Dinoflagellate Phylogeny as Inferred from Heat Shock Protein 90 and Ribosomal Gene Sequences

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Abstract

Background: Interrelationships among dinoflagellates in molecular phylogenies are largely unresolved, especially in the deepest branches. Ribosomal DNA (rDNA) sequences provide phylogenetic signals only at the tips of the dinoflagellate tree. Two reasons for the poor resolution of deep dinoflagellate relationships using rDNA sequences are (1) most sites are relatively conserved and (2) there are different evolutionary rates among sites in different lineages. Therefore, alternative molecular markers are required to address the deeper phylogenetic relationships among dinoflagellates. Preliminary evidence indicates that the heat shock protein 90 gene (Hsp90) will provide an informative marker, mainly because this gene is relatively long and appears to have relatively uniform rates of evolution in different lineages.

Methodology/Principal Findings: We more than doubled the previous dataset of Hsp90 sequences from dinoflagellates by generating additional sequences from 17 different species, representing seven different orders. In order to concatenate the Hsp90 data with rDNA sequences, we supplemented the Hsp90 sequences with three new SSU rDNA sequences and five new LSU rDNA sequences. The new Hsp90 sequences were generated, in part, from four additional heterotrophic dinoflagellates and the type species for six different genera. Molecular phylogenetic analyses resulted in a paraphyletic assemblage near the base of the dinoflagellate tree consisting of only athecate species. However, Noctiluca was never part of this assemblage and branched in a position that was nested within other lineages of dinokaryotes. The phylogenetic trees inferred from Hsp90 sequences were consistent with trees inferred from rDNA sequences in that the backbone of the dinoflagellate clade was largely unresolved.

Conclusions/Significance: The sequence conservation in both Hsp90 and rDNA sequences and the poor resolution of the deepest nodes suggests that dinoflagellates reflect an explosive radiation in morphological diversity in their recent evolutionary past. Nonetheless, the more comprehensive analysis of Hsp90 sequences enabled us to infer phylogenetic interrelationships of dinoflagellates more rigorously. For instance, the phylogenetic position of Noctiluca, which possesses several unusual features, was incongruent with previous phylogenetic studies. Therefore, the generation of additional dinoflagellate Hsp90 sequences is expected to refine the stem group of athecate species observed here and contribute to future multi-gene analyses of dinoflagellate interrelationships.

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Introduction

Dinoflagellates are an extraordinary lineage of protists (unicellular eukaryotes) in regard to overall diversity in cell morphology and nutritional modes (e.g., phagotrophy, ‘kleptophotrophy’, photoautotrophy, mixotrophy, and parasitism) [1–3]. Both heterotrophic and photoautotrophic members of the group are abundant and ecologically important components of marine planktonic communities. Dinoflagellates are morphologically distinct from other eukaryotes in the structure of their (dinokont) flagellar apparatus and (dinokaryotic) nucleus (i.e., permanently condensed chromosomes without typical eukaryotic histones and with an extranuclear spindle that passes through cytoplasmic channels) [1,4,5]. The monophyly of dinoflagellates and their phylogenetic relationships to other alveolate taxa, like ciliates and apicomplexans, have been demonstrated with several different molecular markers [1,4,6–11]. However, the interrelationships of the major subgroups of dinoflagellates are still unresolved using current molecular markers, mainly because of a lack of statistical support (i.e., phylogenetic signal) for the branching order near the phylogenetic backbone of the group [12–14]. The evolutionary relationships of dinoflagellates were initially inferred from a comparison of morphological characters [15], and these data are very important for evaluating weakly resolved branching patterns inferred from molecular markers [13,16]. Accordingly, the poor phylogenetic resolution associated with the molecular markers employed so far prolongs our reliance on morphological
characters when making inferences about dinoflagellate evolutionary history [12,13,16]. As such, inferences based on morphology have yet to be adequately tested with molecular markers that provide sufficient signal at the deepest levels in the dinoflagellate phylogenetic tree.

Ribosomal DNA (rDNA) sequences are most useful for resolving (“genus” level) relationships near the tips of the dinoflagellate tree [12,17–21]. Deeper branches receive either no or poor statistical support in trees inferred from rDNA for several reasons: (1) a large number of highly conserved regions; (2) strong evolutionary rate heterogeneity among sites in variable regions; (3) high levels of compositional heterogeneity among some of the sequences; (4) high levels of homoplasy within variable regions; and (5) non-independently evolving sites in paired helix regions [12–14,22]. Moreover, taxon sample biases and taxon identification are reoccurring problems – fewer than 150 species of about 2,500 known species have so far been sequenced, with a strong bias towards photosynthetic taxa [14]. Although some effort has been made to increase the representation of heterotrophic and uncultivated taxa in the datasets over the past five years [18,19,21,23], the taxon bias remains.

Understanding the phylogeny of athecate (unarmored) dinoflagellates is particularly problematic because (1) their patterns of amphisyimal vesicles are more difficult to discern than in thecate (armored) dinoflagellates, (2) many of them are heterotrophic and uncultivated, (3) they are widely polyphyletic in molecular phylogenetic analyses, and (4) many of them have been misclassified [13,14,21,24,25]. Nonetheless, detailed re-evaluations of morphology combined with molecular phylogenetic studies of several athecate taxa over the past ten years has resulted in descriptions of new genera and improved re-descriptions of existing genera [12,17,20,21,23,26–30].

The phylogenetic position of the (athecate) Noctilucales is especially controversial. These free-living dinoflagellates possess a dinokaryon only during part of their lifecycle and sometimes possess a highly distinctive trophont stage consisting of an inflated balloon-like cell with a feeding tentacle. Molecular phylogenetic analyses of rDNA sequences and heat shock protein gene (Hsp90) sequences plus the absence of a dinokaryon in the trophont stage suggested that *Noctiluca* was an early diverging lineage of dinoflagellates that retained several ancestral states for the group as a whole (e.g., a pre-dinokaryotic nucleus) [31–34]. However, the molecular phylogenetic position of *Noctiluca* is inconsistent in different analyses, and these cells possess several very novel morphological features, so some authors have questioned the interpretation that this lineage is basal among dinoflagellates [13,16].

The major subgroups of dinoflagellates are largely recognized from patterns of either amphisyimal vesicles or thecal plates, called “tabulation patterns”. This morphology-based criterion has been used to identify several monophyletic groups of dinoflagellates, some of which have been corroborated with molecular phylogenetic data, such as the Suessiales, the Gonyaulacales, the Dinophysiales, the Prorocentrales, and the Gymnodiniales sensu stricto [12–14,20,21,24,29,35,36]. Several lineages previously classified within the “Gymnodiniales” have been removed from this subgroup upon closer examination with electron microscopy and molecular phylogenetic analyses [37]. The tabulation pattern found in the Suessiales (represented by *Polarella* and *Symbusiodinium*) forms an intermediate between the tabulation patterns found in some athecate taxa (previously lumped within the Gymnodiniales) and several thecate subgroups, like the Peridiniales and the Gonyaulacales. Although taxon sampling is far from complete, molecular phylogenetic analyses indicate that the Peridiniales is paraphyletic and might form a stem group from which the Gonyaulacales, Dinophysiales and Prorocentrales evolved [15]. Moreover, the highly distinctive morphology of the Prorocentrales indicates that the group is monophyletic, but molecular phylogenetic data did not corroborate this inference [13,14,24,36–40] until analyses of mitochondrial genes were performed [25,41].

Along these lines, molecular phylogenetic analyses of mitochondrial gene sequences (cob + cox1) concatenated with SSU rDNA recover basal positions for *Amphidinium* (athecate) and *Heterocapsa* (thecate) [25]; some paleontological data also support this hypothesis [13]. Although the general morphology of *Amphidinium* and *Heterocapsa* does not immediately indicate a close relationship between them, both genera contain species that possess body scales [42–44]. Scales are unusual in dinoflagellates and are known only in these two genera plus *Leptoiodosimum* [45,46]. Perhaps significantly, *Oxyrrhis*, which is a sister lineage to dinokaryotes (syn. “core” dinoflagellates), also possesses scales on the cell body and the flagella [11,47–49]. The putative phylogenetic distribution of this character suggests that the most recent ancestor of *Oxyrrhis* and dinokaryotes also possessed body scales.

However, inferences about morphological character evolution in dinoflagellates depend on a robust molecular phylogenetic framework, especially at the deepest levels that relate the major subgroups (i.e., “orders”). Accomplishing this requires exploration of different molecular markers, which is the primary aim of this study. We have chosen to significantly expand the current heat-shock protein 90 (Hsp90) dataset for dinoflagellates by more than doubling the taxon sample in a manner that enhances broader representation of the major subgroups. Hsp90 is a highly conserved molecule that functions as a chaperone for protein folding and plays a key role in cellular signal transduction networks in all eukaryotes [50]. Stechmann and Cavalier-Smith [51] predicted that Hsp90 could become an important “universal” phylogenetic marker for eukaryotes because it is relatively long (1,800 bp) and evolves relatively uniformly in very different lineages. These authors advocated that Hsp90 should be sequenced from a broad selection of eukaryotic taxa and included within multi-gene phylogenetic analyses. The relatively homogenous branch lengths in trees inferred from Hsp90 sequences also helps reduce methodological artifacts associated with long-branch attraction.

Hsp90 datasets have been used previously for inferring dinoflagellate relationships [10,33,52,53]. The first dinoflagellate Hsp90 sequences were used to examine the relationships between the three major alveolate subgroups, which resulted in a strongly supported framework [52]; a few subsequent studies have used Hsp90 sequences to address the internal phylogeny of dinoflagellates [10,33,53]. One of these studies used Hsp90 sequences to explore the evolution of plastid diversity within dinoflagellates, which reinforced that there were several independent plastid replacements as suggested earlier using comparative morphology and analyses of rDNA sequences [53,54]. A concatenated analysis of SSU rDNA sequences with Hsp90 sequences demonstrated considerably higher statistical support values for almost all of the deep nodes when compared to trees inferred from SSU rDNA alone [53]. Most recently, Hsp90 gene sequences were used to evaluate the controversial phylogenetic position of *N. scintillans*, and the authors of this study concluded that *N. scintillans* diverges very early within dinoflagellates [33]. However, all of these studies were limited by the very few Hsp90 sequences available at the time.

In an attempt to better resolve some of the earliest branches in the dinoflagellate phylogenetic tree, we sequenced Hsp90 gene
sequences from 17 different species of dinoflagellates, covering as many orders as possible; consequently, the Hsp90 gene data set for dinoflagellates was more than doubled. Moreover, in order to be able to concatenate the Hsp90 data with LSU and SSU rDNA sequences, we supplemented the new Hsp90 sequences with three new SSU rDNA sequences and five new LSU rDNA sequences. All of these data enabled us to address the broad phylogenetic interrelationships of dinoflagellates and will contribute to future analyses using larger multi-gene datasets.

Results

New Hsp90 sequences were generated from dinoflagellates representing seven different orders, including the first sequences from the Phytodiniales and the Suessiales and from the genera *Akashiwo*, *Diplophysalis*, *Peridinium*, *Polarella*, *Protothecodinium*, *Scriptaspela*, *Spiniferodinium*, *Thecadinium* and *Togula*. Only three of the 12 previously known Hsp90 sequences from dinoflagellates were from heterotrophic species, namely *Cryptophyceae*, *Lessardia*, and *Noctiluca*; in this study, we generated four additional sequences from heterotrophic dinoflagellates, namely *Diplophysalis lentiloba*, *Protoperidinium sp.*, *P. steidingeri* and *P. crassus*. An Hsp90 sequence from the phototrophic *Pyrocystis noctiluca* is available in GenBank, but the length of this sequence was too short to include it in our phylogenetic analyses. A sequence representing the type species of each genus is particularly important in dinoflagellates in order to maintain taxonomic stability in the phylogenetic trees. Accordingly, we generated new Hsp90 sequences from six type species: *Akashiwo sanguinea*, *Gymnodinium fucum*, *Polarella glacialis*, *Spiniferodinium galeiformis*, *Thecadinium kofoidii* and *Togula britannica*. All 17 of the new Hsp90 sequences contained the diagnostic indel for dinoflagellates [10]. The new rDNA sequences generated in order to complete the combined phylogenetic analyses represent the first SSU rDNA sequences from *Amphidinium moeotunorum* and *Spiniferodinium galeiformis* and the first LSU rDNA sequence from *Thecadinium kofoidii*.

Six different alignments were constructed and analyzed: (1) SSU rDNA (35 taxa); (2) LSU rDNA (30 taxa); (3) Hsp90 DNA, 3rd codon positions excluded (40 taxa); (4) amino acid sequences inferred from the Hsp90 DNA sequences (40 taxa); (5) Hsp90 DNA, 3rd codon positions excluded, concatenated with LSU rDNA (34 taxa); and (6) Hsp90 DNA, 3rd codon positions excluded, concatenated with LSU rDNA and LSU rDNA (27 taxa). The resulting trees from datasets 3 to 6 are presented as Figures 1, 2, 3, 4, respectively. The statistical support values from the analyses of the SSU rDNA alone (dataset 1, Figure S1) were added to the corresponding nodes in Figure 3 (dataset 5). Analyses of the LSU rDNA sequences alone (dataset 2) resulted in a poorly resolved phylogeny (Figure S2).

The monophyly of dinoflagellates and dinozoans (i.e., the most recent ancestor of dinoflagellates and Perkinsids and all of its descendants) received high support in all of the analyses (Figures 1–4). The statistical support values for basal nodes within dinokaryotes were low in all of the analyses, except for a few basal nodes in the tree inferred from dataset 3 (Hsp90 DNA, 3rd codon positions excluded) (Figure 1). The Gonyaulacales and the Procentrales received modest to strong support in all of the analyses, especially in trees inferred from datasets including rDNA sequences (Figures 3, 4). The *Protothecodinium*/*Diplophysalis* clade and the *Karenia* clade were strongly supported in trees inferred from Hsp90 sequences (Figures 1, 2). *Togula britannica* and *Spiniferodinium galeiformis* formed a strongly supported clade in trees inferred from Hsp90 sequences alone and in trees inferred from datasets including both Hsp90 and LSU rDNA (Figures 1, 2, 3). Unexpectedly, *Polarella glacialis* and *Gymnodinium simplex* did not cluster together in the trees inferred from datasets 3–5 (Figures 1, 2, 3) but did cluster strongly together in the tree inferred from dataset 6 (Figure 4).

Genera of athecate species branched as a paraphyletic assemblage near the base of the dinoflagellate tree in all of the analyses (Figures 1, 2, 3, 4). Moreover, in all of the trees inferred from DNA sequences, the *Karenia*/*Karenia* clade formed the earliest diverging lineage among the dinoflagellates included in the analyses (Figures 1, 3, 4); the tree inferred from amino acid sequences had an anomalous topology, whereby the *Togula*/*Spiniferodinium* clade formed the earliest diverging lineage (Figure 2). *Amphidinium carterae* is a representative of the *Amphidinium* sensu stricto, also branched near the base of the dinoflagellate tree in all of the analyses, albeit with weak statistical support (Figures 1–4). Nonetheless, neither *Noctiluca* nor *Heterocapsa* ever branched in a basal position relative to the other core dinoflagellates (Figures 1, 2, 3, 4). Instead, *Noctiluca* branched in a position that was deeply nested within other lineages of dinokaryotes, especially within the trees inferred from Hsp90 DNA sequences (Figure 1). In order to gain additional insight into how well the Hsp90 data supported the phylogenetic position of *Noctiluca* relative to dinokaryotes, we performed AU tests for comparing the likelihoods of two alternative topologies differing in the relative position of this species: (1) *Noctiluca* positioned as shown in Figure 1, and (2) *Noctiluca* positioned as the nearest sister lineage to all dinokaryotes in the analyses (e.g., after *Oxyrrhis* and before the *Karenia*/*Karenia* clade, Figure 1). Topology 2 was strongly rejected by the AU test in the datasets that incorporated Hsp90 DNA sequences (*P* value for the AU test = 4 × 10^-6^) and topology 1 was supported (*P* value for the AU test = 1.00).

Discussion

General phylogenetic patterns among athecate dinokaryotes

All trees inferred from the data generated in this study have nearly the same taxon composition in order to make the most direct comparison possible between the different phylogenetic markers employed. As outlined in the Results section, several topological differences were detected in trees inferred from Hsp90 sequences (including concatenations with rDNA, Figures 1, 2, 3, 4) and trees inferred from rDNA sequences alone (additional Figure 1 and published trees from previous studies). Some of these differences were also recognized in previous studies that explored Hsp90 as a phylogenetic marker for dinoflagellates [10,53]. Shalchian-Tabrizi et al. [53] also noticed that although the branching order in trees inferred from LSU rDNA and Hsp90 sequences was generally congruent, the statistical support values for most of the deep nodes were considerably higher in the Hsp90 analyses. However, analyses of Hsp90 amino acid sequences produce topologies that are different from those derived from analyses using Hsp90 DNA sequences (excluding the third codon positions), which can be attributed to a more conserved and thus weaker level of phylogenetic signal in the amino acid dataset [53]. The authors of previous molecular phylogenetic studies of rDNA sequences concluded that the Gymnodiniales are polyphyletic and that loss of a theca occurred multiple times independently [13,14,16,54]. Not surprisingly, this scenario is also reflected in our phylogenetic analyses of rDNA sequences and our analyses of Hsp90 DNA sequences concatenated with rDNA sequences (Figures 3, 4). Zhang et al. [25] suggested that either the *Amphidinium* sensu stricto (e.g., *A. carterae*) or *Heterocapsa* occupy the earliest diverging position among dinokaryotes. By contrast, Murray et al. [14] reported that (1) *Noctiluca* formed the earliest diverging branch in trees inferred from SSU rDNA, (2) *Akashiwo*
formed the earliest diverging branch in trees inferred from LSU rDNA and (3) Karlodinium formed the earliest diverging branch in trees inferred from a combination of SSU and LSU rDNA. This last topology is consistent with our studies of Hsp90 sequences, whereby the Karenia/Karlodinium clade formed the earliest diverging branch among dinokaryotes in all of the analyses of DNA sequences (Figures 1, 3, 4).

Our phylogenetic analyses of the Hsp90 amino acids (dataset 4, Figure 2) resulted in athecate genera (i.e., the Gymnodiniales) branching as a paraphyletic assemblage that encompassed the most recent ancestor of all dinokaryotes. Because our study contained 12 species from nine different genera of athecate dinoflagellates, this paraphyletic distribution of athecate dinoflagellates is particularly compelling; this phylogenetic pattern is also consistent with a previous study of Hsp90 sequences that contained representatives of four athecate genera [53]. Therefore, our new sequences and molecular phylogenetic analyses provide additional support for the hypothesis.
that the initial evolutionary radiation of dinoflagellates involved athecate dinoflagellates that subsequently gave rise to several thecate lineages, perhaps independently (e.g., the Prorocenterales, Gonyaulacales and Peridinales). This hypothesis is also consistent with the phylogenetic results derived from dataset 3 (Figure 1), dataset 5 (Figure 3) and dataset 6 (Figure 4); these trees show *Karenia*, *Karodia*, *Gymnodinium*, and *Amphidinium* branching as a paraphyletic assemblage that incorporates the most recent ancestor of all dinokaryotes. In some of the analyses, *Spiniferodinium* and *Akashiwo* were also part of this athecate assemblage (Figures 3, 4). However, the statistical support values for the nodes near the backbone of the trees inferred from all of the datasets were generally modest at best.

*Polarella glucialis* and *Gymnodinium simplex* were not members of the same clade in the trees resulting from datasets 3–5 (Figures 1, 2, 3), but these species formed a robust clade in the tree inferred from a concatenation of all three genes (Hsp90, SSU rDNA and
LSU rDNA, dataset 6) (Figure 4). Other phylogenies suggest that *G. simplex* belongs into the Suessiales [13]; overall, a more confident placement of *G. simplex* requires, in part, a more detailed morphological investigation of this species.

The molecular phylogenetic position of *Noctiluca*

There is significant debate about the phylogenetic position of *Noctiluca scintillans* among dinoflagellates, mainly because this lineage possesses an unusual collection of morphological features.

The molecular phylogenetic analyses published so far (e.g., SSU rDNA, LSU rDNA, β-tubulin, and Hsp90) suggest that *N. scintillans* diverges very early within dinoflagellates, and most studies show this species branching as the nearest sister lineage to dinokaryotes. Some of the morphological features in this lineage (e.g., the absence of a nucleus with permanently condensed chromosomes in the trophont stage) have, accordingly, been interpreted as concordant evidence for a sister relationship between *Noctiluca* and dinokaryotes [31–33]. Moreover, the
published Hsp90 sequence from *N. scintillans* was previously analyzed within the context of other dinoflagellate sequences and shown to be the first branch to diverge from the other taxa in the analyses [33]. However, these analyses were limited by the very small taxon sample available at the time. We were able to re-evaluate these analyses with a much larger sample of Hsp90 sequences from dinoflagellates and show that *N. scintillans* never occupied a basal position and was instead more deeply nested within dinokaryotes (Figures 1, 2, 3). AU tests provided additional support for this inference.

Although the previous molecular phylogenetic analyses suggesting a basal position for *N. scintillans* have been questioned by some authors [13,16], several other authors have used this framework to (mis)interpret different aspects of the biology of *N. scintillans*. For
instance, Fukuda and Endoh [33] stated that Liu and Hastings [56] discovered the most ancestral type of luciferase gene in N. scintillans. However, this was only one of two alternative interpretations posed by Liu and Hastings [56] and was based on the assumption that N. scintillans had already been demonstrated to be among the earliest diverging dinokaryotes. The alternative interpretation posed was that the condition in N. scintillans was a derived state in this lineage: “The ancestral system may have had two genes, which fused in Noctiluca …” [56].

Moreover, Fukuda and Endoh [32,33] attempted to reconstruct the early evolution of dinokaryotes based on the properties of the gametes in N. scintillans; we think this approach is problematic for several reasons. First, a comparison of trees inferred from ribosomal DNA sequences to trees inferred from Hsp90 sequences with a sufficient taxon sample (i.e., this study) demonstrate that the phylogenetic position of this lineage within dinoflagellates has not been confidently established. Thus, at this time, the characters in N. scintillans cannot be interpreted to be ancestral for dinokaryotes as a whole. Second, these authors characterized their observations of N. scintillans as representing the complete life cycle of this species without accounting for previously reported discrepancies [32]. For instance, the authors describe the gametes as being isogamous and having two flagella that are visible with light microscopy [32]. However, TEM was required to demonstrate that the swarmer cells of N. scintillans had a distinctly heteromorph flagellation, with one long flagellum and one very short flagellum oriented to the left side of the cell [57]. The short flagellum is not visible with light microscopy, which is why Zingmark [58] previously described the gametes as being unflagellated.

Contradictory observations in the literature also led Schnepf and Drebes [59] to re-investigate sexual reproduction in N. scintillans and conclude that although a few microgametes with two flagella were present, generally the microgametes possess only a single longitudinal flagellum and do not undergo fusion. Schnepf and Drebes agreed with Uhlig [60], who reasoned that the appearance of gamete fusion and the presence of two long flagella is a consequence of incomplete cytokinesis. The possibility of an isogamous (or nearly oogamous) sexual cycle was also suggested, but the author’s explicitly stated that definitive evidence is unavailable [59]. Until the fusion of gametes and karyogamy is convincingly demonstrated, the mode of sexual reproduction in N. scintillans will remain speculative. The “isogamy hypothesis” and the transformation of the zygote into a mature trophont characterized by Fukuda and Endoh [32,33] also need to be more convincingly described. Perhaps the best way to establish a more confident phylogenetic position and life cycle for the Noctilucales is to move beyond N. scintillans and characterize more species within the “order” at both the ultrastructural and molecular phylogenetic levels [61].

Concluding Remarks

The resolution of interrelationships between the major lineages of dinoflagellates was modest at best when inferred from Hsp90 sequences alone or in concatenation with rDNA sequences. The high degree of sequence conservation and the consistently poor to modest resolution of the deepest nodes in trees inferred from Hsp90 and rDNA sequences supports the hypothesis that dinoflagellates underwent an explosive radiation in morphological diversity relatively recently in their evolutionary history. However, the lack of sufficient phylogenetic signal in the markers analyzed so far for dinoflagellates could be explained in other ways as well (e.g., mutational saturation over a large period of time). Nonetheless, the more comprehensive analysis of Hsp90 sequences presented here enabled us to re-address several phylogenetic interrelationships of dinoflagellates, such as the phylogenetic position of N. scintillans. Currently, there are no Hsp90 sequences available for the Dinophysiales, the Blastodiniales, and the Syndiniales, and the taxon sampling within the other “orders” is far from being an adequate representation for the overall biodiversity within these groups. In our opinion, the Hsp90 dataset for dinoflagellates should be expanded with the inclusion of Dinophysiales species, Pfiesteria-like species, woloszyneziaksoid species, additional noctiluid species (e.g., Spatubulina and Kofodinum), and additional Prorocentrum species that represent the two separate clades inferred from rDNA phylogenies. Moreover, the incorporation of Hsp90 sequences from additional athecate taxa, like Gymnodinium, Polysphoraks, Takayama, and Apicoporus, will help verify the main phylogenetic pattern we observed in this study, namely that athecate dinoflagellates form a paraphyletic assemblage that includes the most recent ancestor of all dinokaryotes. The generation of additional Hsp90 sequences will also contribute significantly to future multi-gene analyses of dinoflagellate interrelationships, and the present study is an essential step in that direction.

Materials and Methods

Strain collection and culture conditions

The strains used in this study were either (1) isolated from natural samples (e.g., the plankton or intertidal sand) and brought into culture or (2) acquired from culture collections and colleagues (see Table 1 and acknowledgments). The strains we isolated were collected from Helgoland, German Bight, North Sea, Germany [62,63]; Boundary Bay, Vancouver, Canada; and Pachena Beach, Vancouver Island, Canada. Cultures were maintained at 17°C under low light conditions in f/2-medium [64].

The cultures of heterotrophic dinoflagellates were grown at room temperature and normal daylight conditions on a plankton wheel at 1–2 rpm and fed with either the diatom Ditylum brightwellii (Diploldis lenticula and Proterodinum steidingeri) or the dinoflagellate Lingulodinium polyedrum (Proterodinum crassipes). Cultures were transferred every 5 to 7 days by pouring approximately one half of the culture into a new flask containing medium and prey cells. The food cultures were grown at 17°C under low light conditions in f/2-medium [64]. See Grubb and Anderson [18,22] for details of the protocol used for strain isolation and culture establishment.

Cells from cultures received from culture collections were harvested immediately for DNA extraction.

DNA extraction, PCR amplification, cloning, and sequencing

Cells were manually isolated or pelleted from the culture medium. Two different methods for DNA extraction were used over the years (Table 1). (1) Collected cells were suspended into 400 µl CTAB extraction buffer (1.12 g Tris, 8.18 g NaCl, 0.74 g EDTA, 2 g CTAB, 2 g Polyvinylpyrrolidone, 0.2 ml 2-mercaptoethanol in 100 ml water) in 1.5 ml Eppendorf tubes. The tube was placed in a heat-block and incubated at 63°C for 20 min with several vigorous shakes in between. After separation with chloroform:isoamyl alcohol (24:1), the aqueous phase was precipitated in 70% ethanol. Distilled water was added to the dry DNA pellets and the samples were stored in the freezer prior to PCR. (2) Genomic DNA was extracted from the cells using the MasterPure complete DNA and RNA purification Kit (EPICENTRE, Madison, WI, USA). The Hsp90, small subunit, and large subunit rDNA sequences were PCR amplified using puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec, Canada), with an error rate of 1 per 20,000–40,000 bases, and primers were used
as reported previously [21] and in Table 2. PCR products of the expected size were gel isolated and cloned into pCR2.1 vector using a TOPO TA cloning kit (Invitrogen Corporation, CA, USA). One clone was completely sequenced with ABI big-dye reaction mix using both vector primers, internal primers in both directions (for SSU) and sometimes specific internal primers designed for the taxon (for Hsp90).

GenBank accession codes of the used new and already published sequences are shown in Table 3.

### Molecular phylogenetic analyses

Six different alignments were constructed for phylogenetic analysis (Table 3): (1) SSU rDNA (35 taxa and 1,381 unambiguously aligned characters); (2) LSU rDNA (30 taxa and 482 unambiguously aligned characters); (3) Hsp90 DNA, first two codon positions (40 taxa and 984 unambiguously aligned characters); (4) amino acid sequences inferred from the Hsp90 DNA sequences (40 taxa and 511 unambiguously aligned characters); (5) Hsp90 DNA, first two codon positions, concatenated with SSU rDNA (34 taxa and 2,365 unambiguously aligned characters); and (6) Hsp90 DNA, first two codon positions, concatenated with SSU rDNA and LSU rDNA (27 taxa and 2,847 unambiguously aligned characters). Unambiguously aligned sequences were confirmed by eye, and all gaps were excluded from the alignments prior to phylogenetic analyses.

### Table 1. Information about the dinoflagellate species from which sequences were generated in this study.

| Taxon                     | Source            | DNA extraction               | PCR primers                |
|---------------------------|-------------------|------------------------------|----------------------------|
| Akashiwo sanguinea        | culture SCCAP K-1503, Helgoland isolate | CTAB                         | F4-R2 (Hsp90)               |
| Alexandrium tamarense     | culture NEPCC 592  | CTAB                         | F4-R2 (Hsp90)               |
| Amphidinium mootonorum    | culture from MH 1, Isolate from Pachena Beach, BC | Master Pure kit             | F4-R2b (Hsp90), PF1-R4 (SSU), D1R-R2 (LSU) |
| Diplospis lenticula       | culture from K. Gribble, M2reiso3 | Master Pure kit             | F4-R2 (Hsp90)               |
| Gymnodinium fuscum        | culture CCMP 1677 | Master Pure kit             | F4-R2b & F4-R2 (Hsp90)      |
| Gymnodinium simplex       | culture SAMS 1117/3 | DNeasy kit, provided by R. Stern | F4-R2b (Hsp90)              |
| Peridinium wiliei         | culture NEPCC 815 | CTAB                         | F4-R2b (Hsp90)              |
| Polarola glacialis        | culture CCMP 1383 | Master Pure kit             | F4-R2 (Hsp90)               |
| Prorocentrum minimum      | culture SCCAP K-1501, Helgoland isolate | Master Pure kit             | F4-R2b (Hsp90)              |
| Protoperidinium crassipes | culture from K. Gribble, MO65-PC-1split1 | Master Pure kit             | F4-R2b & F4-R2 (Hsp90)      |
| Protoperidinium steidingerae | culture from K. Gribble, MV0802-2 | Master Pure kit             | F4-R2b & F4-R2 (Hsp90)      |
| Protoperidinium sp.       | isolate from Bamfield | Phenol/chloro.              | F4-R3 & F6int-R2b (Hsp90)   |
| Scripsiella trochoidea    | culture CCAP K-1502, Helgoland isolate | CTAB                         | F4-R2 (Hsp90)               |
| Spiniferodinium galeiformis | Boundary Bay isolate | CTAB                 | F4-R2b (Hsp90), PF1-R4 (SSU), D1R-R2 (LSU) |
| Thecadinium kofoidii      | culture SCCAP K-1504, Helgoland isolate | CTAB                         | F4-R2b (Hsp90), PF1-R4 (SSU), D1R-R2 (LSU) |
| Thecadinium yashimaense   | culture CCMP1890  | Master Pure kit             | F4-R2b (Hsp90), D1R-R2 (LSU) |
| Togula britannica         | culture from MH 1, Boundary Bay isolate | CTAB                         | F4-R2b & F4-R2 (Hsp90), D1R-R2 (LSU) |

CCMP = Provasoli-Guillard National Centre for Culture of Marine Phytoplankton, Hsp90 = heat shock protein 90 sequence, lsu = large subunit ribosomal DNA sequence, MH = Mona Hoppenrath, NEPCC = North East Pacific Culture Collection (now CCCM = Canadian Center for the Culture of Microorganisms), SAMS = Scottish Association for Marine Science (CCAP = Culture Collection of Algae and Protozoa), SCCAP = Scandinavian Culture Collection of Algae & Protozoa, ssu = small subunit ribosomal DNA sequence, { } = dead/lost.

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### Table 2. PCR primers used in this study.

| Gene    | Primer name | Primer sequence 5'-3'                     | Citation |
|---------|-------------|--------------------------------------------|----------|
| Hsp90   | F4          | GGAGCCTGATHAAYACNTTYTA                     | this study |
|         | F6int       | AAYAARMINAARCCNHINTGGATG                   | this study |
|         | R2          | CGGCCTCATMATCSYCTCACRTRNGC                 | [10]     |
|         | R2b         | GCCTCATDATNXCYXCCATRITT                    | this study |
|         | R3          | GATGACCTNADATYTRTTTYGGTYG                  | [10]     |
| SSU     | PF1         | GCCTATCTGGTTGATCCCTGCC                    | [70] (modified) |
|         | R4          | GATCTCTTCGGACGGTACCTAC                    | [70] (modified) |
| LSU     | D1R         | ACCGGCGTAATTAAGCATTA                      | [71]     |
|         | R2          | ATTCGGCAGGTGAGTTGTTAC                     | [19]     |

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Phylogenetic relationships were inferred from all six alignments using maximum likelihood (ML) and Bayesian inference (BI) methods with the programs RAxML v7.04 [65] and MrBayes v3.12 [66,67], respectively. ML and BI analyses of the nucleotide alignments (i.e., alignments 1–3 and 5–6) were built under a GTR+I+G+8 model as suggested by the criteria implemented in Table 3.

| Taxon                                      | SSU rDNA | LSU rDNA | Hsp90 (Hsp+SSU) | Combined (Hsp+SSU) | Combined (Hsp+SSU+LSU) |
|--------------------------------------------|----------|----------|-----------------|--------------------|------------------------|
| **Ciliates, apicomplexans & Perkinsus (outgroups)** |          |          |                 |                    |                        |
| Blepharisma**                             | M97909   | x        | AY390395        | x                  | x                      |
| Cryptosporidium parvum                    | AF093489 | AE040725 | AY423866        | included           | included               |
| Eimeria tenella                           | EF210325 | x        | AAB97088        | included           | x                      |
| Halteria grandinella                      | AY00744  | x        | AY391253        | included           | x                      |
| Paramecium tetraurelia                    | EF502045 | x        | AAG00569        | included           | x                      |
| Perkinsus marinus                         | AF126013 | AY876319 | AY391259        | included           | included               |
| Tetrathyamina bergeri                     | AF364039 | x        | AY391257        | included           | x                      |
| Tetrathyamina pyriformis                  | EF070254 | x        | AAG00567        | included           | x                      |
| Theileria parva                           | AF013418 | AF218825 | AAA30132        | included           | included               |
| *T*axoplasma gondii                       | M97703   | L25635.1 | AAQ24837        | included           | included               |
| **Dinoflagellates & Oxyrrhis (Ingroup)**   |          |          |                 |                    |                        |
| Oxyrrhis marina                           | x        | x        | AAR27544        | x                  | x                      |
| Akashiwo sanguinea                        | AF276818 | AF260396 | GU295192        | included           | included               |
| Alexanderim tamarasense                   | AB088333 | AY348021 | AM184118        | included           | included               |
| Alex. tamarasene UBC                       | x        | x        | GU295210        | x                  | x                      |
| Amphidinium carterae                      | AF274251 | AY455669 | EU876701        | included           | included               |
| Amphidinium moontorumor                   | GU295202 | GU295205 | GU295199        | included           | included               |
| Cryptochodinum cahnii                     | M64245   | FJ939575 | AAM20974        | included           | included               |
| Diplosalis lentica                        | x        | EF152794 | GU295193        | x                  | x                      |
| Gymnodinium chlorophorum                  | AM184122 | AF200669 | AM184119        | included           | included               |
| Gymnodinium fuscum                        | AF022194 | AF200676 | GU295194        | included           | included               |
| Gymnodinium simplex                       | DQ384666 | AF060901 | GU295211        | included           | included               |
| Heterocapsa triqueta                      | AF022198 | AF260401 | AAR27541        | included           | included               |
| Karenia brevis                            | AF172714 | AF200677 | AM184117        | included           | included               |
| Karenia mikimotai                         | AF022195 | AF200682 | AM184120        | included           | included               |
| Karlodinium micrum                        | AF172712 | AF200675 | AM184121        | included           | included               |
| Kryptoperidinium faiaceum                 | AF274268 | EF052684 | AAV32830        | included           | included               |
| Lessardia elongata                        | AF521100 | x        | AY391256        | included           | x                      |
| Noctiluca scintillans                     | AF022200 | x        | AB297471        | included           | x                      |
| Peridinium willei                         | AF274272 | AF260384 | GU295195        | included           | included               |
| Polarella glacialis                       | AF099183 | AY517373 | GU295196        | included           | included               |
| Prorocentrum micans                       | M14649   | AF260377 | AAR27546        | included           | included               |
| Prorocentrum minimum                      | AY421791 | AF260379 | GU295201        | included           | included               |
| Protoperridinium crassipes                | AB261515 | EF152846 | GU295197        | included           | included               |
| Protoperridinium steidingeri              | x        | DQ444231 | GU295198        | x                  | x                      |
| Protoperridinium sp.                      | x        | x        | GU295212        | x                  | x                      |
| Scripsilla trochoidea                     | AF274277 | AF260393 | GU295213        | included           | included               |
| Spiniferodinium galeiformis               | GU295203 | GU295206 | GU295214        | included           | included               |
| Thecadinium kofoidii                      | GU295204 | GU295207 | GU295215        | included           | included               |
| Thecadinium yashimaense                   | AY238477 | GU295209 | GU295200        | included           | included               |
| Togula britannica UBC                      | x        | GU295216 | GU295216        | included           | included               |
| Togula britannica                         | AY443010 | AY455679 | x                | included           | included               |

**B. intermedium for Hsp90; B. americanum for SSU rDNA. Accession numbers indicated in bold denotes sequences generated in this study. doi:10.1371/journal.pone.0013220.t003**
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Supporting Information

Figure S1 Maximum likelihood (ML) tree inferred from 35 SSU rDNA sequences (dataset 1), 1,381 unambiguously aligned sites and a GTR+I+G+8 model of nucleotide substitutions. Numbers above the branches denote ML bootstrap percentages, and numbers below the branches denote Bayesian posterior probabilities. Black circles denote bootstrap percentages and posterior probabilities of 100% and 1.00, respectively. Found at: doi:10.1371/journal.pone.0013220.s001 (1.6 MB EPS)

Figure S2 Maximum likelihood (ML) tree inferred from 30 LSU rDNA sequences (dataset 2), 482 unambiguously aligned sites and a GTR+I+G+8 model of nucleotide substitutions. Numbers above the branches denote ML bootstrap percentages, and numbers below the branches denote Bayesian posterior probabilities. Black circles denote bootstrap percentages and posterior probabilities of 100% and 1.00, respectively. Found at: doi:10.1371/journal.pone.0013220.s002 (1.38 MB EPS)

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Author Contributions

Conceived and designed the experiments: MH BL. Performed the experiments: MH BL. Analyzed the data: MH BL. Contributed reagents/materials/analysis tools: BL. Wrote the paper: MH BL.
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