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Molecular evidence for cryptic species among the Antarctic fish *Trematomus bernacchii* and *Trematomus hansoni*

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Abstract : The notothenid species *Trematomus bernacchii* has previously been shown, by allozyme analysis, to be a complex of two cryptic species, one of which being more closely related to *T. hansoni* than to the other *T. bernacchii* cryptic species. Two *T. bernacchii* colour morphs, "white blotch" and "brown", at McMurdo Sound, may correspond to these cryptic species. In this study, we present mitochondrial DNA sequences of the 12S and 16S ribosomal regions for six "white blotch" morphs, eight "brown" morphs collected in McMurdo Sound, one individual collected off the Antarctic Peninsula, and two *T. hansoni* individuals from McMurdo Sound. These sequences were compared with those of *T. bernacchii* and *T. hansoni* in the literature. Based on 14 phylogenetically informative sequences, no differences were found between "white blotch" and "brown" morphs. Furthermore, only one substitution separated these sequences from the previously published *T. hansoni* sequence, while 10 substitutions separated them from the previously published *T. bernacchii* sequence. Misidentified specimens, and sequence misreadings may be at the origin of these discrepancies. However, the presence of cryptic species within *T. bernacchii* and *T. hansoni* is not ruled out.

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Introduction

Although the Southern Ocean represents 10% of the world's oceans, its fish fauna is small and less diverse than expected (Eastman 1993). Several hypotheses have been put forth to explain this peculiarity. At the genetic level, nucleotide substitution rates for Antarctic fishes have been shown to be minimal, possibly due to their low metabolism, thus resulting in a reduced level of genetic diversity (Bargelloni *et al.* 1994, Ritchie *et al.* 1996). At the morphological level, a contributing cause for this apparent lack of diversity might simply be due to the misidentification of species, or rather the lack of distinguishing morphological characters between closely related species, that would produce an artificially low number of species. If this were the case, several cryptic species could possibly be present within formerly recognized species.

Allozyme studies have shown that cryptic species may exist within the Antarctic notothenid *Trematomus bernacchii*. Indeed, McDonald *et al.* (1992) showed that out of 66 individuals collected in McMurdo Sound, two clades termed *T. bernacchii-1* and *T. bernacchii-2* could be distinguished at the allozyme level. These two groups represented respectively 59% and 41% of the sampled individuals. *T. bernacchii-2* was found to be more closely related to *T. hansoni* than to *T. bernacchii-1*. In McMurdo Sound, two colour morphs of *T. bernacchii* are found (McDonald *et al.* 1992) one a solid brown colour, while the other form presents a "white blotch" on its nape (Fig. 1). No other merisitic or morphometric features distinguish these two forms which, however, may correspond to the two allozyme groups. At the DNA sequence level, Ritchie *et al.* (1996) have studied several *Trematomus* species and have also concluded that *T. bernacchii* and *T. hansoni* are sister clades. However, no further analysis of the *T. bernacchii-T. hansoni* complex was done.

To determine if cryptic species might be a contributing factor to the apparent lack of species diversity in the Antarctic ichthyofauna, we have analysed the DNA sequences of 14 *T. bernacchii* from McMurdo Sound, six of the "white blotch" morph and eight of the "brown" morph. One *T. bernacchii* individual (brown morph) from the Antarctic Peninsula (off Palmer Research Station), and two *T. hansoni* from McMurdo Sound were also analysed. Previously published sequences (specimens collected in McMurdo Sound) from *T. bernacchii* (Ritchie *et al.* 1996), and *T. hansoni* (Bargelloni *et al.* 1994) were included in our analysis. A previously published sequence from *T. pennellii* (Bargelloni *et al.* 1994) was used as an outgroup.

Materials and methods

Collections and DNA extraction

In this study, we use the conservative generic name *Trematomus* for the species *bernacchii, hansoni,* and *pennellii.* A discussion about their possible inclusion in the genus *Pseudotrematomus,* as proposed by Balushkin (1984), is found in Miller (1993).

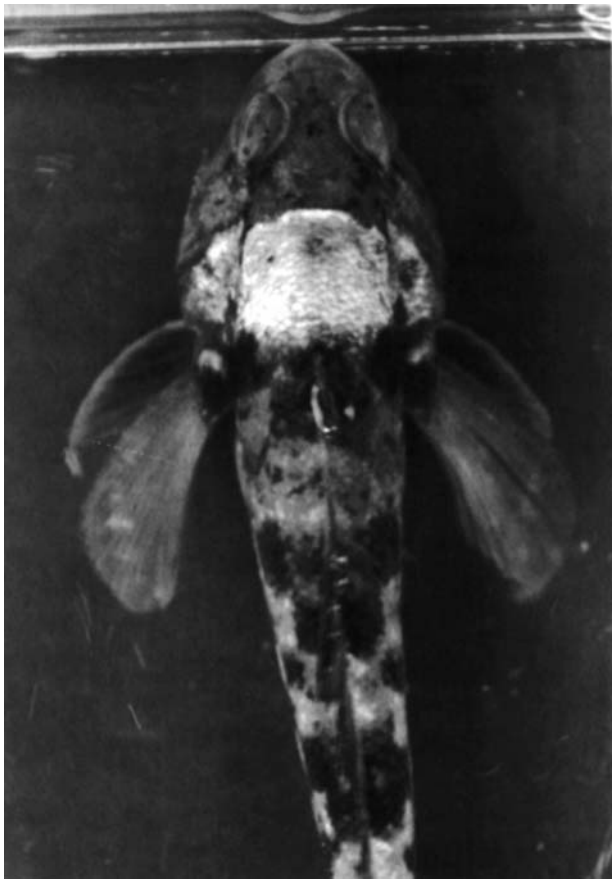

Fig. 1. Dorsal view of *Trematomus bernacchii* "white blotch" colour morph (photo Bernardi). The "white blotch" indicates the large pale patch on the nape, behind the eyes and before the pectoral fins.

Fish were collected by hook and line off McMurdo Research Station and off Palmer Research Station. Muscle tissue was extracted and preserved at -20°C until DNA extraction. Tissues were digested overnight at 55°C in 500 ml of extraction buffer (Tris 10 mM, NaCl 400 mM, EDTA 2 mM, SDS 2%, Proteinase K). The DNA was then purified by chloroform extraction and ethanol precipitation.

Polymerase chain reaction (PCR) amplification

Amplification of the mitochondrial 12S and 16S ribosomal gene regions was accomplished using the primers 12SAL and 12SBH, and 16SAR and 16SBR from Kocher *et al.* (1989). These primer sets amplify a 388bp and 531bp region of the 12S and 16S ribosomal genes respectively. The amplifications (25 µl) contained 10–100 ng of DNA, 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 2.5 units of Taq DNA Polymerase (Perkin-Elmer, Norwalk, Connecticut), 150 mM of each dNTP, and 0.3 mM of each primer, and used a cycling profile of 45 s at 94°C, 45 s at 48°C, 1 min at 72°C, for 35 cycles. Automated sequencing was performed in both directions with the primers used in the amplification using an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA).

Sequence analysis

Sequences were aligned with the aid of the computer program Clustal in Sequence Navigator (Applied Biosystems). Two sequences from the literature were included in our analysis: one sequence from *T. bernacchii* and one sequence from *T. hansoni* (Ritchie *et al.* 1996, Bargelloni *et al.* 1994). Both fish species were collected in McMurdo Sound (P. Ritchie personal communication). In order to determine the relationship between *T. hansoni* and *T. bernacchii,* all available Trematomid sequences were also used (see Ritchie *et al.* 1996, for complete list of sequences). The *T. pennellii* sequence from the literature (Bargelloni *et al.* 1994) was used as an outgroup (following Ritchie 1996). Phylogenetic relationships were assessed using the cladistic Maximum Parsimony (MP) method and the phenetic Neighbor-Joining (NJ) method implemented by the software package PAUP (Phylogenetic Analyses Using Parsimony, version 4.0.d54). Statistical confidence was evaluated using 2000 bootstrap replicates (Felsenstein 1985, Hedges 1992).

Results

Sequences

Out of the 919 aligned base pairs (388 for the 12S region, 531 for the 16S region), 30 were variable, and 14 were phylogenetically informative (Fig. 2). All 14 new *T. bernacchii* sequences from McMurdo individuals were identical regardless of their morphotype ("white blotch" or "brown"). The sequence obtained from the *T. bernacchii* collected at the Antarctic Peninsula differed from these 14 sequences by one substitution. Only one substitution separated both these sequences from the published *T. hansoni* sequence, while 10 substitutions separated them from the published *T. bernacchii* sequence. None of these 10 substitutions were shared with the peninsular *T. bernacchii.* The two *T. hansoni* new sequences presented here differed from each other by one substitution and differed from the *T. bernacchii* clade by an average 13 substitutions (Fig. 3).

Phylogenetic relationships

A single 30 steps (Consistency Index 1.0) most parsimonious tree was obtained as shown in Fig. 3. This tree was topologically identical to the tree obtained using the Neighbor-Joining method. The 14 new McMurdo *T. bernacchii* sequences, the previously published *T. bernacchii* and *T. hansoni* sequences, as well as the *T. bernacchii* sequence from the Antarctic Peninsula formed a robust clade supported

		12S Region	16S Region	Source
		222222333	4555556666667777888	
		1224022689006	3445780112471344427 7	
		0019346165575	3497981195209767852 3	
T. bernacchii				
McMurdo	A1	TTAAATAGACAGG	GCAAATATTAGAGATTTAAC	(1)
	A2	. . . . .	. . . . .	(1)
	A3	. . . . .	. . . . .	(1)
	A4	. . . . .	. . . . .	(1)
	A5	. . . . .	. . . . .	(1)
	A6	. . . . .	. . . . .	(1)
	B1	. . . . .	. . . . .	(1)
	B2	. . . . .	. . . . .	(1)
	B3	. . . . .	. . . . .	(1)
	B4	. . . . .	. . . . .	(1)
	B5	. . . . .	. . . . .	(1)
	B6	. . . . .	. . . . .	(1)
	B7	. . . . .	. . . . .	(1)
	B8	. . . . .	. . . . .	(1)
Palmer	1	. . . . .	. . . . . A	(1)
"T. hansoni"		. . . . .	. . . . . T	(2)
"T. bernacchii"		AGG C	C . TG . . . . . C . . AG	(3)
T. hansoni 1		. . . . TCGA . T . A .	. T . . GC . C . . . . . T	(1)
T. hansoni 2		. . . . TCGA . . . A .	. T . . GC . C . . . . . T	(1)
T. pennellii		. . . T . CGAG . CA .	. . . . GCG . C . . GG . - - - CGT	(2)

Fig. 2. Aligned nucleotide sequences from the 12S and 16S regions. Only variable positions are shown. Points (.) correspond to identical nucleotides. Dashes (-) correspond to insertions/deletions. Numbers above the sequences correspond to the substitution positions. *Trematomus bernacchii* sequences from McMurdo are indicated as McMurdo A, for the "white blotch" morph, and B for the "brown" morph. Sequences labelled (1) are from this study, (2) are from Bargelloni *et al.* (1994), and (3) are from Ritchie *et al.* (1996).

by 100% of the bootstrap replicates. Sequences from this clade also contained a unique 3 base pair insertion in the 16S region (Fig. 2), as compared to the other analysed taxa (and all other Trematomids, not shown).

The two new McMurdo Sound *T. hansoni* sequences formed a robust clade (95% of the boostrap replicates). When using all available Trematomid sequences, *T. hansoni* was found to be the sister group of *T. bernacchii*, although only weakly supported by the bootstrap analysis (67% of the replicates, not shown).

Discussion

T. bernacchii *morphotypes*

T. bernacchii morphotypes ("white blotch" and "brown") were not found to be different at the 12S and 16S region level. PCR contamination was ruled out after running several negative and positive controls, so the absence of difference was not due to a technical artifact. It is, therefore, unlikely that one morphotype is more closely related to *T. hansoni* than the other morphotype, as McDonald *et al.*'s (1992) data indicates. However, since the mitochondrial regions used in this study are conserved (Meyer 1993), it may be possible to find genetic differences between "white blotch" and "brown" morphotypes, in other, more variable regions, such as mitochondrial control regions or nuclear microsatellites.

T. bernacchii *from the Antarctic Peninsula and other sequences*

One substitution difference was found between the *T. bernacchii* individual sampled off the Antarctic Peninsula and the individuals from McMurdo Sound. This one substitution was carefully checked by repeated sequencing on both strands and will be the subject of further investigation as it may reveal population differences.

Ten substitutions were found between the *T. bernacchii* sequence of Ritchie *et al.* (1996) and our McMurdo sequences. All these substitutions were apomorphies, some of them were clustered and were close to the ends of the sequenced regions, which suggest that the differences might in part be attributable to sequencing errors. It is also possible that different haplotypes are found in McMurdo Sound and were not represented in our relatively small sample size.

T. hansoni *sequences*

The *T. hansoni* sequence of Bargelloni *et al.* (1994) was found to be close to our *T. bernacchii* sequences (one substitution difference) and may have resulted from the misidentification of a specimen. Indeed, our new sequences from two large adult *T. hansoni* were shown to be closely related to each other (one substitution) and were placed as the sister group of *T. bernacchii* as was previously suggested by allozyme data (McDonald *et al.* 1992).

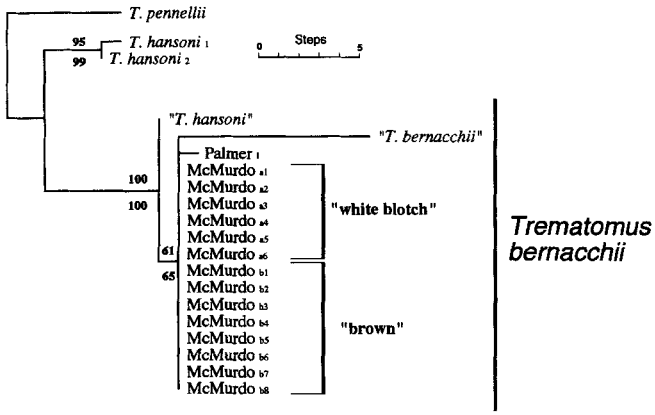

Fig. 3. Single most parsimonious tree based on the 12S and 16S ribosomal regions (identical topology was obtained using the Neighbor-Joining Method). *T. pennellii* was used as an outgroup. Samples of *T. bernacchii* caught off McMurdo Station and Palmer Station are labelled McMurdo and Palmer, respectively. Samples of *T. bernacchii* within the "white blotch" morph are labelled a#, samples in the "brown" morph are labelled b#. The sample labelled "*T. bernacchii*" is from Ritchie *et al.* 1996. Samples labelled "*T. hansoni*", and *T. pennellii* are from Bargelloni *et al.* 1994. Scale bar corresponds to 5 substitutions. Numbers above nodes correspond to percent bootstrap replicates out of 2000 replicates for Maximum Parsimony, numbers under the nodes correspond to percent bootstrap replicates out of 2000 replicates for the Neighbor-Joining analysis. Branches are drawn according to the number of inferred substitutions.

Conclusion

Our study reveals problems and raises several questions about the evolutionary biology of Antarctic fishes. As shown in Fig. 3, it is possible that one previously published sequence (*T. hansoni*) may have been derived from misidentified specimens, and that a previously published *T. bernacchii* sequence contained a few misreadings (as shown by an unusually long branch length in Fig. 3). As mentioned above, Antarctic fish are difficult to identify, particularly at the juvenile stage, and extreme care should be taken in using young individuals. In the case of sequencing errors, most of the time these turn out to be apomorphies and result in unusual branch lengths but rarely alter phylogenetic relationships. However, in a system that is sensitive to substitution rates, such as the Antarctic fish system, where various hypotheses have been proposed to explain unusual rates of evolution (Bargelloni *et al.* 1994), particular care should also be taken in checking every substitution that is encountered.

At this point, one may wonder if there are any cryptic species in the *T. bernacchii–T. hansoni* complex. Two forms of *T. bernacchii* have been found at the allozyme level, one of these forms being more closely related to *T. hansoni* than to the other *T. bernacchii* form (McDonald *et al.* 1992). These fish were collected from the same locality as our own fish, yet fish comprised two different allozyme forms. However, none of the sequences in our "*T. bernacchii*" clade were analogous to the *T. bernacchii* form observed by McDonald *et al.* (1992). Thus two possibilities remain: we either missed this form in our sampling, or this form is a misidentified *T. hansoni*. Assuming that the relative abundance of the two morphs has not changed with time, it is unlikely that one form was missed as indicated by a Chi-square test ($\chi^2 = 20.20$, $P < 0.001$). The possibility of a misidentification, on the other hand, is difficult to rule out. We have found that the sequences from the two *T. hansoni* in this study differ from one another by one substitution. This level of divergence is considerable for regions such as 12S and 16S ribosomal genes that are usually regarded as very conserved (Meyer 1993). It is reasonable to think that within *T. hansoni* sampled in McMurdo Sound, there may be differences at the allozyme level (reflected by the divergence at the 12s and 16S level) that may have previously been interpreted as divergence between *T. hansoni* and a form of *T. bernacchii*.

In conclusion, the presence of cryptic species within the *T. bernacchii–T. hansoni* complex cannot be ruled out. Colour morphs in *T. bernacchii* do not correspond to previously suggested cryptic species. Further studies should determine if the genetic divergences between *T. bernacchii* from McMurdo Sound and the Antarctic Peninsula, and between *T. hansoni* individuals within McMurdo Sound are indicative of the presence of diverse populations or cryptic species.

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