

Reconsideration on the Phylogenetic Position of *Epiclintes* (Ciliophora, Stichotrichia) Based on SSrRNA Gene Sequence and Morphogenetic Data

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Summary. So far, no molecular investigations have been carried out on the systematically uncertain ciliate *Epiclintes* in spite of the recent reviews. In an attempt to solve this problem, we sequenced the small subunit ribosomal RNA gene of two forms of *Epiclintes*: *E. auricularis auricularis* and *E. auricularis rarisetus*. Phylogenetic trees were then constructed by means of bayesian inference (BI), maximum-likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ) methods in order to test previously presumed phylogenetic assignments of *Epiclintes* based on morphological and morphogenetic data. The main results are that the two isolates form a branch that is peripheral to all other typical stichotrichs. *Epiclintes* branched off first in all analyses performed with high BI and moderate MP supporting value (100% BI, 58% MP) while low ML and NJ supporting value (< 50%). This suggests that this genus could represent a unique taxon considering its systematic position. Morphological and ontogenetic investigations on *Epiclintes auricularis* indicates that this genus demonstrates several special features compared with most other related urostylids: (1) several (instead of one) left-most frontal cirri are developed from the FVT anlage I in *E. auricularis rarisetus*; (2) buccal and frontoterminal (or migratory) cirri are lacking at interphase and never occur during divisional processes; (3) each FVT anlage, except the anlage I, develops a transverse cirrus; and (4) anlagen for both marginal rows and dorsal kineties are formed *de novo*. Based on both morphological/morphogenetical and molecular data available, *Epiclintes* might represent an individual taxon at suborder level, i.e., Epiclintina Wicklow, 1983 (nom. nud.) and as a peripheral branch within stichotrichs *s. str.*

Key words: Ciliates, Epiclintina, phylogeny, morphogenesis, SSrRNA.

INTRODUCTION

The ciliate genus *Epiclintes* is unique among traditional hypotrichs *s. l.* regarding the tripartite and highly contractile body, oblique rows of frontoventral

cirri, and lack of differentiated frontoterminal and buccal cirri (Wicklow and Borror 1990, Song and Warren 1996, Berger 2006). Due to the unusual cirral pattern, it was historically classified in various higher taxa (Table 1) because of its bizarre characters in general anatomy and behavior (Wicklow and Borror 1990, Berger 2006). Wicklow and Borror (1990) established a new family Epiclintidae for the genus *Epiclintes* based on ultrastructural and morphogenetic studies on the type species, *E. auricularis*. Later, Tuffrau and Fleury (1994) classified

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the Epiclintidae in the suborder Stichotrichina of the order Oxytrichida. Shi (1999) and Shi *et al.* (1999) argued with this classification and placed *Epiclintes* in Spirofilidae of Stichotrichina. Although Wicklow (1983) established suborder Epiclantina, the taxon Epiclantina is a nomen nudum because no diagnosis or characterization was provided (Berger 2006).

Consequently, the systematic assignment of *Epiclintes* and/or Epiclintidae remains ambiguous (Eigner 2001, Lynn and Small 2002, Berger 2006) though *E. auricularis* has been repeatedly described morphologically (Song and Warren 1996).

Molecular phylogeny based on analyzing small subunit rRNA (SSrRNA) gene sequence proves to be a relatively reliable tool for solving systematic positions of ciliates (Lynn and Sogin 1988). Compared with other spirotrich groups, however, the evolutionary position of *Epiclintes* has not been investigated using molecular methods. This genus still belongs to one of the most contentious groups and needs molecular information for satisfactory identification. For a new survey on evolutionary studies of ciliated protozoa, the SSrRNA genes for two populations of *Epiclintes auricularis* were sequenced. Inferred from sequence information combined with morphological and morphogenetic characters, topological trees were constructed and analyzed to achieve a better interpretation of the systematic position of the genus *Epiclintes* within spirotrichs.

Table 1. Main historical systematic assignment of the genus *Epiclintes* since 1960s.

Author	Date	Ordinal placement	Familial placement
Fauré-Fremiet	1961	Hypotrichida	Keronidae
Borror	1972	Hypotrichida	Urostylidae
Corliss	1979	Hypotrichida	Keronidae
Jankowski	1979	Hypotrichida	Oxytrichidae
Tuffrau	1979, 1987	Hypotrichida	Keronidae
de Puytorac	1994	Oxytrichida	Epiclintidae
Tuffrau and Fleury	1994	Oxytrichida	Epiclintidae
Lynn and Small	2002	Stichotrichida	Epiclintidae

MATERIALS AND METHODS

Ciliate collection and identification

Epiclintes auricularis auricularis (Claparède and Lachmann, 1858) Stein, 1864 and *E. auricularis rarisetus* (Hu *et al.* 2009) were collected from the coast of the Jiaozhou Bay at Qingdao (36°08'N, 120°43'E), northern China in 2007. Observations on living cells were carried out using bright field and differential interference contrast microscopy. Protargol staining method according to Wilbert (1975) was used to reveal infraciliature.

Terminology and systematic classification were according to Lynn (2008).

Extraction of DNA, amplification of SSrRNA gene and DNA sequencing

Total DNA was isolated according to Chen *et al.* (2000). In brief, cells were starved in artificial, sterilized seawater (salinity 33‰) at room temperature overnight to minimize the contaminants from food and other sources. They were harvested by centrifugation at 640 g for 3 min. and treated with lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.6% Tween 20, 0.6% Nonidet P40, 60 µg/ml Proteinase K) at 56°C for 2–3 h, followed by phenol-chloroform purification. Universal oligonucleotide primers (forward 5'-AACCTGGTTGATCCTGCCAGT-3'; reverse 5'-TGATCCTTCTGCAGGTTACCTAC-3') (Medlin *et al.* 1988) were used to amplify the SSrRNA gene. The typical amplification profile consisted of 30 cycles of 1 min. at 94°C, 2 min. at 58°C and 2.5 min. at 72°C, followed by 15 min. at 72°C for final extension. In order to minimize sequence errors, the high-fidelity TaKaRa ExTaq (TaKaRa, Japan) was used for PCR amplification.

Purified PCR product of appropriate size was inserted into the pUCm-T vector (Sangon, Canada). Plasmids were harvested using the plasmid mini-prep spin column kit (Sangon Bio. Co., Canada) and were sequenced by the INVITROGEN sequencing facility in Shanghai, China. Subsequent sequencing was performed in both directions using primer walking.

Sequence availability

Sequences of the SSrRNA gene of other ciliates were obtained from the GenBank/EMBL databases. Accession numbers are listed in Table 2.

Phylogenetic analyses

Data sets were aligned with Clustal W (Thompson *et al.* 1994), and filtered from ambiguously aligned positions with Gblocks_0.91b (Castresana 2000). Sequences with length of 1708 bp were used for further analyses.

A Bayesian inference (BI) analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using the Markov chain Monte Carlo (MCMC) algorithm under the GTR + G + I evolutionary model indicated by MrModeltest v.2 (Nylander 2004). The program was run for 1,500,000 generations with trees sampled every 100 generations and a burn-in of 3,000 trees.

Maximum-likelihood (ML) tree was constructed with the PhyML V2.4.4 program (Guindon and Gascuel 2003), base frequencies and a rate matrix for the substitution model (GTR + G + I) were

Table 2. Classification of the species included in the present study following Lynn (2008).

Subclass	Order	Species	Accession number			
Choreotrichia	Choreotrichida	<i>Pelagostrobilidium neptuni</i>	AY541683			
		<i>Strobilidium caudatum</i>	AY143573			
	Tintinnida	<i>Tintinnopsis dadayi</i>	AY143562			
		<i>Tintinnopsis fimbriata</i>	AY143560			
Oligotrichia	Strombidiida	<i>Omegastrombidium elegans</i>	EF486862			
		<i>Strombidium apolatum</i>	DQ662848			
Stichotrichia	Stichotrichida	<i>Amphisiella magnigranulosa</i>	AM412774			
		<i>Amphisiella annulata</i>	DQ832260			
		<i>Engelmanniella mobilis</i>	AF164134			
	Sporadotrichida		<i>Gastrostyla steinii</i>	AF164133		
			<i>Gonostomum namibiense</i>	AY498655		
			<i>Gonostomum strenuum</i>	AJ310493		
			<i>Oxytricha granulifera</i>	X53486		
			<i>Oxytricha longigranulosa</i>	AM412766		
			<i>Paraurostyla weissei</i>	AJ310485		
			<i>Pattersoniella vitiphila</i>	AJ310495		
			<i>Pleurotricha lanceolata</i>	AF164128		
			<i>Protogastrostyla pulchra</i>	EF194082		
			<i>Sterkiella nova</i>	AF508771		
			<i>Stylonychia lemnae</i>	AF508773		
			<i>Styxophrya quadricornutus</i>	X53485		
			<i>Tetmemena pustulata</i>	X03947		
			<i>Trachelostyla pediculiformis</i>	DQ057346		
			Urostylida		<i>Anteholosticha manca</i>	DQ503578
					<i>Anteholosticha multistylata</i>	AJ277876
					<i>Anteholosticha sp</i>	EF123707
					<i>Apokeronopsis wrighti</i>	EU417963
					<i>Diaxonella trimarginata</i>	DQ190950
					<i>Epiclintes auricularis auricularis</i>	FJ008721
					<i>Epiclintes auricularis rarisetus</i>	FJ008722
					<i>Holosticha bradburyae</i>	EF123706
					<i>Holosticha diademata</i>	DQ059583
					<i>Holosticha heterofoissneri</i>	DQ059582
					<i>Metaurostyloopsis salina</i>	EU220229
					<i>Metaurostyloopsis sinica</i>	EU220227
					<i>Metaurostyloopsis struederkypkeae</i>	EU220228
	<i>Parabirojimia similis</i>	DQ503584				
	<i>Paruroleptus lepisma</i>	AF164132				
	<i>Psammomitra retractilis</i>	EF486865				
<i>Pseudokeronopsis bergeri</i>	DQ777742					
<i>Pseudokeronopsis carnae</i>	AY881633					
<i>Pseudokeronopsis flava</i>	DQ227798					
<i>Pseudokeronopsis qingdaoensis</i>	DQ359728					
<i>Pseudokeronopsis rubra</i>	DQ640314					
<i>Pseudourostyla cristata</i>	DQ019318					
<i>Pseudourostyla franzi</i>	AM412765					
<i>Thigmokeronopsis stoecki</i>	EU220226					
<i>Urostyla grandis</i>	AF508781					
<i>Uroleptus gallina</i>	AF508779					
<i>Uroleptus pisces</i>	AF164131					

Species newly investigated are listed in bold.

detected by MrModeltest v.2. The variable-site gamma distribution shape parameter (G) was 0.5934 and the proportion of invariable sites was 0.5618. The reliability of internal branches was assessed using a nonparametric bootstrap method with 1,000 replicates.

Maximum-parsimony (MP) analyses were performed with the software package PAUP*4.0b10 (Swofford 2002) and the reliability of the internal branches was estimated by using the bootstrap method with 1,000 replicates. During MP analysis, nucleotide sequences were reduced to 393 parsimony informative sites. Parameters for the MP tree were as follows: heuristic search, 100 random addition sequences and TBR branch swapping.

Neighbor-joining (NJ) analysis was performed with the program package MEGA v4.0 (Tamura *et al.* 2007) using the TrN model of substitution (Tamura and Nei 1993) with 1,000 replication steps.

Phylogenetic trees were viewed with TreeView v1.6.6 (Page 1996) and MEGA v4.0 (Tamura *et al.* 2007).

RESULTS

Summary of morphogenetic features in the genus *Epiclintes*

Based on data available (as simply shown in Fig. 1), morphogenetic features in the genus *Epiclintes* can be summarized as below: (1) oral primordium occurs epiapokinetally close to the anterior-most transverse cirrus in the opisthe, while hypoapokinetally in the proter, which develops to replace the proximal part of the old adoral zone of membranelles; (2) no migratory and buccal cirri are formed; (3) one to three left-most frontal cirri are derived from the frontal-midventral transverse cirral anlage (FVT-anlage) I; (4) many oblique FVT-anlagen appear near to the old rows, and only a few old cirri are involved in the formation of these anlagen; moreover, FVT-anlagen appear separately in both dividers; (5) each FVT anlage contributes one transverse cirrus except the anlage I; (6) anlagen for marginal row and dorsal kineties originate *de novo*; and (7) macronucleus evolves in a similar way as in its congener and other hypotrichs.

Deposition of sequences

The SSrRNA gene sequences of two *Epiclintes*-isolates were deposited in the GenBank with accession numbers listed in Table 2.

Phylogenetic trees (Figs 2–3)

Phylogenetic trees produced with different methods generated nearly congruent relationships, in which the subclass Stichotrichia was confirmed to be monophyletic.

Within the Stichotrichia (*sensu* Lynn and Small 2002), two monotypes of *Epiclintes* branched off first in

all analyses performed with high BI and moderate MP supporting value (100% BI, 58% MP) while low ML and NJ supporting value (< 50%) (Figs 2–3). In Bayesian and ML analyses, *Epiclintes* branch was clustered with *Holosticha-Psammomitra* clade with very low supporting value (63% BI, ML < 50%). The genus *Holosticha sensu* Berger (2006) occurred as a monophyletic group in all phylogenetic trees whereas three representatives of *Anteholosticha* were distantly separated.

Four stichotrich species (*Parabirojimia similis*, *Trachelostyla pediculiformis*, *Protogastrostyla pulchra* and *Amphisiella annulata*) occurred as separate branches peripheral to the remaining representatives of Stichotrichia. Their positions could not be resolved in any phylogenetic analyses performed (Figs 2–3). Separation of Urostyloids and Dorsomaginalia (*sensu* Berger 2006), without considering the position of the family Amphisiellidae, are highly supported in BI analysis (98%) (Fig. 2). The Amphisiellidae (*sensu* Lynn and Small 2002), represented by the genera *Amphisiella* and *Gastrostyla*, was considered to be polyphyletic, which is consistent with previous investigations (Schmidt *et al.* 2007, Yi *et al.* 2009).

DISCUSSION

Systematic assignment of *Epiclintes* based on morphological and morphogenetic data

Morphologically, *Epiclintes* demonstrates many common urostylid features, e.g., presence of many fronto-midventral cirral rows and transverse cirri, numerous macronuclear nodules and simple dorsal kinety pattern consisting of 3 bipolar kineties, suggesting that the classification of *Epiclintes* in the Urostylida is reasonable. However, absence of frontoterminal and buccal cirri as two features excludes it from the urostylids.

In terms of morphogenesis, both *Epiclintes* forms also present some urostylid characters: (1) partly rebuilding of the old adoral zone of membranelles in the proter, as shown in many urostylid genera (*Keronella*, *Urostyla*, *Holosticha*, *Anteholosticha*, *Bakuella*, *Pseudourostyla*) (Jerka-Dziadosz 1972, Hemberger 1982, Wiackowski 1985, Ganner 1991, Eigner and Foissner 1992, Song *et al.* 1992, Hu *et al.* 2003, Berger 2006), and (2) appearance of many oblique FVT anlagen. The urostylids are the unique group producing a high number of oblique cirral anlagen, while in other groups (e.g., oxytrichids, amphisiellids, kahliellids), the number is around 6 and

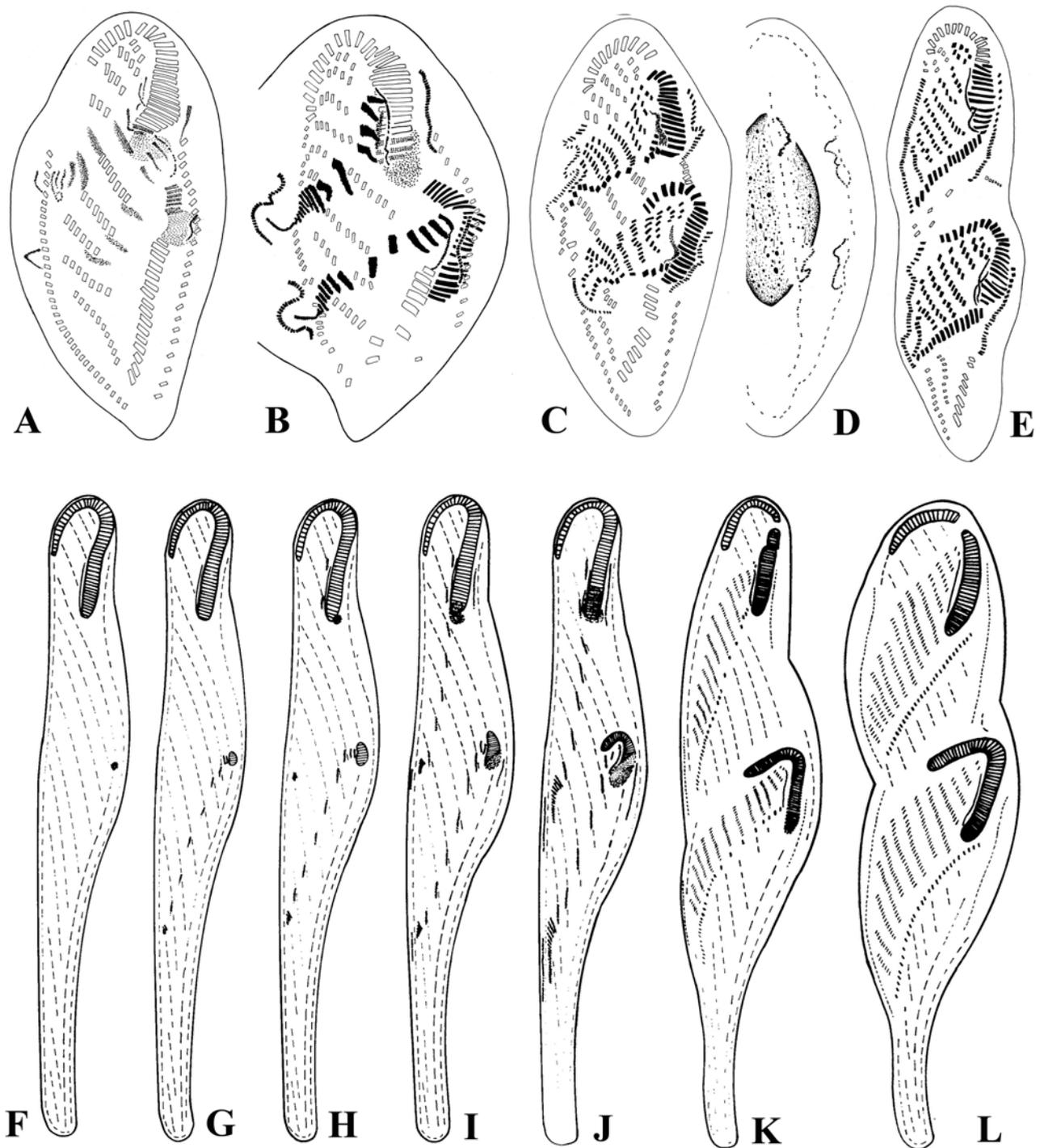


Fig. 1. Morphogenesis of *Epiclintes auricularis rarisetus* (A–E, reproduced from Hu *et al.* 2009) and *E. auricularis auricularis* (F–L from Wicklow and Borror 1990) after protargol impregnation from ventral (A–C, E–L) and dorsal (D) views. A, H, I – early dividers, showing the occurrence of oral primordium in both dividers, and anlagen for frontoventral transverse cirri and marginal cirri at two levels; B, J – early-middle dividers, showing further development of anlagen and primordium as well as differentiation of proximal membranelles; C, K – middle dividers, indicating the formation of new cirri and membranelles; D – the same divider as in C, indicating the fused macronucleus and the *de novo* development of dorsal kineties anlagen; E, L – late dividers, showing the nearly completion of morphogenesis of buccal and somatic ciliature; F, G – very early dividers, showing the appearance of oral primordium and frontal-midventral transverse cirral anlagen in the opisthe.

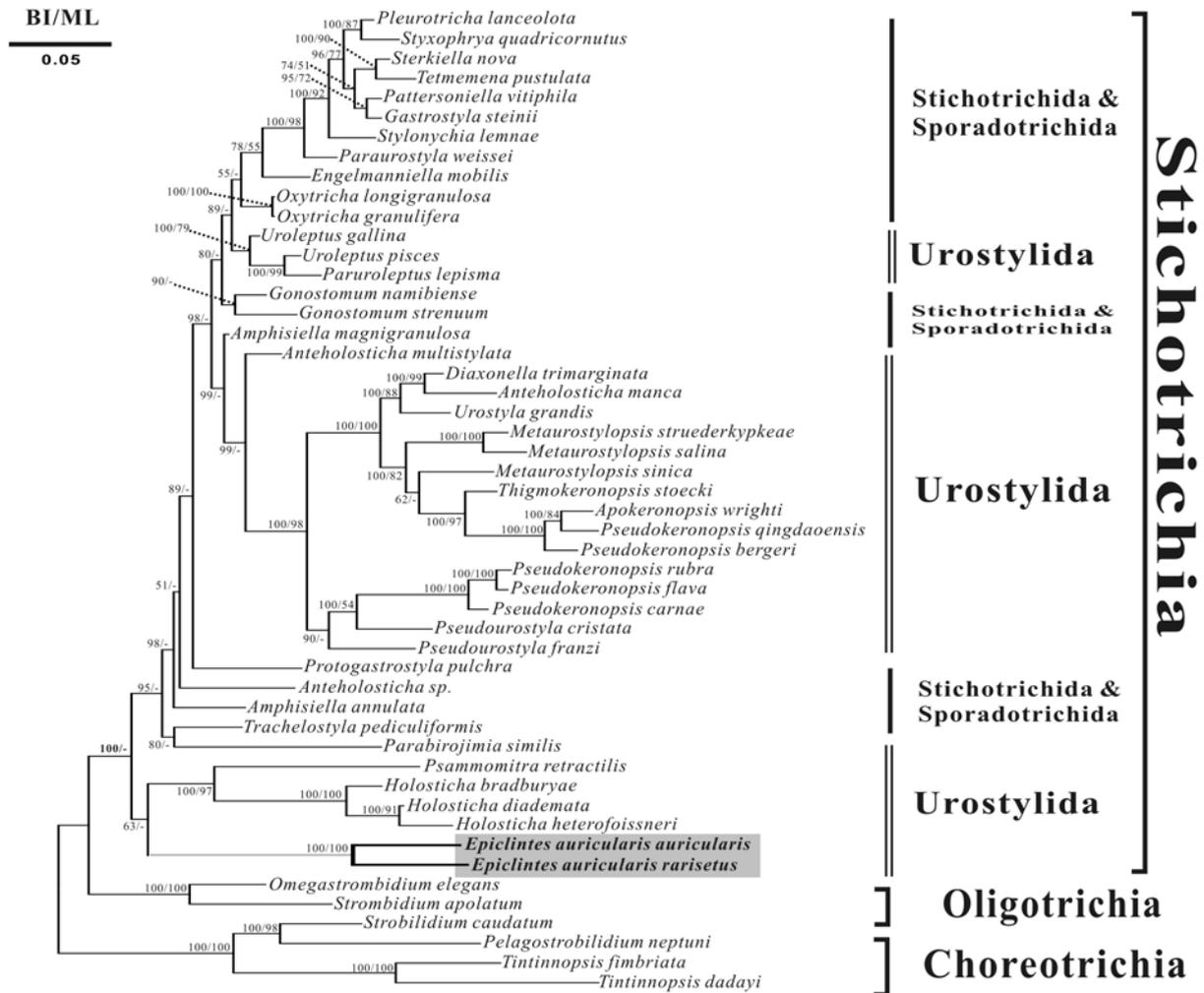


Fig. 2. Phylogenetic tree of SSrRNA sequences showing the positions of *Epiclintes*, by Bayesian inference (BI) and maximum-likelihood (ML) criteria applying the GTR + G + I model. Representatives of the subclasses Choreotrichia and Oligotrichia served as outgroups. Posterior probabilities of BI over 50% and non-parametric bootstrap values of ML over 50% from 1,000 resamples, respectively, are given at the nodes. Disagreements between ML and the reference BI tree at a given node are marked with asterisks (*). The scale bar corresponds to 5 substitutions per 100 nucleotide positions. Species sequenced in the present study are shown in bold type. Systematic classification follows Lynn (2008).

the resulting rows are more or less longitudinally arranged (Berger 1999, Hu and Song 2000). Admittedly, some of the above features are not very convincing. Each FVT anlage, except the anlage I, develops a transverse cirrus, whereas in almost all known urostylids, usually only the posterior anlagen give rise to transverse cirri and the anlage II never forms one transverse cirrus. No formation of true buccal and migratory cirri is also very unique in all known urostylids. Likely, the FVT anlage I unusually develops several frontal cirri in *E. auricularis rarisetus* instead of one as in the urostylids.

Additionally, as concerns the generation mode of marginal rows and dorsal kineties, many urostylids present the type of ‘within proliferation’ (Hu and Song 2001), whereas *Epiclintes auricularis* has a *de novo* formation mode that usually occurs in other groups (e.g., gastrostylids and parabirojimids) (Hu and Song 2000, Yi *et al.* 2009).

In conclusion, morphological and morphogenetic data on the two monotypes of *Epiclintes* support a distinct position of *Epiclintes*/Epiclintidae in the Urostylida. The erection of the suborder Epiclantina by Wick-

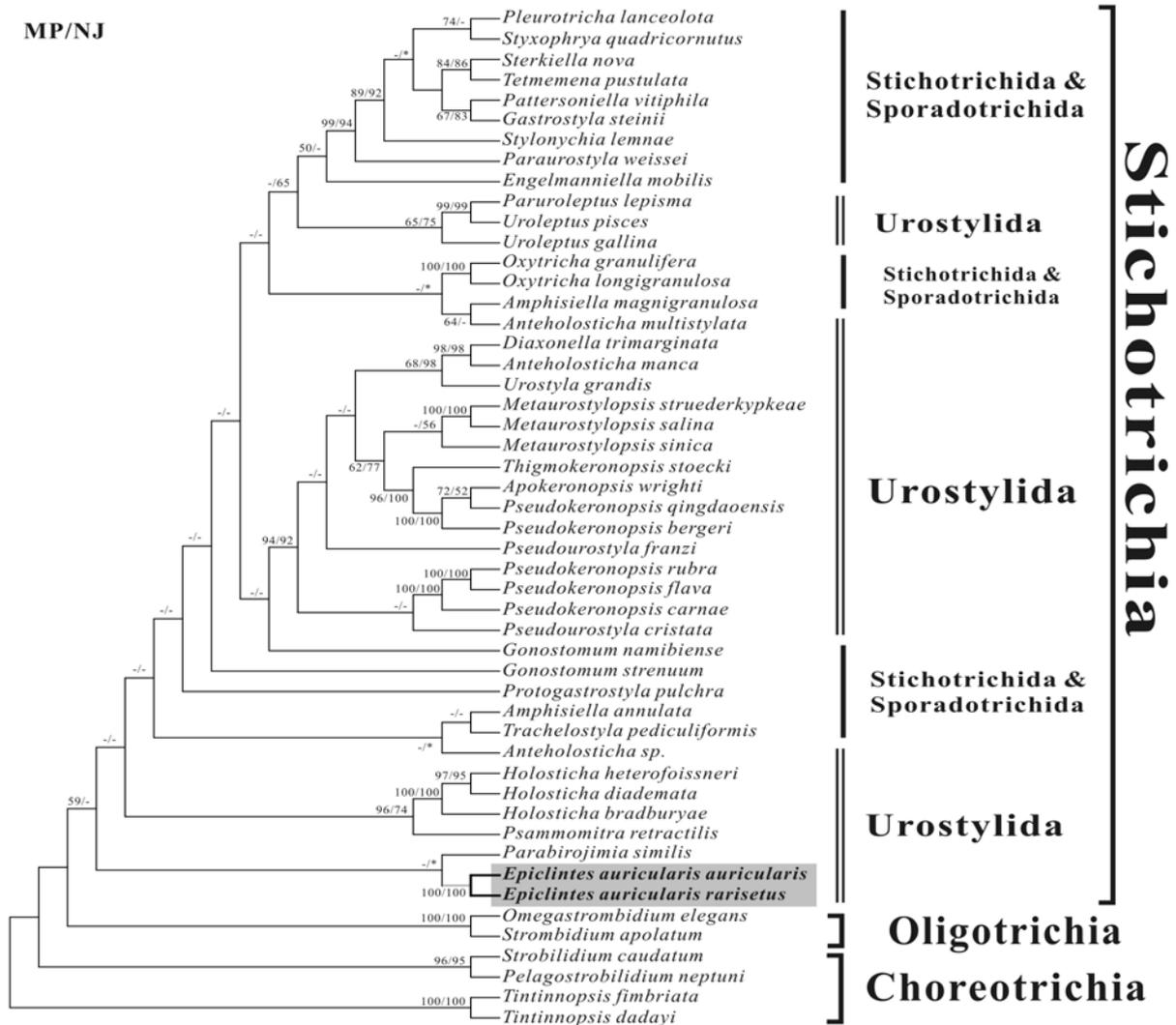


Fig. 3. Phylogenetic tree of SSrRNA sequences showing the positions of *Epiclintes* by Neighbor-joining (NJ) and maximum parsimony (MP) analysis. Representatives of the subclasses Choreotrichia and Oligotrichia served as outgroups. Numbers on branches are values generated from 1000 bootstrap replicates, values below 50 are not shown. Disagreements between NJ and the reference MP tree at a given node are marked with asterisks (*). Species sequenced in the present study are shown in bold type. Systematic classification follows Lynn (2008).

low (1983) also acknowledged this although the taxon is a nomen nudum. We agree with the suggestion of Wicklow (1983).

Systematic assignment of *Epiclintes*/Epiclintidae based on SSrRNA gene sequence

The Urostylida were divided into four families, viz. Holostichidae, Bakuellidae, Urostylidae, and Epiclintidae, without convincing that all these taxa were monophyletic in Berger's review (Berger 2006). Our molecular phylogenetic analyses support the morpho-

logical and morphogenetic data on the monophyly of the *Epiclintes* clade. *Epiclintes* branches at deepest level of stichotrichs in all trees (Figs 2–3), suggesting that *Epiclintes* should be arranged in an isolated position in the Urostylida.

Epiclintes auricularis and *Psammomitra retractilis* were considered as congeneric in the revision of *Epiclintes* by Carey and Tatchell (1983), however, these two species are assigned to separate clusters in all phylogenetic trees. Although Wicklow and Borror (1990) pointed out that *Epiclintes* and *Engelmanniella* were

radiated from *Kahliella*-like stichotrichines, our results indicate that *Epiclintes* and *Engelmanniella* are distantly separated in the molecular phylogenetic trees (Figs 2–3), as *Engelmanniella mobilis* is always clustered with oxytrichids (or Dorsomarginalia *sensu* Berger 2006), the same result with Foissner *et al.* (2004). Because of the absence of molecular data of *Kahliella*, we are not certain about the relationship between *Epiclintes* and *Kahliella*, further molecular data are required in the phylogenetic analysis of stichotrichs.

According to Wicklow (1979, 1983) and Wicklow and Borror (1990), inclusion of *Epiclintes* in any of the stichotrichid suborders (e.g., Urostylina) is artificial. The suborder Epiclintina erected by Wicklow (1983) is also supported by our phylogenetic analyses.

The diagnosis Epiclintina Wicklow, 1983 (nom. nud.) is provided as follows:

The diagnosis of Epiclintina Wicklow, 1983 (nom. nud.)

Elongate, dorso-ventrally flattened body with clearly differentiated adoral zone of membranelles; oblique frontoventral cirral rows numerous; marginal row present in both sides; transverse cirri present or absent; no buccal and migratory cirri formed during morphogenesis; each streak of fronto-midventral transverse cirral anlagen contributing transverse cirrus or cirri except for the left-most one developing a few frontal cirri. Presently one family included.

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