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THE MAGELLAN-ANTARCTIC CONNECTION: LINKS AND FRONTIERS AT HIGH SOUTHERN LATITUDES.
W.E. ARNTZ, G.A. LOVRICH and S. THATJE (eds.)

Cryptic speciation in the giant Antarctic isopod *Glyptonotus antarcticus* (Isopoda: Valvifera: Chaetiliidae)*

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SUMMARY: The genus *Glyptonotus* is most commonly regarded as monospecific, with *Glyptonotus antarcticus* Eights 1852 being its only constituent. Two more forms (*G. acutus, G. antarcticus var. obtusus*) that had been described based on morphological evidence have received little attention in the literature, though no formal attempt to evaluate their taxonomic status has been undertaken. In a survey of within-species genetic diversity, 23 specimens of the benthic Antarctic isopod *Glyptonotus antarcticus* from three sampling areas around the Antarctic had a high genetic variability in their mitochondrial LSU (16S) gene. Eleven unique mitochondrial haplotypes were found, two at the Antarctic Peninsula (AP), two in the Ross Sea (RS) and seven in the Eastern Weddell Sea (EWS). Average haplotype variation within sampling areas (AP, RS, EWS) was one order of magnitude less than between sampling areas. In the EWS, however, two highly differentiated haplotypes co-exist. These four groups of haplotypes may represent cryptic, but reproductively isolated species rather than a single species.

Keywords: sibling species, molecular systematics, biogeography, Antarctic benthos.

RESUMEN: ESPECIACIÓN CRÍPTICA EN EL ISÓPODO GIGANTE ANTÁRTICO *GLYPTONOTUS ANTARCTICUS* (ISOPODA, VALVIFERA, CHAETILIIDAE). – En una investigación sobre diversidad genética intra-específica se registró variabilidad en el gen mitocondrial LSU (16S) de 23 especímenes del isópodo bentónico antártico *Glyptonotus antarcticus* de tres áreas antárticas. Se encontraron once haplotipos mitocondriales únicos, dos en la Península Antártica (AP), dos en el Mar de Ross (RS) y siete en el Mar de Weddell (EWS). La variación media de los haplotipos dentro de las áreas (AP, RS, EWS) fue un orden de magnitud menor que entre ellas. Sin embargo, en el EWS coexisten dos haplotipos altamente diferenciados. Estos cuatro grupos de haplotipos pueden representar especies crípticas reproductivamente aisladas, más que una única especie como se asumía anteriormente.

Palabras clave: especies hermanas, sistemática molecular, biogeografía, bentos antártico.

INTRODUCTION

The giant isopod *Glyptonotus antarcticus* was one of the first isopods described from Antarctic waters (Eights, 1852). It can grow to up to 9 cm long and is one of the most conspicuous and locally abundant components of the High Antarctic megazoobenthos.

Because it is ecologically important and relatively easy to catch and maintain in aquaria, it has become a model organism in several fields of Antarctic biology, including ecology, physiology and biochemistry (Arnaud, 1970; Clarke, 1979, 1982; Dearborn, 1967; Janssen and Hoese, 1993;

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Key and Barnes, 1999; Luxmoore, 1984; Martin, Jaros, Chaigneau and Meyer-Rochow, 1995; Rakusa-Suszczewski and McWhinnie, 1976; Starmans, 1997; Whiteley, Taylor, Clarke and El Haj, 1997; White, 1970, 1975).

Two more species or variants of *Glyptonotus* have been put forward based on morphological characters. Richardson (1906) described *Glyptonotus acutus* from the shallow waters around Wincke and Booth-Wandel islands (Antarctic Peninsula). It differs from *G. antarcticus* mainly by a longer, more acute pleotelson and different proportions. Comparing numerous individuals from both forms, Tattersall (1921) came to the conclusion that many differences between them are a function of body size and age of the individual rather than consistent differences between species. He recommended treating *G. acutus* as a mere variety of *G. antarcticus*. Sheppard (1957) went even further than Tattersall by stating that "...it is impossible to separate *G. acutus*, even as a variety, from *G. antarcticus*".

Another variety, *G. antarcticus* var. *obtusus,* was described by Meyer-Rochow (1980), also based on distinctive features of pleotelson shape and proportions found in a population from the Ross Sea. A more detailed account of the taxonomic history of the genus will be given in a revision of *Glyptonotus* (Held, in prep.), but today the synonymy of all forms described so far is widely accepted among taxonomists (Brandt, 1990; Kussakin, 1982; Wägele, 1991). *Glyptonotus antarcticus* sensu lato is recorded around the High Antarctic shelf and some Subantarctic islands in waters ranging from the shallow subtidal down to more than 600 metres depth.

Molecular data have recently indicated that another widely distributed Antarctic isopod, *Ceratoserolis trilobitoides*, may represent more than one species (Held, 2003), and complimentary morphological data support this interpretation (Held, in prep.). To investigate the taxonomic status and genetic variability of nominate *Glyptonotus antarcticus*, 23 specimens were collected around the Antarctic and a 516 bp fragment of their mitochondrial large subunit gene (16S) was sequenced.

In particular, the following questions were addressed: (1) Is there cryptic speciation in *Glyptonotus antarcticus*? (2) If so, how many species of *Glyptonotus* are present? and (3) How can divergent populations of a single species be separated from several reproductively isolated species?

MATERIAL AND METHODS

Sample collection

Specimens of *Glyptonotus* were collected during the expeditions ANT XIII/3 to the Eastern Weddell

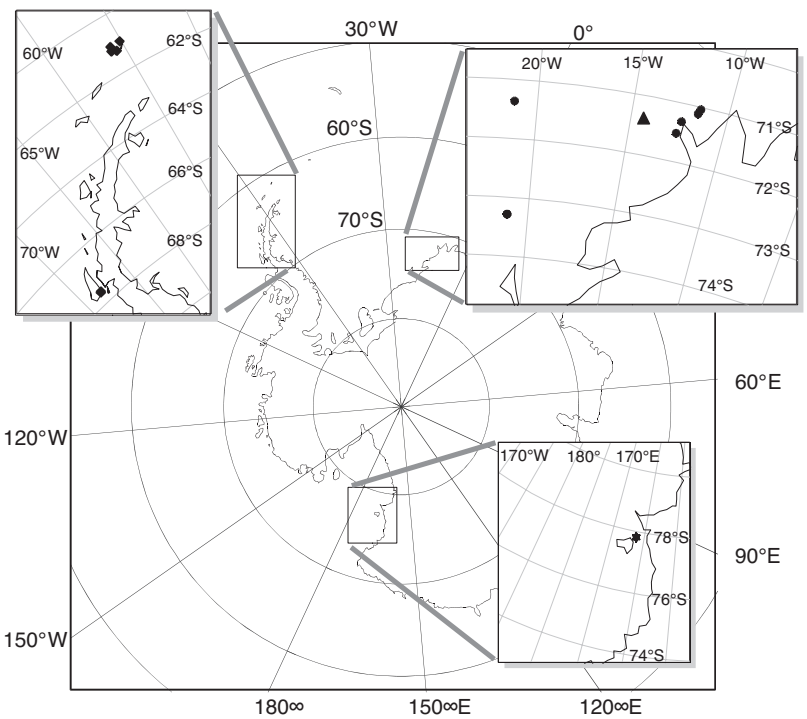

Fig. 1. – The sampling locations of nominate *Glyptonotus antarcticus* in the Southern Ocean The four mitochondrial haplotypes, possibly representing four species, are: Antarctic Peninsula (◆), Eastern Weddell Sea group A (●), Eastern Weddell Sea group B (▲), Ross Sea (✳).

TABLE 1. – Individual codes, GenBank accession numbers and sampling location for the specimens of *Glyptonotus* used in this study. The last column indicates the occurrence of each of the 11 unique 16S haplotypes.

specimen code	accession	region	station	latitude	longitude	depth (m)	haplotype
B088	AM086466	Antarctic Peninsula	Elephant Island	61°11'S	55°58'W	130	H2
B101	AM086464	Antarctic Peninsula	Elephant Island	61°15'S	55°37'W	87	H1
B201	AM086467	Antarctic Peninsula	Elephant Island	61°02'S	55°52'W	148	H1
B233	AM086465	Antarctic Peninsula	Elephant Island	61°01'S	55°07'W	143	H1
B311	AM086468	Antarctic Peninsula	Rothera	67°34'S	68°08'W	10	H1
B312	AM086469	Antarctic Peninsula	Rothera	67°34'S	68°08'W	10	H1
C040	AM086470	Antarctic Peninsula	Anchorage Isl.	67°36'S	68°13'W	10	H1
A126	AM086473	Eastern Weddell Sea	Kapp Norvegia	71°08'S	11°32'W	123	H4
A127	AM086474	Eastern Weddell Sea	Kapp Norvegia	71°08'S	11°32'W	123	H5
A145	AM086475	Eastern Weddell Sea	Kapp Norvegia	71°32'S	12°26'W	504	H6
A146	AM086476	Eastern Weddell Sea	Vestkapp	73°18'S	21°10'W	468	H5
A148	AM086477	Eastern Weddell Sea	Kapp Norvegia	71°19'S	12°17'W	170	H4
A149	AM086479	Eastern Weddell Sea	Vestkapp	73°23'S	21°11'W	338	H4
A151	AM086480	Eastern Weddell Sea	Vestkapp	73°18'S	21°10'W	468	H4
A152	AM086471	Eastern Weddell Sea	Vestkapp	73°18'S	21°10'W	468	H3
A154	AM086481	Eastern Weddell Sea	Drescher Inlet	72°01'S	19°00'W	413	H7
A155	AM086482	Eastern Weddell Sea	Kapp Norvegia	71°03'S	11°26'W	462	H8
A156	AM086472	Eastern Weddell Sea	Kapp Norvegia	71°03'S	11°26'W	462	H3
A157	AM086478	Eastern Weddell Sea	Kapp Norvegia	71°08'S	11°32'W	123	H4
A147	AM086483	Eastern Weddell Sea	Kapp Norvegia	71°23'S	14°20'W	634	H9
A150	AM086484	Eastern Weddell Sea	Kapp Norvegia	71°23'S	14°20'W	622	H9
B359	AM086485	Ross Sea	Arrival Heights	77°49'S	166°39'E	10	H10
B360	AM086486	Ross Sea	Arrival Heights	77°49'S	166°39'E	10	H11

Sea (Arntz and Gutt 1997) and ANT XIV/2 to the Antarctic Peninsula (Kattner, 1998). The catch from towed gear (bottom trawl, Agassiz trawl, Rauschert dredge) was hand-sorted. Specimens were fixed in 80-96% ethanol. To minimize enzymatic degradation of DNA, the ethanol was pre-chilled to -20°C and freshly fixed material was kept at temperatures between -30 and 4°C until the DNA was extracted (Held, 2000a).

Additional material, collected by scuba diving, was kindly made available from the Ross Sea by J. McClintock and co-workers and from the Antarctic Peninsula by M. White, L. Peck and K. Linse. Details about the sampling locations are given in Figure 1 and Table 1.

DNA extraction and sequencing

Sequence data were determined for a total of 23 specimens of *Glyptonotus*. Muscle tissue was dissected from walking legs and transferred to sterile microfuge tubes. Exoskeleton was avoided because of contamination risks with DNA from bycatch organisms (Held, 2000a). The muscle tissue was digested overnight with Proteinase K, and genomic DNA was extracted using spin columns (Qiagen QiaAmp DNA mini) following the animal tissue protocol of the manufacturer. The DNA was finally eluted in 35 μl AE buffer.

Amplification of the mitochondrial 16S ribosomal (LSU) gene was carried out in 25 μl volume (one unit Qiagen Taq polymerase, 2.5 μl 10x PCR buffer, 5 μl Q-buffer, 2.5 μl dNTPs, 0.5-1 μl DNA template, filled to 25 μl with sterile H_2O) as described in Held (2003) using the primers 16Sar 5'-CGCCTGTTTATCAAAAACAT-3' and 16Sbr 5-CCGGTCTGAACTCAGATCACGT-3' (Palumbi *et al.*, 1991). The temperature profile of the amplification on an MWG Primus cycler was as follows: 5 min 94°C initial denaturing, 35 cycles of 45 s 94°C, 45 s 52°C, 80 s 72°C, followed by 7 min final extension. PCR products were column purified (Qiagen Qiaquick) and checked on a 1% ethidium-bromide stained agarose gel for purity, concentration and possible contamination.

Between 0.5 and 1 μl of the purified PCR products were sequenced directly in a dideoxy cycle sequencing reaction on Techne Progene cycler (94°C 2 min initial denaturing, 30 cycles of 25 s 94°C, 25 s 48°C, 35 s 70°C) following the recommendations of the manufacturer (Amersham), except that the reaction volume was reduced to 13 μl. After denaturation (30 s 94°C), the samples were kept on ice and 1.5 μl was loaded and run on an automated sequencer (LiCor models 4000 and 4200). Sequence reads were proofread and contigs from both strands were assembled in the program AlignIR 1.2 (LiCor). The corrected sequences were

TABLE 2. – Maximum-likelihood estimates of pairwise genetic distances (lower triangle) and observed genetic distances (upper triangle) between the 11 unique mitochondrial haplotypes in the LSU gene of *Glyptonotus* in this study. The specimens and sampling localities of each haplotype are given in Table 1. Details about the LRT and the model used are described in the text. Observed distance values should be regarded as rough approximations only because no corrections for multiple substitutions are made. Lower variability within sampling regions was indicate in *italics* and the higher divergence of haplotype H9 in **bold**.

	Ant. Peninsula		Eastern Weddell Sea							Ross Sea	
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11
H1	*	*2*	45	45	43	45	45	45	39	47	46
H2	*0.004091*	*	43	43	41	43	45	43	39	47	46
H3	0.143933	0.133679	*	*4*	*8*	*10*	*6*	*5*	**31**	45	44
H4	0.141898	0.131807	*0.008328*	*	*4*	*6*	*2*	*1*	**30**	44	43
H5	0.133389	0.123621	*0.017215*	*0.008222*	*	*2*	*6*	*5*	**26**	42	41
H6	0.143289	0.133080	*0.022098*	*0.012662*	*0.004071*	*	*8*	*7*	**27**	44	43
H7	0.141898	0.143592	*0.012983*	*0.004129*	*0.012812*	*0.017547*	*	*3*	**29**	43	42
H8	0.141898	0.131807	*0.010504*	*0.002006*	*0.010368*	*0.014903*	*0.006250*	*	**31**	45	44
H9	0.119653	0.121103	**0.086915**	**0.082364**	**0.069076**	**0.073204**	**0.078026**	**0.085804**	*	27	26
H10	0.160591	0.162994	0.145323	0.139167	0.130666	0.140538	0.133283	0.143414	0.072751	*	*1*
H11	0.153961	0.156249	0.139241	0.133283	0.124995	0.134582	0.127591	0.137426	0.068643	*0.002039*	*

aligned with ClustalX (Thompson *et al*., 1997) using the default parameters. On the basis of secondary structure information from *Drosophila melanogaster* (GenBank accession number X53506) as stored in the ribosomal RNA database (Gutell *et al*., 1993), the alignment was corrected resulting in a final alignment length of 505 bp. No data had to be excluded because of alignment difficulties. The alignment is available from the first author upon request.

Data analysis

The number of unique mitochondrial haplotypes was determined with the computer program DnaSP 4 (Rozas *et al*., 2003). To ensure a choice of a model of nuceotide substitution which describes the data accurately, a likelihood-ratio test was carried out with the program Modeltest 3.06 (Posada and Crandall, 1998). The model with the best fit was HKY, with a transition/transversion ratio of 3.7369, gamma distributed rates (shape parameter alpha = 0.2026) and no invariant positions. This model was then used to calculate maximum-likelihood estimates of pairwise genetic distances in Paup 4b10 (Swofford, 1998).

RESULTS

The 23 *Glyptonotus* large ribosomal subunit sequences varied in length between 494 and 505 bp. Among the LSU sequences, 11 unique mitochondrial haplotypes were distinguished (Table 1). Pairwise haplotype differences ranged from one to 47 observed substitutions over the length of the alignment (Table 2). Table 2 also shows distance estimates corrected for multiple substitutions.

Observed, i.e. uncorrected, distances may be a poor estimate of the true number of substitutions, because the identity of the substituted nucleotides is not taken into account. Two pairs of sequences can be separated by an identical number of observed substitutions. If one pair of differences were predominantly due to theoretically rare substitutions, the probability of undetected multiple substitutions is higher than differences due to the most frequently occurring substitution types. For this reason two sequence pairs featuring an identical number of substitutions may not have an identical corrected distance value, particularly at higher degrees of divergence (Table 2, e.g. H6/H10; H3/H11; H8/H11). Therefore, the observed values are only a rough estimate of true genetic divergence.

The largest haplotype diversity was among the *Glyptonotus* from the Eastern Weddell Sea. The two haplotype pairs from the Ross Sea and the Antarctic Peninsula differ only by one and two substitutions respectively. In the Eastern Weddell Sea there are more haplotypes present (7 haplotypes). These show corrected differences of up to 0.022098 (10 substitutions) when haplotype H 9 is ignored and up to 0.086915 (31 substitutions) when H 9 is considered.

For many, but not all, haplotypes the genetic distance scales with the geographic distances between sampling locations. Typically, the haplotypes which are found within each of the three sampling regions (AP, EWS, RS) are about an order of magnitude less divergent than genetic variation between sampling areas (indicate in italics in Table 2).

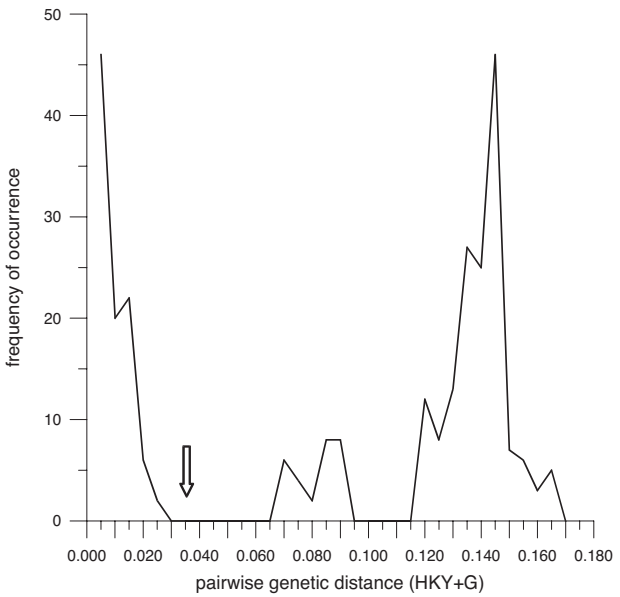

Fig. 2. – Maximum-likelihood estimates of pairwise genetic distances corrected for multiple hits, the mode choice is based on a likelihood-ratio test (for details see text). The arrow marks the smallest interspecific distance in the homologous region of the mitochondrial LSU (16S) gene between the species of marine isopods in Held (2003).

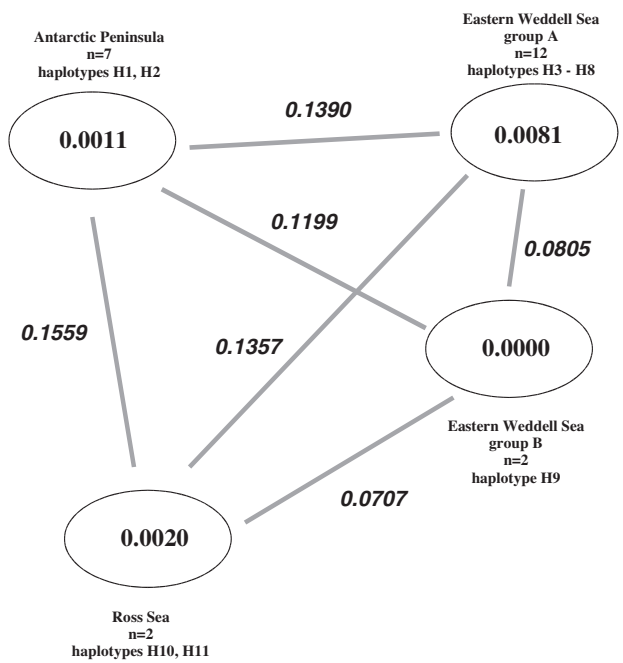

Fig. 3. – Average pairwise genetic distances (HKY + G) within and between four groups of 16S mitochondrial haplotypes of nominate *Glyptonotus antarcticus*; n denotes the number of sequences in the group, Hx indicates unique haplotypes as listed in Table 1.

This is not true, however, for *Glyptonotus* haplotypes in the Eastern Weddell Sea when haplotype 9 is included. The haplotype 9 versus haplotype 3 to 8 distances are in the lower range of values typical of trans-regions several thousand kilometres apart (bold values in Table 2).

The absence of a continuous spectrum of inter-haplotype differences is noteworthy (Fig. 2). Haplotypes are either similar (≤ 0.02210 corrected distance) or different (≥ 0.0691), with no intermediate values. If haplotype H 9 from the Eastern Weddell Sea is ignored, the gap is even more pronounced (≤ 0.02210 vs. ≥ 0.123621).

Only if haplotype H 9 is recognised as a fourth group in addition to the remaining haplotypes grouped according to their region of occurrence a clear pattern emerges.

Within the four groups, the genetic divergence is on average an order of magnitude smaller than any of the average between-group divergence values (Fig. 3).

DISCUSSION

The unexpectedly high genetic variability in nominate *Glyptonotus antarcticus* poses the question whether some or all of the genetic variability is an indication of cryptic speciation, or random, or reflects only locally adaptive differences within one species. There are four sharply distinct groups of mitochondrial haplotypes in *Glyptonotus,* which in the absence of samples connecting the three largely disjunct sampling areas (AP, EWS, RS) could be interpreted as either reproductively isolated species or populations of a single species.

Held (2000b, 2003) developed a set of criteria to provide evidence for cryptic speciation of serolid isopods on the high Antarctic shelf: (1) bimodal distribution of pairwise distance measures with no intermediate values, (2) differentiation at a level known for this gene from other undisputed species pairs closely related to the studied species, and (3) persistence of high levels of genetic differentiation in sympatry.

The *Glyptonotus* data fulfil the first criterion although without further analyses it remains uncertain which of the two gaps in Figure 2 can be regarded as separating the intra- and interspecific distances (see below). Because all distance values between 0.069 and 0.087 in Figure 2 are related to haplotype H9, this amounts to the question of the specific status of the two specimens bearing haplotype H9.

The *Glyptonotus* sequences presented in this study are differentiated at a level that surpasses the other isopods reported by Held (2003) and are in the upper range of typical inter-specific differentiation for this gene in other Crustacea (France and Kocher,

1996; Schubart, Neigel and Felder, 2000 and references therein). By induction from the data for other taxa, the threshold indicating the break between intra- and interspecific values can be assumed to lie between 0.02210 and 0.0691 corrected sequence divergence (position of arrow in Fig. 2).

It should be noted that the last criterion for distinguishing cryptic speciation from genetic plasticity is rigid. In particular, it is not a necessary criterion because different specific status is also possible in allopatric populations.

In the Eastern Weddell Sea specimens with haplotype H9 were caught in two different, but closely spaced, trawls which were situated approximately halfway between locations that were dominated by other haplotypes (Fig. 1). Two of these other haplotypes (H3 and H4) occurred both east and west of the stations where haplotype H9 was caught (Table 1). Although the sampling density—especially further away from the coast—is too low to give a conclusive answer at this time, this can be taken as evidence that haplotype H9 occurs sympatrically or at least in close proximity to other haplotypes while still maintaining a high degree of genetic dissimilarity, which was otherwise only observed between the major sampling areas in this study (AP, RS, EWS).

It is unclear what processes lead to the coexistence of the two putative species of *Glyptonotus* in the Eastern Weddell Sea, but the concept of geographic separation is strongly anthropocentric. France and Kocher (1996) presented data for the giant amphipod *Eurythenes gryllus*, demonstrating that surprisingly little separation in the vertical (a few hundred metres) can yield a genetic separation that is normally associated with horizontal distances, which are many orders of magnitude larger (thousands of kilometres). In *Glyptonotus* haplotype H9 occurs in close proximity to the haplotypes H3 to H8, though both specimens with H9 were the only *Glyptonotus* that were caught deeper than 600 metres (Table 1). It is possible that H9 occurs next to H3-8, but is separated from them vertically.

The molecular data strongly suggest that the four groups of haplotypes, including H9 as a separate group, are candidates for cryptic species, which in the past have been regarded as a single species with a wide distribution range.

Although the present analysis cannot prove the existence of cryptic speciation, it indicates patterns of variation that are not expected in a single species, even in the presence of local differences. In other words, this procedure provides necessary, but not entirely sufficient evidence for cryptic speciation. Nevertheless, it provides a useful starting point for subsequent systematic work aiming to reconcile the existing morphological and molecular data.

It is possible that many accounts of *Glyptonotus antarcticus* in the literature are the result of misidentifications and may refer to reproductively isolated species of the genus. The true distribution range of the putative species of *Glyptonotus* can therefore not be reconstructed from the literature but must refer to museum collections and newly collected material.

We therefore assume that the specimens B088, B101, B201 and B233 which were sampled at the original type locality (King George Island, South Shetlands) represent the species originally described by Eights in 1852. According to this interpretation, *Glyptonotus antarcticus* sensu strictu occurs along the Antarctic Peninsula, with two more species being present in the Eastern Weddell Sea and another in the Ross Sea. It is beyond the scope of this paper to ascertain the identity of one of the other haplotype groups (if any) with the other available names (*G. acutus* and *G. obtusus*). A revision of *Glyptonotus* based on morphological and molecular data from different genes with a formal (re)description of the species and a key is under preparation (Held, in prep.).

These results, if confirmed, also have significant ramifications outside taxonomy and biogeography because *G. antarcticus* is an abundant and conspicuous species of the high Antarctic benthos. It plays a major role as a model organism in ecological, biochemical and physiological studies (see introduction for references). If it comprises four or more species, then previous comparative studies of *G. antarcticus* could have been flawed. In particular, studies dealing with differences in adaptation to environmental parameters such as temperature and seasonality would need to be re-examined.

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