belonging to *C. trilobitoides* sensu Wägele 1986 are split into two well supported groups (Fig. 1), one occuring in the Eastern Weddell Sea and the Antarctic Peninsula region (group 1), the other being confined to the latter (group 2). The genetic differences between within-group (< 0.8 %) and between-group comparisons (> 5.8%) are distinct with no intermediate distances occuring (Fig. 2). This gap between intra- and inter-group distances is equally distinct when no correction for multiple substitutions is applied and only visible nucleotides are used (data not shown). Irrespective of the model of substiution chosen there are no intermediate genetic distances.

The bimodal distribution of sequence similarity does not correlate with geographic distance between sampling sites (Fig. 3). The group 1 individuals are confined to the Antarctic Peninsula with a maximum distance between sampling sites of around 200 km, yet their genetic variability is slightly larger than that of group 2 sequences occuring on either side of the Weddell Sea separated by geographic distances in excess of 2200 km.

On the other hand, the genetic differences between the two groups even in syntopy (e.g. around King George Island: B026 and B027; Table 1), are more than 8-fold larger.

The amount of inter-group genetic differences is in the lower range of genetic distances between undisputed species of serolid isopods (Held 2000b) and also distances between other closely related species of crustaceans reported for this gene (France & Kocher 1996).

DISCUSSION

The molecular data identify two strongly distinct clades of haplotypes for the mitochondrial 16S ribosomal gene within nominal *Ceratoserolis trilobitoides*. The sharp contrast between groups of mitochondrial haplotypes contradict the diffuse boundaries between morphological characters that were found by morphological systematists. It therefore needs to be worked out how this genetic discontinuity can be interpreted in a phylogenetic context.

The molecular data leave little doubt that the provisionally designated groups "1" and "2" (Fig. 1) in reality are two separate species, one of which has been mistaken for *Ceratoserolis trilobitoides* originally described by Eights (1833). The pairwise sequence comparisons show that there is no genetic continuum, but rather two sharply delimited groups with small within-group variation, but large between-

Table 1. Collection data for specimens of *Ceratoserolis trilobitoides* sensu Wägele 1986 included in this study. Specimens are grouped according to their 16S ribosomal RNA gene haplotypes (Figure 1). Numbers identifying individuals, e.g. A031, are referred to in Figure1. BT = bottom trawl, AGT = Agassiz' trawl, SD = scuba diving.

station	gear	lat °S	lon °W	depth [m]	region	specimen
group 1						
39-005	BT	71° 41.1'S	12° 44.3'W	227	Eastern Weddell Sea	A011
39-005	BT	71° 41.1'S	12° 44.3'W	227	Eastern Weddell Sea	A012
39-005	BT	71° 41.1'S	12° 44.3'W	227	Eastern Weddell Sea	A014
39-011	BT	73° 22.6'S	21° 10.6'W	338	Eastern Weddell Sea	A029
39-011	BT	73° 22.6'S	21° 10.6'W	338	Eastern Weddell Sea	A030
39-011	BT	73° 22.6'S	21° 10.6'W	338	Eastern Weddell Sea	A031
39-011	BT	73° 22.6'S	21° 10.6'W	338	Eastern Weddell Sea	A032
39-012	BT	73° 18.1'S	21° 10.1'W	459	Eastern Weddell Sea	A021
39-012	BT	73° 18.1'S	21° 10.1'W	459	Eastern Weddell Sea	A023
39-012	BT	73° 18.1'S	21° 10.1'W	459	Eastern Weddell Sea	A077
39-017	BT	73° 18.0'S	21° 9.9'W	468	Eastern Weddell Sea	A008
39-017	BT	73° 18.0'S	21° 9.9'W	468	Eastern Weddell Sea	A009
39-017	BT	73° 18.0'S	21° 9.9'W	468	Eastern Weddell Sea	A010
39-017	BT	73° 18.0'S	21° 9.9'W	468	Eastern Weddell Sea	A047
39-017	BT	73° 18.0'S	21° 9.9'W	468	Eastern Weddell Sea	A079
42-003	BT	61° 8.4'S	56° 10.8'W	380	Antarctic Peninsula	B001
42-014	BT	61° 14.0'S	54° 38'W	333	Antarctic Peninsula	B027
42-003	BT	61° 8.4'S	56° 10.8'W	380	Antarctic Peninsula	B076
42-023	AGT	61° 6.1'S	56° 7.5'W	371	Antarctic Peninsula	B189
42-023	AGT	61° 6.1'S	56° 7.5'W	371	Antarctic Peninsula	B190
42-027	AGT	60° 58.9'S	55° 58.2'W	415	Antarctic Peninsula	B195
42-027	AGT	60° 58.9'S	55° 58.2'W	415	Antarctic Peninsula	B397
group 2						
42-032	BT	60° 50.2'S	55° 37.3'W	344	Antarctic Peninsula	B202
42-011	BT	61° 14.6'S	55° 37.2'W	87	Antarctic Peninsula	B094
42-012	BT	61° 16.5'S	54° 52.5'W	200	Antarctic Peninsula	B011
42-014	BT	61° 14.0'S	54° 38.0'W	333	Antarctic Peninsula	B026
KGI	SD	62° 14'S	58° 40'W	20	Antarctic Peninsula	A111
KGI	SD	62° 14'S	58° 40'W	20	Antarctic Peninsula	A113

group separation. This bimodal distribution and its persistence around the South Shetland Islands where both groups occur in sympatry indicate two reproductively isolated species rather than races of one species (France & Kocher 1996) . However, the maternal mode of inheritance of mitochondrial genes with no recombination in principle allows an alternative explanation for this situation. The immigration of ovigerous females from a genetically distinct population can in principle also result in the coexistence of two or more different haplotypes in one population. Even in absence of selection, these would be subject to genetic drift leading to the extinction of some types over time, however (Avise 2000). This scenario also fails to provide an adequate explanation for the existence of a conspecific population elsewhere that is differentiated to a degree that is normally found between closely related species of serolid isopods and other crustaceans. The discovery of morphological characters reliably identifying the two species and congruent results from nuclear gene sequences (not shown) further rule out this possibility and corroborate the earlier conclusion that *Ceratoserolis trilobitoides* sensu lato harbours at least one previously overlooked species.

Furthermore, the recognition of a cryptic species within nominal *Ceratoserolis trilbitoides* casts a shadow of doubt on the status of several other morphotypes of this species which have been described from different localities on the Antarctic shelf and also some Subantarctic islands (see introduction). Currently they are regarded as a single species with a high intraspecific variation and morphological plasticity (Wägele 1986). The difficulty to further divide this taxon into smaller units (species) is not the lack of morphological variability but rather its abundance which makes drawing a clear line between species difficult. The identification of a second species leaves both groups with a much smaller degree of morphological variability which may entail the identification of further overlooked entities in this species complex. To this end, a re-evaluation of material, especially from the rather poorly sampled Subantarctic Islands and the East Antarctic shelf, is needed. A complete revision of the genus *Ceratoserolis* based on additional molecular and morphological characters is in preparation.

The biogeography of the two species, with one only known from the South Shetland Islands and the other much more widely distributed in the Eastern Weddell Sea and the Antarctic Peninsula, suggest that their zone of co-occurrence around the South Shetlands may be the result of a secondary contact. With *C. trilobitoides* sensu lato being confined to shallow waters down to approximately 600 meters depth, the most plausible colonization route would be along the Southern Weddell Sea shelf. In this context it is important to clarify the status of the morphologically rather strongly differentiated *C. trilobitoides* found on the Filchner ice shelf (Wägele 1986).

The clarification of the systematics of the genus *Ceratoserolis* is desirable because the abundance and size of these species made them favourite model organisms for a variety of ecological and other studies of invertebrates on the Antarctic shelf. In light of the fact that *C. trilobitoides* sensu lato contains at least one, possibly many more, overlooked species, its appearance in older literature should be viewed with caution.

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REFERENCES

Arntz, W. & Gutt, J. (Eds.) 1997. The expedition ANTARKTIS XIII/3 (EASIZ I) of "Polarstern" to the eastern Weddell Sea in 1996. 148pp.

Avise, J. C. 2000. Phylogeography. The history and formation of species. Harvard University Press, Cambridge, 447pp.

Brandt, A. 1988. Antarctic Serolidae and Cirolanidae (Crustacea: Isopoda): New genera, new species and redescriptions. In: Fricke, R. (Hrsg.): Theses Zoologicae. Koeltz Scientific Books, 143pp.

Brandt, A. 1991. Colonization of the Antarctic shelf by the Isopoda (Crustacea, Malacostraca). Berichte zur Polarforschung 98: 1-240.

Cals, P. 1977. Dérive continentale et spéciation du complex *Ceratoserolis* nov. gen., Crustacés antarctique benthiques connus de l'Arc de la Scotia aux iles Kerguelen. C. R. Acad. Sc. Paris, t. 284, série D: 2273-2277.

Cannone, J. J., Subramanian, S., Schnare, M. N., Collett, J. R., D'Souza, L. M., Du, Y., Feng, B., Lin, N., Madabusi, L. V., Muller, K. M., Pande, N., Shang, Z., Yu, N. & Gutell, R. R. 2002. The Comparative RNA Web (CRW) Site: An Online Database of Comparative Sequence and Structure Information for Ribosomal, Intron, and other RNAs. BioMed Central Bioinformatics 3: 2-2.

Clarke, A. 1984. Lipid composition of two species of Serolis (Crustacea, Isopoda) from Antarctica. British Antarctic Survey Bulletin 64: 37-53.

Clarke, A. & Gore, D. J. 1992. Egg size and composition in *Ceratoserolis* (Crustacea: Isopoda) from the Weddell Sea. Polar Biology 12: 129-134.

Clarke, A. 1982. Temperature and embryonic development in polar marine invertebrates. Int. J. Invert. Repr. Develop. 5: 71-82.

Crandall, K. A. & Fitzpatrick, J. F. j. 1996. Crayfish molecular systematics: Using a combination of procedures to estimate phylogeny. Systematic Biology 45 (1): 1-26.

Eights, J. 1833. Description of a new crustaceous animal found on the shores of the South Shetland Islands. Trans. Albany Inst. 2: 53-57.

France, S. C. & Kocher, T. D. 1996. Geographic and bathymetric patterns of mitochondrial 16S rRNA sequence divergence among deep-sea amphipods, *Eurythenes gryllus*. Marine Biology 126 (4): 633-643.

Held, C. 2000a. Phylogeny and biogeography of serolid isopods (Crustacea, Isopoda, Serolidae) and the use of ribosomal expansion segments in molecular systematics. Molecular Phylogenetics and Evolution 15 (2): 165-178.

Held, C. 2000b. Speziation im Südpolarmeer – Systematik und Biogeographie der Serolidae und Arcturidae (Crustacea: Isopoda). PhD thesis, University of Bielefeld, 144pp.

Hodgson, T. V. 1910. Isopoda. Nat. Antarct. Exped. 1901-1904, 5: 1-77.

Kattner, G. (Eds.) 1998. The expedition ANTARKTIS XIV/2 of RV "Polarstern" in 1996/97. 87pp.

Löytynoja, A. & Milinkovitch, M. C. 2001. SOAP, cleaning multiple alignments from unstable blocks. Bioinformatics 17 (6): 573-574.

Luxmoore, R. A. 1981. The ecology of Antarctic serolid isopods. Council for National Academic Awards, 231 S.

Luxmoore, R. A. 1982. Moulting and growth in serolid isopods. Journal of Experimental Marine Biology and Ecology 56: 63-85.

Luxmoore, R. A. 1984. A comparison of the respiration rate of some Antarctic isopods with species from lower latitude. British Antarctic Survey Bulletin 62: 53-65.

Palumbi, S. R., Martin, A., Romano, S., Mcmillan, W. O., Stice, L. & Grabowski, G. 1991. The simple fool's guide to PCR. A collection of PCR protocols, version 2. University of Hawaii

Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14 (10): 817-818.

Sheppard, E. M. 1933. Isopod Crustacea Part I. The family Serolidae. 'Discovery' Rep. 7: 253-262.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87 (6): 651-701.

Stockton, W. L. 1982. Scavenging amphipods from under the Ross Ice Shelf, Antarctica. Deep Sea Research Part I Oceanographic Research Papers 29 (7A): 819-835.

Swofford, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (* and other methods). version 4.08. Sinauer Associates, Sunderland, Massachusetts.

Tamura, K. & Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10 (3): 512-526.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876-4882.

Wägele, J.-W. 1986. Polymorphism and distribution of *Ceratoserolis trilobitoides* (Eights, 1833) (Crustacea, Isopoda) in the Weddell Sea and synonymy with *C. cornuta* (Studer, 1879). Polar Biology 6: 127-137.

Wägele, J.-W. 1987. On the reproductive biology of *Ceratoserolis trilobitoides* (Crustacea: Isopoda): Latitudinal variation of fecundity and embryonic devolpment. Polar Biology 7: 11-24.