Research

An ITS-based phylogenetic framework for the genus *Vorticella*: finding the molecular and morphological gaps in a taxonomically difficult group

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Vorticella includes more than 100 currently recognized species and represents one of the most taxonomically challenging genera of ciliates. Molecular phylogenetic analysis of *Vorticella* has been performed so far with only sequences coding for small subunit ribosomal RNA (SSU rRNA); only a few of its species have been investigated using other genetic markers owing to a lack of similar sequences for comparison. Consequently, phylogenetic relationships within the genus remain unclear, and molecular discrimination between morphospecies is often difficult because most regions of the SSU rRNA gene are too highly conserved to be helpful. In this paper, we move molecular systematics for this group of ciliates to the infrageneric level by sequencing additional molecular markers—fast-evolving internal transcribed spacer (ITS) regions—in a broad sample of 66 individual samples of 28 morphospecies of *Vorticella* collected from Asia, North America and Europe. Our phylogenies all featured two strongly supported, highly divergent, paraphyletic clades (I, II) comprising the morphologically defined genus *Vorticella*. Three major lineages made up clade I, with a relatively well-resolved branching order in each one. The marked divergence of clade II from clade I confirms that the former should be recognized as a separate taxonomic unit as indicated by SSU rRNA phylogenies. We made the first attempt to elucidate relationships between species in clade II using both morphological and multi-gene approaches, and our data supported a close relationship between some morphospecies of *Vorticella* and *Opisthonecta*, indicating that relationships between species in the clade are far more complex than would be expected from their morphology. Different patterns of helix III of ITS2 secondary structure were clearly specific to clades and subclades of *Vorticella* and, therefore, may prove useful for resolving phylogenetic relationships in other groups of ciliates.

1. Introduction

Vorticella is the largest genus of sessile peritrich ciliates and its members live in a wide assortment of marine, freshwater and terrestrial environments worldwide [1–3]. Their extraordinary variety of habitats and prodigious abilities as suspension feeders suit them to be used widely as biological indicators for assessing the quality of natural bodies of water [4–7]. *Vorticella* and other ciliates are also an integral part of the treatment process in sewage treatment systems worldwide [8]. Species of *Vorticella* are well known as examples of the challenge of identifying ciliates in large genera to the level of species because they have a plastic, highly contractile cell body and show intraspecific variability in size. Also, morphological characters diagnostic for species, including those revealed by protargol and silver nitrate staining, overlap to some degree between putative species. This

has resulted in the naming of numerous species and varieties, many of which are of doubtful taxonomic status [1,9].

During the last few decades, several molecular phylogenetic analyses, based especially on sequences of the gene coding for small subunit ribosomal RNA (SSU rRNA), provided resolution of a number of important questions about evolutionary relationships within several taxa of ciliates. These studies demonstrated that traditional taxonomic classifications are coarse—morphospecies can contain several to many cryptic taxa (e.g. *Tetrahymena pyriformis* [10] and *Paramecium aurelia* [11]). More recently, both morphological and molecular analyses [9,12] have suggested the existence of cryptic species in the *Vorticella* group. However, at present, there are no data at all for genes other than SSU rRNA for almost all named *Vorticella* species. Moreover, genetic distances between SSU rRNA sequences of morphospecies were small [12], and this high degree of conservation makes it difficult to resolve phylogenetic relationships within the genus and make a clear discrimination between morphospecies. Furthermore, the long-standing assumption that the family Vorticellidae was a distinct, stable taxon and that *Vorticella* was equally distinct and stable at the generic level was upset by convincing molecular evidence [12–15]. Given these important systematic uncertainties and the low resolution at the infrageneric level of phylogenies based on SSU rRNA sequences, molecular phylogenies of *Vorticella* that include a broader sample of taxa and additional genes are desirable.

The nuclear internal transcribed spacers (ITSs) ITS1 and ITS2 are the most popular markers for phylogenetic inference at the infrageneric level or between closely related genera [16–18]. The primary sequences of these spacers are subject to lower selective constraints compared to coding regions for the ribosomal subunits and, thus, can be highly variable and possibly unalignable between distantly related species owing to intrasequence nucleotide heterogeneity between sampled taxa. With regard to nuclear ITS2, however, these limitations have been overcome by secondary structure analysis that systematically identifies regions of variability as well as areas of substantial conservation [17,19,20]. More and more studies have suggested that phylogenetic analyses incorporating both primary sequences and secondary structures of ITS2 produce the most robust trees and match resolution provided by analyses of COX1 [18,21–23].

Following this lead, we used increased taxon sampling and genetic markers other than the SSU rRNA gene to create a much more comprehensive phylogenetic analysis of the genus *Vorticella* than has been attempted. In all, samples of 66 populations of *Vorticella* from three continents were collected and both nuclear ITSs from all of them were sequenced as well as the Histone 4 gene from some. Our goals were: (i) to complete a pilot study of *Vorticella* and reveal phylogenetic patterns within the genus; (ii) to test whether entities within *Vorticella* that are extremely similar in morphology are also genetically similar; and (iii) use multi-gene markers to further explore the relationship between morphospecies of *Opisthonecta* and *Vorticella* in one divergent clade.

2. Results

(a) Analyses of sequences and secondary structures

GenBank accession numbers of newly obtained sequences are given in the electronic supplementary material, table S1.

Lengths of ITS1-5.8S-ITS2 and ITS2 sequences of all samples included in the study were 415–432 and 165–169 nt, and averaged 422 and 167 nt, respectively (see the electronic supplementary material, table S1). The GC content of ITS1-5.8S-ITS2 sequences ranged from 33.65 to 41.83%, with a mean value of 36.47%, and that of ITS2 sequences alone ranged from 28.99 to 47.88%, with a mean value of 35.06% (see the electronic supplementary material, table S1).

Ten species were collected more than once, allowing for some intraspecific comparison (see the electronic supplementary material, table S2). As shown in the electronic supplementary material, table S2, the pairwise distance among the ITS1-5.8S-ITS2 sequences of populations from different localities ranged from 0 to 4.23%. The variation between different populations of these 10 species was usually small. There was no variation between populations of *Vorticella convallaria* from China and the USA, and the same result was found in *Vorticella gracilis*-like populations from Austria. Populations of *Vorticella aequilata*, *V. gracilis*, *Vorticella natans* and *Vorticella fusca* varied slightly more than this, with genetic distances ranging from 0.16 to 1.60%. The pairwise distances among *Vorticella similis*, *Vorticella citrina* and *Vorticella campanula*-like populations were 4.23%, 3.06% and 2.24%, respectively. There was no difference between the three collections of *V. campanula* from Wuhan (China) and two from Japan, but Austrian collections differed from all of those from Wuhan by 2.89% at most.

We guided alignment of raw ITS2 sequences and identification of genetic characteristics at the species level and below by comparing ITS2 primary sequences to their secondary structures and determining the compensatory base changes (CBCs and hemi-CBCs) for all samples of *Vorticella*. The general secondary structure of ITS2 in *Vorticella* included three helices (I, II and III), which is congruent with the putative general model for secondary structure in all sessile peritrich ciliates. Our comparisons show that helix III is the most distinctive region of ITS2 in all samples of *Vorticella*, and based on its structure, the genus groups into two markedly divergent clades (I and II), with clade I comprising three clearly demarcated subclades (see the electronic supplementary material, figure S1).

(b) Phylogenetic analyses inferred from ITS1-5.8S-ITS2 and ITS2 regions

The colour coding of each clade and its subclades in figure 1 is based on the ITS2 tree. All tree topologies provided strong statistical support for monophyly of clade II relative to species of *Vorticella* s. str. (clade I). Three genetic clusters distinguished by their ITS2 regions corresponded to the three subclades within clade I seen in topologies based on ITS1-5.8S-ITS2 sequences (figures 1 and 2). In subclade 1, morphological characters were consistent with those that define the *V. convallaria* species complex, which contained the type species of the genus *Vorticella*. Subclade 2 contains representatives of the morphospecies *V. gracilis*, *Vorticella elongata* and *V. campanula* and two unidentified species. Subclade 3 was basal to the other two and consisted of three identifiable and one unidentified species. *Vorticella chlorostigma* was divergent from all subclades and branched basally within clade I. Clade II was basal to clade I (and the entire family Vorticellidae; see [12]), comprising morphospecies of the *Vorticella microstoma*-complexes, *Vorticella astyliformis*-like, *Vorticella infusionum*, *Vorticella striata*?, *Vorticella*
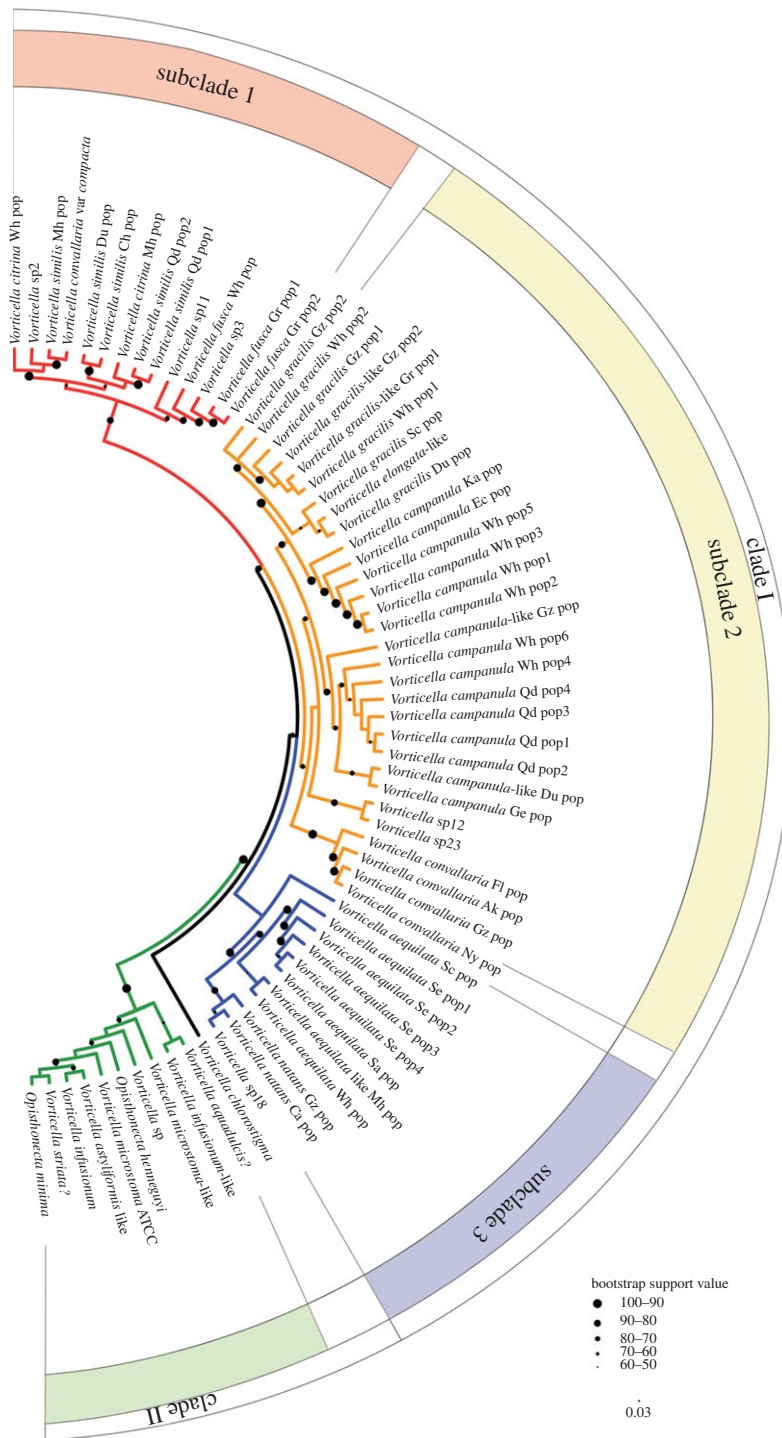
Figure 1. Profile neighbour joining (PNJ) tree constructed with sequence-structure data from the ITS2 region and based on a comprehensive sampling of the genus *Vorticella*. Major phylogenetic clades are highlighted using differential colour-coding. Scale bar, 0.03 substitutions/site. (Online version in colour.)

aquadulcis?, one unidentified species and two species of the stalkless, secondarily free-swimming genus *Opisthonecta*.

Among samples of all morphospecies of *Vorticella* individuals, those of *V. similis* were the most diverse, and representatives could be found in three different clusters within subclade 1. Likewise, the 13 sequences of *V. campanula* were recovered as a paraphyly, with one sequence being a sister to the *V. gracilis* cluster within subclade 2. Of the other morphospecies, none had representatives in more than one clade or subclade. The only differences in topology between trees based on the entire ITS1-5.8S-ITS2 region and those based on the ITS2 region alone were placement of *Vorticella* sp11 and a small cluster of *V. convallaria*. *Vorticella* sp11 nested within subclade1 in the ITS2 tree (figure 1) but occupying a position basal to subclade 2 in the ITS1-5.8S-ITS2 tree (figure 2). In the ITS2 tree

(figure 1), a small cluster of *V. convallaria* (Fl, Ak, Gz, Ny) fell within subclade 2; however, this cluster was associated with subclade 1 in an unresolved position in ITS1-5.8S-ITS2 trees (figure 2).

(c) Phylogenetic relationship of species within clade II inferred from both ribosomal and protein-coding gene markers

We were able to compare topologies of trees inferred from sets of SSU rRNA, ITS1 and ITS2 data from 10 species in clade II with a tree inferred from Histone H4 data from seven of these species (figure 3). Topologies of Bayesian trees constructed with SSU rRNA, ITS1, and H4 sequences (figure 3a−c) and a profile neighbour joining (PNJ) tree constructed with
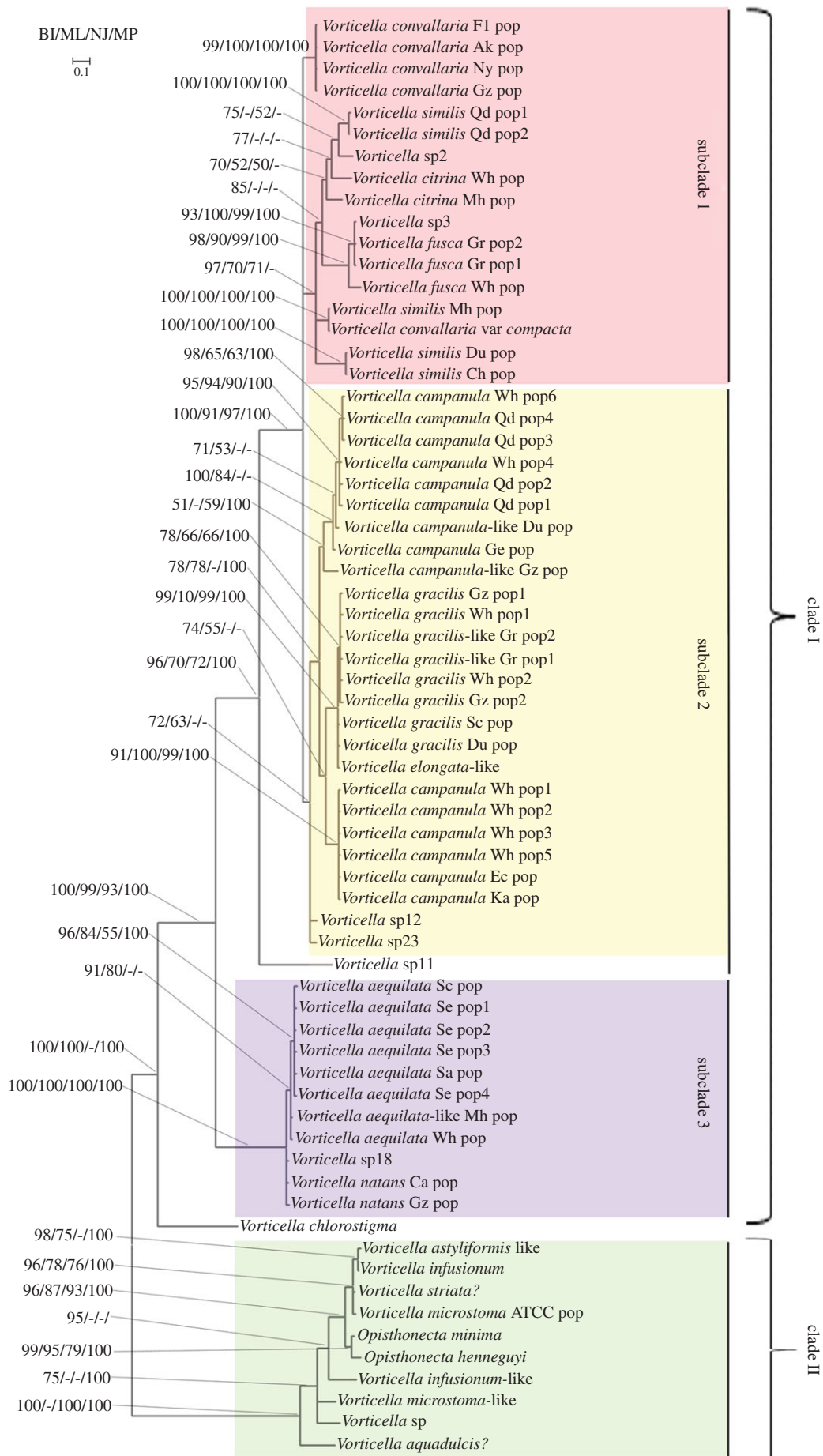
Figure 2. Phylogenetic tree depicting evolutionary relationships among species of *Vorticella* based on ITS1-5.8S-ITS2 sequences. Branch lengths indicate the inferred number of nucleotide substitutions per site. Nodal support values (posterior probabilities for Bayesian inference (BI) and bootstrap values for other methods) are indicated in the following order: BI/maximum likelihood (ML)/neighbour joining (NJ)/maximum parsimony (MP). Scale bar, 0.1 substitutions/site. (Online version in colour.)
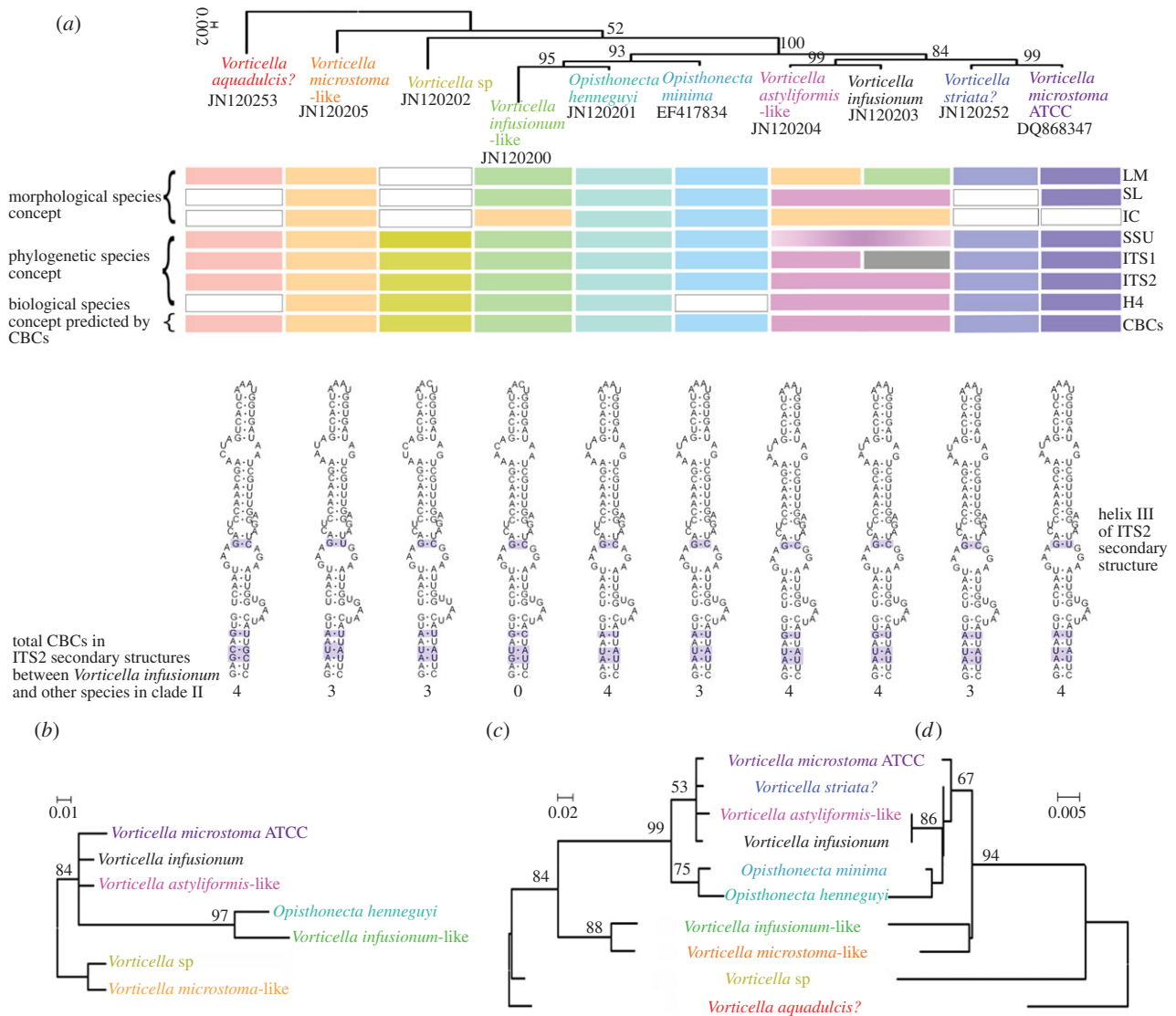
Figure 3. Bayesian trees inferred from (*a*) SSU rRNA, (*b*) Histone 4, and (*c*) ITS-1 sequences and a PNJ tree inferred from (*d*) ITS2 primary sequences and secondary structures. Numbers in each node indicate either the posterior probability (Bayesian trees) or bootstrap values (PNJ tree). (*a*) Horizontal bars show the clustering patterns that can be inferred from gross morphology (LM) and morphology revealed by silver staining (SL and IC), molecular markers (listed on the right side) and sexual reproductive compatibility predicted by CBCs [24]. The blank indicates that corresponding analyses were not carried out. Scale bars: (*a*) 0.002, (*b*) 0.01, (*c*) 0.02 and (*d*) 0.005 substitutions/site. IC, infraciliature; LM, light microscopy; SL, silverline system. (Online version in colour.)

ITS2 sequences and secondary structures (figure 3*d*) were almost identical. In all of these trees, a clade containing *V. astyliformis*-like, *V. infusionum*, *V. microstoma* ATCC and *V. striata*? had a close, well-supported relationship with two members of *Opisthonecta*. However, the relationship of the species of *Opisthonecta* to *V. infusionum*-like was ambiguous. The SSU rRNA phylogeny supported a closer relationship of *Opisthonecta henneguyi* and *V. infusionum*-like than between *O. henneguyi* and *Opisthonecta minima*. Phylogenies based on ITS1 and ITS2 datasets showed *O. henneguyi* was sister to *O. minima*. *Vorticella aquadulcis*? was the most basal taxon in clade II in trees based on ribosomal markers.

3. Discussion

(a) Phylogenetic and taxonomic relationships within the genus *Vorticella* s. l.

Traditionally, morphological identification of *Vorticella* species has been based mostly on characteristics of gross morphology that are visible under the light microscope [1,3]. Morphological variation within species can overlap with variation among species [9], thus, identification using molecular characters is an important alternative for this genus. Information from the ITS1-5.8S-ITS2 region, especially ITS2, has proved to be a source of strong molecular markers for resolving phylogenetic relationships among sessile peritrichs at different taxonomic levels [13,15]. In this work, therefore, we provide a new evaluation of the phylogenetic relationship of *Vorticella* species using both ITS1-5.8S-ITS2 and ITS2 sequences.

Trees made with both ITS1-5.8S-ITS2 sequences and ITS2 sequences alone featured the same subclades of clade I and, furthermore, showed subclades 1 and 2 as sisters with relatively strong support but with support for monophyly of each one being lower (figures 1 and 2). The large number of specific similarities in helix III of ITS2 that exist between subclades 1 and 2 (see the electronic supplementary material, figure S1) explain this high degree of support for their relationship to one another. By contrast, helix III of subclade 3 is clearly distinct from that of both subclades 1 and 2, confirming it as a divergent lineage within clade I (see the electronic

supplementary material, figure S1). The divergent, basal position of *Vorticella chlorostigma* within clade I in all trees suggests that it might be a representative of yet another subclade. Addition of still more species to analyses is probably the only way to resolve subclades 1–3 and test the hypothesis of existence of a fourth *Vorticella chlorostigma* clade.

Topological differences did exist between results derived from ITS1-5.8S-ITS2 and ITS2 data. For example, analysis of ITS1-5.8S-ITS2 data places *Vorticella* sp11 as a basal member of subclade 2 (figure 2), but it is placed within subclade 1 by ITS2 data alone (see the electronic supplementary material, figure S1; figure 1). A possible cause could be the inclusion of phylogenetically distorting characters from the ITS1 region that derive from the relatively high variability in primary sequences among distantly related species of *Vorticella*.

Previous molecular phylogenetic studies of vorticellid peritrichs based on SSU rRNA data alone and including a relatively small number of sequences [13] indicated that morphospecies of *Vorticella* in clade II are not members of the genus *Vorticella* s. str. Sun *et al.* [12] suggested assigning all species in clade II to the family Astylozoidae s. str. on the basis of distinctive molecular signatures in their SSU rRNA sequences in the absence of unifying morphological characteristics and despite differences in gross morphology resulting from loss of stages from the life cycle by *Astylozoon* (telotroch lost) and *Opisthonecta* (trophont lost). In this study, trees based on the ITS1-5.8S-ITS2 and ITS2 regions as well as the distinctive helix 3 of ITS2 in members of clade II provides conclusive support for this taxonomic action and removes any lingering doubt that morphospecies of *Vorticella* in clade II are not congeneric with species of *Vorticella* s. str. in clade I.

The *V. convallaria* species complex includes not only the type species of the genus, but also ciliates that are commonly encountered in both wastewater treatment plants and a variety of natural freshwater habitats worldwide. Historically, this complex consisted of the following taxa: *V. convallaria* Linnaeus, 1758, *V. citrina* Müller, 1773, *Vorticella nebulifera* Müller, 1786, and *V. similis* Stokes, 1887 [9]. *Vorticella nebulifera* differs from the other three species by being found in marine habitats but, otherwise, resembles them in terms of basic morphological features. The three freshwater species are morphologically very similar and difficult to differentiate because of their morphological variability. Consequently, their taxonomic status and phylogenetic relationships have posed a problem for more than a century.

In this study, we sequenced the ITS region for 15 samples belonging to the *V. convallaria* species complex collected from 12 different localities. The relationship between the clade of *V. convallaria* and the one containing *V. similis*, *V. citrina*, *V. convallaria* var *compacta*, *V. fusca* and two unknown species is unresolved (figure 2), but a close relationship between four sequences of *V. convallaria* and sequences of *V. similis*, *V. citrina* and *V. convallaria* var *compacta* is indicated by our molecular analyses, confirming that they all belong to the *V. convallaria* species complex. However, species boundaries within this complex could not be discerned with current data alone—e.g. morphospecies of *V. similis* were spread over three different clusters within subclade 1 (figure 2). In future studies, sequence data from multiple loci should be combined with morphological and ecological evidence to test species boundaries and assess their evolutionary relationships in this species complex.

(b) Pairwise distances between morphospecies of *Vorticella* in clade I

Clade I comprised sequences of 46 populations of 10 morphospecies of *Vorticella* from different localities. The pairwise distance between populations of most of these species was usually small, but *V. citrina*, *V. similis* and *V. campanula* showed a relatively large variation between different populations (see the electronic supplementary material, table S2). *Vorticella campanula* was the most commonly encountered species in our collections, and a genetic distance of 2.89% between the 13 populations from different localities was observed. In both ITS1-5.8S-ITS2 and ITS2 trees (figures 1 and 2), populations of *V. campanula* from Austria, China and Japan were recovered as two separate genetic clusters, with one being sister to the *V. gracilis* cluster. All these results suggest that it is likely that cryptic species exist within the morphospecies *V. campanula*. Thus, future efforts will focus on testing this hypothesis with broader sampling and inclusion of multiple genes in analyses.

(c) Phylogenetic and taxonomic relationships within clade II inferred from multiple datasets

Vorticella microstoma was the only species other than *Opisthonecta* and *Astylozoon* species within clade II that had been included in previous studies, and it appeared to be a phylogenetically isolated organism [15,25]. It is now clear that *V. microstoma* is merely one representative of a 'cloud' of *Vorticella* morphospecies, all with a relatively small body size and narrow peristome. Results of this study (figures 1 and 2) and previous phylogenetic investigations [12] clearly indicate that *Vorticella* s. l. is a paraphyletic assemblage; however, relationships within clade II are far from clear. *Vorticella* morphospecies (see the electronic supplementary material, figure S2*a–c,f–h*) in this clade share a similar gross morphology and differ only slightly in macronuclear shape/orientation and characters revealed by silver staining that overlap to some degree (see the electronic supplementary material, table S3). Species in clade II also have very similar SSU rRNA sequences, with genetic distances among some morphospecies of *Vorticella* being larger than those between species of *Opisthonecta* and other morphospecies of *Vorticella* (see the electronic supplementary material, table S4). In short, morphology appears to reveal little, if anything about phylogenetic relationships among morphospecies of *Vorticella* within clade II, and even generic boundaries are unclear in molecular analyses.

Close relationships were seen between species of *Opisthonecta* and some morphospecies of *Vorticella* in previous studies based only on nuclear SSU rRNA sequences [12]. In this study, we used sets of nuclear SSU rRNA plus the faster evolving genetic markers for H4, ITS1 and ITS2 to assess phylogenetic relationships of the species within clade II. Trees inferred from four different datasets (figure 3) including the same taxa (except the H4 tree, figure 3*b*) revealed a close, well-supported relationship between a clade comprising two species of *Opisthonecta* and a clade containing sequences of four morphospecies of *Vorticella*—*V. astyliformis*-like, *V. infusionum*, *V. microstoma* and *V. striata*? A monophyletic group comprising species of *Opisthonecta* and *V. infusionum*-like was recovered with high support in both SSU rRNA and H4 trees (figure 3*a,b*), and a sister relationship between species of *Opisthonecta* and a clade

containing *V. astyliformis*-like, *V. infusionum*, *V. microstoma* and *V. striata*? was supported in ITS1 and ITS2 trees (figure 3*c*,*d*).

Morphologically, species of *Opisthonecta* are markedly different from all morphospecies of *Vorticella* in clade II, always existing as free-swimming telotrochs (stalkless individuals with a circumferential band of somatic cilia). In most sessile peritrichs, a telotroch is a non-feeding dispersal stage that secretes a stalk and metamorphoses into a trophont by resorbing somatic cilia and reforming oral cilia. In *Opisthonecta*, by contrast, the somatic ciliature persists and individuals are unable to secrete a stalk; and unlike true telotrochs, zooids are able to develop a normal, ciliated oral complex for suspension feeding (see the electronic supplementary material, figure S2*d*,*e*).

At first glance, the loss of the normal trophont morphology appears to be a profound difference between *Opisthonecta* and other species of sessile peritrichs. In reality, this sort of radical evolutionary loss of a developmental stage has happened in other sessile peritrichs and many kinds of organisms [26–28] and could have resulted from a relatively restricted genetic change in the regulation of development without requiring a large degree of divergence in other morphological or physiological characteristics. For example, *Astylozoon* is another genus in clade II [12] that is characterized by loss of a developmental stage (lacks telotroch and thus exists as a stalkless, swimming trophont) and also appears to be significantly different in morphology from morphospecies of *Vorticella*. However, the boundary between them is blurred by the existence of *V. astyliformis* as a typical stalked trophont at some times and a free-swimming, stalkless trophont at other times [29]. A permanently stalkless trophont (i.e. *Astylozoon*) could have evolved from such a species by means of a relatively small genetic change (e.g. only 1.976% genetic distance in SSU rRNA sequences exists between *A. enriquesi* and *V. astyliformis*), and *Opisthonecta* may have evolved equally rapidly without experiencing large-scale genetic divergence.

A close relationship between *V. astyliformis*-like and *V. infusionum* was supported in all trees (figures 1–3). The two species have similar morphological characteristics (e.g. small size of less than 40 μm, rotund body shape, narrow peristome), and subtle morphological differences in characters revealed by silver staining overlapped (see the electronic supplementary material, figure S2 and table S3). There was a 99.935% similarity in their SSU rRNA sequences (see the electronic supplementary material, table S4). Their ITS2 sequences were identical, hence no CBC was found and there was a genetic distance of only 0.671% between their ITS1 sequences (see the electronic supplementary material, table S4). All of these close genetic and morphological similarities prompt us to conclude that *V. astyliformis*-like and *V. infusionum* may be closely related cryptic species or even populations of a single, variable species.

All morphospecies of *Vorticella* in clade II were placed in a new genus *Vorticellides* by Foissner *et al.* [14], partly on the basis of early molecular evidence that one of them—*V. astyliformis*—was not congeneric with species of *Vorticella* s. str. and partly on the basis of a morphological characteristic—presence of two epistomial membranes. Results of this study and that of Sun *et al.* [12] do not support this conclusion. Evolutionary distances between all taxa in clade II (electronic supplementary material, table S4) are small compared with those between taxa in clade I (data were not shown), and there is no support for a single cluster of taxa with stalked trophonts.

Moreover, there are not even clear distinctions between species of *Opisthonecta* and morphospecies of *Vorticella* in clade II. All morphospecies in clade II appear to have a wide geographical distribution owing to their ability to form cysts and disperse between ephemeral aquatic habitats (temporary pools, wet soil); therefore, a much larger set of samples taken from many geographical regions will be required to reveal evolutionary lineages within clade II with more confidence and resolve the taxonomic status of species and genera within it.

(d) Perspectives

Species of *Vorticella*, one of the most commonly encountered groups of ciliates in aquatic ecosystems worldwide, are particularly abundant in sewage treatment systems and aquatic farming ponds where organic matter accumulates. Historically, they were considered to be a monophyletic group for over 200 years. However, the molecular divergence observed in the genus *Vorticella* illustrates that the current catalogue of ciliate genera/classes is incomplete and confirms that it is necessary to recognize a class-level taxon to accommodate all organisms in clade II. Meanwhile, mismatches that were detected between morphological and molecular characters revealed the existence of apparent cryptic species and, possibly, even cryptic genera. Thus, all future workers investigating evolution, biogeography, ecology, conservation, biodiversity and more applied aspects (e.g. water pollution and ecotoxicology) of these *Vorticella* species should be aware of the necessity to make sure of the specific status of their organisms and their possible biological differences. Finally, our results also underscore the need, in terms of both morphology-based systematics and molecular phylogeny, for multiple markers and a broad set of samples from different geographical areas and types of habitats to understand the evolutionary history of any group of ciliates, which are a major group of microbial eukaryotes.

4. Material and methods

(a) Collection, isolation, fixation and identification of organisms

Sixty-six populations of peritrichs matching the morphological description of the genera *Vorticella* and *Opisthonecta* were collected from Europe, Asia and North America and used to obtain sequences of nuclear ITSs. Sequences have been deposited in GenBank with the accession numbers shown in the electronic supplementary material, table S1. Samples were collected from a wide variety of aquatic habitats—streams, irrigation ditches in farmed fields, bogs, lakes, ponds in botanical gardens, ephemeral pools and other areas (see the electronic supplementary material, table S5). Samples were brought directly back to the laboratory, and the cells were confirmed as species of *Vorticella* with live microscopy and silver staining [30]. Some samples we designated as 'Vorticella sp-like', for example *V. astyliformis*-like, because of some uncertainty in identification with overlapping but not identical morphological characters of known species. Cells were isolated under a stereomicroscope from mixed samples with a micropipette or by clipping off bits of the substrate (e.g. aquatic plants, dead leaves and decomposing plant debris) to which they were attached. Isolated cells were observed in wet mounts under a compound microscope (Olympus/Nikon/Zeiss) and photographed with a digital camera. Some cells were cleaned manually by carrying them through three or more changes of syringe-filtered

water and pipetting away contaminants, after which they were fixed with 95% ethanol or transferred to animal tissue lysis buffer (Qiagen, Valencia, CA, USA) for DNA extraction. Some cells were transferred to Petri dishes to start clonal cultures. Cultures of species of *Vorticella* and *Opisthonecta* were grown in a medium made with wheat grains or by adding a 5 g l^{-1} stock solution of boiled and filtered wheat grass powder in small amounts of habitat water to simulate growth of the bacterial community. Cells were picked individually from clonal cultures with a micropipette for DNA extraction. Terminology and systematic classification used in this paper follow Lynn [3].

(b) DNA extraction, gene amplification and gene sequencing

Extraction of DNA from samples was carried out using methods described in [13]. Amplification of the ITS region and a fragment of approximately 150 bp comprising part of the internal H4 region was accomplished according to protocols in [31,32]. Amplicons were cleaned by filtration using a QIAquick PCR Purification Kit (Qiagen Sciences, Germantown, MD, USA) and sequenced in both directions using an ABI 3730-XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence fragments were assembled into contiguous sequences and edited with the SEQUENCHER v. 4.0 software package (GeneCodes Corp., Ann Arbor, MI, USA). All new sequences have been deposited in the GenBank database (see the electronic supplementary material, table S1 for accession numbers).

(c) Predicting secondary structures of ITS2

We relied primarily on the free-energy minimization approach to secondary structure inference, which assumes that the dominant interactions (H-bonding between bases and stacking between adjacent bases) are local and that conformations adopted by RNA are the lowest free-energy conformations at equilibrium [33]. Consensus structures of ITS2 regions were found using the Alifold Server (http://rna.tbi.univie.ac.at/cgi-bin/alifold.cgi), which predicts structures from an alignment of related RNA sequences [34]. With the guidance of these consensus structures, the secondary structures of ITS2 sequences were predicted with MFOLD v. 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) [35] by screening for thermodynamically optimal and suboptimal secondary structures using the default values for folding temperature (37°C), per cent suboptimality (5%), upper bound of computed foldings (50), window parameter (25) and maximum distance between paired bases (no limit). Results for the various species were compared to reveal the folding pattern common to them all, aided by knowledge of the conserved structure of helix II and by the existence of the highly conserved region of DNA on the 5′ side of helix III [20]. This, in turn, established the conserved structural models for sessile peritrichs that revealed evidence of homology useful for

phylogenetic analysis [13]. The ITS2 sequences with homologous structures were synchronously aligned using 4SALE [36]. The alignment with structural information was exported. The resultant filename was changed to a filename with the extension '.xfasta' for subsequent analysis.

(d) Phylogenetic analysis

The alignment output file from 4SALE was imported into PROF-DISTS [37], and 'RNA/DNA structure Profile Neighbor-Joining' was selected from the 'Run' menu (Bootstraps = 1000, Distance Correction Model = General Time Reversible, Ratematrix Q = Q_ITS2.txt). The resulting tree file with node strengths was viewed in PROFDISTS and then visualized and reproduced with FIGTREE v. 1.2.3 [38] and MEGA v. 4.0 [39].

Phylogenetic analyses of 66 strains were also performed with data from the entire ITS1-5.8S-ITS2 region. Sequences of ITS15.8S-ITS2 regions were aligned using CLUSTALW as an accessory application of BIOEDIT v. 7.0.9, then adjusted by eye [40]. The aligned matrix was analysed using four methods (Bayesian inference (BI), maximum likelihood (ML), neighbour joining (NJ) and maximum parsimony (MP)). BI analyses were performed with MRBAYES v. 3.1.2 [41] using the GTR + I + G model selected by MRMODELTEST v. 2 [42] under the Akaike information criterion. Four simultaneous Markov chain Monte Carlo chains were run for 2 500 000 generations sampling every 100 generations. The first 25% trees were discarded as burn-in and the 50% majority-rule consensus tree was determined to calculate the posterior probabilities for each node. The appropriate model GTR + I + G was determined by MODELTEST [43] for ML analysis. An ML tree was constructed with PHYML v. 3.0 (via http://www.atgc-montpellier.fr/phyml), which performed ML analysis with heuristic searches and a 1000-fold bootstrap analysis [44]. The NJ and MP analyses were performed with PAUP* 4.0b10 [45], including 1000 bootstrapping replicates. We also performed BI and PNJ analyses of clade II with four datasets and trees based on these sets of sequences constructed as described above.

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