Does *Kiitricha* (Protista, Ciliophora, Spirotrichea) belong to Euplotida or represent a primordial spirotrichous taxon? With suggestion to establish a new subclass Protohypotrichia

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The genus *Kiitricha* was long assumed to be the most primordial taxon in the Stichotrichia [hypotrichs *sensu lato* (s. l.)] based on its morphological features and was considered to be an intermediate between heterotrichs and the traditional hypotrichous assemblage. In order to evaluate the phylogenetic position of *Kiitricha* within the Hypotrichia, we sequenced the small-subunit rRNA gene and the alpha-tubulin gene for a Qingdao population of *Kiitricha marina*. Phylogenetic trees were constructed and compared to morphological and morphogenetic data. The results show that (i) *Kiitricha* is positioned near *Phacodinium*, both of which always form a sister clade to the assemblage including Stichotrichia, Hypotrichia, Oligotrichia and Choreotrichia, (ii) *Kiitricha*, which may represent an intermediate between heterotrichs (s. l.) and the Stichotrichia–Hypotrichia complex, is probably an ancestor-like form of the latter group and (iii) in contrast to morphological characters, both molecular and ontogenetic data support the separation of *Kiitricha* from the hypotrichs (s. l.). Thus, *Kiitricha* might be placed in the class Spirotrichea at about subclass level, next to Phaconidiidia, Hypotrichia and Stichotrichia, which supports the establishment of a new subclass Protohypotrichia n. subclass within the class Spirotrichea, with characterizations including slightly differentiated somatic ciliature (i.e. cirri on the ventral side generally uniform and non-grouped, no clearly defined marginal cirral rows, ciliature on the dorsal side mixed with cirri and dikinetids, no clearly differentiated dorsal kineties) and a unique but intermediate morphogenetic pattern of cortical structures between Hypotrichia and Stichotrichia.

INTRODUCTION

Spirotrich ciliates, which represent one of the major adaptive radiations within the phylum Ciliophora, are divided into four major ‘classical’ assemblages, the hypotrichs, oligotrichs, choreotrichs and stichotrichs (Lynn, 2008; Lynn & Small, 2002). These groups have been recognized as discrete clades for many years (Corliss, 1979; De Puytorac, 1994; Small & Lynn, 1985). Among them, the hypotrichs and stichotrichs are usually considered together in terms of the evolution of the

sporadically distributed cirri on the ventral surface of ciliates. Kinetosomes distributed in kineties in anterior–posterior rows are assumed to be a feature of the ancestor of hypotrichs and stichotrichs, and this is supported by the inclusion of a ciliate with a simple kinetid organization, *Phacodinium*, which is clustered at the base of the class Spirotrichea (Shin *et al.*, 2000). Furthermore, *Protocruzia*, another ciliate with simple dikinetids arranged in anterior–posterior kineties, is also placed at the base of the spirotrich clade using small-subunit (SSU) rRNA gene sequences, although its position is somewhat variable (Hammerschmidt *et al.*, 1996).

A third genus, *Kiitricha*, has also been considered among the basal hypotrichs *sensu lato* (s. l.). This genus has a unique and probably ancestral pattern of simple ciliated dikinetids for the dorsal ciliature, uniform and slightly differentiated cirri on the ventral side and no differentiation of real marginal rows (Fig. 1) (see also Song & Wilbert, 1997).

Abbreviations: BI, Bayesian inference; DK, dorsal kineties; FVT, frontoventral–transverse; LS, least squares; ML, maximum likelihood; NJ, neighbour joining; s. l., *sensu lato*; SSU, small subunit; UM, undulating membranes.

The GenBank/EMBL/DDBJ accession numbers for the SSU rRNA and partial alpha-tubulin gene sequences of *Kiitricha marina* are AY896768 and EF198664. The accession number for the deduced amino acid sequence of alpha-tubulin from *K. marina* is ABP96884.

Fig. 1. Division morphogenesis in *K. marina* showing stages before (a–f) and after (g–l) cell division. (a, b) Ventral (a) and dorsal (b) views of an early stage divider (same specimen) to show that the oral primordium is formed on the dorsal wall of the buccal cavity. Note that the ventral cirri (arrows) and UM anlage (UMA) are dedifferentiated. (c, d) Ventral (c) and dorsal (d) views of a specimen. Arrows show the cirral anlagen and arrowheads indicate that the anteriormost cirri in each cirral row are still not dedifferentiated. Ma, Macronuclear nodule; Mi, micronucleus; OP, oral primordium. (e, f) Ventral (e) and dorsal (f) views of a mid-stage divider. Arrows show that some dikinetids are more densely arranged, with proliferation of basal bodies in the old DK. In (e), double-arrowheads show that the UMA is prolonged with proliferation of basal bodies. Arrowhead indicates the proximal end of the old adoral zone with membranellae (AZM) dedifferentiated. Arrowheads in (f) demonstrate the old DK. (g, h) Ventral (g) and dorsal (h) views of the same specimen demonstrate the proter after the daughter cells have separated. Arrowheads in (g) show the cirral anlagen and arrows in (h) demonstrate the DK. (i, j) Ventral (i) and dorsal (j) views of a middle divider. In (i), arrows show that the cirral anlagen differentiate the transverse cirral anlagen (TCA) and double-arrowheads indicate the first cirral anlagen. In (j), double-arrowheads show the micronuclei, arrowheads show the anteriormost cirri in each cirral row and arrows indicate the DK. (k, l) Ventral (k) and dorsal (l) views of a very late divider after the daughter cells have separated. Note the division of the macronucleus. In (k), arrows mark the TCA; double-arrowheads indicate that the end of the rightmost ventral cirral row is still an anlage-like structure, which might be the anlage for the left cirral row. In (l), arrows show the DK and arrowheads indicate that the segregation of the cirral anlagen is almost finished. Bars, 50 μ m.

Lynn & Small (1997, 2002) separated all known hypotrichs (*s. l.*) into the two subclasses Stichotrichia and Hypotrichia. The genus *Kiitricha* and its relatives were assigned to the latter, on the basis of a number of features: (i) both basal bodies in each dikinetid of the dorsal kineties (DK) are ciliated, a very unusual character in all known hypotrichs (*s. l.*), and some DK are a mixture of small cirri and dikinetids; (ii) no marginal rows are recognized, but many cirri are distributed onto the dorsal side, unique in all known 'hypotrichs'; and, (iii) except for the transverse

cirri, there is no differentiation of cirri on the ventral side (i.e. cirri are not grouped, but rather are distributed in anterior–posterior files, like those at the segregating stage of cirral anlagen during morphogenesis) (Song & Wilbert, 1997). In addition, *Kiitricha* has more than five transverse cirri, and so is remotely related to species with five frontoventral–transverse (FVT)–anlagen (i.e. all known euplotids). All these features indicate that *Kiitricha* might be an ancestral or 'basal' clade in relation to the 'typical' hypotrichs (*s. l.*).

In order to assess the 'ancestral' position of *Kiitricha* within the Spirotrichea, the SSU rRNA and alpha-tubulin genes of *Kiitricha marina* Nozawa, 1941 were sequenced and analysed. With the combination of morphological and morphogenetic characters, our work provides molecular evidence to ascertain the phylogenetic position of this unusual group.

METHODS

Ciliate collection and identification. *Kiitricha marina* was collected from the coast near Qingdao, China (36° 08' N 120° 43'

E) (Fig. 2a–c, insets). Clonal cultures were established at room temperature for further analysis. Morphological and morphogenetic investigations were performed according to Song *et al.* (2004).

PCR amplification and sequencing. Cells were starved overnight and concentrated with low-speed centrifugation. Genomic DNA was extracted following Li & Song (2006). To minimize PCR amplification errors, high-fidelity TaKaRa ExTaq was used to amplify target genes using the following oligonucleotide primers: for the SSU rRNA gene, 5'-AACCTGGTTGATCCTGCCAGT-3' (forward) and 5'-TGATCCTTCTGCAGGTTACCTAC-3' (reverse), and for the alpha-tubulin gene, 5'-AAGGCTCTCTTGGCGTACAT-3' (forward) and 5'-TGATGCCTTCAACACCTTCTT-3' (reverse). The typical amplification profile consisted of 5 min initial denaturation (95 °C)

Fig. 2. Bayesian tree inferred from nucleotide sequences of the SSU rRNA gene of armophorean, spirotrich and heterotrich ciliates. Numbers at nodes represent support values in the following order: Bayesian credibility value using MrBayes and ML/LS/NJ bootstrap percentages based on 1000 replicates. Asterisks indicate values less than 50%; N indicates that the node was not recovered using this treeing method. Evolutionary distance is represented by the branch length. Bar, 10 substitutions per 100 nucleotide positions. The new sequence for *K. marina* is highlighted in bold. Insets: (a) ventral view of a typical individual of *K. marina in vivo*; (b, c) ventral and dorsal views of the same specimen impregnated by the protargol method.

and 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for 2 min, followed by an additional extension at 72 °C for 10 min. PCR products of the expected size were inserted into the pUCm-T vector (Sangon). A plasmid mini-prep spin column kit (Sangon) was used to harvest and purify plasmid DNA. DNA sequencing was accomplished using an ABI Prism 3730 Automated DNA Sequencer (Applied Biosystems) with three forward and four reverse internal primers (Greenwood *et al.*, 1991). All sequences were confirmed from both strands. Sequence fragments were assembled into continuous sequences and edited with the Sequencher 4.0 software package (GeneCodes Corp.).

Phylogenetic analyses and tree construction. Nucleotide sequences of the SSU rRNA gene were aligned using CLUSTAL W, version 1.80 (Thompson *et al.*, 1994), and then the alignment was refined by removing ambiguous gaps at both termini of the alignment and considering the conserved secondary structures.

A 50 % majority-rule Bayesian inference (BI) tree was constructed with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003) using the Markov chain Monte Carlo (MCMC) algorithm under the GTR + G + I evolutionary model indicated by MRMODELTEST version 2 (Nylander, 2004). The chain length was 1 000 000 generations, with trees sampled every 100 generations. The first 250 000 generations were discarded as burn-in.

A maximum-likelihood (ML) tree was constructed with PAUP (version 4.0b10) (Swofford, 2002) using the TrN + I + G evolutionary model selected by MODELTEST (Posada & Crandall, 1998) and an input file created by performing an accelerated likelihood surface exploration (Nixon, 1999; Vos, 2003) with the program PAUPRAT (Sikes & Lewis, 2001). A 50 % majority-rule consensus ML tree was derived from the output of 200 trees generated by PAUP operating on the input file from PAUPRAT.

PHYLIP version 3.57c (Felsenstein, 1995) was used to calculate sequence similarities and evolutionary distances between pairs of nucleotide sequences using Kimura's two-parameter model (Kimura, 1980). Distance-matrix trees were constructed using the least-squares (LS) method (Fitch & Margoliash, 1967) and the neighbour-joining (NJ) method (Saitou & Nei, 1987). Distance trees were bootstrapped 1000 times.

The topologies of the four trees were almost identical; therefore, the Bayesian tree was chosen as a representative for purposes of illustration. This tree was formatted with MEGA (Kumar *et al.*, 2004) and exported from it as a graphics file for construction of the finished figure. For ease of viewing, it was rooted on 13 species of heterotrichs; this did not affect the topology of the spirotrichous portion of the tree.

Amino acid sequences deduced from alpha-tubulin gene sequences were aligned using CLUSTAL W version 1.80 (Thompson *et al.*, 1994) and trimmed to the same length. MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003) was used to construct a Bayesian tree using the MCMC algorithm under the JTT evolutionary model (Jones *et al.*, 1992) and considering a gamma-shaped distribution of rates of substitution among sites. The chain length for our analysis was 1 000 000 generations with trees sampled every 50 generations. The first 250 000 generations were discarded as burn-in.

RESULTS

SSU rRNA and alpha-tubulin gene sequences of *K. marina*

The SSU rRNA gene sequence of *K. marina* is 1754 nucleotides in length, with a G + C content of 43.9 mol%.

K. marina exhibits the highest sequence similarity to the stichotrichs (84.31–86.09 %). The similarities to other groups are 84.47 % to *Phacodinium*, 82.29–84.4 % to oligotrichs, 81.94–84.68 % to choreotrichs, 82.6–83.55 % to *Protocruzia*, 79.92–81.73 % to *Licnophora*, 78.59–85.6 % to hypotrichs and 78.44–80.65 % to heterotrichs.

The partial alpha-tubulin gene sequence obtained for *K. marina* is 1071 nucleotides in length, encoding 357 amino acids. The deduced alpha-tubulin amino acid sequence of *K. marina* shows 79.35–81.76 % similarity to choreotrichs, 68.72–70.83 % to hypotrichs, 67.74–76.25 % to stichotrichs, 63.47–80.56 % to oligotrichs and 60.01 % to *Phacodinium*.

Morphogenetic features during binary fission (Fig. 1)

Fig. 1 documents the main stages of general cortical development during binary division of *K. marina*, which is characterized by numerous unique features (details will be published separately) that are not observed in any other known spirotrich: (i) no new undulating membranes (UM)-anlage in the opisthe is formed, hence the UM in the opisthe are derived from the division of the de-differentiated parental ones; (ii) no marginal cirral rows are formed; (iii) no typical DK are generated (i.e. several rows of pre-DK consisting of dikinetids with both basal bodies ciliated appear to mix with either isolated small cirri or cirral fragments); (iv) all cirral anlagen are formed interkinetally from the de-differentiated old cirral rows; and, (v) unlike all other known hypotrichs (*s. l.*), the differentiation of ciliature from the primordia occurs mostly after cell division. In addition, the general cirral structures on the ventral side are in a post-anlage pattern: except for the transverse cirri, all other cirri exhibit a primary arrangement (i.e. they are evenly distributed as if they had just been segregated from the anlagen).

Phylogenetic analyses using SSU rRNA gene sequences (Fig. 2)

Phylogenetic trees indicated that *Kiitricha* branches basally from the spirotrichous clade at a very deep level (Fig. 2). In our analyses, the spirotrichs are not a monophyletic taxon: armophorean genera separate *Protocruzia* species from the other spirotrichs. *Licnophora* is a basal lineage within the class Spirotrichea (Lynn & Strüder-Kypke, 2002). Associated with *Phacodinium*, *Kiitricha* branches at the base of the hypotrichs (*s. l.*) and represents a distinct clade from all other spirotrich taxa, with moderate bootstrap support (BI 78 %, ML 76 %, LS 72 %, NJ 62 %).

Bayesian analyses using alpha-tubulin gene sequences (Fig. 3)

The Bayesian tree for alpha-tubulin gene sequences supports the monophyly of the five classes *sensu* Lynn

Fig. 3. Bayesian tree inferred from alpha-tubulin amino acid sequences. The new sequence for *K. marina* is highlighted in bold. Evolutionary distance is represented by branch lengths. Bar, 5 substitutions per 100 amino acid positions.

(2003) from which more than one species representative is available in the alpha-tubulin dataset: Spirotrichea, Oligohymenophorea, Phyllopharyngea, Litostomatea and Heterotrichia. However, there is poor resolution of the three species within the class Heterotrichia. *Kiitricha* diverges after *Phacodinium* within an unresolved trichotomy of spirotrichs.

Cladistic analyses based on morphological and morphogenetic characters (Fig. 4)

A hypothesis on the potential position of *Kiitricha* was predicted based on autapomorphic characters listed in Table 1. As a result of the pattern of stomatogenesis and cirral differentiation, Phacodiniida and Licnophorida appear closer to heterotrichs rather than the euplotid–stichotrich clade. *Kiitricha* represents a distinct branch at

the base of the euplotid–stichotrich clade, with apokinetal stomatogenesis and differentiated cirri but lacking UM-anlage and differentiated marginal cirral rows (Fig. 4). Different from the arrangement suggested by Lynn & Small (2002), discocephalids group with sporadotrichids (i.e. *Stylonychia*, *Oxytricha* etc.) and diverge from the euplotid clade (i.e. *Aspidisca*, *Uronychia*, *Diophrys* and *Euplotes*).

DISCUSSION

The genus *Kiitricha* demonstrates a number of unique features that significantly separate it from other spirotrichs and may represent the type of a higher taxon; Shi *et al.* (1999) suggested the ‘suborder’ Protohypotrichina.

Alignment of sequences clearly showed similarities in primary structure and G+C content of the SSU rRNA gene of *Kiitricha* to those of other ciliates. The G+C content (43.9 mol%) of the SSU rRNA gene of *K. marina* is in the same range as those of other ciliates (Elwood *et al.*, 1985; Sogin & Elwood, 1986; Schlegel *et al.*, 1991; Chen & Song, 2002; Li & Song, 2006). Thus, this aspect of the gene should not bias our phylogenetic analyses. In all our analyses of the SSU rRNA gene, *Kiitricha* grouped with other spirotrichs, switching positions with another unusual spirotrich genus, *Phacodinium*. Thus, there is no doubt that *Kiitricha* is a spirotrich.

Based on ultrastructural features and analyses of some gene sequences, particularly the SSU rRNA and large-subunit rRNA genes, Lynn & Small (1997) presented a revised classification of the phylum Ciliophora, which included 10 classes, 17 subclasses and 57 orders. The class Spirotrichea Bütschli, 1889 contained five subclasses (Protocruziida, Hypotrichia, Choreotrichia, Oligotrichia and Stichotrichia) and four *sedis mutabilis* orders (Armophorida, Clevelandellida, Phaenodileidia and Odontostomatida). Shin *et al.* (2000) placed *Phacodinium* into the spirotrich clade based on its SSU rRNA gene sequence, justifying the establishment of the subclass Phacodiniida. Lynn & Strüder-Kypke (2002) added a seventh monotypic subclass to the spirotrichs when they recognized *Licnophora* as the type for the subclass Licnophoria using SSU rRNA gene

Fig. 4. Phylogenetic position of *Kiitricha* based on morphogenetic and morphological characters (see Table 1). Arrow indicates the evolutionary direction. Apomorphic characters are indicated by filled circles, whereas plesiomorphic characters are indicated by open circles.

Table 1. Autapomorphies considered in assessment of the phylogenetic position of *Kiitricha*

Data were taken from Berger (1999, 2006), Diener *et al.* (1983), Eigner & Foissner (1994), Foissner (1996), Grolière *et al.* (1980), Roque (1970), Shao *et al.* (2006), Song & Wilbert (1997), Song *et al.* (2004), Villeneuve-Brachon (1940) and Wicklow (1982).

Apomorph	Plesiomorph
1. Stomatogenesis in apokinetal mode	Parakinetal mode
2. Cirri present and clearly differentiated	No cirri formed; somatic ciliature uniform and consisting of longitudinal rows of dikinetids
3. Semi-cirri* present in somatic ciliature	Uniform kineties consisting of dikinetids
4. UM in the opisthe formed <i>de novo</i> and together with the oral primordium	No new anlage for UM is formed; it generates from division of the UM-anlage of the proter
5. Marginal rows clearly differentiated	Marginal rows not differentiated
6. First frontal cirrus formed from UM-anlage	No frontal cirrus formed from the UM-anlage
7. Basal bodies in DK in typical hypotrichous pattern	In heterotrichous pattern
8. Somatic ciliature highly reduced or degenerative	Developed or specialized, not degenerative
9. Both ciliature and body shape highly specialized, ectocommensal with adhesive disc	Generally non-specialized, non-commensal
10. Stomatogenesis in subapokinetal mode	In epi-apokinetal mode
11. Number of cirral streaks stable	Variable
12. Five-FVT-cirrus-anlagen mode	Non-five-cirrus-anlagen mode
13. Right marginal row absent	Present
14. DK formed in secondary pattern	In primary pattern
15. Silverline system often highly group-dependent	Non-specialized, non-group-dependent
16. Cirri in mid-ventral rows in zigzag pattern	Not in pattern, low level of organization
17. Cirri in most area stable in number	Mostly variable in number

*Concerns the somatic ciliature of longitudinal rows, in which monokinetids are formed in single-rowed fragments.

sequence analyses, and by confirming that its replication bands clearly demonstrated its affinities with the spirotrichs, but not heterotrichs.

The class Armophorea was suggested by Lynn (2003) as the first 'ribo-class' inferred from SSU rRNA gene sequence analyses and including the orders Armophorida and Clevelandellida. Recently, Stoeck *et al.* (2007) have preliminary evidence that some odontostomatids may be related to the class Plagiopylea.

Lynn & Small (1997, 2002) placed *Kiitricha* in the subclass Hypotrichia Stein, 1859 and its family was the type for the hypotrich order Kiitrichida.

The genus *Kiitricha*, which is clustered basally within the spirotrich clade in all our analyses based on the SSU rRNA and alpha-tubulin gene sequences, represents a distinct clade, remotely related to all other spirotrich taxa. Thus, its position as a type for a larger taxon appears to be justified. Since *Phacodinium* and *Licnophora* both represent types for monotypic subclasses of spirotrichs and *Kiitricha* is similarly isolated, we support Shi *et al.* (1999) and suggest the recognition of a new subclass of spirotrichs, the subclass Protohypotrichia, based on the suborder Protohypotrichina Shi, Song & Shi, 1999. The new taxon contains a single order, Kiitrichida Nozawa, 1941, and could be diagnosed as follows: Spirotrichea with weakly differentiated and non-grouped somatic ciliature (i.e. cirri on ventral side generally uniform, no clearly defined

marginal cirral rows, ciliature on dorsal side mixed with cirri and dikinetids, no clearly differentiated dorsal kineties) and a unique morphogenetic pattern of cortical structures, intermediate between Hypotrichia and Stichotrichia. In contrast to Shi *et al.* (1999), who included two families (i.e. Phacodiniidae and Kiitrichidae) in the suborder Protohypotrichina, we transfer the following taxa to the new subclass as they were assigned to the order Kiitrichida *sensu* Lynn & Small, 2002: Kiitrichidae Nozawa, 1941 and Reichenowellidae Kahl, 1932.

In support of the removal of the kiitrichids from the subclass Hypotrichia, it is useful to review the morphogenetic features of typical euplotids. Euplotids are a monophyletic assemblage united by the following pattern of development of the infraciliature: (i) the FVT-cirri are formed apokinetally in a five-anlagen mode; (ii) marginal rows are present or reduced; (iii) both ventral and dorsal ciliatures have a very stable pattern; (iv) DK are clearly differentiated with typical dikinetids as seen in all other typical spirotrichs (probably except *Licnophora*) and (v) the UM-anlage in the opisthe is formed independently within a subcortical pouch together with the oral primordium (Song & Packroff, 1993; Song *et al.*, 2004). Compared with these euplotid features, all characters revealed during morphogenesis in *K. marina* indicate that this organism should be a primitive form and totally isolated from euplotids and other known hypotrichs (*s. l.*).

Hence, we conclude from both the molecular and morphological/morphogenetic data that the order Kiitrichida represents a distinct taxon within the class Spirotrichea and should be placed in the subclass Protohypotrichia. Indeed, an expanded investigation of the order Kiitrichida is now needed to determine whether the other families and genera assigned to this taxon are properly placed.

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