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Phylogenetic analyses of the dinoflagellate *Noctiluca scintillans* based on β-tubulin and Hsp90 genes

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Abstract

The noctilucid dinoflagellate *Noctiluca scintillans* is an unarmed heterotrophic protist that inhabits the world's oceans and is sometimes responsible for harmful red tides. The phylogenetic position of the noctilucids has been widely disputed because of two alternative views based on morphological characters and phylogenetic analyses using SSU rDNA. Specifically, noctilucids are either placed in a basal position within the dinoflagellates or they are seen as evolutionarily recent derivations descended from unarmored dinoflagellates in the order Gymnodiniales. Thus, the precise relationship of noctilucids to other dinoflagellates is still uncertain. In this study, we isolated β-tubulin and heat shock protein 90 genes from *N. scintillans* to examine this relationship further. The deduced amino acid sequences share commonly substituted amino acids and a deletion with other dinoflagellates, but not with *Perkinsus marinus* or other alveolates. Although Hsp90 analysis did not give robust support, β-tubulin analysis including an AU test, as well as combined analysis of these two amino acid sequences showed that *N. scintillans* is the next earliest branch after *Oxyrrhis marina*, within the dinoflagellates. Given the phylogenetic position of *N. scintillans*, its extremely specialized diploid trophont, and the primitive dinoflagellate-like characteristics of its haploid zoospore, we propose that noctilucids are a possible evolutionary link between ancestral diploid dinoflagellates and haploid core dinoflagellates. This implies that the transition from diploidy to haploidy in trophonts probably occurred via neoteny of a noctilucid-like zoospore.

Keywords: Alveolates; Dinoflagellates; Noctilucae; Zoospore; Ploidy change; Phylogeny

Introduction

Noctiluca scintillans (Ehrenberg) McCartney is a heterotrophic dinoflagellate that inhabits the world's oceans and occasionally causes red tides. The phylogenetic position of noctilucids has been debated. *Noctiluca* was originally classified as a jellyfish until Haeckel (1873) proposed that it should be included in the order Cystoflagellata, within the dinoflagellates. Based on detailed observations of trophont and gamete morphology, Kofoid (1920) later placed *Noctiluca* in the newly created order Noctilucales. This order was regarded as closely related to the order Gymnodiniales, which consists of un-armoured dinoflagellates. This classification was widely accepted. However, in the 1990s, the phylogenetic position of *Noctiluca* was reevaluated using molecular information.

The first phylogenetic analysis that included noctilucids was performed on LSU rDNA sequences of domain 1 and domain 8 (Lenaers et al. 1991). The results of this analysis suggested that *N. scintillans* should be placed in an

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ancestral position within the dinoflagellates. A comprehensive analysis based on SSU rDNA was later performed that also placed *N. scintillans* in an ancestral position (Saunders et al. 1997). Saldarriaga et al. (2004) used a greater number of species to re-analyse the phylogenetic relationships among dinoflagellates including noctilucids. In this case, the placement of *Noctiluca* was inconsistent, depending on the sequences included. One analysis of the SSU rDNA sequences from a diverse collection of eukaryotes suggested that *N. scintillans* should be placed in a basal position among dinoflagellates, while another analysis of SSU rDNA using sequences from only *Perkinsus marina* and dinoflagellates (which allowed for the utilization of many more sites), placed *Noctiluca* within the order Gymnodiniales. The latter analysis suggests a recent branching of noctilucid dinoflagellates (Saldarriaga et al. 2004). In another analysis, *Noctiluca* was placed in a basal position but *Oxyrrhis* was regarded as a specialized species derived from core dinoflagellates (Cavalier-Smith and Chao 2004).

The light-emitting enzyme luciferase (LCF) gene was cloned from *N. scintillans* (Liu and Hastings 2007). According to phylogenetic analysis and a comparison of the genomic structure and domain organization of LCF genes from eight species of dinoflagellates, *N. scintillans* possesses the most ancestral type of LCF gene.

Although some studies tend to place noctilucids in a basal position among dinoflagellates, the lack of broader analyses using protein-coding genes makes it difficult to clarify precise evolutionary relationships within the dinoflagellates.

In this study, we report phylogenetic analyses of *N. scintillans* based on β-tubulin and heat shock protein 90 (Hsp90) gene sequences. These two genes are widely used for phylogenetic analyses among protozoa (e.g. Fast et al. 2002; Harper et al. 2005; Leander and Keeling 2004; Nishi et al. 2005; Saldarriaga et al. 2003; Shalchian-Tabrizi et al. 2006). Our results demonstrate that both *Noctiluca* and *Oxyrrhis* should be placed in a basal position within the dinoflagellates. Taken together with our previous reports on gamete morphology (Fukuda and Endoh 2006), we discuss the origin of core dinoflagellates (which produce haploid trophonts), and propose that they may have evolved from a common haploid ancestor similar to the zoospore of noctilucids (which produce diploid trophonts). This scenario can rationally explain the change in ploidy of trophonts from diploid to haploid during dinoflagellate evolution.

Materials and methods

Cultivation of *Noctiluca scintillans* and isolation of DNA

Cell cultures of *N. scintillans* were established by isolating a mature cell (trophont) from seawater. Strain JNO-11, which is native to Notojima island, Ishikawa Prefecture, Japan, was collected in June 2006. All cells were maintained as previously described (Fukuda and Endoh 2006). Genomic DNA was extracted from gamete cells according to standard procedures to avoid introducing inhibitory material from the trophonts into the samples.

Amplification, cloning, and sequencing

We used degenerate primers for both the β-tubulin gene (expected fragment size ~1200 bp) and the Hsp90 gene (expected fragment size ~1700 bp). Primers used in this study were selected based on previous studies. The primer sequences used for β-tubulin were caRtgYggYaaccaRatYgg (forward) and tccatYtcgtccaRccYtc (reverse; Nishi et al. 2005). The primer sequences used for Hsp90 were acgttYtaYWSNaaYaaRgaRat (forward) and cgccttcatMatNcSYtccaRttNgc (reverse; Leander and Keeling 2004). Polymerase chain reaction (PCR) mixtures (50 µl), which included 0.5 µl DNA solution and 1 U Ex-Taq DNA polymerase (TaKaRa), were subjected to 35 cycles of PCR in a thermal cycler. The PCR conditions for β-tubulin were 60 s at 94 °C, 45 s at 47 °C, and 80 s at 72 °C. PCR conditions for Hsp90 were 60 s at 94 °C, 45 s at 50 °C, and 120 s at 72 °C. All PCR products were purified and cloned into a pT7 Blue T-vector. Sequencing was performed using a Thermosequenase Cycle Sequencing Kit (Amersham), and all samples were analysed with a Lic-4200 DNA analyser (LI-COR).

Phylogenetic analysis

Sequence alignment was performed using CLUSTAL X version 1.81 (Thompson et al. 1997). In total, 376 unambiguously aligned amino acid residues of β-tubulin and 521 amino acid residues of Hsp90 were analysed. All results were additionally edited by hand with BioEdit 7.0.4.1 (Hall 1999). Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel 2003). PhyML employed the WAG amino-acid substitution model (Whelan and Goldman 2001) incorporating the invariable site option and the Gamma distribution (eight categories) option. The proportion of invariable sites and gamma distribution parameter were estimated from datasets. To calculate the bootstrap value, 100 ML trees were constructed using the same datasets. Bayesian analyses were also performed with the WAG amino-acid substitution model, invariable site option and eight gamma categories, using the MrBayes 3.1.2 programme (Ronquist and Huelsenbeck 2003). One cold and three heated Markov chain Monte Carlo (MCMC) chains with default chain temperatures were run for 100,000 generations. Log likelihoods and trees

were sampled at 100-generation intervals. To evaluate the phylogenetic position of *Noctiluca*, we examined the approximately unbiased test value (AU test: Shimodaira 2002) with the Consel programme. The site-wise log likelihood was calculated by the Tree-puzzle-5.2 package (Schmidt et al. 2002) with -wsl option, and tree files were constructed by TreeView [Win32] 1.6.6 (Page 1996). In this study, the borderline of the AU test was 0.05 (below: reject/over: cannot reject).

Results and discussion

Dinoflagellate-specific signatures in β-tubulin and Hsp90 sequences

We determined the sequences of two β-tubulin genes (*NBTU1*: 1143 bp: accession number AB297473 and *NBTU2*: 1150 bp: accession number AB297472) and one heat shock protein gene (*NHSP90*: 1674bp: accession number AB297471) of *N. scintillans*. Among the two β-tubulin genes, *NBTU1* is thought to be a pseudogene because it contains ten nonsense mutations and seven deletion sites that lead to a frameshift mutation. Therefore, we used the other gene sequence (*NBTU2*).

These sequences shared common amino acids at four different sites for β-tubulin (Fig. 1A) and at four sites for Hsp90 (Fig. 1B) with the sequences from dinoflagellates (including *Oxyrrhis*). In addition, one deletion (between amino acid positions 537 and 538) specific to the core dinoflagellates and *Oxyrrhis* was previously reported by Leander and Keeling (2004). This deletion is conserved in the *Noctiluca* Hsp90 gene sequence (Fig. 1B). These common amino acid signatures and deletions are not found in *Perkinsus*. Instead, the *Perkinsus* sequences tend to share signatures with those of apicomplexans and ciliates, which represent other alveolates.

Phylogenetic analyses

The phylogenetic relationships based on Hsp90 amino acid sequences were analysed using sequences derived from alveolates including perkinsids (Fig. 2). In the ML phylogenetic tree, *Oxyrrhis marina* and *N. scintillans* diverged early within the dinoflagellates and are monophyletic with other core dinoflagellates with high bootstrap values (98 and 100, respectively) (Fig. 2A). Monophyly of the core dinoflagellates is, however, only weakly supported (the bootstrap value = 51), so that it is still uncertain if *Noctiluca* diverged before the core dinoflagellates. In addition, the exact order of divergence between the other dinoflagellates is unclear due to low resolution within the core dinoflagellates. To confirm the earlier divergence from the core dinoflagellates, Bayesian analysis was performed. In this analysis,

Alignment of beta-tubulin amino acid sequences

	166	174	182	193	244	252
N. scintillans	TFSIIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
O. marina	SFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
A. corpulentum	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
G.instriatum	TFSIIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
H.triquetra 13	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
H.triquetra 14	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
P. willei	TFSIIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
W. tenuissima	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
Dino CCMP421	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
K. foliaceum 91	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
K. foliaceum 92	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
C. cohnii	TFSVIPSPK		PYNAVLSFHQLV		GQLNCDLRK	
P. marinus 2400	TFSVFPSPK		PYNATLSVYKLV		GQLNADLRK	
P. marinus 2401	TFSVFPSPK		PYNATLSVHQLV		GQLNADLRK	
B. bovis	TFSVVPSPK		PYNATLSVHQLV		GQLNSDLRK	
P. falciparum BA	TFSVVPSPK		PYNATLSVHQLV		GQLNSDLRK	
P. falciparum BB	TFSVFPSPK		PYNATLSVHQLV		GQLNSDLRK	
C. parvum	TFSVFPSPK		PYNATLSIHQLV		GQLNSDLRM	
P. berghei	TFSVFPSPK		PYNATLSVHQLV		GQLNSDLRK	
T. gondii	TFSVFPSPK		PYNATLSVHQLV		GQLNSDLRK	
T. pyriformis BB1	TFSVVPSPK		PYNATLSVHQLV		GQLNSDLRK	
T. pyriformis BB2	TFSVVPSPK		PYNATLSVHQLV		GQLNSDLRK	
T. thermophila A	TFSVVPSPK		PYNATLSVHQLV		GQLNSDLRK	
	TFSVVPSPK					

Alignment of hsp90 amino acid sequences

	55	59	84	88	105	109	174	178	535	540
N. scintillans	IEAQP		TKNEL		EAMSA		IICYL		EDE-KKK	
O. marina	VEAQP		TKNEL		EAMAA		IVCFL		EDE-KKS	
H. triquetra	IEAQP		TKNEL		EAMAA		IICYL		EDE-KKK	
C. cohnii	IEAQP		TKNEL		EAMAA		IICYL		EDE-KKK	
K. foliaceum	IEAQP		TKNEL		EAMAA		IICYL		EDE-KKK	
L. elongata	IEAQP		TKNEL		EAMAA		VICYL		EDE-KKK	
A. tamarense	IEAQP		TKNEL		EAMAA		VICYL		EDE-KKK	
P. micans	IEAQP		TKNEL		EAMAA		IICYL		EDE-KKK	
G. chlorophorum	IEAQP		TKNEL		EAMAA		VICYL		EDE-KKK	
K. micrum	IEAQP		TKNEL		EAMAA		IICYL		EDG-KEK	
K. mikimotoi	IEAQP		TKNEL		EAMAA		VICYL		EDD-KKK	
K. brevis	IEAQP		TKNEL		EAMAA		VICYL		EDD-KKK	
P. marinus	IENEP		TKTEM		EAIQA		VILYL		SDEEKKA	
B. bovis	VEDFP		TKTDL		EAIQA		LILHL		TEEERKN	
T. parva	IEDQP		TKADL		EALQA		LILHL		GEDEKKS	
T. gondii	LKGAP		TKAEL		EALQA		IILHM		DEEEKKK	
E. acervulina	LKTKP		TKADL		EALQA		LILHL		TEEEKKK	
E. tenella	LKTKP		TKADL		EALQA		IILHL		SEEEKKK	
T. thermopila	LEVEP		TKKEL		EALSS		IILHM		TEDEKKK	
T. bergeri	LELEP		TKKEL		EALSS		IILHM		TEDEKKK	
T.pyriformis	LETEP		TKKEL		EALSS		IILHM		SEDEKKS	
P.tetraurelia	LNIEP		TRDEM		EALSS		IILHM		TEDEKKK	
H. grandinella	LETEP		TKAEL		EAISA		IILHM		TEEEKKQ	

Fig. 1. Comparison of β-tubulin and Hsp90 amino acid sequences from *N. scintillans* with representative sequences from alveolates. (**A**) β-tubulin. The four sites (170: I, 186: V, 189: F, and 249: C) shared by dinoflagellates are marked in gray. These dinoflagellate-specific amino acids are not conserved in the *Perkinsus marinus* sequence. (**B**) Hsp90. Amino acids at four sites (at 57: A, 86: N, 107: M, 176: C) and one deletion (at 537–538) are shared by *Oxyrrhis*, *Noctiluca*, and other core dinoflagellates (*P. micans*, *C. cohnii*, *L. elongata*, and *H. triquetra*). *Perkinsus* does not share these signatures with dinoflagellates. Sites in *Perkinsus* are common with other alveolates such as apicomplexans and ciliates. Numbers shown above alignments correspond to the numbers from the amino acid sequences of *Crypthecodinium cohnii*.

N. scintillans was included in the core-dinoflagellate clade, unlike what was found in the ML analysis (Fig. 2B). In both analyses, *O. marina* branched out

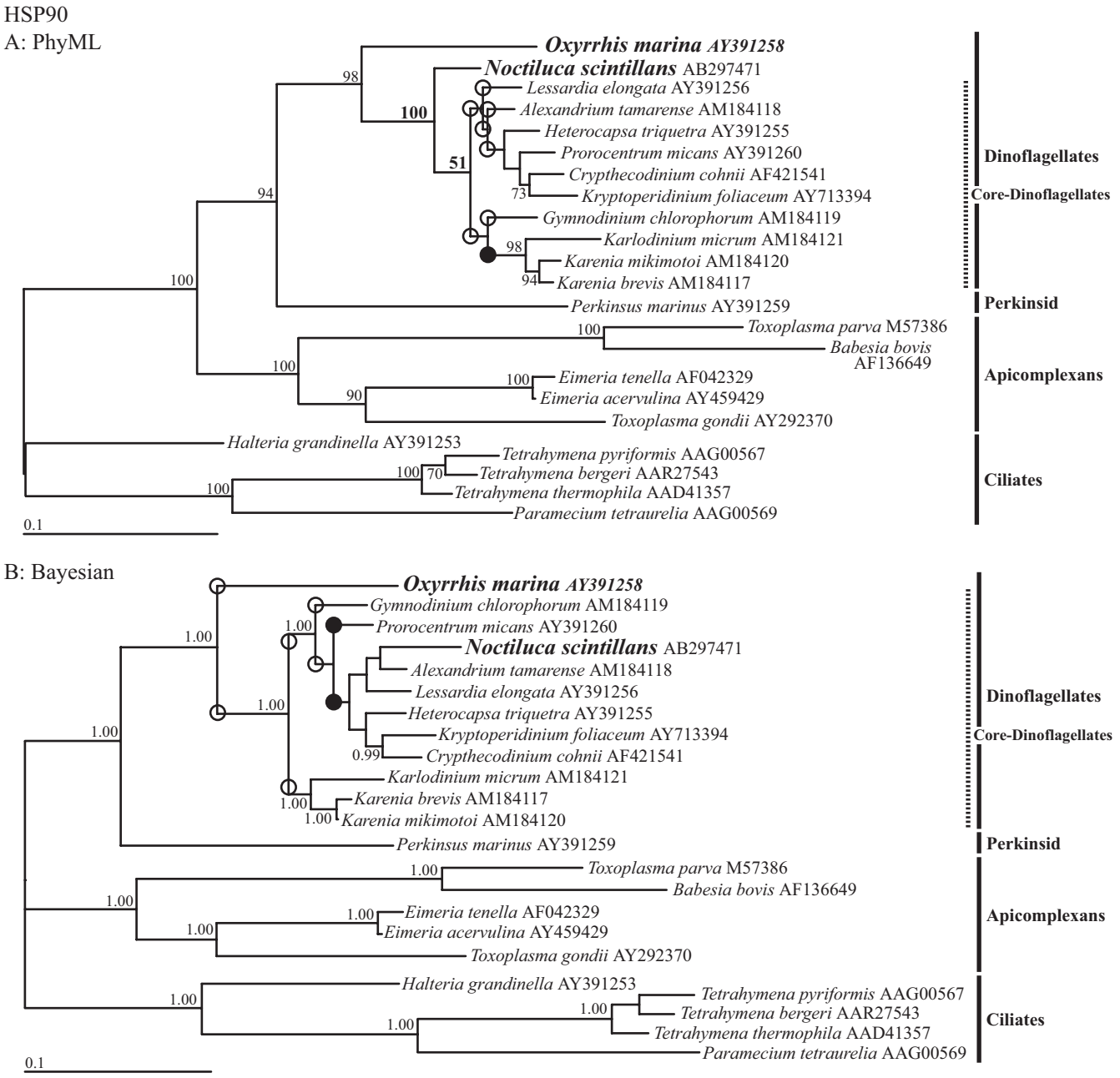

Fig. 2. Phylogenetic tree of Hsp90 constructed by Maximum likelihood (A) and Bayesian analysis (B). Higher-level taxa are named on the right. Circles on branch lines correspond to check points of the AU test. Open circles show rejected positions at confidence levels of >0.05, while filled circles show positions that were not rejected. (**A**) In ML tree, *N. scintillans* is placed at an ancestral position within dinoflagellates, which is only weakly supported. Numbers at the internodes correspond to bootstrap values (only those above 50% shown). (**B**) In this Bayesian analysis, *N. scintillans* is included within core dinoflagellates. Numbers at the internodes correspond to posterior probability (only those above 0.95 are shown).

from an early position, and dinoflagellates and apicomplexans were more closely related to each other than to ciliates (e.g. Fast et al. 2002; Harper et al. 2005; Leander and Keeling 2004). To further evaluate if the Hsp90 data support the relationships of *Noctiluca* to other core dinoflagellates, we carried out AU tests. As shown in Fig. 2, inclusion of noctilucids within the core dinoflagellates was not rejected (open circle) except one group consisting of a few gymnodinialids (closed circle). Thus these Hsp90 analyses could not fully elucidate the exact position of noctilucids within dinoflagellates. The HSP90 sequence probably does not contain enough information to analyse detailed relationships among dinoflagellates.

On the other hand, ML and Bayesian analyses based on β-tubulin amino acid sequences from various

alveolates brought a different conclusion from that of the above-mentioned Hsp90 analyses (Fig. 3). In these analyses, both methods gave the same tree topology. These analyses strongly supported the ancestral position of *Noctiluca* (with *Oxyrrhis*) within dinoflagellates, with fairly high bootstrap value and posterior probability (ML: 72/Bayes: 0.95), i.e. *Noctiluca* diverged before core dinoflagellate radiation. To further confirm the relationships between *Noctiluca* and other core dinoflagellates, we performed the AU tests again. In the AU test, inclusion of *Noctiluca* with most of the core dinoflagellates was rejected, except a possibility that *Noctiluca* forms a monophyly with *Woloszynskia*. Thus, these analyses suggest that noctilucids are one of the earliest lineages to diverge from the main line of the core dinoflagellates.

Finally, we performed ML and Bayesian analyses based on concatenated amino acid sequences of both β-tubulin and Hsp90; again both methods produced the same tree topology. Although fewer taxa were used than in the two analyses above, these analyses showed that *N. scintillans* diverged after *Oxyrrhis* and before the core dinoflagellates (Fig. 4).

Evolutionary origin of noctilucids and core dinoflagellates

Previous phylogenetic analyses based on SSU rDNA have produced two plausible hypotheses concerning the origin of noctilucids within the dinoflagellates. It has been proposed that they are either one lineage of the more ancestral dinoflagellates, or they are evolutionarily recent derivations from the Gymnodiniales (Saldarriaga et al. 2004; Saunders et al. 1997; Shalchian-Tabrizi et al. 2006). Our present study, in particular β-tubulin analysis, supports the hypothesis that noctilucids are placed in an ancestral position within the dinoflagellates.

Ancestral dinoflagellates retain (gain) or lack many characteristics compared to the core dinoflagellates that are assumed to be evolutionarily young (new). *Oxyrrhis* and *Noctiluca* are truly different from the typical dinoflagellates in various aspects. For example, *Oxyrrhis* possesses a histone-containing nucleus, two undeveloped grooves, a large number of long and thin chromosomes, and scales. However, it lacks pusules, a haploid trophont, and dinokaryotic mitosis (Dodge and Crawford 1971a, b; Fensome et al. 1993; Loeblich 1984; Triemer 1982). As previously described, *Noctiluca* trophonts do not retain the shared characters of typical dinoflagellates such as a transverse flagellum and non-condensed chromosomes. In stark contrast to the trophonts, *Noctiluca* gametes maintain primitive dinoflagellate-like characteristics including two grooves, slightly differentiated flagella with different lengths and paraxial rod, and condensed chromosomes (Soyer 1970 1972; Fukuda and Endoh 2006). These gamete characteristics likely reflect the primary, or oldest,

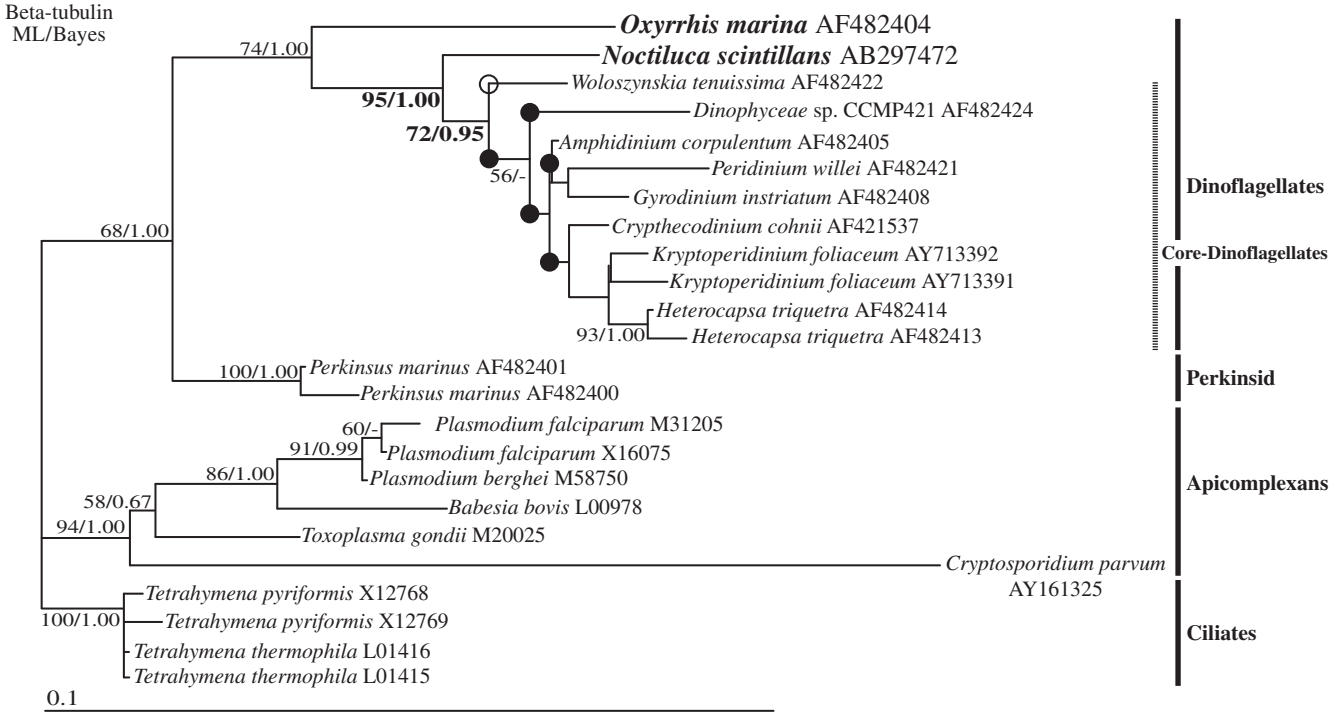

Fig. 3. Phylogenetic tree of β-tubulin constructed by ML and Bayes. In this tree, *N. scintillans* is placed at a basal position in the dinoflagellates similar to what was seen in the Hsp90 ML tree. This result is moderately supported by bootstrap values (ML/Bayesian = 72/0.95). Higher-level taxa are named on the right. Circles correspond to check points of the AU test as shown in Fig. 2.

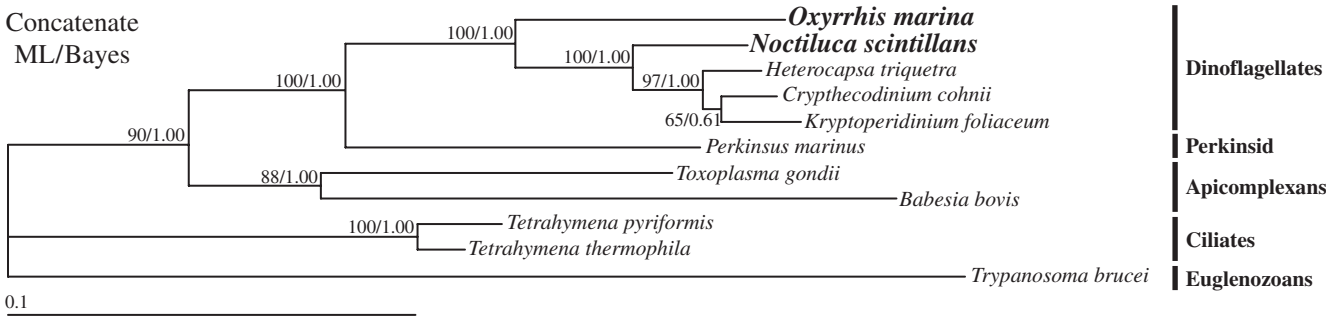

Fig. 4. Phylogenetic tree of concatenated Hsp90 and β-tubulin constructed by ML and Bayes.

attributes. These observations led us to postulate that most dinoflagellates have been derived from an ancestral cell with a haploid nucleus, similar to the *Noctiluca* gametes.

The early evolution of dinoflagellates can be explained by considering the following scenario. Taking account of diploid trophonts in *Oxyrrhis* and *Noctiluca*, the common ancestor of dinoflagellates is likely to be a diploid biflagellate. The organism retained alveoli, two grooves, a specialized longitudinal flagellum with a paraxial rod, and typical eukaryotic nuclei. Furthermore, the ancestral species underwent sexual reproduction during a period of its life cycle. After the ancestral *Oxyrrhis* branched off from the lineage and the ancestral species evolved towards *Noctiluca* (and most of the remaining core dinoflagellates), the descendants of *Oxyrrhis* lost the two grooves. Following these events, *Noctiluca* diverged from the main line of dinoflagellates. While the ancestors of *Noctiluca* displayed a specialized vegetative form (trophont) through the loss of characteristics typical of extant dinoflagellates, characteristics that are derived from the ancient ancestor have been retained by the gametes. By taking ploidy into account (i.e., most dinoflagellates are regarded as haploid), we hypothesize that a common ancestor of the core dinoflagellates has evolved from an ancestor with a haploid nucleus (such as the noctilucid gametes) via neoteny. This made the generation of haploid trophonts (the core dinoflagellates) from diploid ones (*Oxyrrhis* and *Noctiluca*) possible. By the haploidization of the ancestor, the core dinoflagellates would have attained rapid radiation, reflected in low resolution of their phylogenetic relationships. Some (or most) members of the core dinoflagellates acquired the ability to sexually reproduce. This led to a transient post-mating diploid state followed by meiosis. In fact, some species show a very short diploidic period from gamete fusion to meiosis during sexual reproduction. If this scenario is correct, the gametes of *Noctiluca* might be an important intermediate connection between the ancestral species and typical modern dinoflagellates, thereby adding to our current understanding of the origin and early evolutionary history of dinoflagellates. This idea can also contribute to elucidation of alveolate evolution (Cavalier-Smith and Chao 2004; Leander and Keeling 2003).

Note

We obtained partial SSU rDNA sequences from two strains of *N. scintillans* with different geographic origins in Japan (JAJ-1 collected from Awajishima island, Hyogo, and JNO-1 from Notojima island, Ishikawa). Compared to the noctilucid SSU rDNA sequence in GenBank (AF022200), only a few nucleotide substitutions and deletions were detected, suggesting that all strains belong to the same species. Accession numbers are AB297469 (JAJ) and AB297470 (JNO).

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