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ScienceDirect
Molecular Phylogenetics and Evolution 45 (2007) 887–903
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Extensive dinoflagellate phylogenies indicate infrequent marine–freshwater transitions

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Received 23 January 2007; revised 26 July 2007; accepted 9 August 2007
Available online 26 August 2007

Abstract

We have constructed extensive 18S–28S rDNA dinoflagellate phylogenies (>200 sequences for each marker) using Maximum Likelihood and Bayesian Inference to study the evolutionary relationships among marine and freshwater species (43 new sequences). Our results indicated that (a) marine and freshwater species are usually not closely related, (b) several freshwater species cluster into monophyletic groups, (c) most marine–freshwater transitions do not seem to have occurred recently and, (d) only a small fraction of the marine lineages seem to have colonized fresh waters. Thus, it becomes apparent that the marine–freshwater boundary has acted as a barrier during the evolutionary diversification of dinoflagellates. Our results also shed light on the phylogenetic positions of several freshwater dinoflagellates which, to date, were uncertain.

Keywords: Dinoflagellates; Freshwater; Colonization; Phylogeny

1. Introduction

Most major microbial lineages originated in ancient oceans (e.g. Cavalier-Smith, 2006) and subsequently colonized fresh waters. From an evolutionary perspective, oceans can be considered as continents and lakes as islands for aquatic organisms. However, there is an important difference to take into account in this analogy. Marine and fresh waters are two environments which differ in their general physicochemical characteristics, and that has no parallel when continents and islands are compared. For most aquatic animals, the differences in osmotic pressure and ionic concentrations between marine and fresh waters represent a strong barrier that cannot be crossed by most species, which are normally adapted to one environment or the other, but not to both (Lee and Bell, 1999). It is unclear, however, to what extent the physicochemical differences affect the exchange of microbes between marine and fresh waters. In contrast to macroorganisms, most microbes have massive population sizes, high reproductive rates, high genetic diversity (e.g. Snoke et al., 2006) as well as the potential capability for long distance dispersal (Finlay, 2002). These characteristics suggest that frequent migration between marine and freshwater environments as well as rapid ecological diversification within species may be possible.

Traditionally, morphological classifications have suggested that several protist groups are segregated into predominantly marine and freshwater lineages (e.g. Taylor, 1987; Popovsky and Pfiester, 1990; Graham and Wilcox, 2000; Sims et al., 2006). However, the ambiguities of morphological phylogenies precluded further investigations on the phylogenetic relationships between marine and freshwater taxa. Today, molecular phylogenies open a new opportunity for studying the number and timing of marine–freshwater transitions during the evolutionary history

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doi:10.1016/j.ympev.2007.08.005

of a microbial lineage. For instance, the presence of a few monophyletic clusters of freshwater species that are distantly related to all other marine species within an ancestral marine lineage, would indicate that freshwater colonizations are rare and probably not recent events. On the other hand, the presence of many closely related marine and freshwater species or strains would suggest that marine–freshwater transitions have occurred frequently. Recent molecular phylogenies comprising and array of prokaryote and microeukaryote taxa indicate that marine and freshwater species are normally not closely related (Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Saldarriaga et al., 2004; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; von der Heyden and Cavalier-Smith, 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Lefevre et al., 2007). Despite the insight these works have provided, they normally include relatively few freshwater species and/or are mostly focused in other questions than marine–freshwater transitions.

Dinoflagellates, an ancestral marine protist lineage which is present in both marine and fresh waters, constitute a well suited group for investigating marine–freshwater transitions in microbes. DNA sequences (especially 18S and 28S rDNA) from many dinoflagellate taxa are publicly available and there is also a large amount of data on dinoflagellate morphology, physiology, ecology, and fossil record. Dinoflagellates appear to have diverged from ciliates and apicomplexans around 900 million years ago [MYA] (Escalante and Ayala, 1995). At the beginning of the Mesozoic (~250 MYA), dinoflagellates showed a tremendous evolutionary radiation (Fensome et al., 1996, 1999). Dinoflagellates have an enormous diversity of life strategies, with symbionts, parasites, free living planktonic or benthic photosynthesizers, heterotrophs and mixotrophs (Hackett et al., 2004). Usually, dinoflagellates spend most of their life cycles as haploid cells that proliferate by mitotic division (Von Stosch, 1973). Sexuality can be induced by endo- and exogenous factors, and in many cases, results in a diploid resting cyst with environmental resistance and dispersal functions (Pfiester and Anderson, 1987). Dinoflagellates have key roles in the functioning of marine and freshwater ecosystems, and several species are well known toxin producers (e.g. Hallegraeff, 1993).

The phylogenetic relationships between marine and freshwater dinoflagellates have for long been considered unclear (e.g. see Boltovskoy, 1999). To date, most molecular phylogenies have only included a small number of freshwater dinoflagellates, thus contributing little to the clarification of their phylogenetic positions. As a consequence, the number and timing of marine–freshwater transitions in dinoflagellates have so far remained a matter of speculation.

The main objectives of this study are thus: (a) to get insight into the role of the marine–freshwater boundary throughout the diversification of dinoflagellates by analyzing molecular phylogenies, and (b), to shed light into the phylogenetic relationships between marine and freshwater species. Our approach consisted of constructing extensive 18S and 28S (D1/D2) rDNA phylogenies, including 43 new dinoflagellate sequences and publicly available ones. In total, the used sequence dataset represent a major fraction of the dinoflagellate diversity. Our phylogenetic results using Maximum Likelihood and Bayesian Inference suggest that marine–freshwater transitions have been infrequent events during the diversification of dinoflagellates and that in most cases have not occurred recently. In addition, our results suggest the phylogenetic positions of several freshwater dinoflagellate species which to date have been uncertain.

2. Materials and methods

2.1. Morphospecies and DNA extraction

Freshwater and brackish photosynthetic dinoflagellate morphospecies were either obtained from cultures established at our laboratory from plankton samples, or from one of the following sources: Culture Collection of Algae at the University of Cologne, Germany (CCAC); National Institute for Environmental Studies, Japan (NIES); Culture Collection of Algae at the University of Göttingen, Germany (SAG); Culture Collection of Algae and Protozoa, UK (CCAP); The Culture Collection of Algae at the University of Texas at Austin, US (UTEX); private collections from A. Kremp [*Woloszynskia halophila* (Biechler) Kremp et al. WHTV, *Scrippsiella hangoei* (Schiller) Larsen SHTV (brackish)] and E. Kim [*Peridinium limbatum* (Stokes) Lemmermann PLCB, *P. willei* Huitfeld-Kaas PWCL] (Table 1). DNA was extracted from clonal single-cell isolated cultures following Adachi et al (1994).

2.2. Morphospecies identification

The dinoflagellate morphospecies isolated and cultured from our own plankton samples were fixed in formaldehyde 5% for further taxonomic identification. A number of dinoflagellate morphospecies obtained from culture collections were fixed in the same manner for taxonomic corroboration. Dinoflagellate plate detachment between slide and cover slip was carried out with the aid of diluted sodium hypochlorite instillation. Squashed empty thecae and detached plates were analyzed under a Standard 14 Zeiss optical microscope with Nomarsky interference contrast illumination. The morphospecies that were identified or corroborated for this work are specified in Table 1.

2.3. PCR and sequencing

For this study we have chosen the small (18S) and large (28S) subunit ribosomal DNA (SSU and LSU rDNA, respectively) because they have been sequenced for many marine dinoflagellates representing most major taxa, as

Table 1
Freshwater dinoflagellates included in the analyses and the 43 sequences obtained for this work

Morphospecies name	Strain	Geographical origin	GenBank accession number	
			SSU	LSU (D1/D2)
Ceratium hirundinella	None	Lake in San Juan island, Washington, USA	AY443014	—
Ceratium hirundinella	HZ-2004	Mirror Lake, Storrs, CT, USA	AY460574	—
Ceratium sp.	HCB-2005	Old Woman Creek NER, USA	DQ487192	—
Cystodinium phaseoulus	ASW12002	Lake Lunzer, Obersee, Austria	EF058235	—
Dinophyceae	Clone L	Lake Tovel, Italy	—	AY827950
Esoptrodinium gemma	None	University of Aveiro Campus, Portugal	—	DQ289020
Glenodiniopsis steinii	NIES 463	Shizukuishi, Iwate, Japan	AF274257	EF058255
Kryptoperidinium foliaceum	SAG 38.80	Assateague Nat. Wildlife Refuge, USA	—	EF058256
Gloeodinium montanum	CCAC0066	Marburg, Nordeck, Germany	EF058238	EF058258
Gymnodinium impatiens	CCAC0025	Bradenburg; Neuglobsow, Germany	EF058239	EF058259
Gymnodinium palustre	AJC14-732	?	—	AF260382
Gymnodinium sp.	None	Lake Tovel, Northern Italy	AY840208	—
Gymnodinium sp.	LaTo2	Lake Tovel, Northern Italy	AY829527	—
Gymnodinium sp.	Clone M	Lake Tovel, Northern Italy	—	AY829529
Gymnodinium sp.	Clone N	Lake Tovel, Northern Italy	—	AY829530
Gyrodinium helveticum	None	Lake Shikotsu, Hokkaido, Japan	AB120004	—
Hemidinium nasutum	NIES 471	Tsuchiura, Ibaraki, Japan	AY443016	EF058260
Jadwigia applanata	CCAC0021	Biebergemund-Bieber, Lochmuhle, Germany	EF058240	AY950447
Jadwigia applanata	FW 145	?	—	AY950448
Peridiniopsis borgei[a]	PBSK-A	St. kalkbrottsdammen, Skåne, Sweden	EF058241	EF058261
Peridinium aciculiferum	PAER-1	Lake Erken, Sweden	AY970653	AY970652
Peridinium bipes	HY971028T	Korea	—	AY359682
Peridinium bipes f. globossum	NIES495	Lake Onogawa, Fukushima, Japan	EF058242	EF058262
Peridinium bipes f. occultatum	None	Japan	AF231805	—
Peridinium bipes f. occultatum	HYSS0312-04	Korea	—	AY733011
Peridinium centenniale[a]	CCAC0002	Cornwall, England	EF058236	EF058254
Peridinium cf. centenniale[a]	ASW12003	Jakobshaven, Greenland	EF058237	EF058257
Peridinium cinctum	AJC4	?	—	AF260385
Peridinium cinctum	CCAC0102	Spiekeroog, Germany	EF058244	EF058264
Peridinium cinctum	CCAP 1140/1	Plußsee, Germany	DQ166209	EF058263
Peridinium cinctum	None	Kiritappu Moor, eastern Hokkaido, Japan	AB185114	—
Peridinium cinctum[a]	PCGY-4	Lake Gyllebo, Skåne, Sweden	EF058245	EF058265
Peridinium cinctum[a]	SAG2017	Cappeler Weiher, Marburg, Germany	EF058243	EF058266
Peridinium gatunense	CCAP?	?	DQ166208	—
Peridinium gatunense[a]	PGDA-1	Lake Dagstorpsjön, Skåne, Sweden	EF058246	EF058267
Peridinium inconspicuum	CCAP1140/3	Kl. Ukleisee,Germany	EF058247	EF058268
Peridinium inconspicuum	UTEX LB2255	?	AF274271	—
Peridinium limbatum	PLCB-1	Crystal Bog, Oneida County, Wisconsin, USA	—	EF058269
Peridinium palatinum	AJC4cl-a	?	—	AF260394
Peridinium polonicum	NIES 500	Japan	AY443017	—
Peridinium pseudolaeve	AJC6-798	?	—	AF260395
Peridinium sp.	Carolina	USA	DQ166210	—
Peridinium sp.	None	?	AF022202	—
Peridinium sp.	HCB-2005	Old Woman Creek, Ohio, USA	DQ487197	—
Peridinium sp.	None	Lake Tovel, Northern Italy	AY827955	—
Peridinium volzii	NIES501	Pond, Tsuchiura, Ibaraki, Japan	EF058248	EF058270
Peridinium wierzejskii	NIES 502	Tsuchiura, Ibaraki, Japan	AY443018	—
Peridinium willei	PWCL1	Crystal Lake, Oneida County, Wisconsin, USA	EF058250	—
Peridinium willei	NIES366	Pond, Tsuchiura, Ibaraki, Japan	EF058249	EF058273
Peridinium willei	NIES 304	Tsukiyono, Gunma, Japan	AF274272	EF058271
Peridinium willei	NIES 365	Pond, Ajiro, Iwate, Japan	AF274280	EF058272
Peridinium willei	AJC2-675	?	—	AF260384
Peridinium willei	TK007	Kiritappu Moor, eastern Hokkaido, Japan	AB232669	AB232669
Peridinium willei	PWCA-1	Glenmore Reservoir, Calgary, Alberta, Canada	DQ166211	EF058274
Peridinium willei[a]	PWGY-B	Gyllebosjön, Skåne, Sweden	EF375879	—
Phytodinium sp.	ASW12001	Peat bog Neuhauser Moor, Mariazell, Austria	EF058251	—
Prorocentrum foveolatum	PFBL01	Tasmania, Australia	—	AY259172
Prorocentrum playfairii	PPWL01	Tasmania, Australia	—	AY259174
Scrippsiella-like	High-1-a	Highway lake, Vestfold Hills, Antarctica	—	EF058275
Tovellia coronata	Clone F1	Pond near Aneboda, Sweden	—	AY950446
Tovellia leopoliensis	NIES 619	Mitsukaido, Ibaraki, Japan	AY443025	—

(*continued on next page*)

Table 1 (*continued*)

Morphospecies name	Strain	Geographical origin	GenBank accession number	
			SSU	LSU (D1/D2)
Woloszynskia halophila	WHTV-S1	Baltic Sea, Finnish coast	EF058252	—
Woloszynskia pascheri	CCAC0075	Göttingen, Gemany	EF058253	EF058276
Woloszynskia pseudopalustris	AJC12cl-915	?	—	AF260402
Woloszynskia tenuissima	SCCAP K-0666	?	—	AY571374

The sequences obtained for this work start with "EF". Strain abbreviations are specified in Section 2.

[a] Indicates morphospecies identified or corroborated for this work. The complete SSU/LSU sequence datasets are shown in Supplementary Materials.

well as a number of freshwater morphospecies. In addition, the SSU and LSU have slow or moderate evolutionary rates, which allows for the construction of phylogenies of distantly related taxa.

PCR amplifications were done using 25 ng of template genomic DNA, 0.125 mM of each nucleotide, 3.0 mM $MgCl_2$, 1× PCR buffer, 0.4 μM of each primer, and 0.5 U of Taq DNA Polymerase (AmpliTaq®, Applied Biosystems, Foster City, California) in 25 μl total volume reactions. For the SSU PCR we used the combination of the universal primers 4616 (forward) 5′-AACCTGGTTG ATCCTGCCAG-3′ and 4618 (reverse) 5′-TGATCC TTCTGCAGGTTCACCTAC-3′. The SSU PCR started with 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C, 2 min at 72 °C, ending with a final hold of 7 min at 72 °C. For the domains D1/D2 of the LSU rDNA we used the primers DinFi (forward) 5′-GCATATAAGTAMGYGGWGG-3′ and DinRi (reverse) 5′-CCGTGTTTCAAGACGGGTC-3′. The LSU PCR temperature profile was equivalent to the SSU except in that it consisted of 30 amplification cycles with a primer annealing temperature of 50 °C. All PCR amplicons were cleaned using PCR-M™ Clean-Up System (Viogene, Taiwan). LSU fragments were directly sequenced from both sides using the same PCR primers. SSU amplicons were directly sequenced using the PCR primer 4616 plus the sequencing primers 516F 5′-CACATCTAAGGAAGG CAGCA-3′, 528F 5′-CGGTAATTCCAGCTCC-3′, 690F 5′-CAGAGGTGAAATTCT-3′ and 1055F 5′-GGTGG TGCATGGCCG-3′ (Edvardsen et al., 2003). The sequencing reactions were carried out using BigDye (v1.1, Applied Biosystems) chemistry and the products were precipitated following the manufacturer instructions and then loaded into an ABI Prism 3100 sequencer (Applied Biosystems). The obtained SSU and LSU sequences were edited and assembled by analyzing carefully the chromatograms using Bioedit (v7.0.4.1; Hall, 1999). Sequences were deposited in GenBank under the Accession Nos. EF058235–EF058276/EF375879 (Table 1).

3. Phylogenetic analyses

3.1. Alignment construction

SSU and LSU sequences representing most dinoflagellate lineages were downloaded from GenBank (downloaded freshwater sequences are indicated in Table 1; the complete sequence datasets are indicated in Supplementary Materials). After the elimination of identical and apparently erroneous sequences, we created alignments using ClustalX (v1.8; Thompson et al., 1997). We constructed two initial alignments containing 238 sequences and 1850 characters for the SSU and 203 sequences and 619 characters for the LSU. Since in some cases the SSU and LSU were not available for the same species, SSU and LSU alignments differed in species composition.

To check for the potential influence of ambiguously aligned positions and divergent regions on the phylogenies, we constructed more stringent alignments (i.e. with more removed positions) using the program Gblocks (v0.91b, Castresana, 2000) as well as visual examination. The more stringent SSU and LSU alignments consisted of 237 sequences with 1592 characters and 200 sequences with 479 characters, respectively. The elimination of positions can also eliminate phylogenetic signal. For this reason, we ran phylogenetic analyses with alignments which differed in the number of removed positions (see Table 2).

We also constructed alignments with fewer sequences to check if this would have an effect on the phylogenetic reconstructions. The eliminated sequences belonged to significantly supported marine clades with no freshwater relatives. The number of excluded sequences was not high since there are studies indicating that more taxa for a given marker can improve tree reconstruction (see Pollock et al., 2002; Hedtke et al., 2006). The original 238-sequence SSU alignment was reduced to 168 sequences and 1828 characters. In the same manner, the original 203-sequence LSU alignment was reduced to 150 sequences and 619 characters. Gblocks along with visual edition were also used on these sequence-reduced alignments. As a result, we generated a 168-sequence alignment with 1610 characters for the SSU and a 147-sequence alignment with 450 characters for the LSU. All these alignments were used for phylogenetic construction using models that do not consider the secondary structure of the rRNA (see Table 2). Alignments are available upon request.

3.2. Phylogenetic inference without considering rRNA secondary structure

Phylogenies were estimated using Maximum Likelihood (ML) and Bayesian Inference (BI) as implemented in

Table 2
Constructed phylogenies: datasets, evolutionary models, and resulting parameters

Tree #	Dataset			Method	Boot	Model	−lnL (±10)	α (±0.01)	Pinvar
	rDNA	Seq	Char						
1a	SSU	238	1850	BI	—	GTR+Γ+COV	−54950	0.39	—
1b	SSU	238	1850	BI	—	GTR+Γ+COV	−54954	0.39	—
2a	SSU	238	1850	BI	—	GTR+Γ+COV[a]	−55265	0.54	—
2b	SSU	238	1850	BI	—	GTR+Γ+COV[a]	−55260	0.55	—
3a	SSU	237	1592	BI	—	GTR+Γ+COV	−49423	0.38	—
3b	SSU	237	1592	BI	—	GTR+Γ+COV	−49431	0.38	—
4	SSU	238	1850	ML	1000	GTR+Γ+I	−50779	0.66	0.23
5	SSU	237	1592	ML	100	GTR+Γ+I	−46421	0.63	0.20
6a	SSU	168	1828	BI	—	GTR+Γ+COV	−43109	0.33	—
6b	SSU	168	1828	BI	—	GTR+Γ+COV	−43114	0.33	—
7a	SSU	168	1610	BI	—	GTR+Γ+COV	−39726	0.33	—
7b	SSU	168	1610	BI	—	GTR+Γ+COV	−39716	0.33	—
8	SSU	168	1828	ML	100	GTR+Γ+I	−40794	0.66	0.30
9	SSU	168	1610	ML	100	GTR+Γ+I	−36741	0.66	0.32
10a	LSU	203	619	BI	—	GTR+Γ+COV	−36987	0.71	—
10b	LSU	203	619	BI	—	GTR+Γ+COV	−36990	0.71	—
11a	LSU	200	479	BI	—	GTR+Γ+COV	−26825	0.71	—
11b	LSU	200	479	BI	—	GTR+Γ+COV	−26816	0.71	—
12	LSU	203	619	ML	1000	GTR+Γ+I	−33827	0.78	0.04
13	LSU	200	479	ML	1000	GTR+Γ+I	−24004	0.73	0.05
14a	LSU	150	619	BI	—	GTR+Γ+COV	−29792	0.65	—
14b	LSU	150	619	BI	—	GTR+Γ+COV	−29803	0.65	—
15a	LSU	147	450	BI	—	GTR+Γ+COV	−19036	0.66	—
15b	LSU	147	450	BI	—	GTR+Γ+COV	−19031	0.65	—
16	LSU	150	619	ML	1000	GTR+Γ+I	−26458	0.78	0.08
17	LSU	147	450	ML	1000	GTR+Γ+I	−16633	0.75	0.06

BI, Bayesian MCMC Inference; ML, Maximum Likelihood; Seq, number of sequences in the alignment; Char, number of characters in the alignment; Boot, number of bootstrap pseudoreplicates (only ML); GTR, General Time Reversible model; Γ, gamma distributed rate of variation across sites; COV, covarion model; I, proportion of invariable sites model; −lnL, log-likelihood: in ML, highest log-likelihood tree within the bootstrap pseudoreplicates; in BI, harmonic mean of the log-likelihood of the cold chain after the burn-in phase; α, shape parameter of the gamma distribution: in ML, alpha corresponds to the highest log-likelihood tree; in BI: alpha corresponds to the mean value after the burn-in phase; pinvar, proportion of invariable sites (only for ML, corresponds to the highest log-likelihood tree).
All Bayesian MCMC analyses were run for 5×10^6 generations with seven Markov chains and a burn-in of 2×10^6 generations.
a and b indicate replicates of each Bayesian run.
[a] Doublet and 4by4 models for the stem and loop areas of the SSU correspondently.

GARLI (serial version, v0.942; Zwickl, 2006) and MrBayes (v3.1.2 parallel version [MPI]; Metropolis-coupled Markov Chain Monte Carlo model [MCMC] approach for approximation of Bayesian posterior probabilities [PPs]; Huelsenbeck and Ronquist, 2001; Altekar et al., 2004). The hierarchical likelihood ratio test (Huelsenbeck and Crandall, 1997) and the Akaike information criterion (Akaike, 1974), as implemented in ModelTest (v3.7; Posada and Crandall, 1998) indicated that the General Time Reversible (GTR) model of nucleotide substitution, with a Gamma (Γ) distributed rate of variation across sites and a proportion of invariable sites (I) was the most appropriate evolutionary model for our SSU and LSU datasets. In ML and BI analyses, the shape parameter (α) of the Gamma (Γ) distribution and the proportion of invariable sites (I) were estimated from the datasets using default options in the programs GARLI and MrBayes.

All Bayesian MCMC analyses were run with seven Markov chains (six heated chains, one cold) for 5×10^6 generations and the trees were sampled every 100 generations, which resulted in 5×10^4 sampled trees. Each analysis used default (flat) priors and was repeated at least twice from random starting trees. The evolutionary model used in Bayesian analyses was the GTR+Γ+COV. The Covarion Model (COV) allows substitution rates to change across positions through time (Miyamoto and Fitch, 1995; Huelsenbeck, 2002). The COV model can be regarded as a general case of the proportion of invariable sites model (Huelsenbeck, 2002) permitting sites to change between invariable and variable states in an "on–off" fashion independently from each other (as implemented in MrBayes). The covarion model was used since previous phylogenetic analyses with dinoflagellate rDNA (Shalchian-Tabrizi et al., 2006b), along with studies in other taxa, indicate that this model gives a better explanation of rDNA data (Galtier, 2001; Huelsenbeck, 2002; Shalchian-Tabrizi et al., 2006a). The obtained posterior probability (PP) values for the branching patterns as well as marginal likelihoods for the tree reconstructions were compared to ensure convergence. Consensus trees and PPs were calculated using the 3×10^4 trees after the log-likelihood stabilization (burn-in phase).

ML analyses in GARLI were run with 100 or 1000 bootstrap (Felsenstein, 1985) pseudoreplicates. All parameters were used in default options, except for the number of generations that the program should run with no significant improvements in the scoring of the topology, which was set to 5000. All analyses in GARLI were run under the GTR+Γ+I model, since the covarion model is not implemented. Consensus trees from the bootstrap output were generated using MrBayes.

Phylogenetic analyses with MrBayes and GARLI were run at the University of Oslo Bioportal (http://www.bioportal.uio.no/). The trees generated with MrBayes and GARLI were visualized in TreeView (v1.6.6; Page, 1996) and further edited in MEGA (v 3.1; Kumar et al., 2004).

3.3. Phylogenetic inference considering rRNA secondary structure

The rRNA molecule has a complex secondary structure that comprises stems (paired nucleotides) and loops (unpaired nucleotides) (Hillis and Dixon, 1991). To maintain this secondary structure, mutations in stem nucleotides are usually compensated by other stem mutations, therefore nucleotide variation in stem areas can be correlated, violating an assumption made by most models of nucleotide substitution. The effect of differential variation in loop and stem areas for phylogenetic construction has been investigated (e.g. Dixon and Hillis, 1993) and models that take into account this covariation have been proposed (e.g. Schoniger and von Haeseler, 1994; Muse, 1995). Some researchers have reported considerable improvements in phylogenetic reconstructions after implementing models which consider nucleotide covariation (e.g. Murray et al., 2005; Telford et al., 2005). In order to investigate the effects of nucleotide covariation in our study, we have constructed SSU phylogenies considering the secondary structure. The SSU rRNA secondary structure of the dinoflagellate *Peridinium aciculiferum* was estimated with MFOLD (Zuker et al., 1999) and used as a general model for estimating the covariating sites in the SSU alignment consisting of 238 sequences and 1850 characters. This secondary structure was used only for estimating the covariating SSU sites and not for trying to improve the alignments. Partitioned Bayesian MCMC phylogenetic analysis were carried out using the Doublet model (based on the SH model of Schoniger and von Haeseler, 1994) for the covariating stem areas and the 4by4 for the loop areas. For stem and loop areas, the GTR+Γ+COV model was used. Bayesian MCMC analyses were run with seven Markov chains (one cold and six heated) for 5×10^6 generations, and the trees were sampled every 100 generations, which resulted in 5×10^4 sampled trees. Consensus trees and PPs were calculated from the 3×10^4 trees after the log-likelihood stabilization. The generated trees were visualized and edited in the same way as indicated above. The comparison between the model considering the secondary structure (H_1 = doublet+4by4) and the one not considering it (H_0 = 4by4) was done using a Bayes factor (B_{10}), which is equal to the ratio of the posterior probabilities of H_1 and H_0 given that the prior probabilities for H_1 and H_0 are equal. The marginal likelihood for each model was estimated using the harmonic mean of the likelihood values obtained during the stationary phase of the MCMCs, as suggested by Newton et al. (1994). Bayes factors were interpreted as suggested by Kass and Raftery (1995). We did not carry out secondary structure analysis with the LSU since the results with the SSU did not indicate that major changes occur by using this model.

4. Results

4.1. Phylogenetic results

All the data presented here represent a summary of the results obtained from a total of 26 constructed phylogenies (Table 2). Except when specified, we do not refer to a clade seen in a single consensus tree, but to clades which were shared among all or most of the different phylogenies. The trees in Figs. 1 and 2 correspond to trees 1a and 10a in Table 2, respectively. These trees are intended to represent the entire set of phylogenetic results instead of a single phylogenetic reconstruction. Posterior Probabilities (PPs) and Bootstrap support values (BVs) for some clades recurrently obtained across the phylogenies are presented in Table 3.

Around 70% of the analyzed freshwater SSU (19 out of 27) and LSU (24 out of 30) sequences clustered with other freshwater sequences (Figs. 1 and 2; sequences from the same morphospecies were counted only once in the calculation of the percentage). Only two freshwater sequences (*Peridinium aciculiferum* and *Woloszynskia pseudopalustris*) affiliated very closely with marine species (Figs. 1 and 2). Some freshwater clades and single species did not show any consistent affiliation with any other lineage (e.g. the clades PESS, PBOR, TOVE [see Table 3 for definitions], *Hemidinium nasutum*), while other freshwater clades or species affiliated to marine groups (e.g. WOLO, *Gyrodinium helveticum*, *Gymnodinium palustre*) (Figs. 1 and 2). Several freshwater sequences or clades displayed long branches in the phylogenies (e.g. TOVE, PESS, WOLO, *Hemidinium nasutum*; Figs. 1 and 2).

Most SSU and LSU phylogenies had weakly defined backbone topologies but several well supported internal clades (Figs. 1 and 2, Table 3). In most SSU and LSU trees, several clades were repeatedly obtained using different datasets, tree-reconstruction methods and evolutionary models (examples of recurring clades in Table 3). There were some cases where moderately or well supported clades by one tree reconstruction technique or dataset were poorly supported, or not present at all, using different methods or datasets (e.g. clades 9 and 14 in Table 3).

The implementation of the model not considering the SSU secondary structure [4by4] (H_0) was ~310 lnL units

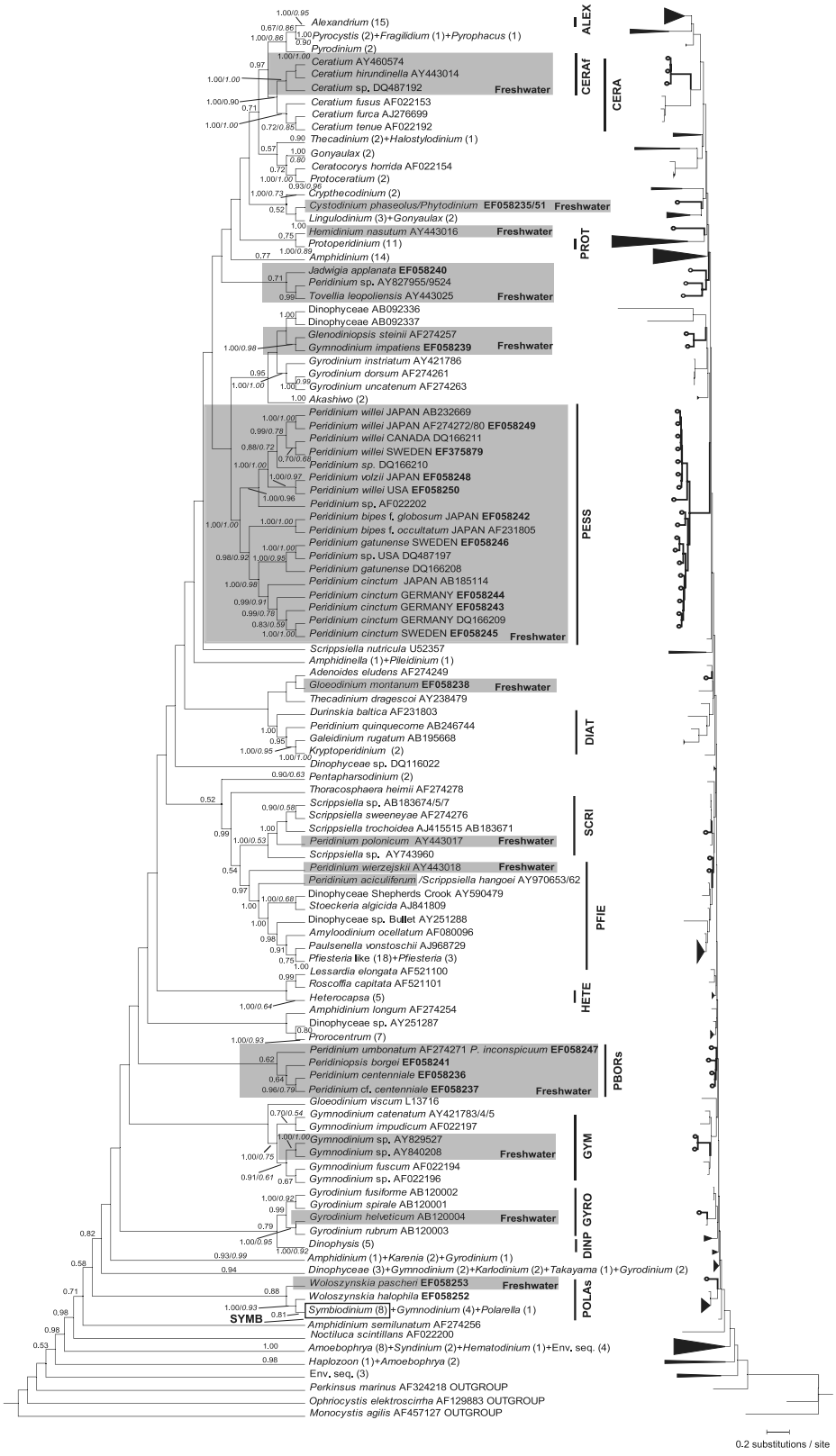
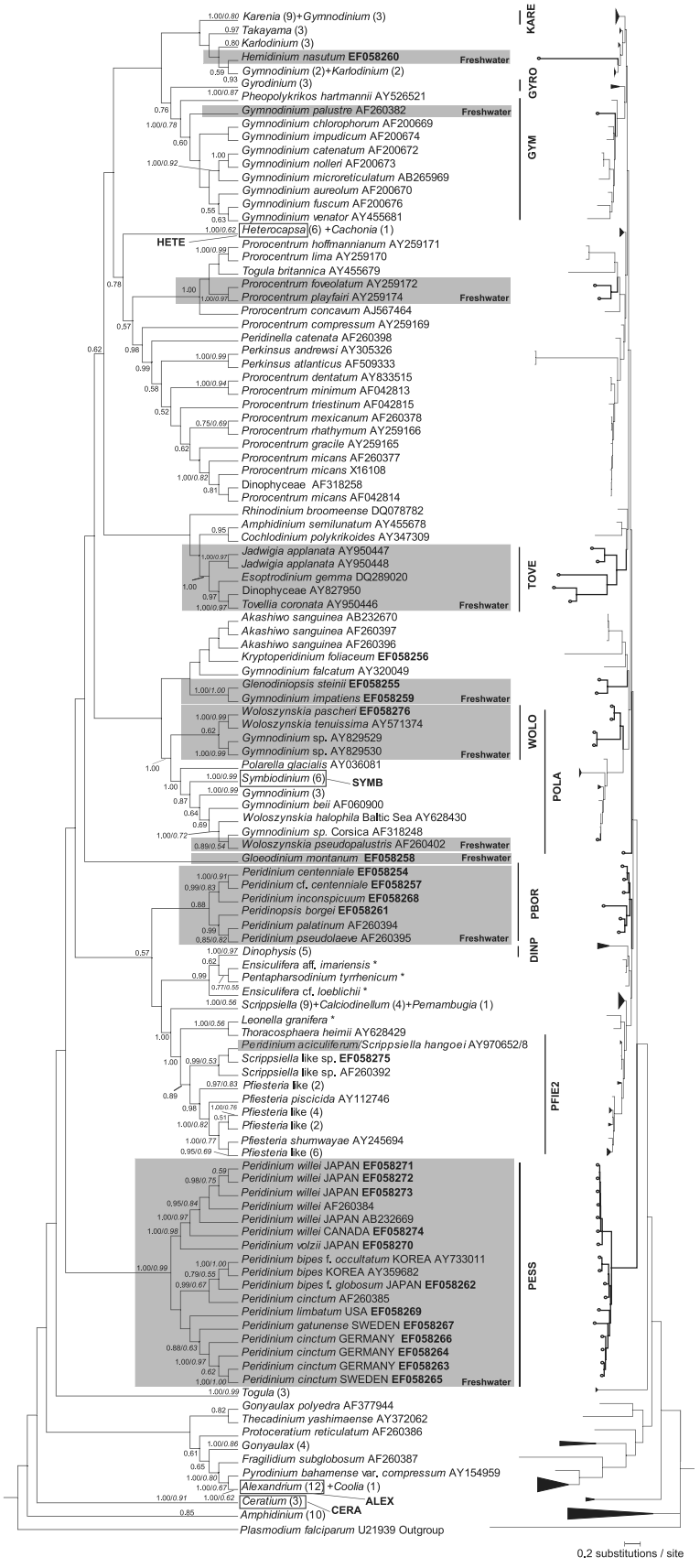


Fig. 1. Consensus SSU rDNA phylogeny constructed from an alignment with 238 sequences and 1850 characters under the GTR+Γ+COV (Bayesian Inference) and GTR+Γ+I (Maximum Likelihood) models. This consensus phylogeny corresponds to the trees 1a and 4 (Table 2) and intends to represent the whole set of constructed SSU trees (Table 2). The tree on the left shows the topology and posterior probabilities (normal print)/bootstrap support (italics) values >0.50. The tree on the right shows the evolutionary distances obtained by Bayesian MCMC Inference. Freshwater taxa and clusters are shown within frames in the cladogram (left) and indicated with a small terminal circle in the phylogram (right). The triangles in the phylogram correspond to marine clades that were collapsed. The number of collapsed sequences is indicated within parenthesis. The clusters indicated with the vertical lines or rectangles are described in Table 3 or Section 4.

better than the Doubles+4by4 model (H_1), thus producing a Bayesian factor which indicates that there is no phylogenetic improvement considering the rDNA secondary structure (Table 2).



In most cases, ML and BI phylogenies using SSU and LSU supported (BVs and PPs > 0.80) the monophyly of some major dinoflagellate genera (Table 3, Figs. 1 and 2). In general, the PP values were higher than the bootstrap values (Table 3). This is most probably related to the fact that BVs are normally more conservative than PPs (see Cummings et al., 2003; Simmons et al., 2004). In several cases, there was a slight decrease in the PPs and BVs of clades in trees constructed with reduced character datasets (Table 3).

4.2. Phylogenetic relationships between marine and freshwater dinoflagellates

In the SSU and LSU phylogenies using ML and BI, there was always a high support (BVs and PPs > 0.97) for a clade comprising freshwater morphospecies of the *Peridinium* sensu stricto (=PESS) group (i.e. *P. willei* Huitfeld-Kaas, *P. cinctum* Ehrenberg, *P. gatunense* Nygaard, *P. bipes* Stein, *P. limbatum* (Stokes) Lemmermann, *P. volzii* Lemmermann) [group proposed by Boltovskoy (1979, 1999)]. The PESS clade was separated by a relatively longer branch from other marine and freshwater groups (Figs. 1 and 2). The internal topology of PESS was well defined in BI and ML analyses for both the SSU and LSU (Figs. 1 and 2).

Another cluster, mostly freshwater, that was suggested by the SSU and LSU phylogenies is PBOR [composed of: *Peridinium umbonatum* Stein, *P. inconspicuum* Lemmermann, *Peridiniopsis borgei* Lemmermann, *P. centenniale* (Playfair) Lefèvre, *P.* cf. *centenniale*, *P. palatinum* Lauterborn and *P. pseudolaeve* Lefèvre (Figs. 1 and 2; Table 3)]. Within this group all species are fully freshwater, except for *Ps. borgei*, which is normally found in brackish limnic habitats, but not marine brackish environments. The PBOR clade did not show consistent affiliation to any other marine or freshwater clade.

Another clade indicated by the LSU and SSU phylogenies is POLA (Table 3; Figs. 1 and 2). Within POLA, the freshwater LSU sequences *Woloszynskia pascheri*, *W. tenuissima*, and the gymnodinioids AY829529–AY829530 formed the subclade WOLO (Fig. 2). In the ML and BI LSU phylogenies, the freshwater *W. pseudopalustris* grouped with significant support with a *Gymnodinium* from the Mediterranean (Corsica) and *W. halophila* from the Baltic Sea (PPs > 0.90, BVs > 0.70; Fig. 2). *W. pseudopalustris* was very closely related (very short branch lengths) to the Mediterranean *Gymnodinium* sp. and *W. halophila*. This cluster was distantly related to the WOLO subclade (Fig. 2).

SSU and LSU phylogenies suggested the clusters PFIE and PFIE2 (see Figs. 1 and 2) which included the freshwater morphospecies *Peridinium wierzejskii* Woloszynska and *P. aciculiferum* Lemmermann. The cluster PFIE and PFIE2 received significant PPs (>0.80), but was poorly supported by ML phylogenies (see Table 3). In the SSU phylogenies, *P. wierzejskii* and the pair *P. aciculiferum*/*Scrippsiella hangoei* (these species share identical rDNA; Gottschling et al., 2005; Logares et al., 2007) appeared in basal positions (Fig. 1). In the LSU BI and ML trees (which did not include *P. wierzejskii*) the freshwater *P. aciculiferum* (along with the brackish *S. hangoei*) clustered with marine-brackish *Scrippsiella*-like (EF058275 and AB260392) morphospecies (not available for SSU phylogenies) with a relatively high PP support (>0.90) but low BVs [~0.50] (Fig. 2).

SSU phylogenies also suggested the clade SCRI (Fig. 1), which included the freshwater *Peridinium polonicum* (Woloszysnka) Bourrelly. SCRI received high PPs (>0.90) but low BVs (~0.39) (Table 3).

All SSU and LSU phylogenies strongly supported a clade composed of sequences from *Ceratium* morphospecies (PPs and BVs > 0.90). In all analyses, the three freshwater SSU *Ceratium* sequences formed a highly supported subclade (=CERAf) distinctively separated from the marine cluster (PPs and BVs > 0.90) (Fig. 1).

The ML and BI LSU phylogenies suggested a freshwater cluster (=TOVE) composed by *Jadwigia applanata* Moestrup et al., *Esoptrodinium gemma* Javornicky, *Tovellia coronata* (Woloszynska) Moestrup et al., and the dinoflagellate sequence AY827950 (Fig. 2). TOVE received variable support (ranging from high to very low) in the LSU phylogenies (Clade 18, Table 3). In the SSU phylogenies, which included less sequences within TOVE than the LSU, the freshwater *J. applanata* sometimes affiliated to the freshwater pair *Tovellia leopoliensis* (Woloszynska) Moestrup et al. and *Peridinium* AY827955 (Fig. 1). The clustering of *T. leopoliensis* and *Peridinium* AY827955 was highly supported across BI phylogenies (PP > 0.90).

In the phylogenies, the freshwater *Gymnodinium palustre* Schilling (LSU; Fig. 2) and the freshwater sequences *Gymnodinium* sp. AY829527–AY840208 (SSU; Fig. 1) clustered with marine *Gymnodinium* species (Figs. 1 and 2; GYM clade) with significant support (PPs and BVs > 0.70) [In LSU phylogenies, *Pheopolykrikos hartmannii*

Fig. 2. Consensus LSU rDNA phylogeny constructed from an alignment with 203 sequences and 619 characters under the GTR+Γ+COV (Bayesian Inference) and GTR+Γ+I (Maximum Likelihood) models. This consensus phylogeny corresponds to the trees 10a and 12 (Table 2) and intends to represent the whole set of constructed LSU trees (Table 2). The tree on the left shows the topology and posterior probabilities (normal print)/bootstrap support (italics) values >0.50. The tree on the right shows the evolutionary distances obtained by Bayesian MCMC Inference. Freshwater taxa and clusters are shown within frames in the cladogram (left) and indicated with a small terminal circle in the phylogram (right). The triangles in the phylogram correspond to marine clades that were collapsed. The number of collapsed sequences is indicated within parenthesis. The clusters indicated with the vertical lines or rectangles are described in Table 3 or Section 4. These sequences have been finally published by their authors. Since they are not pivotal for this study, their accession numbers could be still shown in the Supplementary materials. The accession numbers for these sequences are shown in Supplementary Materials.

Table 3
PP and BV support for 18 selected clades which were normally found across most LSU and SSU phylogenies

	Clade #																			PP_m BV_m	SD
	Tree #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
SSU	1a	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.97	0.62	1.00	0.88	0.99	1.00		—	—	—	**0.96**	**0.11**
	2b	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.67	0.61	1.00	1.00	0.79	0.92	—	—	—	—	**0.92**	**0.14**
	3a	1.00	0.88	1.00	1.00	1.00	1.00	—	1.00	0.97	0	1.00	0.97	0.94	1.00	—	—	—	—	**0.90**	**0.27**
	4	*0.89*	*0.95*	*0.92*	*0.97*	*0.90*	*1.00*	—	*0.64*	*0.10*	*0.24*	*0.45*	*0.23*	*0.40*	*0.53*	—	—	—	—	***0.63***	***0.33***
	5	*0.90*	*0.62*	*0.94*	*0.98*	*0.88*	*1.00*	—	*0.58*	*0.11*	*0.10*	*0.46*	*0.25*	*0.38*	*0.37*	—	—	—	—	***0.58***	***0.33***
	6a	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.56	0.92	1.00	0.99	0.99	1.00	—	—	—	—	**0.95**	**0.12**
	7b	1.00	1.00	1.00	1.00	1.00	1.00	—	1.00	0.95	0	1.00	0.90	0.96	1.00	—	—	—	—	**0.91**	**0.27**
	8	*0.95*	*1.00*	*0.96*	*0.98*	*0.89*	*1.00*	—	*0.85*	*0.15*	*0.24*	*0.46*	*0.23*	*0.52*	*0.13*	—	—	—	—	***0.64***	***0.36***
	9	*0.89*	*1.00*	*0.91*	*1.00*	*0.91*	*1.00*	—	*0.80*	*0.09*	*0*	*0.35*	*0.24*	*0.44*	*0.54*	—	—	—	—	***0.63***	***0.37***
LSU	10a	—	1.00	1.00	1.00	1.00	1.00	1.00	1.00	—	—	—	—	1.00	—	0.88	1.00	0.99	1.00	**0.96**	**0.09**
	11b	—	1.00	1.00	1.00	1.00	1.00	0.71	0.82	—	—	—	—	1.00	—	0.66	1.00	1.00	0	**0.85**	**0.30**
	12	—	*0.62*	*0.97*	*0.99*	*0.91*	*0.94*	*0.80*	*0.62*	—	—	—	—	*0.87*	—	*0.27*	*0.56*	*0.23*	*0.18*	***0.66***	***0.30***
	13	—	*0.59*	*0.96*	*0.98*	*0.94*	*0.86*	*0.76*	*0.44*	—	—	—	—	*0.87*	—	*0.26*	*0.56*	*0.35*	*0.12*	***0.64***	***0.30***
	14a	—	1.00	1.00	1.00	1.00	1.00	1.00	0.67	—	—	—	—	1.00	—	0.92	1.00	0.89	0.58	**0.92**	**0.14**
	15b	—	1.00	0	1.00	1.00	1.00	0.98	0.98	—	—	—	—	1.00	—	0.41	1.00	0.61	0.79	**0.81**	**0.32**
	16	—	*1.00*	*1.00*	*1.00*	*0.99*	*0.97*	*0.80*	*0.61*	—	—	—	—	*0.96*	—	*0.24*	*0.51*	*0.23*	*0.22*	***0.71***	***0.33***
	17	—	*0.99*	*0*	*1.00*	*1.00*	*0.98*	*0.59*	*0.42*	—	—	—	—	*0.90*	—	*0.20*	*0.44*	*0.12*	*0.16*	***0.57***	***0.39***
	%	100	100	88	100	100	100	100	100	100	66.7	100	100	100	100	100	100	100	100		
	PP_m	**1.00**	**0.99**	**0.89**	**1.00**	**1.00**	**1.00**	**0.92**	**0.94**	**0.82**	**0.43**	**1.00**	**0.95**	**0.97**	**0.98**	**0.71**	**1.00**	**0.87**	**0.59**		
	SD	0	0.04	0.33	0	0	0	0.14	0.12	0.19	0.41	0	0.05	0.07	0.03	0.23	0	0.18	0.43		
	BV_m	***0.91***	***0.85***	***0.83***	***0.99***	***0.93***	***0.97***	***0.73***	***0.62***	***0.11***	***0.15***	***0.43***	***0.24***	***0.67***	***0.39***	***0.24***	***0.52***	***0.23***	***0.17***		
	SD	*0.03*	*0.2*	*0.34*	*0.01*	*0.05*	*0.05*	*0.10*	*0.15*	*0.03*	*0.12*	*0.05*	*0.01*	*0.25*	*0.19*	0.03	0.06	0.09	0.04		

Tree #, corresponds to trees in Table 2. In BI analyses, only the replicate with the highest −lnL harmonic mean was considered. Clades 1–8 (clades include all analyzed species within each genus and nothing else): 1, PROT (*Protoperidinium* spp.); 2, ALEX (*Alexandrium* spp.); 3, DINP (*Dinophysis* spp.); 4, SYMB (*Symbiodinium* spp.); 5, CERA (*Ceratium* spp.); 6, PESS (*Peridinium* sensu stricto spp., *P. willei*, *P. limbatum, P. gatunense*, *P. cinctum*, *P. bipes*, *P. volzii*); 7, KARE (*Karenia* spp., includes some sequences misnamed *Gymnodinium*); 8, HETE (*Heterocapsa* spp.). Clades 9–18 (clades include at least all mentioned species/sequences): 9, PFIE (*Peridinium wierzejskii, P. aciculiferum-Scrippsiella hangoei*, *Pfiesteria*-like, *Pfiesteria* spp.); 10, PBORs (*Peridinium umbonatum, P. inconspicuum*, *Peridiniopsis borgei*, *P.* cf. *centenniale,*, *P. centenniale*); 11, DIAT (*Durinskia baltica*, *Peridinium quinquecorne*, *Galeidinium rugatum*, *Kryptoperidinium foliaceum*); 12, POLAs (*Woloszynskia pascheri*, *W. halophila*, *Symbiodinium* spp., *Gymnodinium* spp., *Polarella glacialis*); 13, GYRO (*G. helveticum*, *G. fusiforme, G. spirale, G. rubrum, G. dominans*); 14, SCRI (*Peridinium polonicum*, *Scrippsiella* sp. AB183674, *S. sweeneyae*, *S. trochoidea*, *Scrippsiella* sp. HZ2005); 15, PBOR (clade 10 + *Peridinium palatinum*, *P. pseudolaeve*); 16, POLA (clade 12 + *Woloszynskia tenuissima*, *W. pseudopalustris, Gymnodinium* sp. AY829529, AY829530); 17, PFIE2 (*Peridinium aciculiferum- Scrippsiella hangoei*, *Scrippsiella*-like EF058275, *Scrippsiella* sp. AF260392, *Pfiesteria* sp., *Pfiesteria*-like spp.); 18, TOVE (*Jadwigia applanata*, *Esoptrodinium gemma*, *Tovellia coronata*, Dinophyceae AY827950). 0, clade broken (no support); —, clade not applicable for the specified tree; BVs, bootstrap values (in italics); PPs, posterior probability values (in regular fonts); %, presence of a given clade across the phylogenies in percentage; PP_m and BV_m (values in bold), median PPs or BVs for a clade across the phylogenies (columns) or for all the selected clades along one phylogeny (files); SD, standard deviation.

(Zimmermann) Matsuoka & Fukuyo is included in GYM; Fig. 2]. In both cases, the freshwater *Gymnodinium* species were not closely related to the marine *Gymnodinium* species (Figs. 1 and 2).

In the SSU analyses the freshwater *Gyrodinium helveticum* (Penard) Takano & Horiguchi clustered with significant PP support (>0.75) within a clade (=GYRO) composed by other marine *Gyrodinium* species (Fig. 1). Within GYRO, *G. helveticum* had a noticeable degree of sequence divergence from the marine species (Fig. 1).

In the LSU phylogenies, the freshwater *Prorocentrum playfairii* Croome & Tyler and *P. foveolatum* Croome & Tyler clustered with high PP (>0.90) support to other marine *Prorocentrum* sequences as well as the marine *Togula britannica* (Herdman) Flø Jørgensen et al. (=PRO1 clade) (Fig. 2). Within PRO1, *P. playfairii* and *P. foveolatum* always clustered together with high PP and BV support (>0.90).

The freshwater *Glenodiniopsis steinii* (Lemmermann) Woloszynska and *Gymnodinium impatiens* Skuja were strongly associated (PPs and BVs > 0.90) throughout all the SSU and LSU phylogenies (Figs. 1 and 2). In SSU and LSU phylogenies, the pair did not show consistent affiliation to any other clade.

A number of solitary freshwater sequences appeared in very different positions depending on the marker or the method used for tree reconstruction. In several cases, those solitary sequences displayed long branches. The morphospecies associated to these sequences are *Cystodinium phaseolus* Pascher ASW12002, *Hemidinium nasutum* Stein NIES 471 and *Gloeodinium montanum* Klebs CCAC0066 (Figs. 1 and 2).

In the LSU phylogenies *Perkinsus* sp. appeared in different positions depending on the method for phylogenetic reconstruction and alignment dataset. This is almost certainly an artifact (*Perkinsus* is considered basal to dinoflagellates, as the SSU phylogenies indicate) and will not be further considered.

5. Discussion

Here, we have investigated the role of the marine–freshwater boundary on the historical diversification of dinoflagellates. Our results indicated that (a) marine and freshwater dinoflagellates are usually not closely related, (b) several freshwater species cluster into monophyletic groups, (c) only a small fraction of the marine lineages seem to have colonized fresh waters, and (d) most marine–freshwater transitions do not seem to have occurred recently. Our results also gave insight on the phylogenetic positions of several freshwater dinoflagellate species, which so far have been unclear.

5.1. Marine and freshwater taxa were normally not closely related

In the SSU and LSU phylogenies, ~70% of the investigated freshwater species appeared more closely related to other freshwater species than to marine species. In some cases, freshwater sequences could be related to marine clades (the opposite was not observed). However, a number of freshwater sequences did not affiliate to any other of the analyzed marine and freshwater dinoflagellates (*Cystodinium phaseolus* Pascher ASW12002, *Hemidinium nasutum* Stein NIES 471 and *Gloeodinium montanum* Klebs CCAC0066). In several cases, the freshwater species/clades displayed a significant degree of sequence divergence (long branches) in comparison to the marine species/clades. Altogether, this indicates the segregation of marine and freshwater dinoflagellates, and also that freshwater invasions may have occurred long time ago or that the rate of molecular divergence has increased in invading lineages. Morphological data also suggest the separation between marine and freshwater lineages (see Taylor, 1987; Popovsky and Pfiester, 1990), although this separation can be ambiguous due to the uncertainties of morphology-based phylogenies. A historical segregation of marine and freshwater dinoflagellates is also suggested by the fossil record. Several fossil freshwater dinoflagellates are not identifiable with any known marine fossil genera or species (Batten, 1989). The segregation of marine and freshwater taxa can also be observed in other microbial phylogenetic studies (e.g. Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Lefevre et al., 2007). However, these works for the most part include few freshwater species and/or investigate questions different than marine–freshwater transitions. Interestingly, long branches separating freshwater species/clades from marine taxa are also observed in other microbial groups (e.g. Hoef-Emden et al., 2002; Von der Heyden et al., 2004). These long branches could be product of ancient divergences, an increase of the evolutionary rates during marine–freshwater transitions, or a mix of both.

5.2. Several freshwater species clustered into monophyletic groups

A central result of this study is that several freshwater dinoflagellate species clustered into monophyletic groups. In particular, our results strongly supported the monophyly of the freshwater *Peridinium* sensu stricto group [=PESS], which was proposed by Boltovskoy (1979) on a morphological basis (e.g. number of cingular plates, sulcal structure, ecdysial openings, capsulate cysts), but which monophyly was still under debate (Figs. 1 and 2). The relatively long branch separating PESS from the rest of the dinoflagellates suggests an ancient divergence of this clade from the other taxa (Figs. 1 and 2), or an increase of the evolutionary rates during the marine–freshwater transition. Interestingly, there are two fossil dinoflagellate morphospecies, *Palaeoperidinium pyrophorum* and *Pa. cretaceum*,

from the early Cretaceous (145-135 MYA) which are considered to be members of the ancestral group from which the modern PESS have derived (see Boltovskoy, 1979; Bujak and Davies, 1983; Harding, 1990). *Pa. pyrophorum* was found in marine sediments while *Pa. cretaceum* was obtained from brackish paleoenvironments. These data suggest that *Pa. cretaceum* could have been an intermediate species during the marine–freshwater transition that eventually led to PESS. In contrast to many dinoflagellate groups, the internal topology of the PESS clade was highly resolved, with subgroups that correspond to morphospecies definitions (Figs. 1 and 2) [morphologically, the six species within PESS are distinguished by their outline body shapes; degree of dorso-ventral compression; bilateral symmetry in the plate disposition; relative position, shape and size of plates; and presence or absence of the apical pore complex and of apical and antapical horns and lists (Evitt and Wall, 1968; Boltovskoy, 1973, 1975, 1976; Imamura and Fukuyo, 1990a, b)]. In addition, both the SSU and LSU indicated a significant degree of sequence divergence within most of these morphospecies (cryptic diversity). This suggests that each morphospecies could actually constitute a species complex, as was reported for other dinoflagellates (e.g. Scholin et al., 1994; Montresor et al., 2003; Kim et al., 2004; Lilly et al., 2005). Our results also show that a number of freshwater and marine morphospecies currently assigned to *Peridinium* are evolutionary unrelated to PESS (*Peridinium* sensu stricto). These include *Peridinium polonicum*, *P. pseudolaeve*, *P. palatinum*, *P. inconspicuum*, *P. umbonatum*, *P. aciculiferum*, *P. wierzejskii*, *P. centenniale*, and *P. quinquecorne*.

Other monophyletic freshwater groups which are indicated by our analyses are PBOR (except for *Peridiniopsis borgei* that is present in limnic-brackish habitats), TOVE, CERAf, and WOLO (Figs. 1 and 2). The clade PBOR does not seem to have been reported before, however, results from other authors have already suggested the existence of the TOVE clade (Lindberg et al., 2005; Calado et al., 2006; Moestrup et al., 2006; Hansen et al., 2007). In the phylogenies, the marine and freshwater *Ceratium* appeared clearly separated (Fig. 1), suggesting that this lineage has not invaded fresh waters recently. Some morphological and biological differences between the marine and freshwater *Ceratium* also suggest that these lineages did not diverge recently: the freshwater species of *Ceratium* have six plates composing the cingulum in contrast to marine species which have five; freshwater *Ceratium* species produce resting cysts, whereas the far more numerous marine species do not encyst (Wall and Evitt, 1975). An early freshwater colonization for the CERAf lineage is suggested also by fossil data, which indicate the presence of shared morphological characteristics between living freshwater *Ceratium* and Cretaceous *Ceratium*-like marine fossils (as the presence of pendant horns instead of the upswept horns observed in many modern marine *Ceratium*, the development of a fourth horn, and the presence of the sixth cingular plate) (Wall and Evitt, 1975). The present study is probably one of the first to report the freshwater WOLO subclade (within the POLA clade) (Fig. 2). The clear separation between the WOLO clade and other marine species within POLA suggests an early freshwater invasion by the WOLO lineage (Fig. 2).

5.3. An indication of infrequent freshwater colonizations by a few ancestrally marine lineages

The clustering of several freshwater sequences into monophyletic groups suggests that freshwater colonizations were not common during the diversification of dinoflagellates and that the diversification of several freshwater taxa occurred after the invasion of fresh waters. If freshwater colonizations would have been more common, a relatively higher number of evolutionary distantly related freshwater taxa would have been expected (i.e. several freshwater colonization lines from a spectrum of marine lineages). In addition, in our phylogenies, several marine groups did not include any freshwater relative, suggesting that relatively few of the living marine dinoflagellate lineages have colonized fresh waters. Morphological studies also suggest that only a handful dinoflagellate lineages have colonized fresh waters. It is estimated that out of 14 dinoflagellate orders, only five have freshwater representatives that are distributed within about 12 families. Four of those families comprise more than 90% of the freshwater morphospecies (estimated from Taylor (1987) and Popovsky and Pfiester (1990)). In our study, we have covered ~70% of the freshwater genera, with two or more species, that are recognized in Popovsky and Pfiester (1990). Despite that a more extensive taxon sampling will most probably reveal new freshwater dinoflagellate lineages, their total number will most likely continue to be much lower than the total number of marine lineages. A similar pattern to ours was observed in diatoms, where apparently only a fraction of the marine lineages have been successful in colonizing fresh waters (Mann, 1999).

5.4. Most freshwater colonizations do not seem to have occurred recently

Except for two cases (*Peridinium aciculiferum* and *Woloszynskia pseudopalustris*), our results indicate that most of the studied freshwater dinoflagellates belong to lineages that have colonized fresh waters a long time ago. This is suggested by (a) the very small number of closely related marine and freshwater species, and (b) by the presence of relatively long branches separating several freshwater species/clades from other marine groups (Figs. 1 and 2). Nevertheless, the observed long branches could also be partially explained by a putative increase in the evolutionary rates during freshwater colonizations.

An increased taxon sampling will most probably identify other freshwater species which are a product of recent freshwater colonizations. Yet, the number of species

belonging to lineages that have colonized fresh waters long time ago will most likely be much higher than the number of species that have colonized freshwater recently. Early freshwater colonizations are also indicated by the fossil record. The freshwater dinoflagellate fossil record extends back until the Mesozoic (~140 MYA), with several freshwater fossil morphospecies that are not identifiable with marine fossils (Batten, 1989). This indicates the occurrence of ancient freshwater invasions by marine dinoflagellates and the posterior evolution of truly freshwater species. The changes in the sea levels that occurred during the Mesozoic and Cenozoic flooded large continental areas (Haq et al., 1987). This could have promoted the early invasion of continental waters by some dinoflagellate lineages. Sims et al. (2006) proposed a similar scenario for the invasion of continental waters by some marine diatom lineages.

5.5. Other freshwater colonization lines

Apart from the discussed freshwater clades, we have identified several other freshwater colonization lines that were represented in most cases by only one or two species. For instance, the freshwater *Peridinium polonicum* has probably diverged from marine *Scrippsiella* species (Fig. 1). *P. aciculiferum* has probably colonized fresh waters very recently, since it is very closely related to the marine-brackish *Scrippsiella hangoei*, and both morphospecies cluster with marine-brackish species (see Logares et al., 2007) (Figs. 1 and 2). According to the SSU phylogenies, the freshwater *P. wierzejskii* is related to a group of mostly marine-brackish dinoflagellates which includes *Pfiesteria* and associated species (Fig. 1), although this relationship is not supported by morphology (different plate patterns). The freshwater species *Gymnodinium* AY829527, *Gymnodinium* AY840208 and *G. palustre* most probably represent the freshwater colonization of a marine *Gymnodinium* clade (GYM clade; Figs. 1 and 2). Likewise, the freshwater *Gyrodinium helveticum* represents a freshwater colonization line of the marine *Gyrodinium* clade (altogether forming the clade GYRO; Fig. 1). Similarly, the freshwater *Prorocentrum playfairii* and *P. foveolatum* represent a freshwater colonization line of a marine *Prorocentrum* clade (Fig. 2). Within the clade POLA, the freshwater *Woloszynskia pseudopalustris* clustered with a marine *Gymnodinium* and the brackish *W. halophila* (Fig. 2). In this case, the freshwater colonization seems to have occurred recently, since the three morphospecies were very closely related. The freshwater *Glenodiniopsis steinii* and *Gymnodinium impatiens* did not show any clear affiliation to any other lineage across the phylogenies (the fact that this and other *Gymnodinium* species did not cluster together in the phylogenies clearly indicates that this genus is polyphyletic). There were also other solitary freshwater sequences which drifted across the phylogenies not affiliating with any other group or species (i.e. *Cystodinium phaseolus, Hemidinium nasutum*, *Gloeodinium montanum*).

Altogether, each one of these freshwater colonization lines could represent undersampled freshwater lineages, which colonized fresh waters a long time ago. If the marine–freshwater transitions leading to these species had happened more recently, then those species would most probably have affiliated with marine relative groups. However, despite our extensive taxon sampling of marine lineages, it is also possible that the marine relatives of these solitary freshwater sequences have not been sequenced yet.

5.6. Methodological comments on phylogeny reconstruction

Several clades were significantly supported by the SSU and LSU phylogenies (e.g. PESS, DINP, SYMB, ALEX, CERA; see Table 3). In general, at the genus level, our molecular phylogenies agreed with morphological classifications (the agreement between molecular phylogenies and morphological classifications in dinoflagellates has been addressed in other studies [e.g. Saldarriaga et al., 2004; Taylor, 2004] and will not be discussed here). On the other hand, we have obtained a low support for the backbone of most phylogenies. Both patterns agree with other studies (e.g. Saldarriaga et al., 2004; Zhang et al., 2005; Shalchian-Tabrizi et al., 2006b). The low resolution of the treeś backbones does not represent a problem for this work, which does not intend to resolve deep phylogenetic relationships.

Despite that other studies have reported an improvement in the phylogenetic reconstructions when considering the SSU secondary structure (e.g. Murray et al., 2005; Telford et al., 2005), the results of our analyses did not differ significantly whether or not the secondary structure was considered. This could be the consequence of a non-optimal modeling of the SSU secondary structure or that the Doublets model was not the most appropriate for the analyses. Another possibility is that the use of an extensive taxon sampling or the covarion model improved the quality of the trees that did not consider the secondary structure (see Galtier, 2001; Huelsenbeck, 2002; Pollock et al., 2002; Hedtke et al., 2006; Shalchian-Tabrizi et al., 2006b), which then showed similar results to the ones that considered the secondary structure.

A fraction of the clades present in most of the phylogenies received significant BVs and PPs (e.g. ALEX, DINP, SYMB, CERA, PESS; Table 3), whereas other clades received high PPs and low BVs (e.g. PFIE, PFIE2, POLA, SCRI). This contradiction is puzzling. A number of studies indicate that Bayesian Inference (BI) overestimates and bootstrap underestimates support values (e.g. Cummings et al., 2003; Simmons et al., 2004). In our results, it seems that the differences are too large and consistent to be only a product of over- or underestimation. Another possibility is that these clades represent attractive topologies for Bayesian analyses and were visited more often in the MCMC explorations of the parameter space, giving them a higher support (see Cummings et al., 2003). However, some clades

that obtained low-moderate BVs and PPs with the SSU obtained higher BVs and PPs in the LSU phylogenies, which happened to included more sequences within them (compare POLAs and POLA, PBORs, and PBOR; Table 3). This suggests that the low support values could have been the product of reduced phylogenetic signal caused by the presence of fewer sequences.

The diversity of dinoflagellates is still being uncovered, and new species and lineages are being discovered regularly (e.g. Burkholder et al., 1992; Montresor et al., 1999; Lopez-Garcia et al., 2001; de Salas et al., 2003; Jeong et al., 2005; Lindberg et al., 2005; Lin et al., 2006). We have considered if future analyses including newly discovered freshwater and marine dinoflagellates could lead to different patterns (e.g. closely related freshwater and marine species in several lineages), and concluded that this possibility is unlikely. In this study, we have included 88 SSU and LSU sequences associated to around 40 freshwater dinoflagellate morphospecies (out of ~220 recognized). This covers ~70% of the freshwater genera [as recognized in Popovsky and Pfiester (1990)] which contain more than two species. The SSU and LSU have been sequenced for numerous marine dinoflagellates and sequences representing most lineages have been included in our analyses. If closely related marine and freshwater dinoflagellates were common in nature, then we should have detected a higher number of them (more than only two cases) due to our extensive taxon sampling.

The existence of an enormous hidden diversity of marine and freshwater dinoflagellates that if analyzed could lead to radically different conclusions is also an unlikely scenario. Conspicuous (>10 μm) marine and freshwater dinoflagellates have been widely studied by taxonomists, and there is no indication that the total number of recognized species will increase by orders of magnitude after new taxonomic descriptions. In contrast, a potentially high diversity could be hidden in small (<10 μm) and poorly studied dinoflagellates. Environmental DNA surveys of marine and freshwater small (<10 μm) microeukaryotes found new dinoflagellate lineages and species. However, these studies do not indicate that the unknown diversity of small dinoflagellates is enormous (e.g. Lopez-Garcia et al., 2001; Moon-van der Staay et al., 2001; Richards et al., 2005; Lin et al., 2006; Lefevre et al., 2007). In particular, not many freshwater picodinoflagellates (i.e. pico-sized) were found in environmental DNA surveys of two freshwater lakes in Europe and North America, suggesting that the unknown amount of picodinoflagellate diversity might not be very high (Richards et al., 2005; Lefevre et al., 2007). Interestingly, the freshwater picodinoflagellates detected by Richards et al. (2005) and Lefevre et al. (2007) clustered into two clades, and are apparently not evolutionary closely related to marine picodinoflagellates. Thus, as it seems to be the case for several picoeukaryote lineages (see Richards et al., 2005), freshwater and marine picodinoflagellates might also be separated into marine and freshwater lineages.

5.7. Evolutionary remarks

In summary, our molecular results, along with fossil and morphological data, indicated that during the evolution of dinoflagellates, the marine–freshwater margin has acted as a barrier that relatively few lineages have been able to cross. Molecular data from other microbial lineages also support the contention that marine–freshwater transitions have not been frequent during the evolution of microbes (Methe et al., 1998; Zwart et al., 1998, 2002; Hoef-Emden et al., 2002; Pawlowski and Holzmann, 2002; Holzmann et al., 2003; Warnecke et al., 2004; Katz et al., 2005; Richards et al., 2005; Figueroa and Rengefors, 2006; Scheckenbach et al., 2006; Sims et al., 2006; Alverson et al., 2007; Lefevre et al., 2007). Altogether, these data indicate that habitat differences prevent the free interchange of microbes between marine and fresh waters in a similar manner as it does with metazoans (Lee and Bell, 1999). Likewise, several studies on multicellular organisms also indicate that only a few marine lineages have been successful in colonizing fresh waters (e.g. Lee and Bell, 1999; Miller and Labandeira, 2002; Vermeij and Wesselingh, 2002; Lovejoy et al., 2006). Despite that the development of complex osmoregulatory mechanisms is probably one of the main barriers for the colonization of freshwater by metazoans, it is unclear what constitutes the barrier that prevents marine microbes from the constant recolonization of fresh waters and the reverse. Contrary to metazoans, microbes normally have massive population sizes, high reproductive rates, enormous genetic variability (e.g. Snoke et al., 2006) and capabilities for long distance dispersal (Finlay, 2002). Due to these characteristics, it would be expected that microbial strains from several lineages would acquire the necessary mutations to make the environmental transition. Future studies will need to investigate if the marine–freshwater barrier is generated only by salinity gradients or there are other factors, like competitive exclusion by adapted residents (De Meester et al., 2002) or high extinction rates in small limnic habitats (island biogeography theory), playing a role.

Acknowledgments

The Swedish Research Council (to K.R.), the SEED project contract, GOCE-CT-2005-003875 (European Commission Directorate General Research) to K.R., and The Royal Physiographic Society in Lund provided financial support (K.R. and R.L.). A. Kremp and E. Kim provided cultures and DNA. Special thanks to the Bioportal (UiO) bioinformatics staff, in particular T.A. Ruden. We thank the staff at the "Workshop on Molecular Evolution 2006", Marine Biological Laboratory, for valuable discussions and suggestions that helped to improve our analyses. S. Bensch, N. Rodríguez-Ezpeleta, K. Lindberg, the MPE associate editor and anonymous reviewers provided valuable comments and suggestions that helped to improve this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.08.005.

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