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Phylogenetic footprints of an Antarctic radiation: The Trematominae (Notothenioidei, Teleostei)

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ABSTRACT

The teleost suborder Notothenioidei is restricted to the Southern Ocean and has been described as a species flock spanning the whole of it. Within the suborder, the subfamily Trematominae is important for coastal Antarctic ecosystems. The eleven *Trematomus* species occupy a large range of ecological niches. The genus is monophyletic if the genus *Pagothenia* (two additional species) and *Cryothenia amphitreta*, also nested within it, are included. Although the Trematominae have received much interest, the relationships among these fourteen species are still unclear.

Several recent studies have tried to resolve these interrelationships; however no complete and clear picture has emerged, probably because of the use of a low number of insufficiently variable markers. The only common results places *T. scotti* as the sister-group of the rest of the subfamily and *T. loennbergi* close to *T. lepidorhinus*. We use here more variable markers. Four nuclear markers, two of which are new, and a mitochondrial marker for the biggest trematomine sampling ever gathered (14 species, 78 specimens). We found that several nuclear haplotypes are shared by several species (mostly in very closely related species). The haplotype patterns coupled with the cytogenetics of the subfamily suggest that a phenomenon of incomplete lineage sorting (ILS) is likely to be at play. Using a calibration linked to fossil evidence, we evaluate the relative ages of each clade within the Trematominae to assess the proximity of the speciation events to one another. The main trematomine diversification was recent and sudden.

1. Introduction

Antarctic regions are particularly interesting for biologists working on evolutionary processes because freezing temperatures of coastal waters demand remarkable adaptations for survival. A key adaptation in the local ectotherm teleosts is antifreeze glycoproteins. They protect the hyposmotic body fluids of Antarctic notothenioids from freezing (Eastman, 1993; Clarke and Johnston, 1996; DeVries and Cheng, 2005). Moreover, the surface waters of the Southern Ocean are isolated from the Indian Ocean, the Atlantic Ocean and the Pacific Ocean by the Antarctic Circumpolar Current (ACC). Therefore, in spite of their vastness, Antarctic waters can be considered as a closed basin (Eastman and Clarke, 1998). This isolation had lead to the evolution of unique characteristics that exist only in the Antarctic fish fauna (Cheng and Detrich, 2007; Chen et al., 2008).

Many studies have argued that the suborder Notothenioidei (Teleostei) has undergone a remarkable adaptive radiation (Bargelloni et al., 1994; Clarke and Johnston, 1996; Johns and Avise, 1998) and that this radiation is equivalent to a species flock (Eastman and Eakin, 2000). But within the suborder, the antarctic-endemic subfamily Trematominae (Nototheniidae) might also have undergone a rapid diversification (Janko et al., 2011). The subfamily is the dominant component of the Antarctic shelf fish fauna (DeWitt, 1971; Kock, 1992). *Trematomus* species occupy a large range of ecological niches. They have been widely studied for their biogeography, evolutionary biology and physiology (Eastman, 1993). However, their interrelationships are still unclear. Part of the problem is the relatively recent diversification of the group, estimated from 2 to 9.8 Ma depending on the study (for a review see Near, 2004). For such recent species, systematic investigations can be complicated by hybridisation, introgression, incomplete lineage sorting, in addition to the classical methodological problems of phylogenetic reconstruction also present for deeper divergences

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(Kubatko and Degnan, 2007; Degnan and Rosenberg, 2009; Janko et al., 2011).

The monophyly of Trematominae has always been recovered in every large-scale notothenioid study, as was the position of *T. scotti* as a sister species to all others (Bargelloni et al., 1994; Ritchie et al., 1996; Ritchie et al.1997; Stankovic et al., 2002; Near et al., 2004; Sanchez et al., 2007). Similarly, *T. loennbergii* and *T. lepidorhinus* are always in the same clade (Ritchie et al., 1996; Near et al., 2004; Sanchez et al., 2007; Near and Cheng, 2008; Kuhn and Near, 2009), and *T. bernacchii* and *T. vicarius* are also closely related (Sanchez et al., 2007; Janko et al., 2011). The genus *Pagothenia* (*P. borchgrevinki* and *P. brachysoma*) is repeatedly found within the genus *Trematomus* (Ritchie et al., 1996; Ritchie et al., 1997; Bargelloni et al., 2000; Near et al., 2004; Sanchez et al., 2007; Kuhn and Near, 2009; Lautredou et al., 2010). The genus *Cryothenia* includes two species (*Cryothenia amphitreta* and *Cryothenia peninsulae*). *Cryothenia amphitreta*, recently described, nests within *Trematomus* based on both morphological and molecular data (Cziko and Cheng, 2006; Kuhn and Near, 2009). The phylogenetic position of the other species, *Cryothenia peninsulae* is not yet clear. The genus *Trematomus* might therefore contain fourteen species: eleven currently classified in the genus *Trematomus*, two currently in the genus *Pagothenia* and at least one of the two species currently in genus *Cryothenia*.

Several recent molecular-based studies have tried to resolve the relationships within Trematominae. Apart from the few species with a repeated position, the results are not congruent from one study to another, and there is even disagreement between datasets within a single study. However, these studies had either a low number of markers (Near et al., 2004; Sanchez et al., 2007; Near and Cheng, 2008; Kuhn and Near, 2009; Lautredou et al., 2010), or a low number of specimens per species (Near et al., 2004; Sanchez et al., 2007; Near and Cheng, 2008; Janko et al., 2011). A low number of specimens per species might be an issue as some previous studies using nuclear markers (for instance S7 in Kuhn and Near (2009)) do not recover the monophyly of all the species, hinting at intraspecific polymorphism. This suggests that an adequate sampling of species and including a higher number of individuals per species is necessary as well to explore all the aspects of the history of this group.

Moreover, the variability of the markers used is an important issue. Microsatellites have been developed for *Trematomus newnesi* and five other species: *Pagothenia borchgrevinki*, *Trematomus bernacchii*, *Trematomus eulepidotus*, *Trematomus hansoni* and *Trematomus scotti* (Van Houdt et al., 2006; Van de Putte et al., 2009). While these are very variable and useful to compare individuals within a species, they are problematic as phylogenetic characters across multiple species (Ellegren et al., 1997). Within this group, it is hard to find nuclear markers variable enough to resolve the relationships among the Trematominae and to strongly support the tree (Janko et al., 2011). The mitochondrial markers are generally more adapted to work on recent species, but rarely allow unveiling complex histories including deep coalescence, hybridisation or introgression.

The goal of this paper is to get a better insight into the complex history of the recent speciation of Trematominae by increasing simultaneously the number of specimens per species and the number of variable markers. It is the largest species sampling to date for the group. It was sequenced for five markers, one mitochondrial (CO1: cytochrome oxidase 1) and four single-copy nuclear markers: Pkd1 (Lautredou et al., 2010, polycystic kidney disease), Rhodopsin retrogene (Chen et al., 2003) as well as two markers never used before for phylogenetics, HECW (E3 ubiquitin-protein ligase HECW2) and SSRP (structure-specific recognition protein 1).

2. Materials and methods

2.1. Taxon sampling

Only three previous studies included multiple specimens per species in Trematominae phylogeny (Kuhn and Near, 2009; Lautredou et al., 2010; Janko et al., 2011). We include here all the species from the genus *Trematomus*. For each of the two genera *Pagothenia* (*Pagothenia borchgrevinki* and *Pagothenia brachysoma*) and *Cryothenia* (*Cryothenia amphitreta* and *Cryothenia peninsulae*), only *Cryothenia amphitreta* and *Pagothenia borchgrevinki* were included, as the other two species are extremely rare. The taxonomic sampling is summarised in Table 1.

Morphological identifications were performed during the sampling cruise, and checked in the lab in the case of discrepancies between morphological and molecular results by Ozouf-Costaz and Denys, using their taxonomic expertise and the characters listed in DeWitt et al. (1990) and Fisher and Hureau (1987). The *C. amphitreta* sequenced here is the type specimen (Cziko and Cheng, 2006). For *T. lepidorhinus* and *T. loennbergii* specimens which are difficult to distinguish, we retained the original identifications performed on board.

The four outgroups (*Gobionotothen gibberifrons*, *Lepidonotothen squamifrons*, *Notothenia coriiceps*, *Patagonotothen guntheri*) form three non-monophyletic outgroups according to Near et al. (2004) and Sanchez et al. (2007). The clade formed by *Lepidonotothen* and *Patagonotothen* was the sister group of *Trematomus* in both of these studies.

2.2. Gene sampling

Five markers were selected for this study (Table 2). CO1 (cytochrome oxidase 1), Rhodopsin retrogene and Pkd1 (polycystic kidney disease) had already been used in Trematominae (Lautredou et al., 2010). HECW (E3 ubiquitin-protein ligase HECW2) and SSRP (structure-specific recognition protein 1) are new, and were developed with a protocol modified from Dettai and Lecointre (2008) and Li et al. (2009). Genome/genome filtering the ENSEMBL database (http://www.ensembl.org/) release 41 using the Biomart mining tool (Haider et al., 2009) yielded a list of protein-coding sequences common to at least three of the available teleost genomes including Tetraodon nigroviridis, Takifugu rubripes and Danio rerio. Long coding sequences were investigated manually to find exons larger than 500pb, starting with the sequences with high sequence divergence between *T. nigroviridis* and *T. rubripes* to maximise our chances to identify markers that provide information at a smaller scale. Sequences of interest were downloaded and aligned using BioEDIT (Hall, 1999).

Conserved regions of length superior to 20 bp were identified by eye in the sequence alignments, and imported in OLIGO 4.1 to refine them into primers.

Several pairs of primers were ordered and tested for each new marker, and only the primers and markers that could be amplified easily were retained. The sequenced region of the SSRP gene spans an intron, but all the other markers are partial sequences from exons of protein coding genes. This is the first time that HECW and SSRP have been used for a phylogenetic study.

2.3. Molecular data

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

Table 1
Specimens included in this study. Specimen information, GenBank and BOLD Accession numbers are listed. The accession numbers are the 'consensus' sequences of each individual, the sequences before phasing each sequence using the FastPhase algorithm implemented in DnaSP v5.

Species	Latitude	Longitude	Depth (m)	Voucher number	BOLD Accession NbCOI	GenBank Accession Nb Pkd1	GenBank Accession Nb Rhodopsin	GenBank Accession Nb HECW	GenBank Accession Nb SSRP
Cryothenia amphitreta	−77.85	166.65	20	USNM 385901	EATFR002	JQ063253	JQ063279	JQ063175	JQ063312
Pagothenia borchgrevinki					GU997395	GU997454	GU997248	JQ063176	JQ063313
Trematomus bernacchii					GU997403	GU997458	GU997257	JQ063177	JQ063314
					GU997404	GU997459	GU997258	JQ063178	JQ063315
					GU997406	GU997461	GU997260	JQ063179	JQ063316
					GU997407	GU997462	GU997261	JQ063180	JQ063317
					GU997410	GU997463	GU997264	JQ063181	JQ063321
					GU997411	GU997464	GU997265	JQ063182	JQ063322
	−66.559853	140.797323	361	MNHN 2009-1310	EATF345	GU997466	GU997269	JQ063183	JQ063318
	−66.559853	140.797323	361	MNHN 2009-1311	EATF346	GU997467	GU997270	JQ063184	JQ063319
	−66.559853	140.797323	361	MNHN 2009-1312	EATF347	GU997468	GU997271	JQ063185	JQ063320
Trematomus eulepidotus	−66.3202	143.649	570	MNHN 2009-1354	EATF046	GU997469	GU997272	JQ063186	JQ063329
	−66.534813	141.982677	520	MNHN 2009-1232	EATF110	GU997474	JQ063280	JQ063187	JQ063330
	−66.534813	141.982677	520	MNHN 2009-1233	EATF111	GU997475	GU997277	JQ063188	JQ063331
	−66.534813	141.982677	520	MNHN 2009-1235	EATF113	GU997476	GU997279	JQ063189	JQ063332
	−66.534813	141.982677	520	MNHN 2009-1236	EATF114	JQ063254	JQ063281	JQ063190	JQ063333
	−66.534813	141.982677	520	MNHN 2009-1237	EATF115	GU997477	GU997280	JQ063191	JQ063334
	−66.5348	141.983	520.4	MNHN 2009-1238	EATF116	GU997478	GU997281	JQ063192	JQ063335
	−66.534813	141.982677	520	MNHN 2009-1239	EATF117	GU997479	GU997282	JQ063193	JQ063336
	−66.534813	141.982677	520	MNHN 2009-1240	EATF118	JQ063255	JQ063282	JQ063194	JQ063337
	−66.539917	145.290892	403	MNHN 2009-1263	EATF211	JQ063256	GU997283	JQ063195	JQ063338
	−65.869947	143.001547	430	MNHN 2009-1271	EATF253	GU997481	JQ063283	JQ063196	JQ063339
	−65.869947	143.001547	430	MNHN 2009-1272	EATF254	GU997482	JQ063284	JQ063197	JQ063340
	−65.912427	143.966988	370	MNHN 2009-1287	EATF284	GU997485	GU997284	JQ063198	JQ063341
	−66.335097	141.272662	207	MNHN 2009-1298	EATF325	GU997486	GU997285	JQ063199	JQ063342
	−66.516823	140.001423	176	MNHN 2009-1314	EATF358	GU997491	JQ063286	JQ063200	JQ063343
	−66.148263	140.649927	213	MNHN 2009-1343	EATF425	GU997500	JQ063287	JQ063201	JQ063344
	−66.1691	139.932	149.9	MNHN 2009-1358	EATF480	GU997501	JQ063288	JQ063202	JQ063345
Trematomus hansoni					GU997412	GU997503	GU997300	JQ063203	JQ063323
	−66.667	140.017	25	MNHN 1962-	EATF595	GU997506	GU997303	JQ063204	JQ063324

(*continued on next page*)

Table 1 (*continued*)

Species	Latitude	Longitude	Depth (m)	Voucher number	BOLD Accession NbCOI	GenBank Accession Nb Pkd1	GenBank Accession Nb Rhodopsin	GenBank Accession Nb HECW	GenBank Accession Nb SSRP
				1037					
	−66.6634	140.034817	230		GU997417	GU997508	GU997306	JQ063205	JQ063325
	−66.683033	139.998933			GU997420	GU997511	GU997309	JQ063206	JQ063326
	−74.717	164.133	143–174	MNHN 1999-0388	EATF586	GU997512	GU997312	JQ063207	JQ063327
					GU997423	GU997513	GU997313	JQ063208	JQ063328
Trematomus tokarevi	−66.338398	140.02921	510	MNHN 2009-1345	EATF442	GU997593	GU997387	JQ063229	JQ063383
	−66.1706	139.353	673.5	MNHN 2009-1357	EATF474	GU997594	JQ063307	JQ063230	JQ063384
Trematomus vicarius	−54.30183	−37.4217		SAIAB75107	EATF588	GU997595	GU997388	JQ063248	JQ063385
Trematomus newnesi					GU997431	GU997570	GU997348	JQ063223	JQ063360
	−66.661833	139.989667	46		GU997428	GU997563	GU997349	JQ063224	JQ063361
	−66.5599	140.797	360.9	MNHN 2009-1369	EATF533	GU997562	JQ063294	JQ063225	JQ063362
Trematomus lepidorhinus	−66.534813	141.982677	520	MNHN 2009-1242	EATF120	GU997525	GU997323	JQ063209	JQ063346
	−66.7505	143.95	640.9	MNHN 2009-1247	EATF162	JQ063257	JQ063289	JQ063210	JQ063347
	−66.54375	143.990627	787	MNHN 2009-1254	EATF194	GU997531	JQ063290	JQ063211	JQ063348
	−66.738715	144.307023	904	MNHN 2009-1258	EATF202	GU997533	GU997330	JQ063212	JQ063349
	−66.738715	144.307023	904	MNHN 2009-1259	EATF203	GU997534	GU997331	JQ063213	JQ063350
	−66.315523	143.301408	693	MNHN 2009-1269	EATF241	GU997537	JQ063291	JQ063214	JQ063351
	−65.869947	143.001547	430	MNHN 2009-1276	EATF257	GU997539	GU997334	JQ063215	JQ063352
	−66.338398	140.02921	510	MNHN 2009-1351	EATF449	GU997559	GU997346	JQ063216	JQ063353
Trematomus loennbergii	−67.046928	145.15082	1267	MNHN 2009-1248	EATF166	GU997529	JQ063292	JQ063217	JQ063354
	−66.38878	140.428852	791	MNHN 2009-1322	EATF376	GU997545	JQ063293	JQ063218	JQ063355
	−66.38878	140.428852	791	MNHN 2009-1325	EATF379	GU997548	GU997338	JQ063219	JQ063356
	−66.338398	140.02921	510	MNHN 2009-1348	EATF446	GU997556	GU997343	JQ063220	JQ063357
Trematomus nicolai					GU997441	GU997575	GU997359	JQ063221	JQ063358
					GU997437	GU997572	GU997356	JQ063222	JQ063359
Trematomus pennellii	−66.308813	142.29392	217	MNHN 2009-1376	EATF095	GU997578	GU997368	JQ063226	JQ063363
	−66.308813	142.29392	217	MNHN 2009-1378	EATF097	GU997580	JQ063295	JQ063227	JQ063364
	−71.267	−13.067	186	MNHN 1991-0563	EATF592	GU997576	GU997372	JQ063228	JQ063365
Trematomus scotti	−66.052413	142.763643	452	MNHN 2009-1289	EATF002	JQ063258	JQ063296	JQ063231	JQ063366
	−66.052413	142.763643	452	MNHN 2009-1252	EATF017	JQ063259	JQ063297	JQ063232	JQ063367

	−66.052413	142.763643	452	MNHN 2009-1253	EATF018	JQ063260	GU997379	JQ063233	JQ063368
	−66.052413	142.763643	452	MNHN 2009-1260	EATF020	JQ063261	GU997587	JQ063234	JQ063369
	−66.052413	142.763643	452	MNHN 2009-1266	EATF021	JQ063262	GU997381	JQ063235	JQ063370
	−66.000458	143.297105	473	MNHN 2009-1293	EATF031	JQ063263	GU997382	JQ063236	JQ063371
	−66.000458	143.297105	473	MNHN 2009-1297	EATF032	JQ063264	JQ063298	JQ063237	JQ063372
	−66.003943	143.716085	426	MNHN 2009-1320	EATF037	JQ063265	GU997383	JQ063238	JQ063373
	−66.333198	143.357078	702	MNHN 2009-1371	EATF077	JQ063266	GU997384	JQ063239	JQ063374
	−66.308813	142.29392	217	MNHN 2009-1379	EATF098	JQ063267	JQ063299	JQ063240	JQ063375
	−66.308813	142.29392	217	MNHN 2009-1227	EATF099	JQ063268	JQ063300	JQ063241	JQ063376
	−66.308813	142.29392	217	MNHN 2009-1228	EATF100	JQ063269	JQ063301	JQ063242	JQ063377
	−66.538527	144.972508	441	MNHN 2009-1262	EATF210	JQ063270	JQ063302	JQ063243	JQ063378
	−65.912427	143.966988	370	MNHN 2009-1285	EATF282	JQ063271	JQ063303	JQ063244	JQ063379
	−66.38878	140.428852	791	MNHN 2009-1331	EATF386	JQ063272	JQ063304	JQ063245	JQ063380
	−65.706925	140.597385	423.9	MNHN 2009-1367	EATF531	JQ063273	JQ063305	JQ063246	JQ063381
	−65.706925	140.597385	423.9	MNHN 2009-1368	EATF532	JQ063274	JQ063306	JQ063247	JQ063382
Notothenia coriiceps						JQ063166	JQ063249	JQ063275	JQ063171	JQ063308
Lepidonotothen squamifrons						JQ063168	JQ063252	JQ063278	JQ063174	JQ063311
Gobionotothen gibberifrons						JQ063167	JQ063250	JQ063276	JQ063172	JQ063309
Patagonotothen guntheri						JQ063169	JQ063251	JQ063277	JQ063173	JQ063310

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.

Gene	Frag. size	Name	Sens	Primers	*T*° of hyb.	Sources
Mitoch.						
COI	≈650 bp	FishF1	F	5′-TCAACCAACCACAAAGACATTGGCAC-3′	52 °C	Ward et al. (2005)
		FishR1	R	5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′		
		FishF2	F	5′-TCGACTAATCATAAAGATATCGGCAC-3′		
		FishR2	R	5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′		
Nuclear						
Rhodo.	≈800 bp	RhF193	F	5′-CNTATGAATAYCCTCAGTACTACC-3′	50 °C	Chen et al. (2003)
		RhR1039	R	5′-TGCTTGTTCATGCAGATGTAGA-3′		
Pkd1	≈800 bp	Pkd1F62	F	5′-CATGAGYGTCTACAGCATCCT-3′	50 °C	Lautredou et al. (2010)
		Pkd1R952	R	5′-YCCTCTNCCAAAGTCCCACT-3′		
HECW	≈670 bp	HECWF160	F	5′-GCTTGTTACTATGNAGAYGACAG-3′	55 °C	This study
		HECWR838	R	5′-CTCACCTGAATGGGKGAAAG-3′		
SSRP	≈560 bp	SSRP34	F	5′-GCAAACTGAGCTATGGTTGT-3′	55 °C	This study
		SSRP600	R	5′-RTTTCCTTGAAGCGCAGGTG-3′		

For the five markers, DNA amplification was performed by PCR in a final 21 μl volume containing 1 μl of DMSO, 1 μl of BSA, 0.80 μl of dNTP 6.6 mM, 0.12 μl of Taq DNA polymerase (MP Biomedicals or Qiagen), using 2 μl of the buffer provided by the manufacturer, 0.32 μl of each of the two primers at 10 pM (primer sequences in Table 2); 1 μl of DNA extract was added.

After denaturation for 2 min, the PCR was run for 50 cycles of (20 s, 94 °C; 20 s, see Table 2 for hybridisation temperatures; 50 s to 1 min 10 s, 72 °C) using a Biometra trioblock cycler (T3000). The results were visualised on ethidium-bromide stained agarose gels. Sequencing was performed by the National Center for Sequencing (Génoscope) at Evry using the same primers.

All markers were sequenced in both directions and sequence chromatograms were checked manually using Sequencher 4.8 (Gene Codes Corporation). The sequences were aligned by hand using BioEdit (Hall, 1999), and were controlled against sample mix-ups and contaminations by pairwise comparison.

The CO1 sequence of *Cryothenia amphitreta* was deposited in BOLD with their accompanying information. The nuclear 'consensus' sequences were deposited in GenBank (accession numbers in Table 1).

2.4. Datasets and alignment

All the CO1 sequences of Trematominae available in our laboratory and in the BOLD were used to make a larger dataset of 222 sequences. The sequences available for the nuclear markers were both analysed separately and used to make a concatenated dataset of all the protein-coding sequences. All the sequences were aligned manually.

2.5. Phylogenetic analyses

For the Maximum Likelihood analyses (ML) and Bayesian phylogenetic inference (BI), we used the sequences of each individual without phasing them. Thus, each individual is a terminal in the trees.

Each of the five datasets was analysed separately with both Maximum Likelihood and Bayesian Inference. Each marker was partitioned by codon position, except for SSRP which is partly non-coding. Thus, three partitions were made for COI, HECW, Pkd1 and Rhodopsin and one partition for SSRP.

We also performed ML and BI analyses of the concatenated dataset for all taxa. Once again, each marker was partitioned by codon position, except SSRP. Thirteen partitions were therefore used for the ML and BI analyses of the five concatenated markers.

Maximum Likelihood analyses (ML): The ML analyses were run with RaxML 7.2.7 on the Cipres Science Gateway online web server (Miller et al., 2010), with the GTR + G model implemented. The robustness of the nodes of the cladograms was estimated by the bootstrap method (Felsenstein, 1985) with 1000 replicates for each analysis.

Bayesian phylogenetic inference (BI): Using MrModeltest 2.3 (Posada and Crandall, 1998), we found the recommended model for partition is GTR + I + G under the AIC criterion. All the parameters were estimated during the analyses. BI analyses were conducted with Mr. Bayes v. 3.1.2 (Huelsenbeck and Ronquist, 2005) on each dataset. Four chains (three heated and one cold) were run for 10,000,000 generations for each of the two independent runs for each dataset (one tree out of 100 sampled). All the trees were pooled after a burn-in of 5%, after checking it was sufficient for convergence.

Multispecies coalescent analysis and dating: *BEAST v1.6.1 (Bayesian Evolutionary Analysis Sampling Trees) (Heled and Drummond, 2010) was used to estimate the species tree. For these analyses, we phased each sequence using the FastPhase algorithm implemented in DnaSP v5 (Librado and Rozas, 2009) to estimate more precisely the nucleotide diversity during the coalescence analysis.

In *BEAST, thirteen different trait sets were defined according to the species determination for each individual. The GTR + I + G was set for each separate dataset, and the model parameters were estimated with the tree topologies in the analysis. In addition to the substitution model, a random local clock model and tree topologies were estimated independently for each marker. We used a mitochondrial tree model for CO1 and an autosomal model for nuclear loci, with randomly generated starting trees in all cases.

Four runs of 150,000,000 generations were performed, sampling once every 1000 tree. Posterior probabilities of the nodes were computed for all Bayesian analysis across the sampled trees after burn-in. The burn-in was set as 5000 trees (5000,000 generations). The effective sample sizes (ESS) of parameters of interest (gene trees, species tree, root age) were all above 200. *BEAST XML data file was deposited in the Dryad Repository: http://dx.doi.org/10.5061/dryad.gd365jq4.

The dating procedure is not so much aimed at determining the absolute age of the root of the Trematominae but to evaluate the relative ages of each clade within this group. By comparing these relative ages, the number of divergence events in trematomines can be evaluated. *BEAST v1.6. automatically calibrates the root to a relative age of 1.0, that we scaled *a posteriori* to 24.1 Ma following Near (2004). As some limitations and potential inconsistence could be introduced in the results with secondary calibration (Shaul and

Graur, 2002; Heads, 2005; but see Hedges and Kumar, 2004) we did not directly use the 24.1 Ma point in the analysis. Using this approach allowed the different substitution rates to converge in an independent posterior distribution.

3. Results and discussion

Multiple individuals were included for each species except for *Pagothenia borchgrevinki* and *Cryothenia amphitreta*. The length of the CO1 dataset is 516 bp, the Rhodopsin retrogene dataset is 678 bp, the Pkd1 dataset is 720 bp, the HECW dataset is 363 bp and SSRP is 421 bp long (Table 3). The sequences of the four coding markers display no stop codons and the alignment was straightforward. This confirms that they are single-copy markers in trematomines, as they are in the five teleosts with completely sequenced genomes (*Danio rerio, Tetraodon nigroviridis, Takifugu rubripes, Oryzias latipes Gasterosteus aculeatus*). These five markers are located on different chromosomes in the genome of the stickleback, the available genome closest to notothenioids.

3.1. Variability of the four markers and constitution of the datasets

Table 3 summarises the variability of the markers included here and the variability of the markers used in Janko et al. (2011). Unsurprisingly, the only mitochondrial marker (CO1) is the most variable marker for the number of variable sites and the number of parsimony informative sites. Within nuclear markers, ITS1 is the most variable for the number of variable sites and the number of parsimony informative sites. Nuclear loci highly variable for Trematominae are rare. Within the subfamily, only two of the eight markers of Janko et al. (2011) have a variability equal to or above 3%, whereas all the markers in this study have a variability equal or above 3%.

Haplotype diversity: All nuclear markers have multiple haplotypes for each species. These haplotypes are however shared mainly between sister species pairs, excluding extensive hybridization as the source (Hartl and Clark, 2007). HECW exhibits the highest haplotype diversity (60 haplotypes) and Rhodopsin the lowest (22 haplotypes), with CO1, Pkd1 and SSRP showing 47, 45 and 35 haplotypes respectively. The number of among-species shared haplotypes do not show a clear correlation with haplotype diversity, with HECW, CO1, Pkd1, SSRP and the Rhodopsin harbouring six, two, two, seven and five haplotypes shared among species, respectively (see Fig. 2 and Table 4).

Table 3
Evaluation of the number of variable sites and the number of parsimony informative sites. One specimen per species was retained. The percentage of variable sites or number of parsimony informative sites is indicated in parentheses and is calculated from the total length of the sequences for every marker.

	Genes	Dataset length	Number of variable sites	Number of parsimony informative sites
Mitoch.				
	COI	516	115 (22%)	104 (20%)
Nuclear				
This study	Rhodopsine	678	34 (5%)	30 (4%)
	Pkd1	720	47 (6%)	35 (5%)
	HECW	363	30 (8%)	10(3%)
	SSRP	421	59 (14%)	19(4%)
Janko et al. (2011)	RPS7	621	77 (12%)	20 (3%)
	GNRH	381	20 (5%)	4(1%)
	IRBP1	757	48 (6%)	11(1%)
	ITS1	524	81 (15%)	25 (5%)
	ITS2	410	34 (8%)	8 (2%)
	T1	621	33 (5%)	11 (2%)
	T5	346	35 (10%)	7 (2%)
	T16	272	8 (3%)	1 (0.4%)

3.2. Gene tree incongruence

In the separate analyses of ML and BI of the five markers, Trematominae are monophyletic for the COI and Rhodopsin topologies. However, the relationships among the species are not recovered from one tree to another. Moreover, for some species all individuals are not always grouped together, although the affected species are not always the same depending on the marker. For example, in the Pkd1 tree, *T. newnesi* is not monophyletic. There are no problematic species in the CO1 and the Rhodopsin trees (Fig. 1A) whereas the lack of monophyly affects all the species in the SSRP and HECW trees (Fig. 1B).

Several hypotheses can explain this gene tree incongruence: hybridisation, introgression, incomplete lineage sorting (ILS), and errors due to stochastic character sampling. The last hypothesis has a lower probability in the present study because of the relatively high variability of the markers used (Table 3) (Chen et al., 2003; Lautredou et al., 2010). Stochastic character sampling errors could explain discrepancies among separate trees when the number of mutational events and the number of variable positions are both very low (Swofford and Olsen, 1996) but it is not the case here.

The hybridisation hypothesis can probably also be rejected. The number of chromosomes within *Trematomus* varies a great deal from one species to another, which probably presents a substantial barrier against at least some of the inter-specific hybridisation. Chromosome diploid numbers and formulae of trematomines are species-specific (except for *T. loennbergii* which presents several formula, however a more in depth study with a higher number of individuals would be necessary). They range from $2n = 24$ (*T. eulepidotus)* to $2n = 58$ (*T. nicolai*) and variations could be accounted for by Robertsonian fusion or fission events (review in Pisano and Ozouf-Costaz (2002)).

Moreover, there are no close associations of haplotypes frequencies among species for the markers used, as would be expected if there are preferential genetic material exchanges between some species pairs. Some haplotypes of *Trematomus* species are shared with other species belonging to the outgroup (red haplotype of SSRP marker). *Trematomus* species therefore probably exhibit the traces of widespread ILS (Fig. 2). Deep coalescence leading to ILS is considered to be the most frequent explanation for gene tree incongruence in recently diverged species (Edwards, 2009). ILS in Trematominae has already been invoked to explain incongruence among gene trees from independent loci or to explain trees in which species are not monophyletic (Janko et al., 2011). This explanation has also been proposed for a number of other teleost species flocks (Moran and Kornfield, 1993; McMillan and Palumbi, 1995; Strecker et al., 1996; Sullivan et al., 2002; Near et al., 2006). It is to date the most reasonable explanation of the pattern of haplotypes we find among the trematomine fishes, taking into account what we know about their cytogenetics. Different coalescence times are also observed according to the expected fixation rates. For example, CO1 exhibits the lowest number of shared haplotypes, in agreement with the high fixation rate generally found in mitochondrial loci (Bensasson et al., 2001).

Analyses of the group therefore need to take into account the ILS phenomenon which has rarely been considered in its phylogenetic studies. Janko et al. (2011) was the only study thus far to have performed this type of analyses whereas all the remaining studies (Ritchie et al., 1997; Near et al., 2004; Sanchez et al., 2007; Near and Cheng, 2008; Kuhn and Near, 2009) did either not include sufficient sampling or did not assess incomplete lineage sorting. When substantial disagreement in individual gene trees is observed and is believed to be due to incomplete lineage sorting,

Table 4
Haplotypes found in the Trematominae species for each locus. The numbers of sequences of each haplotype harboured by species are shown in parenthesis. Shared haplotypes in bold.

Species	CO1	HECW	Pkd1	Rhodopsin	SSRP
N. coriceps	Hap 1(2)	Hap_1(2)	Hap_1(2)	**Hap** 1(2)	Hap 1(2)
G. giberriffrons	Hap_2(2)	Hap_2(2)	Hap_2(2)	Hap_2(2)	Hap_2(2)
P. guntheri	Hap 4(2)	Hap_3(2)	**Hap_3(1)**; Hap_4(1)	Hap_3(2)	Hap_3(2)
L. squamifrons	Hap_3(2)	Hap_3(2)	Hap_4(1); Hap_5(1)	**Hap_3(2)**	Hap_4(1); Hap_5(1)
Hap_4(2) *amphitreta*	Hap_5(2)	**Hap_6(2)**	Hap_5(2)	Hap_6(2)	Hap_5(1); Hap_6(1)
P. borchgrevinki	Hap_6(2)	Hap_7(2)	Hap_6(2)	**Hap_7(2)**	
Hap_4(1); Hap_7(1)					
bernachii	Hap_7(10); **Hap_8(8)**	**Hap_6(9)**; Hap_8(4); **Hap_9(3)**; Hap_10(1); Hap_11(1)	Hap_7(7); Hap_8(8); Hap_9(1); Hap_10(1); Hap_11(1)	**Hap_8(16)**; Hap_9(1); Hap_10(1)	**Hap_4(6)**; **Hap_8(11)**; Hap_9(1)
T. hansoni	Hap_18(2); Hap_19(4); Hap_20(2); Hap_21(2); Hap_22(2)	**Hap_6(10)**; Hap_27(2)	Hap_17(5); Hap_18(2); Hap_19(3); Hap_20(1); Hap_21(1)	Hap_13(12)	Hap_10(3); Hap_11(9)
T. eulepidotus	Hap 9(4); Hap 10(14); Hap_11(4); Hap_12(2); Hap_13(2); Hap_14(2); Hap_15(2); Hap_16(2); Hap_17(2)	**Hap_6(8)**; **Hap_9(4)**; Hap_12(1); Hap_13(1); Hap_14(1); Hap_15(2); Hap_16(2); Hap_17(3); Hap_18(1); Hap_19(4); Hap_20(1); Hap_21(1); Hap_22(1); Hap_23(1); Hap_24(1); Hap_25(1); Hap_26(1)	Hap_12(7); Hap_13(2) Hap_14(8); Hap_15(14); Hap_16(3)	Hap_11(31); Hap_12(3)	**Hap_4(4)**; Hap_12(1); Hap_13(2); Hap_14(3); **Hap_15(6)**; Hap_16(8); Hap_17(1); Hap_18(1); **Hap 19(2)**; Hap 20(2); Hap_21(2); Hap_22(2)
T. lepidorhinus		Hap_23(2); Hap_24(2); Hap_25(2); Hap_26(2); Hap_27(2); Hap_28(2); Hap_29(2); Hap_30(2)	**Hap_6(7)**; **Hap_28(1)**; Hap_29(1); **Hap_30(3)**; Hap_31(1); Hap_32(1); Hap_33(2)	Hap_22(1); Hap_23(2); **Hap_24(10)**; Hap_25(1); Hap_26(1); Hap_27(1)	Hap_14(6); **Hap_15(4)**; **Hap_16(6)**
Hap_4(2;	**Hap_19(2)**; Hap_23(4); **Hap_24(3)**; Hap_25(1); Hap_26(2); Hap_27(2)				
T. loennbergii	Hap_31(2); Hap_32(2); Hap_33(2)	**Hap_6(5)**; **Hap_28(1)**; **Hap_30(1)**; **Hap_34(1)**	**Hap_24(8)**	**Hap_15(1); Hap_16(7)**	**Hap_15(2)**; Hap_20(2); **Hap_24(2)**; Hap_28(1); Hap_29(1)
T. newnesi	Hap_34(2); Hap_35(4)	Hap_35(4); Hap_36(2)	Hap_28(3); Hap_29(2); Hap_30(1)	**Hap_7(1)**; Hap_17(5)	**Hap_4(4)**; **Hap_8(2)**
T. nicolai	Hap_36(4)	**Hap_6(1)**; **Hap_34(3)**	Hap_31(2); Hap_32(2)	Hap_18(4)	Hap_30(3); Hap_31(1)
T. penellii	Hap_37(4); Hap_38(2)	**Hap_6(2)**; Hap_37(2); Hap_38(1); Hap_39(1)	Hap_33(2); Hap_34(1); Hap_35(3)	Hap_19(1); Hap_20(5)	Hap_32(4); Hap_33(2)
T. tokarevi	Hap_46(4); Hap_47(2)	Hap_40(1); Hap_41(3)	Hap_43(3); Hap_44(1)	**Hap_1(2)**; Hap_22(2)	**Hap_10(4)**
T. vicarius	**Hap_8(2)**	Hap_59(1); Hap_60(1)	Hap_45(2)	**Hap_8(2)**	**Hap_4(1)**; **Hap_8(1)**
T. scotti	Hap 39(20); Hap_40(2); Hap_41(2); Hap_42(2); Hap_43(4); Hap_44(2); Hap 45(2)	Hap_42(1); Hap_43(2); Hap 44 (3); Hap_45(10); Hap_46(1); Hap_47(2); Hap 48(1); Hap_49(1); Hap_50(1); Hap_51(1); Hap 52(1); Hap_53(3); Hap_54(1); Hap_55(1); Hap 56(1); Hap_57(2); Hap 58(2)	Hap_36(18); Hap 37(1); Hap_38(10); Hap_39(2); Hap_40(1); Hap_41(1); Hap_42(1)	Hap 21(34)	Hap_34(32); Hap_35(2)

sampling more individuals per species is probably beneficial (Maddison and Knowles, 2006). The taxonomic sampling used here is composed of 13 of the known 14 species and multiple individuals per species.

3.3. Species tree inferences

Coalescent analysis of the five markers: The *BEAST tree (Fig. 2) recovers Trematominae monophyly, and *T. scotti* is the first to diverge from the other species.

T. bernacchii and *T. vicarius* are sister-groups as in previous studies (Sanchez et al., 2007; Lautredou et al., 2010) and form the clade A. This clade is strongly supported. *T. hansoni* is the sister-group of clade A.

T. loennbergii and *T. lepidorhinus* are grouped in our tree like in previous studies (Ritchie et al., 1996; Sanchez et al., 2007; Near and Cheng, 2008; Kuhn and Near, 2009). This grouping of *T. lepidorhinus* and *T. loennbergii* is strongly supported by the posterior probabilities.

T. eulepidotus is the sister group of *T. loennbergii*/*T. lepidorhinus* forming the clade B. In 2000, based on morphological data, Bargelloni and colleagues proposed that *Trematomus newnesi* was the sister taxon to *Pagothenia* and *Cryothenia*. In our tree, *T. newnesi* is the sister-group of *P. borchgrevinki*. This relationship is also recovered by other sequence data (Near and Cheng, 2008), but not well supported, neither here nor in other studies. The positions of *C. amphitreta*, *T. nicolai*, *T. tokarevi* and *T. pennellii* are not repeated and not well supported, and are therefore still unclear.

ML and BI combined analyses of the five markers (Fig. 3): In BI, the monophyly of trematomines is not supported, whereas it is in the ML. Similarly to the *BEAST analysis, the ML and the BI trees recover *T. eulepidotus* as the sister-group of *T. lepidorhinus*–*T. loennbergii*,

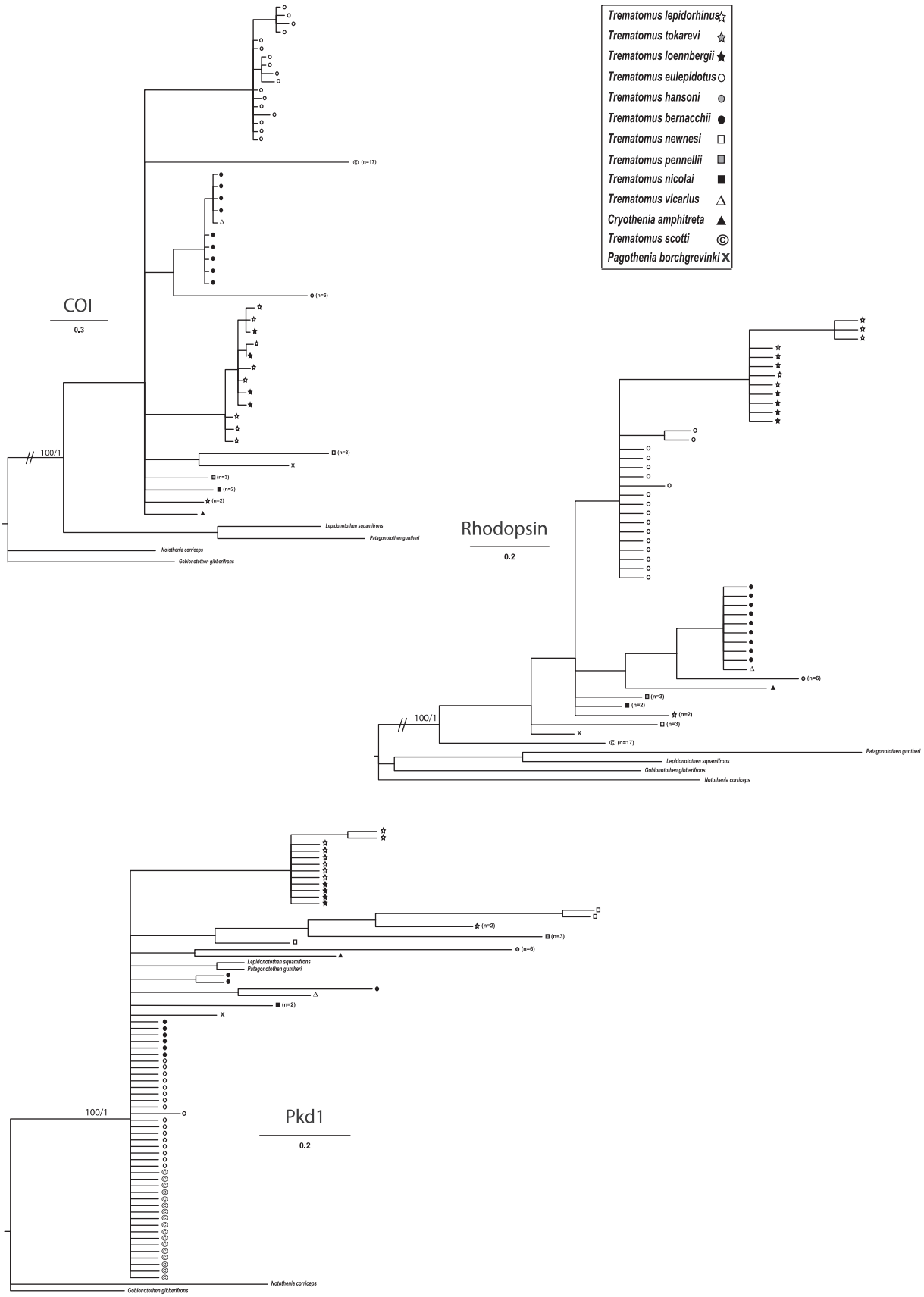

Fig. 1. (A) Bayesian and maximum likelihood trees obtained by the analyses of the CO1, Rhodospin and Pkd1 markers. Nodes with no indication have bootstrap values or posterior probabilities below 70 or 0.95. Each symbol represents a species. (B) Bayesian and maximum likelihood trees obtained by the analyses of the SSRP and HECW markers. Nodes with no indication have bootstrap values or posterior probabilities below 70 or 0.95. Each symbol represents a species.

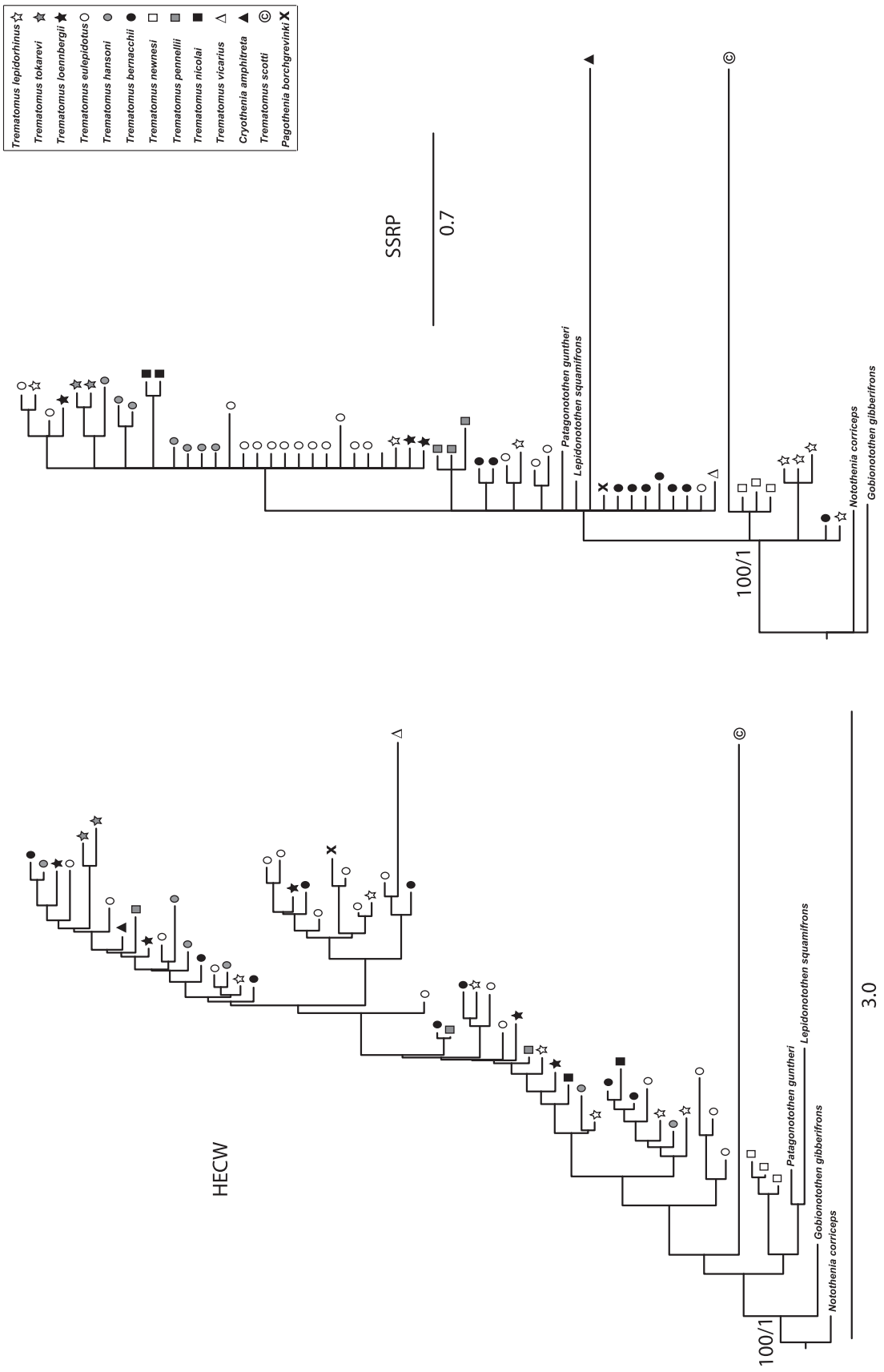

Fig. 1 (*continued*)

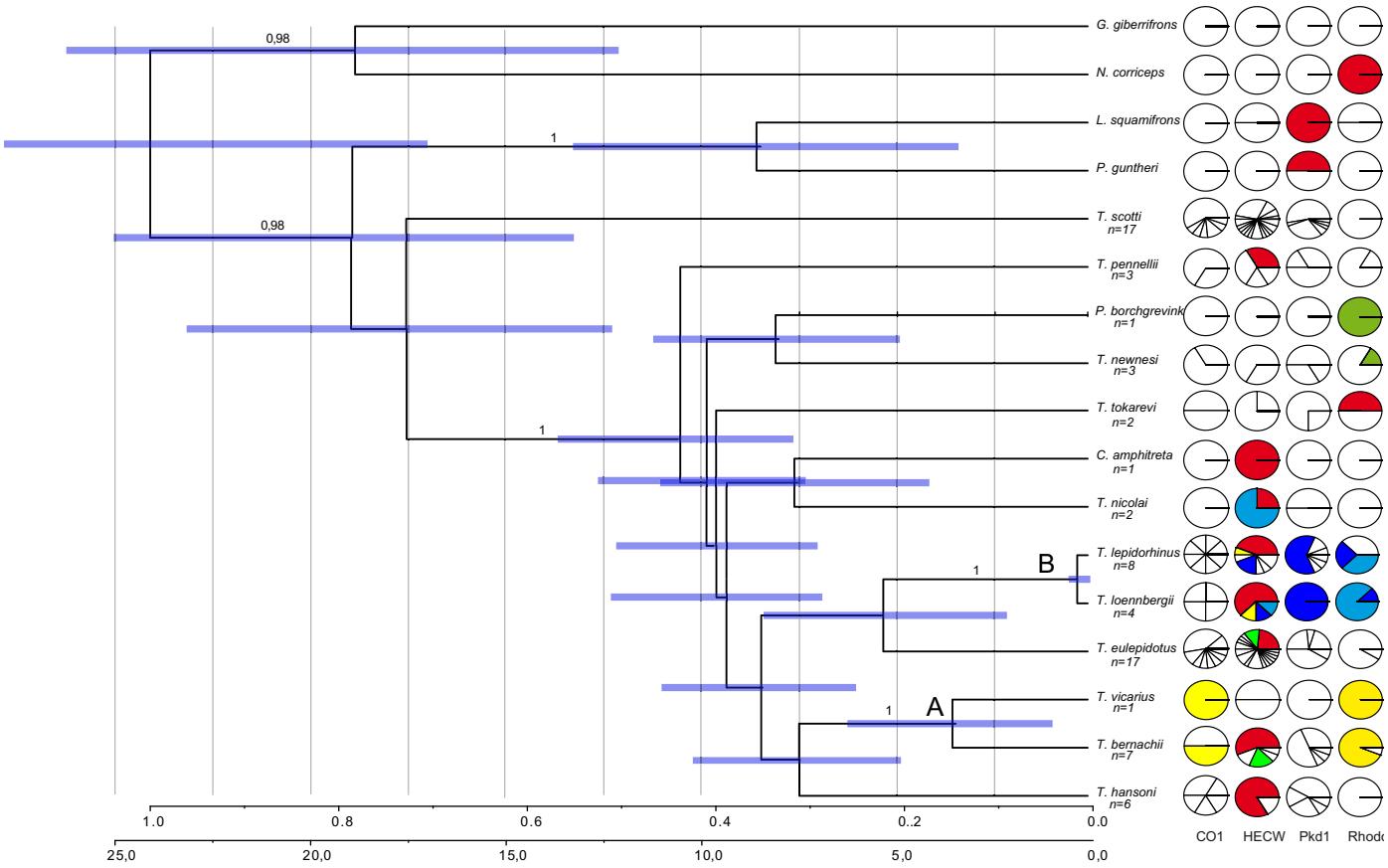

Fig. 2. Bayesian chronogram of the Trematominae (*BEAST sofware). The pies represent the proportions of the different haplotypes for each locus. Shared haplotypes for a marker are indicated by a shared colour across species. For legibility, only the shared haplotypes among species are coloured. Confidence intervals for node date estimates in blue. Nodes with no indication have posterior probabilities below 0.95.

and *T. hansoni* as the sister-group of *T. bernachii* and *T. vicarius*. These relationships are well supported by posterior probabilities in BI but not by bootstrap values in ML. In both analyses, *C. amphitreta* is sister-group to the *T. hansoni–T. bernachii–T. vicarius* clade, although the support is very low in ML. The ML analysis recovers the *T. newnesi –P. borchgrevinki* clade from the *BEAST analysis, but with moderate bootstrap support. The positions of *T. nicolai*, *T. tokarevi* and *T. pennellii* are not repeated between the two analyses.

If histories from independent genes are shared, it is probably because they are the traces left by species cladogenesis. It is highly unlikely that several independent gene histories resemble each other purely by chance (Miyamoto and Fitch, 1995). Including all the datasets in a single analysis is recommended to enhance the extraction of the shared signal (Barrett et al., 1991; Bull et al., 1993; de Queiroz 1993; Kluge, 2002). However, this is not always optimally exploited by classical combined analyses, that can be biased by one of the included markers (Chen et al., 2003) or rendered inconsistent when substantial ILS results from short internal branches in the species trees (Kubatko and Degnan, 2007; Degnan and Rosenberg, 2009; Heled and Drummond, 2010).

In the present study, the BI tree of the concatenated dataset divides *T. eulepidotus* in two clades well supported by posterior probabilities (these clades are also present, but with shorter branches and less support, in the ML tree). This grouping in two clades is not supported by any of the separate analyses. *T. eulepidotus* specimens are either grouped within the same clade (COI, Rhodopsin, Pkd1, Fig. 1A), or all over the tree (SSRP, HECW, Fig. 1B). However, the combined analyses "average" these very different positions reflective of different histories to produce well-supported artefactual groups with long branch lengths.

Repetition of information coming from different markers is more valuable to extract reliability. With the use of the *BEAST software, we avoid this problem. Indeed, the species tree is determined using coalescent produced gene trees that statistically match the species tree (Heled and Drummond, 2010). This way, *BEAST extracts the concordant information from each locus with the produced species tree, using MCMC (Drummond and Rambaut, 2007; Heled and Drummond, 2010).

Trematomus lepidorhinus and loennbergii complex: In the studies where several individuals identified as *T. lepidorhinus* and *T. loennbergii* are included, they do not form two distinct groups (Kuhn and Near, 2009; Lautredou et al., 2010). Moreover, they are the only species pair to share haplotypes for all nuclear genes.

Specimens recognised as *T. lepidorhinus* on the basis of morphological characters all have 48 chromosomes, whatever the locality of capture is. For the specimens recognised as *T. loennbergii* several karyomorphs have been observed, among and within different localities ranging from 30 to 46 chromosomes and showing extensive re-patterning (Pisano and Ozouf-Costaz, 2002 and Ozouf-Costaz, comm. pers.). As already stated in Lautredou et al. (2010), studies including these two species should provisionally list them as *T. loennbergii*/*T. lepidorhinus* complex until further investigations.

Position of Cryothenia peninsulae: The genus *Cryothenia* includes two species: *Cryothenia amphitreta* (Cziko and Cheng, 2006) and *Cryothenia peninsulae* on which the genus has been described (Daniels, 1981).

In the CO1 tree (data not shown), *C. peninsulae* does not nest within Trematominae, but is placed with the outgroups *Gobionotothen gibberifrons* and *Notothenia coriiceps*. The single CO1 sequence (EU326339) available for *C. peninsulae* is very similar (above 99%)

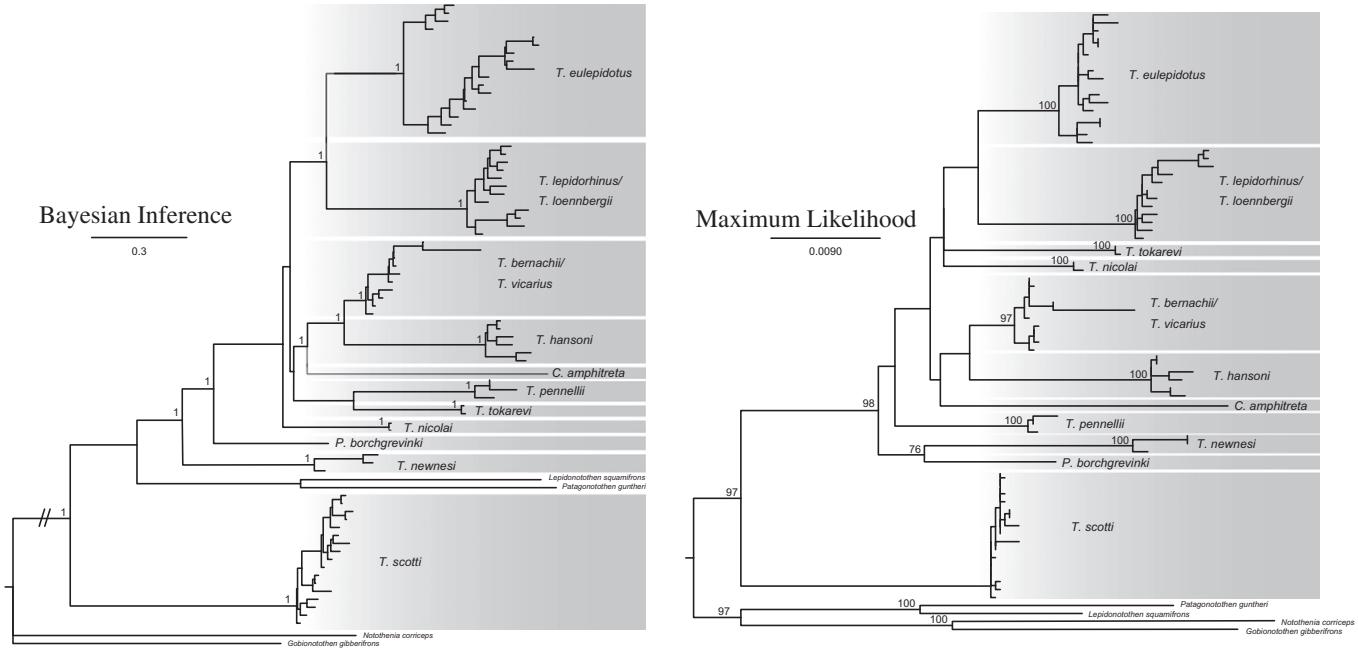

Fig. 3. Bayesian and maximum likelihood trees of the simultaneous analysis of the five markers. Nodes with no indication have bootstrap values or posterior probabilities below 70 (ML analysis) or 0.95 (Bayesian analysis).

to the CO1 sequences of *Aethotaxis mitopteryx*, a morphologically and molecularly distinct notothenid species. Previous studies have shown that the CO1 sequences of most antarctic teleosts are distinct even between closely related species (Lautredou et al., 2010; Dettai et al., 2011), which is not the case here: sequence contamination or misidentification are therefore the most probable hypotheses. The identification of the sequenced specimen must be checked before it is possible to conclude that *C. peninsulae* does not belong to Trematominae.

3.4. Classification of the Trematominae

Trematomus taxa were split into two different genera by Balushkin (1982): *Trematomus* and *Pseudotrematomus*. Only *Trematomus newnesi* was retained in the genus *Trematomus*, and all other remaining *Trematomus* species were placed in *Pseudotrematomus*.

This generic division was based on several morphological criteria of the pectoral fins. Based on the position of *T. newnesi* in this study and all the other previous molecular studies, the separation of this species does not appear to be supported. As *Trematomus* (Boulenger, 1902) is older than *Pseudotrematomus* (Jones et al., 2008), *Trematomus* is the only valid name for the species belonging to Trematominae.

The position of *Pagothenia borchgrevinki* is consistent with many morphological and molecular studies (Bargelloni et al., 1994; Ritchie et al., 1996; Ritchie et al., 1997; Sanchez et al., 2007; Lautredou et al., 2010). Moreover, *Pagothenia borchgrevinki* had initially been included within the genus *Trematomus* but later moved into the genus *Pagothenia* based on morphological characters (DeWitt, 1962).

There are two species in the genus and although we were unable to test the position of *Pagothenia brachysoma*, we assume that *Pagothenia brachysoma* is also a *Trematomus* species.

Cziko and Cheng (2006) suggested that *Cryothenia amphitreta* belongs to the T*rematomus*.

As *Pagothenia borchgrevinki* and *Cryothenia amphitreta* are again included within *Trematomus* in this study, we hereby propose to change their name to *Trematomus borchgrevinki* and *Trematomus amphitreta*, as has already been proposed by Sanchez et al. (2007) and Kuhn and Near (2009).

3.5. Radiation of the Trematominae: a species flock?

All divergence events within the subfamily occurred around ≈10 millions years (between 0.3 and 0.5 time units, corresponding to 7 and 12 Ma) except for the clades A and B and *T. scotti*. This is congruent with the most recent literature (9.8 ± 0.4 according to Near (2004), Rutschmann et al. (2011) and Near et al. (2012)). In older papers, the main divergence (all Trematominae except *T. scotti*) was estimated at 3.4 Ma (Ritchie et al., 1996) using a rate of 0.14% of transversional changes per million year for mitochondrial ribosomal DNA. Bargelloni et al. (1997) estimated this divergence from 2.5 to 4.5 Ma while Near (2004), using a fossil-based calibration estimated it at 7.4 ± 0.3 Ma. The mid-Miocene Climatic Optimum (when a warming trend reduced the extent of Antarctic ice, 15 Ma) episode was followed by a gradual cooling and reestablishment of a major ice-sheet on Antarctica by 10 Mya (Poulin et al., 2002). The emergence of the Trematominae appears to coincide with the re-establishment of the ice sheet in the Antarctic shelf.

Radiations in freshwater fishes are well documented (Salzburger and Meyer, 2004) but there are far less studies on marine fishes radiations (Johns and Avise, 1998; Eastman and McCune, 2000; McCartney et al., 2003; Duda and Rolan, 2005). The rapid increase in the number of lineages through time previously shown (Janko et al., 2011) favours the hypothesis of a radiation event as highlighted by the Fig. 2. The ILS, the weak support and short internodes in all the trees from multiple markers are the footprints of a rapid radiation event, and are all found in every phylogenetic trees of the Trematominae. The trematomine radiation should be interpreted as a soft polytomy (in the sense of Maddison (1989)). Some hints about this diversification might be found in the chromosomal or the ecological specificities of the group. The *Trematomus* and *Pagothenia* species exhibit the highest chromosomal diversity among the notothenioid clades (Pisano and Ozouf-Costaz, 2002). Chromosomal barriers can set up efficient barriers to reproduction within a short time frame, and could be an explanation for the high number

of speciation events in the group (White et al., 2010; Nunes et al., 2011). This would have to be investigated further. Two recent studies suggested an ecological radiation in Trematominae, showing several colonisation to all available niches during the ice-sheet expansion (Matschiner et al., 2011; Near et al., 2012).

Species flocks: The Antarctic continental shelf has been described as a giant species flock generator (Eastman and McCune, 2000), because of a number of physical similarities with ancient lakes where species flocks were found (isolation, depth and age). Several studies have argued that the Trematominae is a species flock (Clarke and Johnston, 1996; Eastman and Clarke, 1998; Johns and Avise, 1998). It is also possible to recognise the Trematominae as a species flock by using the criteria of Eastman and McCune (2000). Starting from both the definition of Ribbink (1984), and the one of Greenwood (1984), Eastman and McCune (2000) proposed five criteria to identify (or detect) such a radiation: monophyly, high number of species, high level of endemism, morphological and ecological diversification, and habitat dominance (in terms of biomass).

With fourteen species, all endemic to the coastal areas of the Southern Ocean, the Trematominae minus *T. scotti* corresponds to a sudden burst of diversification (Janko et al., 2011, and Fig. 2). The group is monophyletic (Sanchez et al., 2007; Near et al., 2004; Near and Cheng, 2008), and there is a noticeable degree of ecological diversity which does not fit the phylogeny (Eastman, 1993; Klingenberg and Ekau, 1996; Ritchie et al., 1996; Sanchez et al., 2007). They represent an important part of the biomass of the coastal ichtyofauna. The number of species is the weakest criterion (14 species, i.e. more than 10% of the notothenioids, 134 species) however it reflects the relatively low number of teleost species in the Southern Ocean compared to other oceans (Eastman, 1993, 2005).

4. Conclusion

In this study, we have included two additional nuclear markers (HECW and SSRP) with a high variability, while also increasing the number of specimens. Those markers can directly be used for phylogenetic studies on different groups of acanthomorph fish at small scale. The present approach to select new markers shows its usefulness here again. It had already led to the identification of several new nuclear markers before (Dettai and Lecointre, 2004, 2008; Li et al., 2009). It could easily be used in routine to find new variable markers for future phylogenetic work.

These two new markers are among the most variable nuclear markers used for studies on the Trematominae. Other recently defined nuclear markers (myh6, Ptr and tbr1, Li et al., 2007) have one, three and six differences over the whole sequence between *Trematomus nicolai* and *Trematomus newnesi* (Rutschmann et al., 2011; Near et al., 2012), whereas HECW and SSRP have seven and eight respectively.

The challenges associated with inferring evolutionary relationships of recently diverged species differ significantly from those for deep phylogenetic divergence. At shallow time depths, random events such as ILS predominate (Sanderson and Shaffer, 2002; Kubatko and Degnan, 2007; Janko et al., 2011). This is the first time that ILS is strongly supported in Trematominae even if it could be suspected from the S7 tree of Kuhn and Near (2009) and was suggested in the study of Janko et al. (2011). This is why looking at the separate analyses of the five markers is an essential step in the data exploration, especially in small scale phylogenetic studies, as well as including a high number of specimens per specie and using appropriate methods. Taking into account biological data on Trematominae such as the pattern of among-species haplotype distribution and their chromosomal diversification, we conclude that ILS is the best interpretation of the incongruence observed among the gene trees in Trematominae. Powerful methods (Knowles, 2009; Liu et al., 2009) have been developed to deal with ILS-provoked incongruence. Unfortunately, this is only possible with the analysis of a very large number of nuclear markers (Knowles, 2009; Liu et al., 2009), and might not be possible even then in case of rapid lineage diversification (McCormack et al., 2009).

The rapid increase in the number of lineages through time (present study, Janko et al., 2011; Near et al., 2012) is in favour of a radiation event in the subfamily, maybe driven by ecological diversification (Matschiner et al., 2011; Near et al., 2012). All the trees from multiple markers display the hallmarks of a rapid radiation event in Trematominae: ILS, weak support and short internodes.

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References

Balushkin, A.V., 1982. Classification of Antarctic Trematomine fishes [In Russian]. Biology of the Shelf Zones of the World Ocean, Vladivostok, pp. 9–10.

Bargelloni, L., Ritchie, P.A., Patarnello, T., Battaglia, B., Lambert, D.M., Meyer, A., 1994. Molecular evolution at subzero temperatures – mitochondrial and nuclear phylogenies of fishes from Antarctica (Suborder Notothenioidei), and the evolution of antifreeze glycopeptides. Mol. Biol. Evol. 11, 854–863.

Bargelloni, L., Patarnello, T., Ritchie, P.A., Battaglia, B., Meyer, A., 1997. Molecular phylogeny and evolution of notothenioid fish based on partial sequences of 12S and 16S ribosomal RNA mitochondrial genes. In: Battaglia, B., Valencia, J., Walton, D.W.H. (Eds.), Antarctic Communities: Species, Structure & Survival. Cambridge University Press, Cambridge, pp. 45–50.

Bargelloni, L., Marcato, S., Zane, L., Patarnello, T., 2000. Mitochondrial phylogeny of notothenioids: a molecular approach to Antarctic fish evolution and biogeography. Syst. Biol. 49, 114–129.

Barrett, M., Donoghue, M.J., Sober, E., 1991. Against consensus. Syst. Zool. 40, 486–493.

Bensasson, D., Zhang, D.-X., Hartl, D.L., Hewitt, G.M., 2001. Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends Ecol. Evol. 16 (6), 314–321.

Boulenger, G.A., 1902. Pisces. Report on the collections of natural history made in the Antarctic regions during the voyage of the "Southern Cross" (ix, 344p, 53 leaves of plates)

Bull, J.J., Huelsenbeck, J.P., Cunningham, C.W., Swofford, D.L., Waddell, P.J., 1993. Partitioning and combining data in phylogenetic analysis. Syst. Biol. 42, 384–397.

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.

Chen, Z., Cheng, C.-H.C., Zhang, J., Cao, L., Chen, L., Zhou, L., Jin, Y., Ye, H., Deng, C., Dai, Z., Xu, Q., Hu, P., Sun, S., Shen, Y., Chen, L., 2008. Transcriptomic and genomic evolution under constant cold in Antarctic notothenioid fish. Proc. Nat. Acad. Sci. USA 103, 10491–10496.

Cheng, C.-H.C., Detrich III, H.W., 2007. Molecular ecophysiology of Antarctic notothenioid fishes. Philos. Trans. R. Soc. Lond. B 362, 2215–2232.

Clarke, A., Johnston, I.A., 1996. Evolution and adaptive radiation of Antarctic fishes. Trends Ecol. Evol. 11, 212–218.
Cziko, P.A., Cheng, C.H.C., 2006. A new species of Nototheniid (Perciformes: Notothenioidei) fish from McMurdo Sound, Antarctica. Copeia, 752–759.
Daniels, R.A., 1981. *Cryothenia peninsulae*, a new genus and species of nototheniid fish from the Antarctic Peninsula. Copeia 3, 558–562.
De Queiroz, A., 1993. For consensus (sometimes). Syst. Biol. 42, 368–372.
Degnan, J.H., Rosenberg, N.A., 2009. Gene tree discordance, phylogenetic inference and the multispecies coalescent. Trends Ecol. Evol. 24 (6), 332–340.
Dettai, A., Lecointre, G., 2004. In search of notothenioid (Teleostei) relatives. Antarc. Sci. 16 (1), 58–71.
Dettai, A., Lecointre, G., 2008. New insights into the organization and evolution of vertebrate IRBP genes and utility of IRBP gene sequences for the phylogenetic study of the Acanthomorpha (Actinopterygii: Teleostei). Mol. Phylogenet. Evol. 48, 258–269.
Dettai, A., Adamowizc, S.J., Allcock, L., Arango, C.P., Barnes, D.K.A., Barratt, I., Chenuil, A., Couloux, A., Cruaud, C., David, B., Denis, F., Denys, G., Diaz, A., Eleaume, M., Feral, J.P., Froger, A., Gallut, C., Grant, R., Griffiths, H.J., Held, C., Hemery, L.G., Hosie, G., Kuklinski, P., Lecointre, G., Linse, K., Lozouet, P., Mah, C., Monniot, F., Norman, M.D., O'Hara, T., Ozouf-Costaz, C., Piedallu, C., Pierrat, B., Poulin, E., Puillandre, N., Riddle, M., Samadi, S., Saucede, T., Schubart, C., Smith, P.J., Stevens, D.W., Steinke, D., Strugnell, J.M., Tarnowska, K., Wadley, V., Ameziane, N., 2011. DNA barcoding and molecular systematics of the benthic and demersal organisms of the CEAMARC survey. Polar Sci. 5, 298–312.
DeVries, A.L., Cheng, C.-H.C., 2005. Antifreeze proteins and organismal freezing avoidance in polar fishes. In: Farrell, A.P., Steffensen, J.F. (Eds.), The Physiology of Polar Fishes. Elsevier Academic Press, San Diego, pp. 155–201.
DeWitt, H.H., 1962. A new Antarctic notothenii fishes with notes on two recently described notothenii forms. Copeia 4, 819–833.
DeWitt, 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.
DeWitt, H.H., Heemstra, P.C., Gon, O., 1990. Nototheniidae. In: Gon, O., Heemstra, P.C. (Eds.), Fishes of the Southern Ocean, pp. 279–399.
Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol 7, 214. http://dx.doi.org/10.1186/1471-2148-7-214.
Duda, T.F., Rolan, E., 2005. Explosive radiation of Cape Verde *Conus*, a marine species flock. Mol. Ecol. 14 (1), 267–272.
Eastman, J.T., 1993. Antarctic Fish Biology. Academic Press, San Diego.
Eastman, J.T., 2005. The nature of the diversity of Antarctic fishes. Polar Biol. 28, 93–107.
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.
Eastman, J.T., Eakin, R.R., 2000. An updated species list for notothenioid fish (Perciformes; Notothenioidei), with comments on Antarctic species. Arch. Fish. Mar. Res. 48 (1), 11–20.
Eastman, J.T., McCune, A.R., 2000. Fishes on the Antarctic continental shelf: evolution of a marine species flock? J. Fish Biol. 57, 84–102.
Edwards, S.V., 2009. Is a new and general theory of molecular systematics emerging? Evolution 63, 1–19.
Ellegren, H., Moore, S., Robinson, N., Byrne, K., Ward, W., Sheldon, B.C., 1997. Microsatellite evolution – a reciprocal study of repeat lengths at homologous loci in cattle and sheep. Mol. Biol. Evol. 14, 854–860.
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.
Greenwood, P.H., 1984. What is a species flock? In: Echelle, A.A., Kornfield, I. (Eds.), Evolution of Fish Species Flocks. University of Maine at Orono Press, Orono, pp. 13–19.
Haider, S., Ballester, B., Smedley, D., et al., 2009. BioMart Central Portal unified access to biological data. Nucleic Acid. Res. 37, W23–W27.
Hall, T.A., 1999. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis. Department of Microbiology, North Carolina State University.
Hartl, D.L., Clark, A.G., 2007. Principles of Population Genetics, fourth ed. Sinauer, Sunderland, MA.
Heads, M., 2005. Dating nodes on molecular phylogenies: a critique of molecular biogeography. Cladistics 21, 62–78.
Hedges, S.B., Kumar, S., 2004. Precision of molecular time estimates. Trends Genet. 20, 242–247.
Heled, J., Drummond, A.J., 2010. Bayesian inference of species trees from multilocus data. Mol. Biol. Evol. 27, 570–580.
Huelsenbeck, J.P., Ronquist, F., 2005. Bayesian analysis of molecular evolution using MrBayes. In: Nielsen, R. (Ed.), Statistical Methods in Molecular Evolution. Springer, New York.
Janko, K., Marshall, C., Musilova, Z., Van Houdt, J., Couloux, A., Cruaud, C., Lecointre, G., 2011. Multilocus analyses of an Antarctic fish species flock (Teleostei, Notothenioidei, Trematominae): phylogenetic approach and test of the early-radiation event. Mol. Phylogenet. Evol. 60, 305–316.
Johns, G.C., Avise, J.C., 1998. Tests for ancient species flocks based on molecular phylogenetic appraisals of Sebastes rockfishes and other marine fishes. Evolution 52, 1135–1146.
Jones, C.D., Anderson, M.E., Balushkin, A.V., Duhamel, G., Eakin, R.R., Eastman, J.T., Kuhn, K.L., Lecointre, G., Near, T.J., North, A.W., Stien, D.L., Vacchi, M., Detrich, H.W., 2008. Diversity, relative abundance, new locality records and population structure of Antarctic demersal Wshes from the northern Scotia Arc islands and Bouvetøya. Polar Biol. 31, 1481–1497.
Klingenberg, C.P., Ekau, W., 1996. A combined morphometric and phylogenetic analysis of an ecomorphological trend: pelagization in Antarctic fishes (Perciformes: Nototheniidae). Biol. J. Linn. Soc. 59, 143–177.
Kluge, A.G., 2002. Distinguishing "or" from "and" and the case for historical identification. Cladistics 18, 585–593.
Knowles, L.L., 2009. Estimating species trees: methods of phylogenetic analysis when there is incongruence across genes. Syst. Biol. 58 (5), 463–467. http://dx.doi.org/10.1093/sysbio/syp061.
Kock, K.H., 1992. Antarctic Fish and Fisheries. Cambridge Univ. Press, Cambridge.
Kubatko, L.S., Degnan, J.H., 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. Syst. Biol. 56 (1), 17–24.
Kuhn, K.L., Near, T.J., 2009. Phylogeny of Trematomus (Notothenioidei: Nototheniidae) inferred from mitochondrial and nuclear gene sequences. Antarc. Sci. 21, 565–570.
Lautredou, A.C., Bonillo, C., Denys, G., Cruaud, C., Lecointre, G., Rey, O., Dettai, A., 2010. Molecular taxonomy and identification of genus Trematomus (Notothenioidei, Teleostei): how good is barcoding with COI? Polar Sci. 4, 333–352.
Li, C., Orti, G., Zhang, G., Lu, G., 2007. A practical approach to phylogenomics: the phylogeny of ray-finned fish (Actinopterygii) as a case study. BMC Evol. Biol. 7, 44.
Li, B., Dettai, A., Cruaud, C., Couloux, A., Desoutter-Meniger, M., Lecointre, G., 2009. RNF213, a new nuclear marker for acanthomorph phylogeny. Mol. Phylogenet. Evol. 50, 345–363.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Liu, L., Yu, L., Pearl, D.K., Edwards, S.V., 2009. Estimating species phylogenies using coalescence times among sequences. Syst. Biol. 58 (5), 468–477. http://dx.doi.org/10.1093/sysbio/syp031.
Maddison, W., 1989. Reconstructing character evolution on polytomous cladograms. Cladistics 5, 365–377.
Maddison, W.P., Knowles, L.L., 2006. Inferring phylogeny despite incomplete lineage sorting. Syst. Biol. 55 (1), 21–30.
Matschiner, M., Hanel, R., Salzburger, W., 2011. On the origin and trigger of the notothenioid adaptive radiation. PLoS ONE 6 (4), e18911.
McCartney, M.A., Acevedo, J., Heredia, C., Rico, C., Quenoville, B., Berminghams, E., Mc Millan, W.O., 2003. Genetic mosaic in a marine species flock. Mol. Ecol. 12, 2963–2973.
McCormack, J.E., Huang, H., Knowles, L.L., 2009. Maximum likelihood estimates of species trees: how accuracy of phylogenetic inference depends upon the divergence history and sampling design. Syst. Biol. 58 (5), 501–508.
McMillan, W.O., Palumbi, S.R., 1995. Concordant evolutionary patterns among Indo-West Pacific butterflyfishes. Proc. Biol. Sci. 260, 229–236.
Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. Gateway Computing Environments Workshop (GCE), New Orleans, pp. 1–8.
Miyamoto, M.M., Fitch, W.M., 1995. Testing species phylogenies and phylogenetic methods with congruence. Syst. Biol. 44, 64–76.
Moran, P., Kornfield, I., 1993. Retention of an ancestral polymorphism in the Mbuna species flock (Teleostei, Cichlidae) of Lake Malawi. Mol. Biol. Evol. 10, 1015–1029.
Near, T., 2004. Estimating divergence times of notothenioid fishes using a fossil calibrated molecular clock. Antarc. Sci. 16, 37–44.
Near, T.J., Cheng, C.H., 2008. Phylogenetics of notothenioid fishes (Teleostei: Acanthomorpha): inferences from mitochondrial and nuclear gene sequences. Mol. Phylogenet. Evol. 47, 832–840.
Near, T.J., Pesavento, J.J., Cheng, C.H., 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.
Near, T.J., Parker, S.K., Detrich, H.W., 2006. A genomic fossil reveals key steps in hemoglobin loss by the Antarctic icefishes. Mol. Biol. Evol. 23 (11), 2008–2016.
Near, T.J., Dornburg, A., Kuhn, K.L., Eastman, J.T., Pennington, J.N., Patarnello, T., Zane, L., Fernández, D.A., Jones, C.D., 2012. Ancient climate change, antifreeze, and the evolutionary diversification of Antarctic fishes. PNAS, pp. 1–6.
Nunes, A.C., Catalan, J., Lopez, J., da Graca Ramalhinho, M., da Luz Mathias, M., Britton-Davidian, J., 2011. Fertility assessment in hybrids between monobrachially homologous Rb races of the house mouse from the island of Madeira: implications for modes of chromosomal evolution. Heredity 106, 348–356.
Pisano, E., Ozouf-Costaz, C., 2002. Cytogenetics and evolution in extreme environment: the case of Antarctic Fishes. In: Val, A., Kapoor, G. (Eds.), Fish Adaptation. Oxford IBH Publishing, New Delhi, pp. 311–338.
Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.
Poulin, E., Palma, A.T., Feral, J.P., 2002. Evolutionary versus ecological success in Antarctic benthic invertebrates. Trends Ecol. Evol. 7 (5), 218–222.
Ribbink, A.J., 1984. Is the species flock concept tenable? In: Echelle A.A., et Kornfield, I. (Eds.), Evolution of Fish Species Flocks, pp. 21–25.
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.

Ritchie, P.A., Lavoue, S., Lecointre, G., 1997. Molecular phylogenetics and the evolution of Antarctic Notothenioid fishes. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 118, 1009–1025.

Rutschmann, S., Matschiner, M., Damerau, M., Muschick, M., Lehmann, M.F., Hanel, R., Salzburger, W., 2011. Parallel ecological diversification in Antarctic notothenioid fishes as evidence for adaptive radiation. Mol. Ecol. 20, 4707–4721.

Salzburger, W., Meyer, A., 2004. The species flocks of East African cichlid fish: recent advances in molecular phylogenetics and population genetics. Naturwissenschaften 91, 277–290.

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

Sanderson, M.J., Shaffer, H.B., 2002. Troubleshooting molecular phylogenetic analyses. Annu. Rev. Ecol. Syst. 33, 49–72.

Shaul, S., Graur, D., 2002. Playing chicken (*Gallus gallus*): methodological inconsistencies of molecular divergence date estimates due to secondary calibration points. Gene 300, 59–61.

Stankovic, A., Spalik, K., Kamler, E., Borsuk, P., Weglenski, P., 2002. Recent origin of sub-Antarctic notothenioids. Polar Biol. 25, 203–205.

Strecker, U., Meyer, C.G., Sturmbauer, C., Wilkens, H., 1996. Genetic divergence and speciation in an extremely young species flock in Mexico formed by the genus Cyprinodon (Cyprinodontidae, Teleostei). Mol. Phylogenet. Evol. 6, 143–149.

Sullivan, J.P., Lavoue, S., Hopkins, C.D., 2002. Discovery and phylogenetic analysis of a riverine species flock of African electric fishes (Mormyridae: Teleostei). Evolution 56, 597–616.

Swofford, D.L., Olsen, G.J., 1996. Phylogeny reconstruction. In: Hillis, D.H., Moritz, C. (Eds.), Molecular Systematics. Sinauer Associates, Sunderland, MA, pp. 411–501.

Van de Putte, A.P., 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.

Van Houdt, J.K.J., Hellemans, B., Van de Putte, A., Koubbi, P., Volckaert, F.A.M., 2006. Isolation and multiplex analysis of six polymorphic microsatellites in the Antarctic notothenioid fish, *Trematomus newnesi*. Mol. Ecol. Notes 6, 157–159.

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

White, T.A., Bordewich, M., Searle, J.B., 2010. A network approach to study karyotypic evolution: the chromosomal races of the common shrew (*Sorex araneus*) and house mouse (*Mus musculus*) as model systems. Syst. Biol. 59 (3), 262–276.

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