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Molecular taxonomy and identification within the Antarctic genus *Trematomus* (Notothenioidei, Teleostei): How valuable is barcoding with COI?

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Abstract

The Trematominae are a particularly interesting subfamily within the antarctic suborder Notothenioidei (Teleostei). The 14 closely related species occupy a large range of ecological of niches, extremely useful for evolutionary and biogeography studies in the Antarctic Ocean. But some *Trematomus* species can be difficult to identify by using morphological criteria, specially young stages and damaged specimens. Molecular identification would therefore be highly useful, however the suitability of the cytochrome oxidase I gene in a barcoding approach needs to be assessed. We evaluated species delineation within the genus *Trematomus* comparing morphological identification, nuclear markers (the rhodopsin retrogene and a new nuclear marker pkd1: polycystic kidney disease 1) and COI. We show that *Trematomus vicarius* is not distinguishable from *Trematomus bernacchii* with the molecular markers used, and neither is *Trematomus loennbergii* from *Trematomus lepidorhinus*. We suggest that until this is investigated further, studies including these species list them as *T. loennbergii/T. lepidorhinus* group, and keep voucher samples and specimens. Generally, COI gives a congruent result with the rhodopsin retrogene, and except for the previously cited species pairs, COI barcoding is efficient for identification in this group. Moreover pkd1 might not be suitable for a phylogenetic study at this scale for this group.

Keywords: Barcode; Molecular taxonomy; Southern Ocean; *Pagothenia*

1. Introduction

The highly endemic suborder Notothenioidei (Teleostei) is one of the most successful groups in the freezing waters of the Southern Ocean (Ritchie et al., 1996), representing 35% of the "'fish" species and 90% of the biomass on the continental shelf (De Witt,

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1971; Eastman and Clarke, 1998; Kock and Jones, 2005).

The subfamily Trematominae (Nototheniidae) is central to our understanding of the coastal Antarctic ecosystem, although it contains only 14 species. Genus *Trematomus* includes 11 of them, two are in genus *Pagothenia* (Sanchez et al., 2007; Kuhn and Near, 2009). A recently described species, *Cryothenia amphitreta*, appears to also belong within the subfamily (Cziko and Cheng, 2006). *Trematomus* species occupy a large range of ecological niches, and are known for their high diversity and plasticity in habitat distribution (Eastman, 1993). Thus, they are particularly interesting notothenioids and they could be an extremely useful genus for evolutionary and biogeography questions in the Antarctic.

However ecologists working on these species are hindered by identification problems (Koubbi pers. com.). In fact, several *Trematomus* species are very similar morphologically. For instance, *Trematomus lepidorhinus* and *Trematomus loennbergii* only differ by the presence or absence of scales on the lower jaw and preorbital, respectively (De Witt et al., 1993). *Trematomus vicarius* and *Trematomus bernacchii* have many features in common up to number of pores on the supratemporal canal, which is usually a diagnostic character for the group (De Witt et al., 1993; Fisher and Hureau, 1987). They only differ by their lateral scale series (Norman, 1938). Some species like *Trematomus newnesi* exhibit a certain degree of phenotypic plasticity (Eastman and DeVries, 1997).

Moreover, some types of samples used in ecological studies present additional identification problems. Generally, stomach contents are very damaged and therefore impossible to identify precisely. In the case of fragmented animals, only certain parts such as heads or eyes can be used for identification (Brenner et al., 2001). Also, ontogenetic changes during the larval stages of these species hinder their identification, and require a considerable time and effort even from an experienced taxonomist. Teleost larvae identification is based on observation of pigments and morphology under microscope (Koubbi et al., 2007). Many species of nototheniids look remarkably similar at young stages which may lead to confusions (Rock et al., 2008). The confirmation of larval identification by laboratory spawning experiments (Webb et al., 2006) is generally impossible to perform for these groups. Last, larvae are easily damaged and vouchers are often not in an optimal state for morphological identification as many conservation media degrade pigments.

Wrong identifications could mislead our views of speciation, diversity, phylogeny, niche partitioning, and many other features of ecosystems. Nevertheless, a large proportion of the studies in ecology (62.5% in 80 papers) lack supporting information justifying or guaranteeing the correct identification of the organisms studied or manipulated (Bortolus, 2008). Ecologists need a reliable tool to avoid all these problems. The molecular barcode and the Barcoding of Life Initiative have been suggested as a tool for precise identification of specimens. Barcoding is already a fast technique, and as the efficiency of molecular tools continues to increase at a rapid pace, it will become even more so. This method relies on the amplification and sequencing of a short standardized region of the mitochondrial genome, and the comparison of the specimen sequence to a multispecies database of reference sequences. When successful, this allows assignment of the specimen to an already known species, genus or family (Hebert et al., 2003a,b). A fragment (655 bp) of the mitochondrial gene coding for cytochrome C oxidase subunit 1 (COI) has been selected as a universal marker. Reference sequences of COI are available in the Barcode of Life Database (BOLD) (http://barcoding.si.edu/) in open access. Ultimately, the goal is to have all species represented by sequences from multiple specimens in the database.

Since the publications of Hebert et al. (2003a,b), COI has been used among others to identify Australian marine fishes (Ward et al., 2005), North American birds (Hebert et al., 2003b), insects (tropical Lepidoptera; Hajibabaei et al., 2006) or primates (Lorenz et al., 2005). Pegg et al. (2006) confirmed the utility of barcoding to identify fish larvae from Australian waters, and it is being implemented for the identification of stomach contents (Suzuki et al., 2008), so testing it on *Trematomus* is interesting. Despite this, the barcoding approach is still much debated (De Salle et al., 2005; Dasmahapatra and Mallet, 2006; Rubinoff et al., 2006; De Salle, 2006; Buhay, 2009) and might not be applicable in all cases and all groups (see the list in Dasmahapatra and Mallet, 2006). For a successful molecular identification, all the specimens from a given species must cluster together in the analysis (unique COI clusters for each species, Steinke et al., 2009). However, sometimes our knowledge of the species boundaries is faulty. In these cases, no successful identification can be performed, as the clustering and the previous knowledge will be in conflict. Investigating the validity of the species is thus the first step. However, no single marker is enough to evaluate this isolation (De Salle et al., 2005; Rubinoff

et al., 2006), and the results for mitochondrial markers like COI must be compared to one, or better, several nuclear markers. The mitochondrial COI gene is only inherited through females; events such as hybridization and introgression cannot always be detected. We therefore decided to test the congruence of nuclear and mitochondrial species delineation in the genus *Trematomus*, as a previous study (Kuhn and Near, 2009) had failed to recover monophyletic *Trematomus* species using S7, a nuclear marker.

However, the use of nuclear genes is not devoid of problems; they evolve on average much slower than the mitochondrial genes. Thus most are not variable enough at such a small scale, and they must be chosen very carefully. We retained two nuclear markers: the first one is the retrogene of the rhodopsin (Bellingham et al., 2003) as this marker has already been used to study nototheniid relationships (Sanchez et al., 2007). The second one is the pkd1 gene (polycystic kidney disease 1). This last marker had never been used before for either phylogenetics or molecular taxonomy, but is present in single copy in all the available complete teleost genomes, is easily amplifiable for a wide notothenioid sampling, and is expected to be more variable than the rhodopsin retrogene.

The efficiency of identification also depends very much on the completeness of the database: when a sequence highly similar to the sequence of interest is not present in the database (Webb et al., 2006). At present, BOLD contains 73 COI sequences for the genus *Trematomus*, representing 10 species (on September 2009).

Finally, COI variability is a problem in some groups. In fact, species identification is based on the prerequisite that intra-specific variability should always be much smaller than the inter-specific variability. This has been shown in case studies for numerous groups (in Annelida, Arthropoda, Chordata, Echinodermata, Mollusca, Nematoda, Platyhelminthes, Hebert et al., 2003c) but exceptions have been found in several groups (in Cnidarians, Hebert et al., 2003c; in marine gastropods, Meyer and Pauley, 2005; in sponges, Park et al., 2007). The limit between the values for inter-specific divergences and intra-specific divergences has been suggested to be around 2% divergence among COI sequences or 3.5% for "fishes" (Ward et al., 2009) but the value of this threshold is debated, as well as the relevance of even using a threshold (Meyer and Pauley, 2005; De Salle et al., 2005; De Salle, 2006; Rubinoff et al., 2006; Hickerson et al. 2006; Dasmahapatra and Mallet, 2006; Buhay, 2009).

The aim of this work is first to explore the species delineations in the genus *Trematomus*. This will allow to improve the estimation of the inter- and intra-specific variabilities in the genus, and to check that the conditions for a good identification by barcoding are present, with both methods available on the BOL website: species clusters on a distance tree and using the divergence rates between DNA sequences.

2. Materials and methods

2.1. Taxon sampling and morphological identifications

There are only two studies including multiple specimens per species on Trematominae barcode or molecular taxonomy. The sampling of these studies is very limited concerning the number of species as well as the number of individuals per species, causing a lack of reliability of the results for such a recent species separation (Ritchie et al., 1996). The study of Rock et al. (2008) on Antarctic fish barcoding concerns only seven of the species, and very few specimens for each. The richest study is from Kuhn and Near (2009), and includes most species including the recently described *C. amphitreta*, with the number of specimens varying from one to seven depending on species. However, this study only involves sequences from the mitochondrial markers ND2 and 16S rDNA, and consequently does not provide information on the relevance of the COI.

Our study includes the largest number of representatives per species published yet (Table 1). 220 specimens have been included in this work representing 12 species. As in Near et al. (2004) study, this sampling also includes individuals representing distant geographic areas whenever possible, collected during the ICOTA, ICEFISH 2004, CEAMARC and EPOS 1989 cruises. Several individuals come from Weddell Sea (1), Terre Adélie (2), South Georgia (3) and Terra Nova Bay (4) (Fig. 1). This probably optimizes the representativity of the intra-specific divergence, and would allow to check whether individuals identified as belonging to the same species can be differentiated according to their geographical origin (see Table 1). However, most specimens were collected during the recent CEAMARC (Collaborative East Antarctic MARine Census) cruises (see Table 1). All of the CEAMARC specimens and a few from previous campaigns were kept as vouchers. To test the possibility of using COI for egg identification, we have included in the sampling eggs collected in a sponge during the CEAMARC cruise. A preliminary

Table 1
Specimens included in this study. Specimen information, GenBank and BOLD Accession numbers are listed. 1 = Weddell Sea, 2 = Terre Adélie, 3 = South Georgia and 4 = Terra Nova Bay.

Species	Local tag	Zone of capture	Latitude	Longitude	Depth (m)	Voucher number	BOLD accession Nb COI	GenBank accession Nb pkd1	GenBank accession Nb rhodopsin
Pagothenia borchgrevinki	TA219PAB03	2							GU997239
	TA263PABO1	2	−66.665	139.994	40	MNHN 2002-1711	EATF594-10		GU997240
	TA391	2	−66	140		MNHN 2009-0678	EATF596-10	GU997453	GU997241
	TA392	2					GU997389		GU997242
	TA537PAB1	2					GU997390		GU997243
	TA537PAB2	2					GU997391		GU997244
	TA568PAB1	2					GU997392		GU997245
	TA568PAB2	2					GU997393		GU997246
	TA568PAB3	2					GU997394		GU997247
	TA582PABO1	2					GU997395	GU997454	GU997248
	TA582PABO4	2					GU997396		GU997249
	TA582PABO6	2					GU997397	GU997455	GU997250
	TA582PABO7	2					GU997398		GU997251
	TA582PABO8	2					GU997399		GU997252
	TA593PABO1	2					GU997400		GU997253
Trematomus bernacchii	Bern7NS	2					GU997401		GU997254
	Bern8NS	2					GU997402	GU997456	GU997255
	Bern8S	2						GU997457	GU997256
	Bern9S	2					GU997403	GU997458	GU997257
	TA20	2					GU997404	GU997459	GU997258
	TA21	2					GU997405	GU997460	GU997259
	TA22	2					GU997406	GU997461	GU997260
	TA126	2					GU997407	GU997462	GU997261
	TA650BE1	2					GU997408		GU997262
	TA657BE3	2					GU997409		GU997263
	TA657BE4	2					GU997410	GU997463	GU997264
	TA657BE5	2					GU997411	GU997464	GU997265
	TA657BE6	2							GU997266
	si126n786	2	−66.553653	142.636368	139	MNHN 2009-1244	EATF125-10		GU997267
	si350n2559	2	−66.559853	140.797323	361	MNHN 2009-1310	EATF345-10	GU997466	GU997269
	si351n2560	2	−66.559853	140.797323	361	MNHN 2009-1311	EATF346-10	GU997467	GU997270
	si352n2561	2	−66.559853	140.797323	361	MNHN 2009-1312	EATF347-10	GU997468	GU997271
Tremaomus eulepidotus	si46n	2	−66.3202	143.649	570	MNHN 2009-1354	EATF046-10		
	si48n369	2	−66.3202	143.649	570	MNHN 2009-1359	EATF048-10		
	si94n566	2	−66.308813	142.29392	217	MNHN 2009-1374	EATF093-10		GU997274
	si108n666	2	−66.534813	141.982677	520	MNHN 2009-1229	EATF107-10		GU997275
	si110n656	2	−66.534813	141.982677	520	MNHN 2009-1231	EATF109-10	GU997473	GU997276
	si111n657	2	−66.534813	141.982677	520	MNHN 2009-1232	EATF110-10		
	si112n658	2	−66.534813	141.982677	520	MNHN 2009-1233	EATF111-10		GU997277
	si113n659	2	−66.534813	141.982677	520	MNHN 2009-1234	EATF112-10		GU997278
	si114n660	2	−66.534813	141.982677	520	MNHN 2009-1235	EATF113-10	GU997476	GU997279

	si115n661	2	−66.534813	141.982677	520	MNHN 2009-1236	EATF114-10		
	si116n662	2	−66.534813	141.982677	520	MNHN 2009-1237	EATF115-10		
	si117n663	2	−66.5348	141.983	520.4	MNHN 2009-1238	EATF116-10	GU997478	GU997281
	si118n664	2	−66.534813	141.982677	520	MNHN 2009-1239	EATF117-10		
	si119n665	2	−66.534813	141.982677	520	MNHN 2009-1240	EATF118-10		
	si216n1665	2	−66.539917	145.290892	403	MNHN 2009-1263	EATF211-10		GU997283
	si217n1688	2	−66.5399	145.291	403.5	MNHN 2009-1264	EATF212-10		
	si218n1689	2	−66.539917	145.290892	403	MNHN 2009-1265	EATF213-10	GU997480	
	si258n2025	2	−65.869947	143.001547	430	MNHN 2009-1271	EATF253-10		
	si259n2026	2	−65.869947	143.001547	430	MNHN 2009-1272	EATF254-10		
	si260n2027	2	−65.869947	143.001547	430	MNHN 2009-1274	EATF255-10		
	si288n2184	2	−65.912427	143.966988	370	MNHN 2009-1286	EATF283-10		
	si289n2185	2	−65.912427	143.966988	370	MNHN 2009-1287	EATF284-10	GU997485	GU997284
	si330n2440	2	−66.335097	141.272662	207	MNHN 2009-1298	EATF325-10	GU997486	GU997285
	si331n2441	2	−66.335097	141.272662	207	MNHN 2009-1299	EATF326-10	GU997487	GU997286
	si332n2442	2	−66.335097	141.272662	207	MNHN 2009-1300	EATF327-10		GU997287
	si342n2496	2	−66.5618	141.262	176.9	MNHN 2009-1391	EATF337-10	GU997489	GU997288
	si346n2530	2	−66.563722	141.255738	170	MNHN 2009-1307	EATF341-10		GU997289
	si347n2531	2	−66.563722	141.255738	170	MNHN 2009-1308	EATF342-10		GU997290
	si348n2532	2	−66.563722	141.255738	170	MNHN 2009-1309	EATF343-10		GU997291
	si363n2624	2	−66.516823	140.001423	176	MNHN 2009-1314	EATF358-10		
	si364n2625	2	−66.516823	140.001423	176	MNHN 2009-1315	EATF359-10		
	si365n2626	2	−66.516823	140.001423	176	MNHN 2009-1316	EATF360-10	GU997493	GU997292
	si366n2627	2	−66.516823	140.001423	176	MNHN 2009-1317	EATF361-10		GU997293
	si367n2628	2	−66.516823	140.001423	176	MNHN 2009-1318	EATF362-10		GU997294
	si426n2949	2	−66.148263	140.649927	213	MNHN 2009-1338	EATF421-10		GU997295
	si427n2950	2	−66.148263	140.649927	213	MNHN 2009-1339	EATF422-10		
	si428n2951	2	−66.148263	140.649927	213	MNHN 2009-1340	EATF423-10	GU997498	GU997296
	si429n2952	2	−66.148263	140.649927	213	MNHN 2009-1341	EATF424-10		GU997297
	si430n2953	2	−66.148263	140.649927	213	MNHN 2009-1343	EATF425-10		
	si487n3152	2	−66.1691	139.932	149.9	MNHN 2009-1358	EATF480-10		
	si490n3229	2	−65.9894	139.995	192.1	MNHN 2009-1360	EATF482-10		
	si491n3189	2	−65.989378	139.994898	192	MNHN 2009-1361	EATF483-10		
	W61	1	−75.217	−27.017	280	MNHN 1990-1371	EATF584-10		
	W60	1	−75.217	−27.017	280	MNHN 1990-1370	EATF585-10		GU997298
Trematomus hansoni	1F300	1						GU997515	
	si109n717	2	−66.534813	141.982677	520	MNHN 2009-1230	EATF108-10		GU997299
	TA19	2					GU997412	GU997503	GU997300
	TA60	2					GU997413		GU997301
	TA61	2					GU997414	GU997505	
	TA101	2	−66.667	140.017	25	MNHN 1962-1037	EATF595-10	GU997506	GU997303
	TA346TRHA1	2					GU997415	GU997507	GU997304
	TA388	2					GU997416		GU997305
	TA606HA1	2					GU997417	GU997508	GU997306
	TA646HA1	2					GU997418	GU997509	GU997307

(*continued on next page*)

Table 1 (*continued*)

Species	Local tag	Zone of capture	Latitude	Longitude	Depth (m)	Voucher number	BOLD accession Nb COI	GenBank accession Nb pkd1	GenBank accession Nb rhodopsin
	TA651HA1	2					GU997419	GU997510	GU997308
	TA651HA2	2					GU997420	GU997511	GU997309
	TH1	3					GU997421		GU997310
	TH2	3					GU997422	GU997514	GU997311
	TNB244	4	−74.717	164.133	143−174	MNHN 1999-0388	EATF586-10	GU997512	GU997312
	TNB248	4					GU997423	GU997513	GU997313
	W162	1	−71.1	−12.567	499−515	MNHN 1990-1327	EATF587-10	GU997516	
Trematomus tokarevi	si171n1296	2	−66.750233	145.534688	526	MNHN 2009-1250	EATF169-10	GU997591	
	si396n2711	2	−66.38878	140.428852	791	MNHN 2009-1333	EATF391-10	GU997592	
	si447n3011	2	−66.338398	140.02921	510	MNHN 2009-1345	EATF442-10	GU997593	GU997387
	si481n310	2	−66.1706	139.353	673.5	MNHN 2009-1357	EATF474-10		
Trematomus newnesi	1036	2					GU997431	GU997570	GU997348
	TA50TRNE1	2					GU997428	GU997563	GU997349
	TA355TRNE3	2					GU997429	GU997564	GU997350
	TA355TRNE4	2					GU997430	GU997565	GU997351
	TA390TRNE11	2					GU997432	GU997566	GU997352
	TA398TRNE13	2					GU997433	GU997567	GU997353
	TA399TRNE7	2					GU997434	GU997568	GU997354
	TA403TRNE2	2					GU997569	GU997355	
	si542n2570	2	−66.5599	140.797	360.9	MNHN 2009-1369	EATF533-10		
Trematomus lepidorhinus	1965								GU997318
	1156 805								GU997316
	1368	1					GU997424	GU997518	GU997317
	si26n197	2	−66.00264	142.9521	465	MNHN 2009-1281	EATF026-10		
	si47n302	2	−66.0039	143.716	425.6	MNHN 2009-1356	EATF047-10	GU997522	GU997321
	si85n492	2	−66.3357	143.036	683.6	MNHN 2009-1372	EATF085-10		
	si120n724	2	−66.534813	141.982677	520	MNHN 2009-1241	EATF119-10	GU997524	GU997322
	si121n725	2	−66.534813	141.982677	520	MNHN 2009-1242	EATF120-10		GU997323
	si122n726	2	−66.534813	141.982677	520	MNHN 2009-1243	EATF121-10	GU997526	GU997324
	si164n1122	2	−66.7505	143.95	640.9	MNHN 2009-1247	EATF162-10		
	si197n1485	2	−66.54375	143.990627	787	MNHN 2009-1254	EATF194-10	GU997531	
	si198n1486	2	−66.54375	143.990627	787	MNHN 2009-1255	EATF195-10		
	si204n1575	2	−66.738715	144.307023	904	MNHN 2009-1257	EATF201-10	GU997532	GU997329
	si205n1576	2	−66.738715	144.307023	904	MNHN 2009-1258	EATF202-10	GU997533	GU997330
	si206n1577	2	−66.738715	144.307023	904	MNHN 2009-1259	EATF203-10	GU997534	GU997331
	si211n1618	2	−66.538527	144.972508	441	MNHN 2009-1261	EATF208-10	GU997535	GU997332
	si242n1922	2	−66.318845	143.63217	566	MNHN 2009-1268	EATF237-10	GU997536	
	si246n1945	2	−66.315523	143.301408	693	MNHN 2009-1269	EATF241-10	GU997537	
	si261n2028	2	−65.869947	143.001547	430	MNHN 2009-1275	EATF256-10	GU997538	GU997333
	si262n2029	2	−65.869947	143.001547	430	MNHN 2009-1276	EATF257-10	GU997539	GU997334
	si263n2030	2	−65.869947	143.001547	430	MNHN 2009-1277	EATF258-10	GU997540	GU997335
	si267n2062	2	−65.823	142.955	774.9	MNHN 2009-1278	EATF262-10		

	si268n2064	2	−65.823045	142.955393	775	MNHN 2009-1279	EATF263-10		
	si343n2497	2	−66.561803	141.261932	177	MNHN 2009-1306	EATF338-10	GU997543	
	si360n2569	2	−66.5599	140.797	360.9	MNHN 2009-1313	EATF355-10	GU997544	GU997336
	si431n2967	2	−66.1483	140.65	213	MNHN 2009-1399	EATF426-10		
	si453n3023	2	−66.338398	140.02921	510	MNHN 2009-1350	EATF448-10	GU997558	GU997345
	si454n3024	2	−66.338398	140.02921	510	MNHN 2009-1351	EATF449-10	GU997559	GU997346
	TNB238	4					GU997425	GU997520	GU997320
	W96	1	−74.667	−29.517	602	MNHN 1991-5982	EATF589-10		
Trematomus loennbergii	139						GU997426		GU997314
	427	2					GU997427	GU997517	GU997315
	TA63	2	−66.033	139.85	290	MNHN 1996-0325	EATF590-10	GU997519	GU997319
	TA80	1	−66.033	139.833	450	MNHN 1996-0326	EATF591-10		
	si23n123	2	−66.013913	142.715945	433	MNHN 2009-1267	EATF023-10		
	si137n860	2	−66.549847	142.958825	867	MNHN 2009-1245	EATF136-10	GU997527	GU997325
	si159n923	2	−66.570203	143.377362	810	MNHN 2009-1246	EATF158-10		GU997326
	si168n1264	2	−67.046928	145.15082	1267	MNHN 2009-1248	EATF166-10	GU997529	
	si170n1265	2	−67.046928	145.15082	1267	MNHN 2009-1249	EATF168-10		GU997327
	si174n1339	2	−66.750233	145.534688	526	MNHN 2009-1251	EATF172-10	GU997530	GU997328
	si381n2697	2	−66.38878	140.428852	791	MNHN 2009-1322	EATF376-10		
	si382n2698	2	−66.38878	140.428852	791	MNHN 2009-1323	EATF377-10		GU997337
	si383n2699	2	−66.38878	140.428852	791	MNHN 2009-1324	EATF378-10		
	si384n2700	2	−66.38878	140.428852	791	MNHN 2009-1325	EATF379-10	GU997548	GU997338
	si385n2701	2	−66.38878	140.428852	791	MNHN 2009-1326	EATF380-10		
	si386n2702	2	−66.38878	140.428852	791	MNHN 2009-1327	EATF381-10	GU997550	GU997339
	si387n2703	2	−66.38878	140.428852	791	MNHN 2009-1328	EATF382-10	GU997551	GU997340
	si388n2704	2	−66.38878	140.428852	791	MNHN 2009-1329	EATF383-10	GU997552	GU997341
	si398n2696	2	−66.38878	140.428852	791	MNHN 2009-1334	EATF393-10	GU997553	
	si450n3021	2	−66.338398	140.02921	510	MNHN 2009-1347	EATF445-10	GU997555	GU997342
	si451n3022	2	−66.338398	140.02921	510	MNHN 2009-1348	EATF446-10	GU997556	GU997343
	si452n3027	2	−66.338398	140.02921	510	MNHN 2009-1349	EATF447-10	GU997557	GU997344
	si461n3064	2	−66.403927	139.794363	903	MNHN 2009-1353	EATF455-10	GU997560	
	si473n3095	2	−66.17064	139.353133	683	MNHN 2009-1355	EATF467-10	GU997561	GU997347
Trematomus nicolai	1369wed002	1					GU997438		
	NI2	2					GU997439	GU997573	GU997357
	NI5	2					GU997440	GU997574	GU997358
	NI6	2					GU997441	GU997575	GU997359
	TA222TRNI1	2					GU997435	GU997571	GU997360
	TA619TRNI1	2					GU997436		
	TNB214	4					GU997437	GU997572	GU997356
Trematomus pennellii	TA42TRPE1	2					GU997442	GU997583	
	TA657PE2	2					GU997443		GU997362
	TA657PE22	2							GU997363
	TA657PE23	2					GU997444		GU997364
	TA657PE24	2					GU997445	GU997584	GU997365
	TA657PE25	2						GU997585	GU997366
	TA1998	2					GU997446	GU997577	GU997361

(*continued on next page*)

Table 1 (*continued*)

Species	Local tag	Zone of capture	Latitude	Longitude	Depth (m)	Voucher number	BOLD accession Nb COI	GenBank accession Nb pkd1	GenBank accession Nb rhodopsin
	si95n563	2	−66.308813	142.29392	217	MNHN 2009-1375	EATF094-10		GU997367
	si96n612	2	−66.308813	142.29392	217	MNHN 2009-1376	EATF095-10		GU997368
	si97n614	2	−66.308813	142.29392	217	MNHN 2009-1377	EATF096-10		
	si98n615	2	−66.308813	142.29392	217	MNHN 2009-1378	EATF097-10		
	si333n2444	2	−66.335097	141.272662	207	MNHN 2009-1301		GU997581	GU997369
	si334n2445	2	−66.335097	141.272662	207	MNHN 2009-1302			GU997370
	si340n2443	2	−66.335097	141.272662	207	MNHN 2009-1304		GU997582	GU997371
	W40	1	−71.267	-13.067	186	MNHN 1991-0563	EATF592-10	GU997576	GU997372
Eggs	si494n3235	2				MNHN 2009-1362	EATF486-10		
Trematomus vicarius	1213	3	−54.30183	−37.4217		SAIAB75107	EATF588-10	GU997595	GU997388
Trematomus scotti	867						GU997447		GU997373
	1371	1					GU997448		GU997374
	SCO1	2					GU997449	GU997586	GU997375
	SCO2	2					GU997450		GU997376
	SCO3	2					GU997451		GU997377
	si2n27	2	−66.052413	142.763643	452	MNHN 2009-1289	EATF002-10		
	si17n78	2	−66.052413	142.763643	452	MNHN 2009-1252	EATF017-10		
	si18n98	2	−66.052413	142.763643	452	MNHN 2009-1253	EATF018-10		GU997379
	si19n99	2	−66.0081	142.685	432.8	MNHN 2009-1256	EATF019-10		GU997380
	si20n100	2	−66.052413	142.763643	452	MNHN 2009-1260	EATF020-10	GU997587	
	si21n101	2	−66.052413	142.763643	452	MNHN 2009-1266	EATF021-10		GU997381
	si31n232	2	−66.000458	143.297105	473	MNHN 2009-1293	EATF031-10		GU997382
	si32n233	2	−66.000458	143.297105	473	MNHN 2009-1297	EATF032-10		
	si33n223	2	−66.000458	143.297105	473	MNHN 2009-1303	EATF033-10		
	si37n278	2	−66.003943	143.716085	426	MNHN 2009-1320	EATF037-10	GU997588	GU997383
	si77n437	2	−66.333198	143.357078	702	MNHN 2009-1371	EATF077-10	GU997589	GU997384
	si99n615	2	−66.308813	142.29392	217	MNHN 2009-1379	EATF098-10		
	si100n616	2	−66.308813	142.29392	217	MNHN 2009-1227	EATF099-10		
	si101n617	2	−66.308813	142.29392	217	MNHN 2009-1228	EATF100-10		
	si213n1625	2	−66.538527	144.972508	441	MNHN 2009-1262	EATF210-10		
	si287n2182	2	−65.912427	143.966988	370	MNHN 2009-1285	EATF282-10		
	si321n2417	2	−66.00072	141.353593	233	MNHN 2009-1294	EATF316-10	GU997590	GU997385
	si326n2424	2	−66.00072	141.353593	233	MNHN 2009-1295			GU997386
	si327n2425	2	−66.00072	141.353593	233	MNHN 2009-1296	EATF322-10		
	si391n2714	2	−66.38878	140.428852	791	MNHN 2009-1331	EATF386-10		
	si432n2930	2	−66.1483	140.65	213	MNHN 2009-1400	EATF427-10		
	si540n3616	2	−65.706925	140.597385	423.9	MNHN 2009-1367	EATF531-10		
	si541n3617	2	−65.706925	140.597385	423.9	MNHN 2009-1368	EATF532-10		
	W68	1	−75.15	−27.55	404	MNHN 1990-1281	EATF583-10		GU997378
	W77	1					GU997452		
	W151	1	−71.65	−12.2	330	MNHN 1990-1347	EATF593-10		

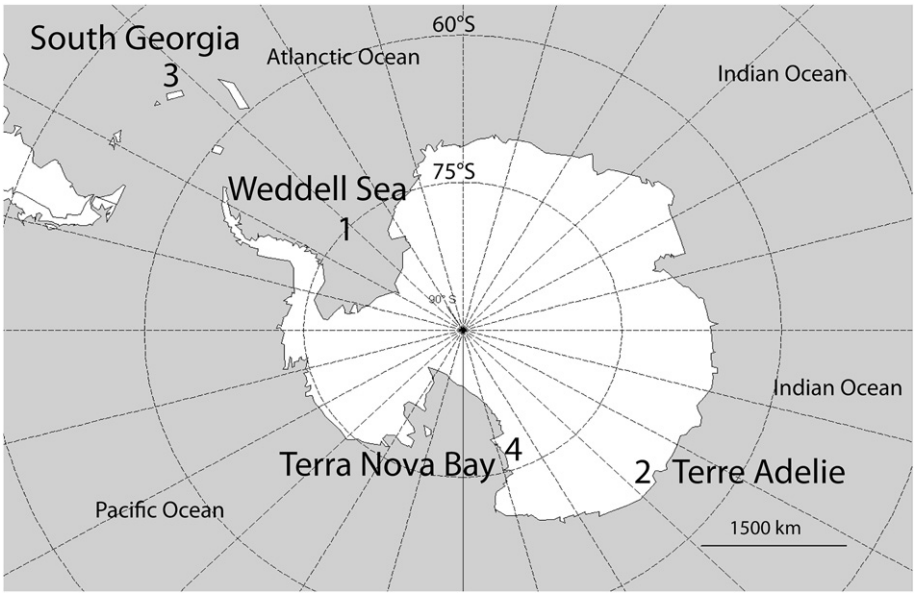


Fig. 1. General sampling map. The numbers represent the different geographic areas where the sampling was made.

study on stomach content sequencing was also performed on several specimens (TA582PABO1, TA582PABO4, TA582PABO6, TA582PABO7 and TA582PABO8) extracted from the stomach of a *Gymnodraco acuticeps* (TA582) and identified tentatively using morphology as *Pagothenia borchgrevinki*. Morphological identifications were first performed on board, and then checked in the lab in the case of discrepancies between morphological and molecular results by Ozouf and Denys using their experience with the taxa and the characters listed in De Witt et al. (1993) and Fisher and Hureau (1987).

2.2. DNA extraction, amplification and sequencing

For each sample, a small piece of muscle tissue was stored at −24 °C or fixed in 70% ethanol at 3 °C. All DNA extraction followed a classical CTAB protocol with a chloroform isoamylalcohol step (Winnpennminck et al., 1993).

For the stomach content specimens, a tissue sample was collected from inside the specimens to avoid the contamination by the stomach cells of *Gymnodraco victori* or by fragments of the other ingested individuals.

pkd1 was selected from a list of coding genes shared by *Tetraodon nigroviridis*, *Takifugu rubripes*, and *Danio rerio*, extracted from the Ensembl database release 40 using *T. nigroviridis* as a query for the Biomart mining tool of the Ensembl Portal (Hubbard et al., 2005). Only genes having unique best hits were retained. The genes presenting the lowest similarity between the two tetradontids were checked for divergence and exon length through the Ensembl Portal on all the available teleost genomes. The sequence coding for exon 18 of the gene pkd1 (ref. ENSTNIG00000014075) was long (2618 base pairs) and had a promising divergence level (*p*-distances is 0.182 for the selected fragment vs. 0.074 for the same taxa for the rhodopsin retrogene). We used the BLAST tool (Altschul et al., 1997) to search all available teleost genomes, and check whether it was single copy in all of the genomes. All available sequences for acanthomorph species were recovered from GenBank and used for primer design after alignment with BioEdit (Hall, 1999).

For the three markers, DNA amplification was performed by PCR in a final 25 μL volume containing 5% DMSO, 1 μL of dNTP 6.6 mM, 0.15 μL of Taq DNA polymerase (MP Biomedicals or Qiagen), using 2.5 μL of the buffer provided by the manufacturer, 100 u μl^{-1} and 0.4 μL of each of the two primers at 10 pM (see Table 2); 1 μL of DNA extract was added. After denaturation for 2 min, the PCR was run for 40–50 cycles of (20 s, 94 °C; 20 s, see Table 2 for hybridization temperature; 50s to 1 min 10s, 72 °C) using a Biometra trioblock cycler (T3000). The result was visualised on ethidium-bromide stained agarose gels. Sequencing was performed by the National Centre for Sequencing (Génoscope) at Evry using the same primers.

Table 2
List of the primers used in this study. Frag. Size is the size of the fragment expected; F = Forward; R = Reverse; T° of hyb. is the temperature of hybridization used to amplify every marker. The line in bold indicates the threshold of 2%.

	Gene	Frag. Size	Name	Sens	Primers	T° of hyb. (°C)	Sources
Mitoch.	COI	≈650 bp	FishF1	F	5′-TCAACCAACCACAAAGACATTGGCAC-3′	52	Ward et al., 2005
			FishR1	R	5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′		
			FishF2	F	5′-TCGACTAATCATAAAGATATCGGCAC-3′		
			FishR2	R	5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′		
Nuclear	Rhodo.	≈840 bp	RhF193	F	5′-CNTATGAATAYCCTCAGTACTACC-3′	50	Chen et al., 2003
			RhR1039	R	5′-TGCTTGTTCATGCAGATGTAGA-3′		
	pkd1	≈840 bp	pkd1F62	F	5′-CATGAGYGTCTACAGCATCCT-3′	50	This study
			pkd1R952	R	5′-YCCTCTNCCAAAGTCCCACT-3′		

2.3. Data management and sequence alignment

All markers were sequenced in both directions and checked manually against their chromatogram using Sequencher 4.8 (Gene Codes Corporation). The sequences were aligned by hand using BioEdit (Hall, 1999), and were controlled for mix-ups and contaminations by pairwise comparison. This yielded three datasets: partial COI, partial rhodopsin retrogene and partial pkd1-coding gene.

All new COI sequences for which voucher were available were deposited in BOLD with their accompanying information. The other nuclear sequences and the mitochondrial sequences without vouchers were deposited in GenBank (http://www.ncbi.nlm.nih.gov) (accession numbers listed in Table 1).

2.4. Phylogenetic and distance analyses

All phylogenetic analyses were performed using the software PAUP* 4.10b (Swofford, 1999).

To allow comparison with the NJ trees provided by the BOL Data System (Ratnasingham and Hebert, 2007), each dataset was analyzed by the NJ distance method with the Kimura 2 parameter model (Kimura, 1980).

To test the delimitation of the species of the genus *Trematomus*, parsimony analyses were also performed on each dataset. These analysis helped us to avoid some of the problems stemming from the pitfalls of distance analyses (De Salle et al., 2005). The low divergences for all the markers hint at a low level of homoplasy in the sequences, so the use of sequence evolution models is probably not necessary. For each analysis, two outgroups were chosen: *Lepidonotothen squamifrons* (Bouvet Island 30L54) and *Patagonotothen ramsayi* (PR3I2004).

2.4.1. Maximum parsimony analyses

Considering the number of taxa and of the size of the datasets, a heuristic search with 1000 replicates starting from a random tree and with rearrangements of branches by TBR (Tree Bisection Reconnection) was performed. As the number of trees was saturating the memory buffer, the dataset was reduced. Multiple identical sequences were reduced to a single representative for this analysis.

2.4.2. Bootstrap values

The robustness of the nodes of the cladograms was estimated by the bootstrap method (Felsenstein, 1985) with 1000 replicates (same settings as before for both NJ and maximum parsimony) for each analysis. Bootstrap values were then indicated on the corresponding branches of the trees obtained by the distance and the maximum parsimony analyses.

2.4.3. Divergence levels among sequences

The intra-specific distances (mean and maximum), as well as the inter-specific distance (mean and minimum, Meier et al., 2008) from the closest species cluster, were calculated using MEGA 4 (Tamura et al., 2007).

2.5. The barcoding of life database (BOLD)

One sequence from each of the obtained clusters was used to query the BOL Data System with the "Identify specimen" tool using both the complete database of all records ("unvalidated dataset") and then the validated database.

3. Results

Not all sequences could be obtained for all markers. 200 sequences of the COI gene, 101 sequences of the pkd1 gene and 147 sequences of the rhodopsin retrogene have been obtained. The amplification of *Trematomus pennellii* samples for the COI gene was especially difficult, although the same samples posed no problems for the other markers.

Variability of the three markers – The COI gene is much more variable and contains more informative sites for maximum parsimony than the two nuclear genes. For this mitochondrial gene, the third codon positions are more variable and largely more parsimony-informative than the first and second positions. The difference is less marked for the nuclear genes, both in variability and in content in parsimony-informative sites between the third codon positions and the first and second. Contrary to what was expected from the divergences observed in *T. nigroviridis* and *T. rubripes*, in Trematominae the variability of pkd1 gene is not higher than the variability of the rhodopsin retrogene (Table 3).

Distance and parsimony trees are congruent for COI and rhodopsin (see Figs. 2, 3a and 4a,b). However, the maximum parsimony trees are not well resolved (Fig. 4). When comparing trees for each marker built using the same method, the branches are less supported by the bootstrap values for the rhodopsin than for the COI gene (Figs. 2, 3a and 4a,b).

3.1. Molecular taxonomy

3.1.1. Distance analyses

The sequences for all specimens identified as belonging to the same species are clustered together for COI (Fig. 2), with the exception of two pairs of species. The single specimen of *T. vicarius* is included among the *T. bernacchii*, and *T. lepidorhinus*–*T. loennbergii* form a single cluster of highly similar terminals (Fig. 2). A visual inspection of the sequences of the COI gene shows that eight non exclusive sites support the segregation of *T. vicarius*–*T. bernacchii* but they do not permit the grouping of all *T. bernacchii* to the exclusion of *T. vicarius*. *T. vicarius* and *T. bernacchii* could however be two subspecies, as they are not found in sympatry. No such site could be detected separating the specimens that were first identified as *T. lepidorhinus* and *T. loennbergii* with the COI gene.

Sequences are also clustered by species on the rhodopsin distance tree (Fig. 3a) except for the same two pair of species and *T. pennellii*. The non-clustering of *T. pennellii* is the result of an artefact of distance-method reconstruction because no site support the paraphyly of this species (see Leclerc et al., 1998). *T. vicarius* is again inside the *T. bernacchii* cluster. *T. lepidorhinus* and *T. loennbergii* are segregated in two clusters, but both clusters contain specimens first identified as *T. lepidorhinus* and specimens first identified as *T. loennbergii* (Fig. 3a). These two species are also together and forming two clusters for the pkd1 distance tree, but the clusters for the rhodopsin retrogene tree and for the pkd1 distance tree do not include the same individuals. There is also no correlation between collection depth and molecular clusters for these species, as might be expected if there was an ongoing bathymetric separation and speciation process. In the pkd1 distance tree, not all individuals identified as belonging to the same species cluster together, although most do. One *T. bernacchii* and the *T. vicarius* form a distinct cluster from the rest of the *T. bernacchii* sequences. A single *Trematomus hansoni* sequence is associated with the *P. borchgrevinki* sequences, and one *T. newnesi* is included within the *T. bernacchii* cluster, although with a long branch (Fig. 3b, specimens indicated by arrows). Geographical origin has no influence on the grouping of the individuals within species whatever the marker (not shown on the trees).

3.1.2. Maximum parsimony analyses

Using the maximum parsimony method, there are many unresolved nodes. For COI, all individuals identified as belonging to the same species (and only them) are grouped in the same clade, except for the same two species pair as previously (Fig. 4a). For the rhodopsin tree, the pattern is the same, except that there is a lack of resolution so the *T. newnesi* and *P. borchgrevinki* specimens are not grouped (Fig. 4b). For pkd1, *T. bernacchii* and *Trematomus eulepidotus* are not resolved, and a single specimen of *T. hansoni* does not group with the others of the same species (Fig. 4c). There are no distinct clades within either rhodopsin or COI trees within *T. bernacchii* or *T. newnesi*, in agreement with the results

Table 3
Evaluation of the number of variable informative and not informative sites for maximum parsimony for every marker for all positions and by codon positions. Pos. 1, Pos. 2 and Pos. 3 corresponds to the first, second and third codon positions. The percentage of informative or not informative sites for the parsimony is indicated in parentheses and is calculated from the total length of the sequences for every marker.

		Dataset length	Number of variable parsimony non-informative sites				Number of parsimony-informative sites			
			Pos. 1	Pos. 2	Pos. 3	All sites	Pos. 1	Pos. 2	Pos. 3	All sites
Mitoch.	COI	657	17 (3%)	11 (2%)	129 (20%)	157 (24%)	13 (2%)	2 (0.3%)	118 (18%)	133 (20%)
Nuclear	Rhodo	714	17 (2%)	10 (3%)	24 (10%)	51 (7%)	11 (2%)	5 (1%)	15 (2%)	31 (4%)
	pkd1	834	19 (2%)	12 (1%)	22 (3%)	53 (6%)	16 (2%)	11 (1%)	18 (2%)	45 (5%)

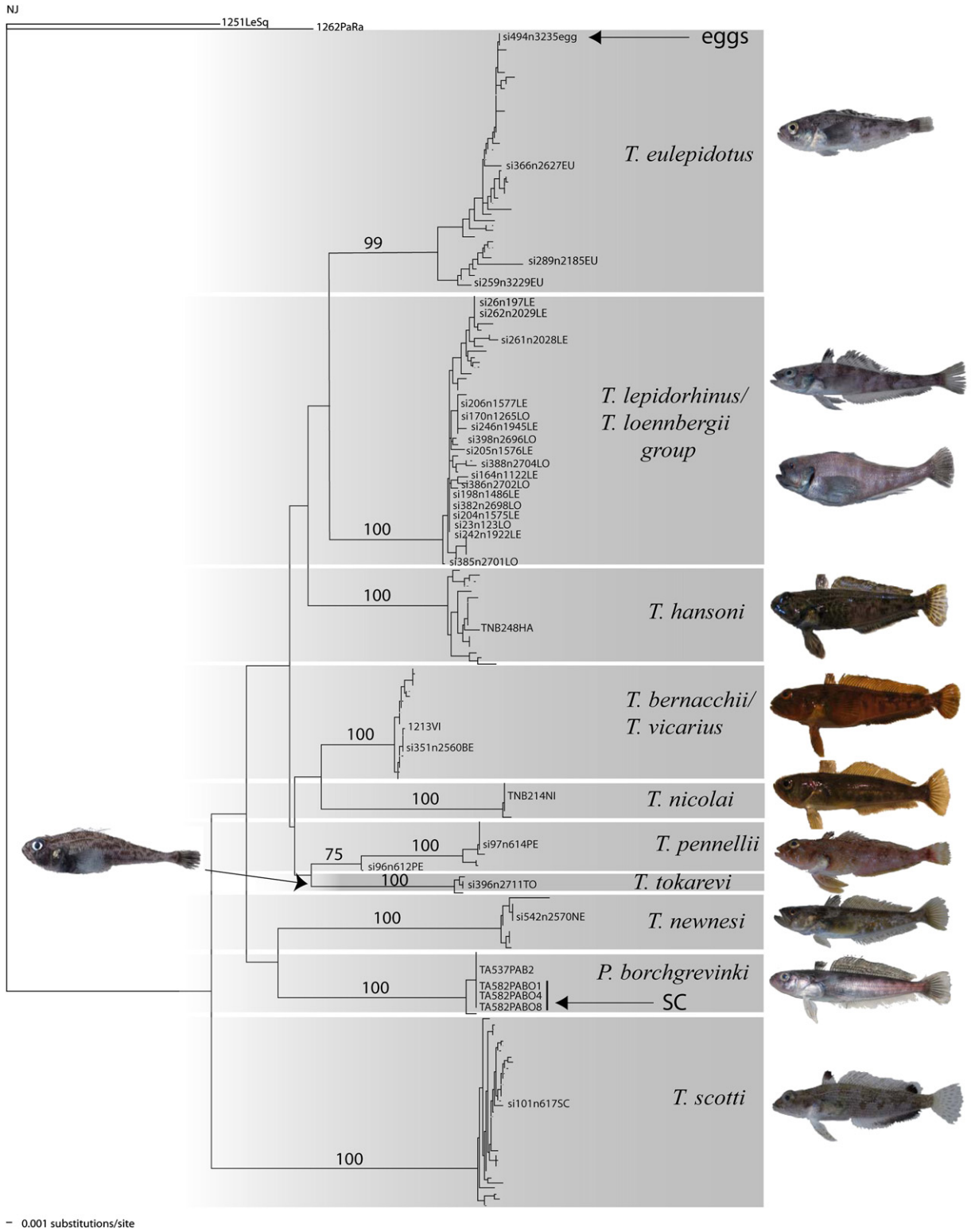


Fig. 2. Trematominae NJ K2P tree for the COI gene. Distance tree for 217 sequences of 657 bp of the partial COI gene. As the sequences cluster by species, only the sequence reference numbers that are essential to the understanding have been represented. SC = Stomach contents.

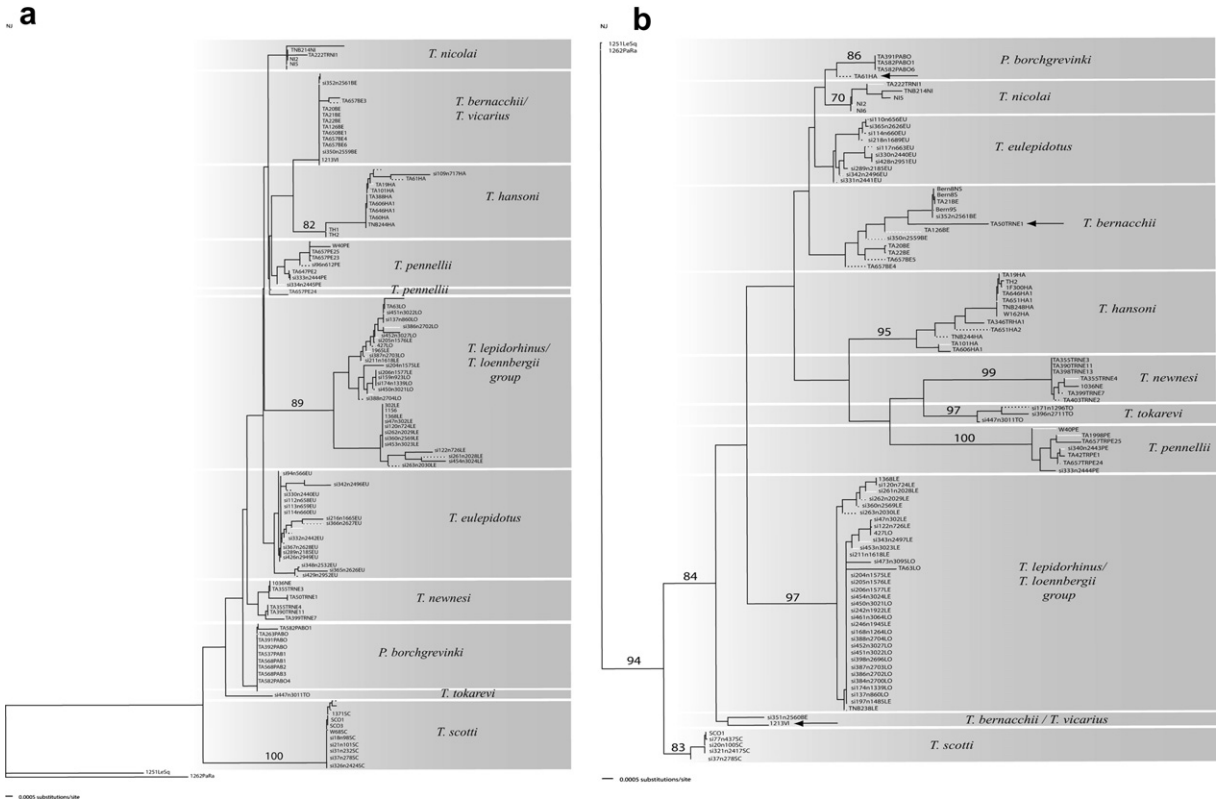


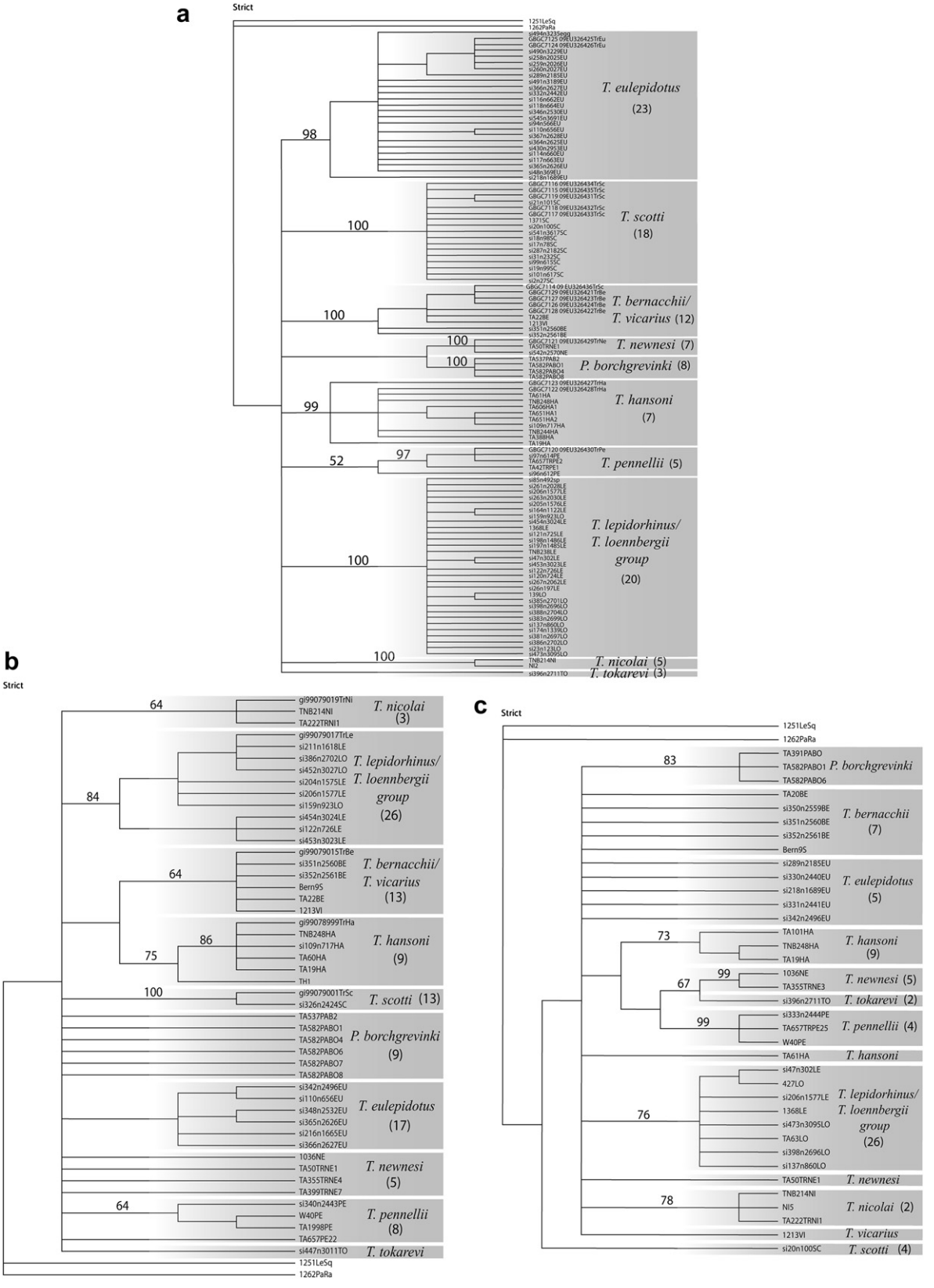
Fig. 3. Trematominae NJ K2P trees for the COI gene, the rhodopsin retrogene and the pkd1 gene. a – Distance tree for 154 sequences of 714 bp for the partial rhodopsin retrogene. As the sequences cluster by species, only the sequence reference numbers that are essential to the understanding have been represented. b – Distance tree for 103 sequences of 834 bp of the partial pkd1 gene. Bootstrap values above 50 are indicated on the branches of the trees.

of Bernardi and Goswami (1997). However, the presence of both the proposed morphs in our sampling (Eastman and DeVries, 1997; Piacentino and Barrera-Oro, 2009) could not be checked.

Most relationships among species are not resolved, and the few that are, are not well supported by bootstrap. With COI, there is a *T. newnesi*–*P. borchgrevinki* clade (Fig. 4a). For the rhodopsin retrogene, there is a *T. bernacchii/T. vicarius*–*T. hansoni* clade (Fig. 4b). For pkd1, *T. newnesi*–*Trematomus tokarevi* is the sister-group of *T. pennellii*, and the three are the sister-group of *T. hansoni*, contradicting the relationships present in the trees from the other two markers (Fig. 4c).

No separate clades are recovered for *T. lepidorhinus* and *T. loennbergii* using maximum parsimony with the pkd1 or COI genes (Fig. 4a,c), but there are two distinct clades in the rhodopsin tree (Fig. 4b). As there was no correspondence between the morphological identifications and the rhodopsin clusters, the corresponding specimens were re-identified morphologically. The original descriptions of these two species were re-examined in De Witt et al. (1993). Most morphological and meristic characters overlap between the two species, the distinction in the key to species is only based on the presence of "scales on preorbital and on at least proximal part of the lower jaw" in *T. lepidorhinus*, while "lower jaw and preorbital are naked (a few preorbital scales being present on large *T. loennbergii*)". The only two characters listed as characteristic of one species in the key and description in De Witt et al. (1993) are therefore present in combination with characters distinctive of the other species on some specimens. This suggests the necessity of re-examining a large batch of these species and selecting suitable characters for morphology and possibly morphometry in order to verify whether they should be really split into two species or put into synonymy. The lack of correlation between the clades and their depth of divergence contradicts the hypothesis that it could be two subspecies distributed according to the depth range.

For the two pairs of species *T. lepidorhinus/T. loennbergii*, *T. bernacchii/T. vicarius*, a species delineation problem cannot be excluded, so they will not be


a
Strict
T. eulepidotus (23)
T. scotti (18)
T. bernacchii/ T. vicarius (12)
T. newnesi (7)
P. borchgrevinki (8)
T. hansoni (7)
T. pennellii (5)
T. lepidorhinus/ T. loennbergii group (20)
T. nicolai (5)
T. tokarevi (3)
b
Strict
T. nicolai (3)
T. lepidorhinus/ T. loennbergii group (26)
T. bernacchii/ T. vicarius (13)
T. hansoni (9)
T. scotti (13)
P. borchgrevinki (9)
T. eulepidotus (17)
T. newnesi (5)
T. pennellii (8)
T. tokarevi
c
Strict
P. borchgrevinki
T. bernacchii (7)
T. eulepidotus (5)
T. hansoni (9)
T. newnesi (5)
T. tokarevi (2)
T. pennellii (4)
T. hansoni
T. lepidorhinus/ T. loennbergii group (26)
T. newnesi
T. nicolai (2)
T. vicarius
T. scotti (4)

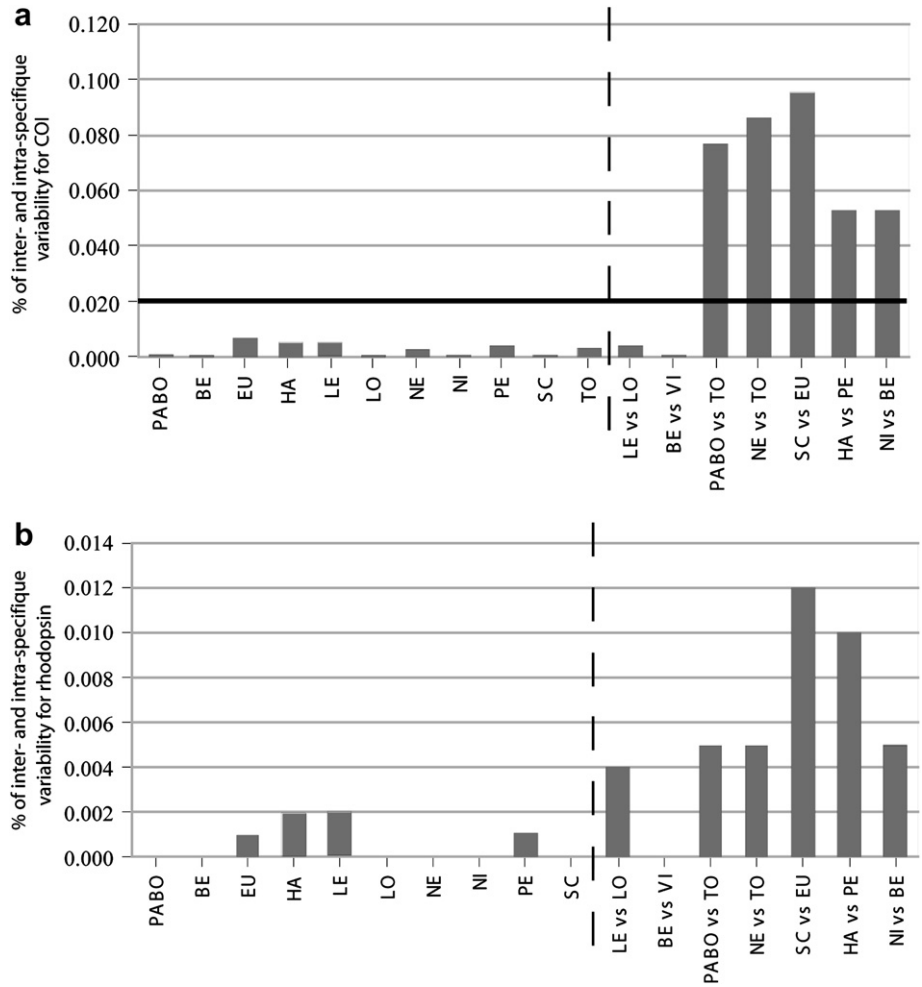


Fig. 5. Inter- and intra-specific variability of the COI gene and the rhodopsin retrogene. The intra-specific distances as well as the inter-specific distance were calculated from the closest species cluster. BE = *T. bernacchii*, PABO = *P. borchgrevinki*, EU = *T. eulepidotus*, HA = *T. hansoni*, LE = *T. lepidorhinus*, LO = *T. loennbergii*, NE = *T. newnesi*, NI = *T. nicolai*, PE = *T. pennellii*, SC = *T. scotti*, VI = *T. vicarius* and TO = *T. tokarevi*. The solid line indicates the previously proposed threshold of 2% between intra-specific divergence from inter-specific divergence. The dashed line helps to demarcate between inter-specific comparisons and intraspecific comparisons.

included to test the efficiency of the barcoding approach on Trematominae.

3.1.3. Peculiarities of the pkd1 gene

The sequences of some individuals presented a few double peaks on their chromatograms. Re-extracting and re-sequencing yielded the exact same peaks at the exact same place. The double peaks were species-specific, and may represent different alleles. Some specimens belonging to *T. hansoni*, *T. bernacchii* and *T. newnesi*, which were clustered by species on COI and rhodopsin trees, are not together with the other specimens from their species on the pkd1 distance tree (see Fig. 3b, specimens indicated by arrows). In the parsimony tree, these specimens are not with the other individuals of the same species but they are not clearly associated with specimens of another species either. Moreover, their positions in the trees are never supported by high bootstrap values. The hypothesis of contaminations could be excluded as all these

Fig. 4. Maximum parsimony analyses for the unique sequences of each dataset. a – Strict consensus of 156 869 trees of 453 steps based on 109 terminals for the COI gene. b – Rhodopsin parsimony tree. Strict consensus of 108 239 trees of 89 steps based on 50 terminals. c – Strict consensus of 3979 trees of 89 steps based on 76 terminals for the pkd1 gene. Bootstrap values above 50 are indicated on the branches of the trees. Multiple identical sequences were reduced to a single representative for the analyses. Number of identical sequences removed is indicated in parentheses.

specimens were sequenced again yielding the same sequence, and none of them is identical to the sequence from another specimen from our sampling.

3.2. Inter- and intra-specific variations for the three markers

3.2.1. COI gene (see Fig. 5a)

The intra-specific variation is always lower than the inter-specific variation for all species except for *T. vicarius*–*T. bernacchii* and *T. lepidorhinus*–*T. loennbergii*. There is no overlap between inter-specific and intra-specific distance ranges (minimal inter-specific distance 0.045, maximal intra-specific distance 0.024). The average inter-specific variability is always greater than the proposed threshold of 2% and the average intra-specific variability is lower. However, the sole use of averages for the intra- and inter-specific distance is not relevant and has to be supported by the minimum and the maximum of intra- and inter-specific distance (Meier et al., 2008). In this case, there is an exception for *T. eulepidotus* because the maximum intra-specific distance (0.024) is higher than 2%. The "inter-specific" variability between specimens first identified as *T. lepidorhinus* and *T. loennbergii* (0.003) is in the same range than intra-specific variabilities in other trematomine species. For *T. vicarius* and *T. bernacchii*, the average intra-specific distance of *T. bernacchii* is 0.001 (there is just one specimen for *T. vicarius*) and the inter-specific variability is the same (0.001).

The distance within the two problematic pairs is more than 30 times smaller than the minimal inter-specific distance for the other species (average = 0.07; min = 0.05; max = 0.104). These two inter-specific variations, if the two pairs indeed represent four distinct species, are in the same order of magnitude as the intra-specific variations of the others species.

3.2.2. Rhodopsin gene (see Fig. 5b)

The variability of this nuclear gene is much lower than the variability of the mitochondrial COI. The intra-specific variation is always lower than the inter-specific variation for all species except for *T. bernacchii* and *T. vicarius*. In fact, for the rhodopsin gene, all sequences of *T. bernacchii* and *T. vicarius* are identical.

Contrary to the COI, the inter-specific variability among *T. lepidorhinus* and *T. loennbergii* specimens (0.004%) is in the same order of magnitude as the others inter-specific values (average = 0.007; min = 0.005; max = 0.016).

There is an overlap between inter-specific and intra-specific distance ranges with this marker. The minimal inter-specific distance is 0.005 and the maximal intra-specific distance is 0.007.

3.3. Identification with the COI gene

3.3.1. Identification using the distance tree

All individuals of the same species are clustered together except for the two problematic species pairs (Fig. 2), so identification using the position in the distance tree should work.

Three stomach contents in five could be sequenced (TA582PABO1, TA582PABO4, TA582PABO8) with COI. They are placed with the other individuals belonging to the species *P. borchgrevinki*, which is in agreement with the preliminary morphological identification. Three distinct eggs from the egg batch could be sequenced and were identified as *T. eulepidotus* (see Fig. 2). This is in agreement with the analysis of the video filmed at the time of the egg collection, as *T. eulepidotus* adults were detected near the sponge where the eggs were found.

3.3.2. Identification with the database

For the sequences used as test, *Trematomus scotti* (si101n617SC), *T. pennellii* (si97n614PE), *Trematomus nicolai* (TNB214NI), *T. newnesi* (si542n2570NE), *T. hansoni* (TNB248HA) and *T. bernacchii* (si351n2560BE), the correct identification was recovered whatever the database used.

For *T. tokarevi* (si396n2711TO), the stomach contents (TA582PABO1, TA582PABO4, TA582PABO8) and *P. borchgrevinki* (TA537PAB2), no identification could be provided, and the closest species using the unvalidated database are *T. pennellii* and *T. vicarius* (94%).

This is easily explained because there is no sequence of *P. borchgrevinki* and *T. tokarevi* in the database, and the results have no meaning for the species identification.

For *T. eulepidotus*, the si366n2627 sequence nested within one of the *T. eulepidotus* clusters in the NJ tree provided by the BOL Data System, and was identified as *T. eulepidotus* with 100% of similarity.

For the eggs (si494n3235), no identification could be provided using the validated database but they were identified as *T. eulepidotus* using the unvalidated database.

For the two problematic species pair, the BOLD search tool returned "erroneous" identifications with no warning of an alternative choice using the validated database. For the *T. lepidorhinus/T. loennbergii* group (si206n1577LE and si398n2696LO), the validated database returned *T. loennbergii*. For *T. vicarius* it returned *T. bernacchii* using the validated database.

4. Discussion

4.1. Species delimitation and importance of nuclear markers

Comparing mitochondrial and nuclear data for molecular taxonomy has allowed us to detect a problem of delimitation for two pairs of species. *T. vicarius* and *T. bernacchii* could actually be a single species, or two geographical forms not presenting enough differences to be discriminated yet by the markers used. The rhodopsin sequences show there is a unique variable site for *T. vicarius*–*T. bernacchii* and it does not permit the segregation of two groups by species. The fact that the rhodopsin also fails to discriminate these two groups would rather corroborate this hypothesis, but additional specimens of *T. vicarius* and more variable markers would be needed. Moreover, *T. vicarius* had been first described as subspecies of *T. bernacchii* by Loennberg (1905). They are very similar morphologically (Norman, 1938). A re-analysis of the morphological data is necessary to explore this, and cytogenetic studies are ongoing to determine the karyotype of these two species. This could give indications on their potential breeding abilities. In that case, the lack of divergence between their sequences could simply reflect their recent separation. Nonetheless, this delimitation is very interesting, because *T. vicarius* has a restricted distribution which is more northern than the circum-Antarctic distribution of *T. bernacchii* according to FishBase (Froese and Pauly, 2009). Indeed, one of the key concerns raised against barcoding is that DNA sequence variation in COI may not be detectable for very closely related and/or recently diverged species (Hickerson et al., 2006; Mallet and Willmot, 2003). However, as long as the taxonomic issue is not solved, it is not possible to consider that these two species pose a problem for the reliability of the barcode for this group.

Being able to separate *T. lepidorhinus* and *T. loennbergii* with the barcode would have been even more interesting as these two species can be found in sympatry. However, the monophyly of these species cannot be recovered with any of the molecular markers used here, and the morphological characters on the same specimens do also not give a clear picture. All of this suggests a delineation problem, or at the very least an inadequacy of the characters used.

T. lepidorhinus and *T. loennbergii* have been karyotyped in different sectors of the Antarctic continental shelf. In Prydz Bay and the Weddell Sea, *T. lepidorhinus* had a diploid number of 48 chromosomes (Ozouf-Costaz et al., 1991). A specimen from Terre Adélie identified as *T. loennbergii* by Hureau also showed a diploid number of 48 chromosomes and the same formula (Ozouf-Costaz et al., 1999). This specimen kept in the MNHN collection was later reassigned to the species *T. lepidorhinus* by Marino Vacchi. This well demonstrates the possible confusions between the two species at morphological level. In the Ross Sea, specimens identified as *T. loennbergii* displayed two karyomorphs in the same locality (off Terra Nova Bay) with diploid numbers of 28 and 30, respectively. However the arm number is 52 for both morphs, as well as for *T. lepidorhinus*, suggesting important Robertsonian rearrangements are occurring within this group, leading to chromosome instability. We suggest that until this is investigated further, ecology studies including these species list them as *T. loennbergii/T. lepidorhinus* group, and keep voucher samples and specimens.

T. lepidorhinus and *T. loennbergii* could have been a very good example to illustrate a criticism often made of barcoding: the difference between the sequences of very close species can be too weak to allow their discrimination by employing a fixed threshold (Meyer and Pauley, 2005). But in this case, the use of nuclear markers completes the information given by COI and allows choosing among competing explanatory hypotheses. The morphology and the nuclear genes suggest that there is a delimitation problem at play. It will have to be investigated further before these examples can be used to support or question the use of barcode for trematomine species identification.

The size and representativity of the sampling is also very important. In this case, the inclusion of several individuals per species for the analyses permitted us to detect a potential reminiscent polymorphism problem with the pkd1 gene. It might have retained ancestral polymorphism in some of the species, making its use problematic for phylogeny within the Trematominae. The structure of the tree obtained with the S7 gene in Kuhn and Near (2009) hints that this gene could have a similar problem. This will have to be considered for future reconstruction using nuclear genes on this group.

4.2. Validation of the use of molecular barcode for the species of the genus Trematomus

4.2.1. COI as a tool for identification

COI appears to be effective at recovering morphologically identified species for the genus *Trematomus*. The position on the distance tree appears to be suitable for identification.

The stomach contents could not be identified precisely by making a query in BOLD, probably because there is no sequence of *P. borchgrevinki* in this database.

This illustrates very well the necessity of having the most complete database possible. The three sequences of stomach contents obtained with COI are almost identical (TA582PABO7 differs with one base and TA582PABO8 differs with two bases from the others sequences) to other sequences of *P. borchgrevinki* from our sampling and are placed as such in our trees (Figs. 2–4). We can therefore confirm that this was indeed the DNA from the stomach contents, and that there was no contamination by the specimen whose stomach they come from (*G. acuticeps*). The success of the amplification and the sequencing of the most degraded stomach contents with the rhodopsin is encouraging as the nuclear genes are more difficult to amplify than the mitochondrial genes. It appears that the incomplete digestion in this case had little effect on the amplification or sequencing. But there are currently few publications on the subject, and more cannot be concluded from these results because the number of successful amplifications is too small. For future studies of totally destroyed and mixed specimens, cloning techniques should allow the separation of mixed DNAs when the risk of contamination is too high.

4.2.2. *Inter- and intra-specific variability*

In genus *Trematomus*, there is a clear difference between the intra-specific variabilities (max value = 0.024) and inter-specific variabilities (min value = 0.045) when the two pairs of species *T. vicarius*/*T. bernacchii* and *T. loennbergii*/*T. lepidorhinus* are excluded.

For *Trematomus eulepidotus*, the intra-specific divergence is above 2%. It is therefore more cautious for identifications of *Trematomus* samples to use the position on the tree and not rely on the threshold.

If we accept the threshold, it would be necessary to consider that *T. loennbergii* and *T. lepidorhinus* form a single species. For the nuclear gene rhodopsin, their inter-specific variation is of the same order of magnitude as the other inter-specific variations, but more importantly they cannot be separated on any of our trees. The rhodopsin retrogene could be used as a marker for molecular identification for Trematominae. It had already been used as a reference sequence in the Fish-trace project (www.fishtrace.org). Even so, it cannot be considered to be completely reliable because of its low variability, but is most useful used in addition to COI. A more variable nuclear marker would be interesting for the study of this genus. However, the problem is to find a nuclear fragment of 600–1000 bp evolving quickly enough to allow the distinction between close species (Dasmahapatra and Mallet, 2006), but posing no technical problems. The nuclear genes evolve in average ten times slower than the mitochondrial genes; furthermore, the presence of introns can change their size. The results of the pkd1 marker highlight other problems of the use of nuclear marker for recent divergences: the problem of incomplete lineage sorting, and the presence of multiple alleles in a single individual. In fact, this gene seems to have a potential problem of reminiscent polymorphism for this group, just like the nuclear gene of Kuhn and Near (2009).

5. Conclusion

The very large sampling of the present study allowed the addition of 129 supplementary sequences in BOLD (against 73 before this study). Almost all *Trematomus* species are now present, including for the first time, for COI, *T. tokarevi* and *P. borchgrevinki*. Several promising areas for study could be highlighted that had not been identified using smaller samplings and fewer genes. The status of the *T. bernacchii*/*T. vicarius* and the *T. loennbergii*/*T. lepidorhinus* pairs will have to be investigated with additional specimens and more variable markers.

The barcode using COI is thus a promising tool of identification for the species of the genus *Trematomus*. This result has a major importance for the ecologists working on Antarctic marine ecosystems as it provides a reliable tool to identify even a large number of specimens, including difficult to identify larvae and eggs. In fact, in spite of some problems, COI appears to be the best available marker to this day for identification for this genus, for ease of amplification as well as for the availability of the reference sampling.

The sequences of COI are also relatively easy to obtain compared to the other markers (rhodopsin), except for *T. pennellii* samples. In fact, it is even possible to obtain sequences up to the degree of degradation of the stomach content specimens studied here. Considering the wide variety of the species of teleostean "barcoded" fishes (Ivanova et al., 2007; Ward et al., 2005, 2009) and this study, the approach seems promising whatever the type of samples, but needs to be corroborated by other types of data, whether morphological or molecular, for each group of interest. It is also necessary to complete the databases to obtain routinely reliable identification.

The inclusion of a nuclear marker like the rhodopsin retrogene would permit to perform in parallel barcode identifications and molecular taxonomy through the comparison of the results for both marker using more reliable phylogenetic reconstruction methods. However, the search for new more variable markers remains necessary, as the currently available nuclear datasets might not be informative enough. Microsatellites have already been proposed in Trematominae (Van de Putte et al., 2009) and could be very useful to solve this

problem of variability. pkd1, while bringing interesting information on some of the species, is less variable than it first appeared on *T. nigroviridis* and *T. rubripes*. It might have retained ancestral polymorphism in some of the species, making its use problematic for phylogeny within the Trematominae.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, k Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402.

Bellingham, J., Tarttelin, E.E., Foster, R.G., Wells, D.J., 2003. Structure and evolution of the teleost extraretinal rod-like opsin (errlo) and ocular rod opsin (rho) genes: is teleost rho a retrogene? J. Exp. Zool. Part B Mol. Dev. Evol. 297 (1), 1–10.

Bernardi, G., Goswami, U., 1997. Molecular evidence for cryptic species among theAntarctic fish *Trematomus bernacchii* and *Trematomus hansoni*. Antarct. Sci. 9 (4), 381–385.

Bortolus, A., 2008. Error cascades in the biological Sciences: the unwanted consequences of using bad taxonomy in ecology. Ambio 37 (2), 114–118.

Brenner, M., Buck, B.H., Cordes, S., Dietrich, L., JacobMintenbeck, K., Schröder, A., Brey, T., Knust, R., Arntz, W., 2001. The role of iceberg scours in niche separation within the Antarctic fish genus *Trematomus*. Polar Biol. 24, 502–507.

Buhay, J.E., 2009. "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. J. Crust. Biol. 29 (1), 96–110.

Chen, W.J., Bonillo, C., Lecointre, G., 2003. Repeatability of clades as a criterion of reliability: a case study for molecular phylogeny of Acanthomorpha (Teleostei) with larger number of taxa. Mol. Phylogenet. Evol. 26, 262–288.

Cziko, P., Cheng, C.H., 2006. A New species of nototheniid (Perciformes: Notothenioidei) fish from McMurdo Sound, Antarctica. Copeia 4, 752–759.

Dasmahapatra, K.K., Mallet, J., 2006. DNA barcodes: recent successes and future prospects. J. Hered. 97, 254–255.

De Salle, R., 2006. Species discovery versus species identification in DNA barcoding efforts: response to Rubinoff. Conserv. Biol. 20 (5), 1545–1547.

De Salle, R., Egan, M.G., Siddall, M., 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Phil. Trans. R Soc. B 360, 1905–1916.

De Witt, H.H., 1971. Coastal and deep-water benthic fishes of the Antarctic. In: Bushnell, V.C. (Ed.), Antarctic Map Folio Series Folio, vol. 15. American Geographical Society, New York, pp. 1–10.

De Witt, H.H., Heemstra, P.C., Gon, O., 1993. Nototheniidae. In: Gon, O., Heemstra, P.C. (Eds.), Fishes of the Southern Ocean, pp. 279–399.

Eastman, J.T., DeVries, A.L., 1997. Biology and phenotypic plasticity of the Antarctic nototheniid fish *Trematomus newnesi* in McMurdo Sound. Antarct. Sci. 9, 27–35.

Eastman, J.T., 1993. Antarctic Fish Biology. Academic Press, San Diego.

Eastman, J.T., Clarke, A., 1998. A comparison of adaptative radiations of Antarctic fishes with those of non-Antarctic fish. In: di Prisco, G., Pisano, E., Clarke, A. (Eds.), Fishes of Antarctica, a Biological Overview. Springer-Verlag, Milano, pp. 3–26.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fisher, W., Hureau, J.C., 1987. Océan austral. FAO 2, 371–389.

Froese, R., Pauly, D., 2009. FishBase. World Wide Web Electronic Publication. www.fishbase.org.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W., Hebert, P.D.N., 2006. DNA barcodes distinguish species of tropical Lepidoptera. Proc. Natl. Acad. Sci. U.S.A. 103 (4), 968–971.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Res. Symp. Ser. 41, 95–98.

Hebert, P.D.N., Cywinska, A., Ball, S.L., Dewaard, J.R., 2003a. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. Ser. B Biol. Sci. 270, 313–322.

Hebert, P.D.N., Ratnasingham, S., deWaard, J.R., 2003c. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B (Suppl.) 270, S96–S99.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S., Francis, C.M., 2003b. Identification of birds through DNA barcodes. PLoS Biol. 2, 1657–1663.

Hickerson, M.J., Meyer, C.P., Moritiz, C., 2006. DNA barcoding will often fail to discover new animal species over broad parameter space. Syst. Biol. 55, 729–739.

Hubbard, T., Andrews, D., Caccamo, M., Cameron, G., Chen, Y., Clamp, M., Clarke, L., Coates, G., Cox, T., Cunningham, F., Curwen, V., Cutts, T., Down, T., Durbin, R., Fernandez-Suarez, X.M., Gilbert, J., Hammond, M., Herrero, J., Hotz, H.,

Howe, K., Iyer, V., Jekosch, K., Kahari, A., Kasprzyk, A., Keefe, D., Keenan, S., Kokocinsci, F., London, D., Longden, I., McVicker, G., Melsopp, C., Meidl, P., Potter, S., Proctor, G., Rae, M., Rios, D., Schuster, M., Searle, S., Severin, J., Slater, G., Smedley, D., Smith, J., Spooner, W., Stabenau, A., Stalker, J., Storey, R., Trevanion, S., Ureta-Vidal, A., Vogel, J., White, S., Woodwark, C., Birney, E., 2005. Ensembl 2005. Nucleic Acids Res. 33, 447−453. http://dx.doi.org/10.1093/nar/gki138.

Ivanova, N.V., Zemlak, N.V., Hanner, R.H., Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. 7 (4), 544−548.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111−120.

Kock, K.H., Jones, C.D., 2005. Fish stocks in the Southern Scotia Arc region − a review and prospects for future research. Res. Fish. Sci. 13, 75−108.

Koubbi, P., Duhamel, G., Hecq, J.H., Beans, C., Loots, C., Pruvost, P., Tavernier, P.E., Vacchi, M., Vallet, C., 2007. Ichthyoplankton in the neritic and coastal zone of Antarctica and Subantarctic islands: a review. J. Mar. Syst. 78 (4), 547−556.

Kuhn, K.L., Near, T.J., 2009. Phylogeny of *Trematomus* (Notothenioidei: Nototheniidae) inferred from mitochondrial and nuclear gene sequences. Antarct. Sci., 1−6.

Leclerc, M.C., Barriel, V., Lecointre, G., De Réviers, B., 1998. Low divergence in rDNA ITS sequences among five species of *Fucus* (Phaeophyta) suggests a very recent radiation. J. Mol. Evol. 46, 115−120.

Loennberg, E., 1905. Pelagische von der schwedischen Südpolar −Expedition erbeutete Fishe. Zool. Anz. 28 (23). In: Gon, O., Heemstra, P.C. (Eds.), Fishes of the Southern Ocean. Grahamstown. J.L.B. Smith Inst. Ichthyol., South Africa, pp. 762−766.

Lorenz, J.G., Jackson, W.E., Beck, J.C., Hanner, R., 2005. The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. Phil. Trans. R Soc. B 360 (1462), 1869−1877.

Mallet, J., Willmot, K., 2003. Taxonomy: renaissance or Tower of Babel? Trends Ecol. Evol. 18, 57−59.

Meier, R., Zhang, G., Ali, F., 2008. The use of mean Instead of Smallest interspecific distances exaggerates the size of the "Barcoding Gap" and leads to misidentification. Syst. Biol. 57 (5), 809−813.

Meyer, C.P., Pauley, G., 2005. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 3 (12), 2229−2238.

Near, T.J., Pesavento, J.J., Cheng, C.C., 2004. Phylogenetic investigations of Antarctic notothenioid fishes (Perciformes: Notothenioidei) using complete gene sequences of the mitochondrial encoded 16S rRNA. Mol. Phylogenet. Evol. 32, 881−891.

Norman, J.R., 1938. Coast fishes. Part 3. The Antarctic zone. Discovery Rep. 18, 1−104.

Ozouf-Costaz, C., Hureau, J.C., Beaunier, M., 1991. Chromosome studies on fish of the suborder Notothenioidei collected in the Weddll Sea during EPOS 3 cruise. Cybium 15 (4), 271−289.

Ozouf-Costaz, C., Pisano, E., Thaeron, C., Hureau, J.C., 1999. Karyological survey of the notothenioid fish occurring in Adelie Land (Antarctica). In: Seret, B., Sire, J.Y. (Eds.), Proc. 5th Indo-Pac. Fish Conf., Nouméa. Soc. Fr. Ichtyol., Paris, pp. 427−440.

Park, M.H., Sim, C.J., Baek, J., Min, G.S., 2007. Identification of genes suitable for DNA barcoding of morphologically Indistinguishable Korean Halichondriidae sponges. Mol. Cell. 23 (2), 220−227.

Pegg, G.G., Sinclair, B., Briskey, L., Arpden, W.J., 2006. MtDNA barcode identification of fish larvae in the southern Great Barrier Reef, Australia. Sci. Mar. 70 (Suppl. 2), 7−12.

Piacentino, G.L.M., Barrera-Oro, E., 2009. Phenotypic plasticity in the antarctic Wsh *Trematomus newnesi* (Nototheniidae) from the South Shetland islands. Polar Biol. 32, 1407−1413.

Ratnasingham, S., Hebert, P.D.N., 2007. BOLD: the barcode of life data system. Mol. Ecol. Notes 7, 355−364.

Ritchie, P.A., Bargelloni, L., Meyer, A., Taylor, J.A., MacDonald, J.A., Lambert, D.M., 1996. Mitochondrial phylogeny of trematomid fishes (Nototheniidae, Perciformes) and the evolution of antarctic fish. Mol. Phylogenet. Evol. 5, 383−390.

Rock, J., Costa, F.O., Walker, D.I., North, A.W., Hutchinson, W.F., Carvalho, G.R., 2008. DNA barcodes of fish of the Scotia Sea, Antarctica indicate priority groups for taxonomic and systematics focus. Antarct. Sci. 20 (3), 253−262.

Rubinoff, D., Cameron, S., Will, K., 2006. A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. J. Hered. 97 (6), 581−594.

Sanchez, S., Dettai, A., Bonillo, C., Ozouf-Costaz, C., Detrich, B., Lecointre, G., 2007. Molecular and morphological phylogenies of the Antarctic teleostean family Nototheniidae, with emphasis on the Trematominae. Polar Biol. 30, 155−166.

Steinke, D., Zemlak, Tyler S., Hebert, Paul D.N, 2009. Barcoding Nemo: DNA-based identifications for the ornamental fish trade. PLoS One 4 (7), 1−5.

Suzuki, N., Hoshino, K., Murakami, K., Takeyama, H., Chow, S., 2008. Molecular Diet analysis of Phyllosoma larvae of the Japanese Spiny Lobster *Panulirus japonicus* (Decapoda: Crustacea). Mar. Biotechnol. 10, 49−55.

Swofford, D., 1999. PAUP*, Phylogenetic Analysis Using Parsimony, Version 4.0b10.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596−1599.

Van de Putte, Anton Pieter, Van Houdt, J.K.J., Maes, G.E., Janko, K., Koubbi, P., Rock, J., Volckaert, F.A.M., 2009. Species identification in the trematomid family using nuclear genetic markers. Polar Biol. 32, 1731−1741.

Ward, R.D., Hanner, R., Hebert, P.D., 2009. The campaign to DNA barcode all fishes, FISH-BOL. J. Fish. Biol. 74, 329−356.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D.N., 2005. DNA barcoding Australia's fish species. Phil. Trans. R Soc. B 360 (1462), 1847−1857.

Webb, K.E., Barnes, D.K.A., Clark, M.S., Bowden, D.A., 2006. DNA barcoding: a molecular tool to identify Antarctic marine larvae. Deep Sea Res. II 53, 1053−1060.

Winnpennminck, B., Backeljaut, T., Watcher, R.D., 1993. Extraction of high molecular weight DNA from molluscs. Trends Genet. 9, 407.

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