The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Morphology and Molecular Phylogeny of Two Poorly Known Species of *Protocruzia* (Ciliophora: Protocruziida)

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Keywords
Ciliate; morphogenesis; SSU rDNA; taxonomy.

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Received: 26 January 2016; revised 29 June 2016; accepted June 29, 2016.
Early View publication July 28, 2016

doi:10.1111/jeu.12344

ABSTRACT

The ciliate genus *Protocruzia* is a highly confused group, which was formerly placed in the class Heterotrichea or Karyorelictea, and is according to the most recent system tentatively assigned to the class Spirotrichea. In the present study, the morphology, ciliary pattern, and molecular phylogeny of two poorly known species, *Protocruzia tuzeti* Villeneuve-Brachon, 1940, and *Protocruzia granulosa* Kahl, 1933, isolated from coastal waters of China, were investigated. *Protocruzia tuzeti* differs from its congeners mainly in possessing 6 adoral membranelles, 8–11 somatic kineties, and postoral dikinetids. *Protocruzia granulosa* is characterized by its extremely slender body, three postoral kineties, and 13 or 14 somatic kineties. The morphogenesis of *P. granulosa* is similar to that of *P. tuzeti*, especially in the parakinetal mode of stomatogenesis and the reorganization of the parental paroral membrane; however, more than one somatic kinety joins in the formation of the oral primordium in *P. granulosa*. Phylogenetic analyses based on small subunit ribosomal RNA gene revealed that six *Protocruzia* species form a fully supported clade that does not belong to any ciliate class; therefore, our data support the establishment of the class Protocruziea Gao et al. (*Sci. Rep.*, 6, 2016, 24874).

THE ciliophora are a large group of protists with probably the greatest diversity of cell structure, physiological behavior, and ecological adaptation among all eukaryotic microorganisms (e.g. Bharti et al. 2015; Corliss 1979; Foissner et al. 2014; Jung et al. 2015; Kahl 1931; Kumar et al. 2015; Lynn 2008; Pan et al. 2015). Over 10,000 nominal species have been reported, including free-living, commensal, and parasitic forms (Song et al. 2009). *Protocruzia* is a relatively small genus, but its systematic placement is uncertain because of the unusual nuclear complex, the poorly developed alveoli, the lack of replication bands, and the chromosome-like structures which are reminiscent of the polytene chromosomes that appear during the development of the "higher" spirotrich macronuclear anlage following conjugation (Corliss 1979; Gentekaki et al. 2014; Grolière et al. 1980; Kahl 1932; Li et al. 2010; Lynn 2008). Eight nominal species are included in the genus so far (Ammermann 1968; Grolière et al. 1980; Kahl 1931, 1932, 1933; Song and Wilbert 1997; Villeneuve-Brachon 1940). The descriptions of most species are rather incomplete, e.g. superficial observations on living cells that lack information about the ciliary pattern. There is only one description of cell division (Grolière et al. 1980), which was considered in the revision by Foissner (1996). Thus, most *Protocruzia* species need detailed investigations on their morphology, morphogenesis, and gene sequences.

In this article, we described two species, *Protocruzia tuzeti* and *Protocruzia granulosa*, based on populations isolated from Chinese coastal waters. Additionally, the morphogenesis of *P. granulosa* was partially investigated. The molecular phylogeny of both species was analyzed based on small subunit ribosomal RNA gene (SSU rDNA) sequence data.

MATERIAL AND METHODS

Sampling site and cultivation

Protocruzia tuzeti was collected from costal seawater (temperature 21 °C, pH 8.0, and salinity 29‰) of the Daya Bay in Shenzhen (22°43′N, 114°32′E), southern China (Fig. S1A) on April 22, 2009. *Protocruzia granulosa* was collected from the coastal waters (temperature 22 °C, pH 7.9, and salinity 32‰) in the Clear Water Bay, Hong Kong (22°17′N, 114°17′E), China (Fig. S1B) on January 7, 2010. Glass slides were used as artificial substrate to collect the ciliates. Briefly, the slides were fixed to a frame and immersed in the sea to a depth of 1 m for 7–10 days to allow colonization by ciliates. The slides were transferred to Petri dishes containing water from the sampling site. Rice grains were added to enrich the bacterial food. Isolated specimens were cultured in filtrated seawater in Petri dishes for several days.

Morphological and morphogenetic methods

Live cells were observed under a Nikon Eclipse 80i microscope equipped with differential interference contrast (Nikon Instruments Inc., Tokyo, Japan) at magnifications of 100–1,000X. The protargol staining method according to Wilbert (1975) was used to reveal the ciliary pattern and nuclear apparatus. Measurements of stained specimens were performed, using an ocular micrometer. Drawings of live cells were based on live observations and microphotography; those of impregnated specimens were conducted with the help of a camera lucida.

DNA extraction, PCR amplification, and gene sequencing

Genomic DNA was extracted from three to five cleaned cells, using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO). PCR amplification, cloning, and sequencing of the SSU rDNA were performed according to Gao et al. (2014).

Phylogenetic analyses

The newly characterized sequence of *P. tuzeti* and other related sequences obtained from the NCBI GenBank database were aligned, using GUIDANCE algorithm (Penn et al. 2010a) with default parameters in the GUIDANCE web server (http://guidance.tau.ac.il/, Penn et al. 2010b). Both ends of the alignment were trimmed and ambiguous columns were removed based on confidence scores calculated by GUIDANCE. The final alignment of 1,656 characters was then used for the construction of phylogenetic trees. Karyorelictea and Heterotrichea were used as outgroups.

The maximum likelihood (ML) tree was constructed by RAxML-HPC2 on XSEDE v8.1.11 (Stamatakis 2006; Stamatakis et al. 2008) on the online server CIPRES Science Gateway (Miller et al. 2010) with the GTR + I + G model as selected by Modeltest v.3.4 (Posada and Crandall 1998). Support came from a majority rule consensus tree of 1,000 replicates. Bayesian inference (BI) analysis was performed, using MrBayes on XSEDE v 3.2.6 (Ronquist and Huelsenbeck 2003) on CIPRES Science Gateway with the best-fit model GTR + I + G as indicated by the Akaike information criterion (AIC) in the program MrModeltest v2 (Nylander 2004). Markov chain Monte Carlo (MCMC) simulations were run for 4,000,000 generations with a sampling frequency of every 100 generations and discarding the first 10,000 trees. The remaining trees were used to calculate the posterior probabilities (PP) of a 50% majority rule consensus tree.

Terminology

Terminology and classification follow Song and Wilbert (1997) and Lynn (2008), respectively.

RESULTS

Morphological description of *Protocruzia tuzeti* Villeneuve-Brachon, 1940 based on Qingdao population

Living cells approximately 30–40 μm × 12–25 μm in size in the fresh sample, sometimes up to 60-μm long in cultures; slightly contractile, ratio of length to width 2.5–3:1. Body flexible, outline thus highly variable, ranging from ellipsoidal to ovoidal in lateral view (Fig. 1A, 2A, C). Anterior end bluntly pointed, posterior end broadly rounded (Fig. 1A, 2A). Laterally flattened ca. 2:1, right side flat, left side convex with frontal region gradually set off from the flat anterior end (Fig. 1E). Ciliary rows on right side extend in shallow and thus inconspicuous longitudinal furrows (Fig. 2A). Numerous colorless cortical granules (ca. 0.2-μm across) densely arranged around and between somatic ciliary rows on right side (Fig. 1C,

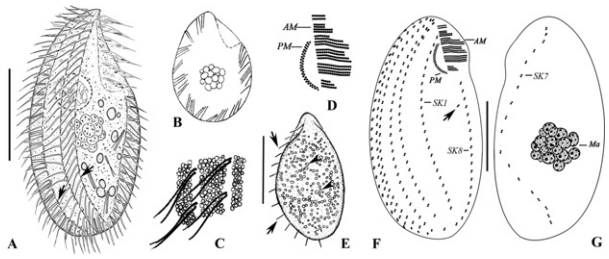

Figure 1 *Protocruzia tuzeti* from life (**A–C, E**) and after protargol impregnation (**D, F, G**). (A) Right lateral view of a representative individual, note the longitudinal furrows with the ciliary rows (arrows). (B) Right lateral view to show the extrusomes. (C) Arrangement of the cortical granules. (D) The oral ciliature. (E) Left lateral view to show the cortical granules (arrowheads) and the stiff cilia (arrows). (F, G) Right (F) and left (G) lateral views of the same specimen. The arrow marks a postoral dikinetid. AM, adoral membranelles; Ma, macronucleus; PM, paroral membrane; SK1, 2, 8, somatic kinety 1, 2, 8. Scale bars = 15 μm (A, E–G).

2E), but irregularly scattered on left side (Fig. 1E, 2B). Macronucleus usually centrally positioned, composed of 13–19, closely packed globular nodules (Fig. 1G, 2H). Micronucleus invisible neither in vivo nor after protargol impregnation.

Cytoplasm hyaline, colorless or slightly grayish. One large food vacuole containing diatoms or flagellates frequently near buccal area (Fig. 1A). Extrusomes rod-shaped, straight, about 3 μm × 0.1 μm in size in vivo, slightly clustered, arranged sparsely in the cell periphery, except for buccal area (Fig. 1B, 2D). Movement slowly, usually crawling on substrate, occasional swimming.

Invariably eight somatic kineties (Fig. 1F; Table 1) comprising dikinetids. Kineties 1–6 and 8 on right side, composed of narrowly spaced dikinetids; kinety 7 on left side, conspicuously curved to dorsal side (Fig. 1G, 2I). Kineties 1–7 almost bipolar, row 8 commencing at cytostome level, extending to rear end. One to three isolated dikinetids positioned postorally near anterior portion of kinety 8 (Fig. 1F, 2F, G), which is invisible in vivo. Both dikinetidal basal bodies bear flexible, about 7-μm long cilia, except for those of kinety 7, in which the dikinetids have associated only one stiff cilium with each anterior basal body (Fig. 1E, 2B).

Buccal apparatus inside a conspicuous oral cavity, located in anterior one-third of cell. Invariably six adoral membranelles, cilia about 7-μm long in vivo. Membranelles 1 (anterior-most one) and 6 composed of two and three rows of basal bodies, respectively; other membranelles composed of four rows (Fig. 1D). Paroral membrane curved, composed of two rows of basal bodies, which are arranged in a zigzag pattern (Fig. 1D, 2F).

Morphological description of *Protocruzia granulosa* Kahl, 1933 based on Guangdong population

Living cells approximately 60–100 × 10–15 μm in vivo in size (Fig. 3A, 4A, B). Body highly contractile, usual linearis-leaf-shaped in extended state to broadly rounded when contracted (Fig. 3B, I, J). Figure 4B shows the maximally contracted, middle, and usual (extended) forms of the same cell, which are about 65-, 80-, and 100-μm long,

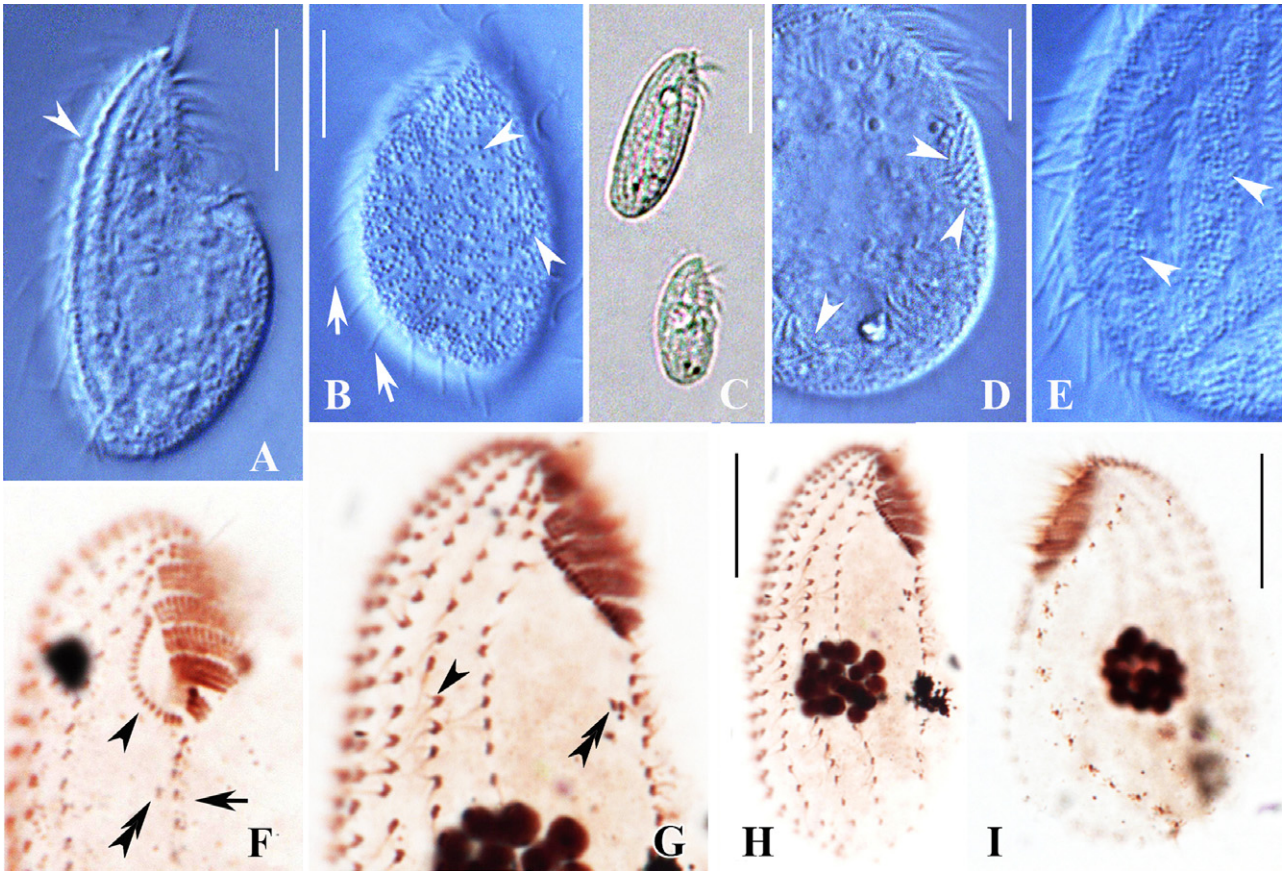

Figure 2 *Protocruzia tuzeti* from life (**A–E**) and after protargol impregnation (**F–I**). (A) Right lateral view of a representative individual, note the furrows with the somatic kineties (arrowhead). (B) Left lateral view to show the cortical granules (arrowheads) and the stiff cilia (arrows). (C) Right lateral view of different cells. (D) Right lateral view to show the extrusomes (arrowheads). (E) Detailed view of the cortical granules (arrowheads). (F) Left anterior cell portion. The arrowhead points to the paroral membrane, the arrow denotes somatic kinety 1, the double-arrowhead marks a postoral dikinetid. (G) Left anterior cell portion. The arrowhead points to a dikinetid bearing two cilia, the double-arrowhead denotes the postoral dikinetids. (H, I) Right (H) and left (I) lateral views. Ma, macronucleus. Scale bars = 15 μm (A), 10 μm (B, H, I), 30 μm (C), 5 μm (D).

Table 1. Morphometric data of *Protocruzia tuzeti* (first line) and *Protocruzia granulosa* (second line)

Characters	Min	Max	Mean	SD	CV	*n*
Body length (μm)	27	51	40.6	5.2	12.7	25
	40	68	51.3	7.5	14.7	25
Body width (μm)	19	30	23.6	2.5	10.8	25
	25	40	29.8	3.2	10.8	25
Buccal length (μm)	9	12	10.4	0.9	8.3	25
	8	15	11.7	1.6	13.8	25
Number of adoral membranelles	6	6	6.0	0	0	25
	6	6	6.0	0	0	25
Number of somatic kineties	8	8	8.0	0	0	25
	13	14	13.0	0.2	1.5	25
Number of macronuclear nodules	13	19	16.2	1.8	10.9	22
	17	28	21.6	3.8	17.6	24
Macronuclei length	8	12	10.1	1.0	9.9	21
	7	12	10.1	1.3	12.7	24
Macronuclei width	7	10	8.6	1.0	12.0	21
	5	11	8.6	1.5	17.4	24

Measurements based on protargol-impregnated specimens.
CV, coefficient of variation in %; Max, maximum; Mean, arithmetic mean; Min, minimum; *n*, number of cells investigated; SD, standard deviation.

respectively. Both body ends pointed. In some individuals, anterior end with a small projection slightly bending to the ventral side (Fig. 4D). Laterally flattened. No remarkable furrows along ciliary rows. Cortical granules colorless, 0.2–0.3 μm across, conspicuous at 400X magnification (Fig. 3D, 4G), relatively sparsely between ciliary rows. Macronucleus in the middle cell portion, about 11 μm across in vivo, composed of 17–28, closely packed globular nodules (Fig. 3F, 4F). Micronucleus invisible in vivo and after protargol impregnation.

Cytoplasm hyaline, colorless, often with numerous tiny (1–3 μm across) greasily shining globules and short (ca. 1-μm long) crystals (Fig. 4F) rendering the posterior cell portion slightly grayish or opaque. Food vacuole containing diatoms or flagellates in middle cell portion (Fig. 3A, 4C, D). Extrusomes rod-shaped, 3- to 5-μm long in vivo, widely spaced in whole cell periphery except for buccal area (Fig. 3C, 4F). Movement slowly, usually crawling on substrate.

Thirteen or fourteen somatic kineties (Fig. 3E, F; Table 1) comprising dikinetids. Kineties n, n-1, and n-2 positioned postorally and thus shortened anteriorly, about two-third of cell length; kinety n-3 beginning left of membranelle 2, extending to posterior cell end; other kineties almost bipolar. Somatic cilia about 8-μm long in vivo (Fig. 4E).

Buccal field occupies about anterior one-fourth to one-sixth of live cell length (Fig. 4A–D) depending on contraction state. Invariably six adoral membranelles; cilia about 8-μm long in vivo. Membranelles 1 (anterior-most one) and 6 composed of two and three rows of basal bodies, respectively; other membranelles composed of four rows (Fig. 3E). Paroral membrane curved, composed of two rows of basal bodies, which are arranged in a zigzag pattern (Fig. 4H).

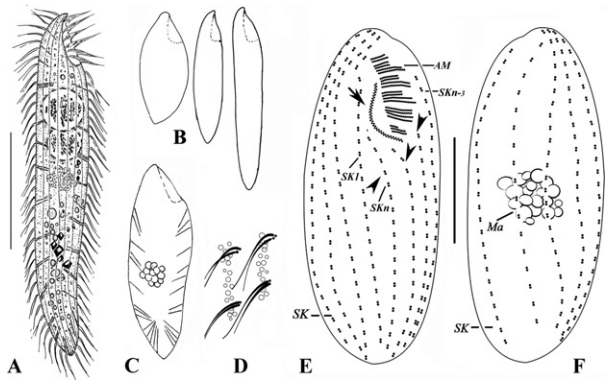

Figure 3 *Protocruzia granulosa* from life (**A–D**) and after protargol impregnation (**E, F**). (A) Right lateral view of a representative individual. (B) Shape variants in the same individual. (C) Arrangement of extrusomes. (D) Detail of somatic kineties showing the cortical granules. (E, F) Ventrolateral (E) and dorsolateral (F) views of the same specimen. The arrowheads mark the postoral kineties, the arrow denotes the paroral membrane. AM, adoral membranelles; Ma, macronucleus; SK, somatic kineties; SK1, n, n-3, somatic kinety 1, n, n-3. Scale bars = 30 μm (A), 20 μm (E, F).

Morphogenesis of *Protocruzia granulosa* Kahl, 1933

Only a few division stages were available, which demonstrate the following ontogenetic features: (i) the oral primordium of the opisthe appears within and to the left of the middle portion of postoral kinety SKn-2 (Fig. 5A, E), which generates new membranelles in the next stages; the adjacent postoral kinety (SKn-1) proliferates basal bodies that contribute to the formation of the paroral membrane (Fig. 5B, F, G); (ii) the parental adoral membranelles remain intact; (iii) the parental paroral membrane is reorganized and several extra dikinetids are possibly resorbed successively; (iv) the parental somatic kineties are renewed by intrakinetal proliferation of basal bodies; (v) each nodule of the macronuclear complex simply elongates and individually divides (Fig. 5C, H, I). In a later divider, the nuclear apparatus was found to be divided into two sets (Fig. 5D, J).

SSU rDNA sequence and phylogenetic analyses

The SSU rDNA sequence of *P. tuzeti* was deposited in the GenBank database with the accession number KU500620. The length and GC content are 1,741 bp and 43.08%, respectively. The SSU rDNA sequence of *P. granulosa* was deposited in the GenBank database by Huang et al. (2012) as *Protocruzia* sp. with the accession number JF694044, the length is 1,736 bp, and the GC content is 41.85%. The DNA from which the SSU rDNA was amplified by Huang et al. (2012) was extracted from

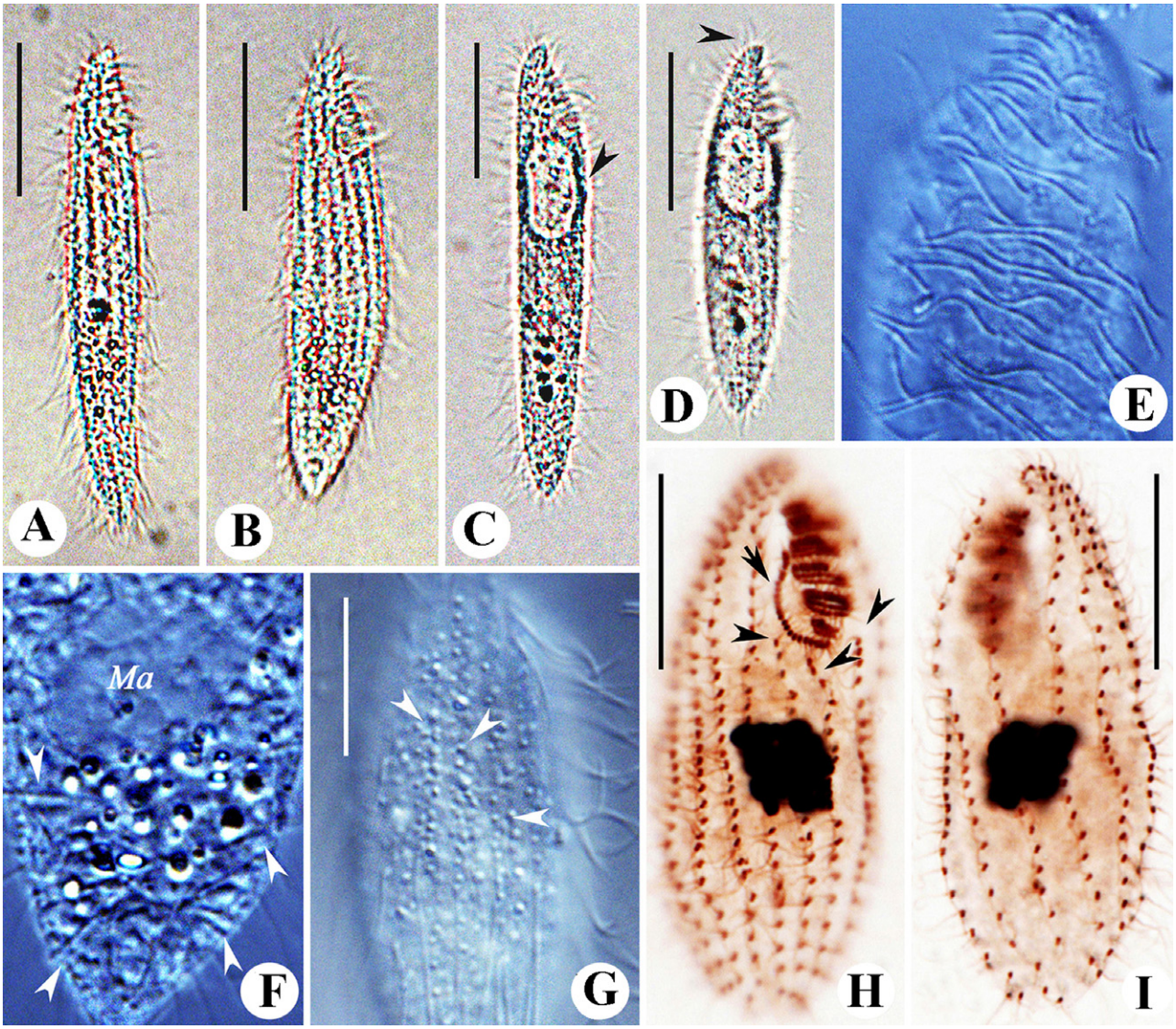

Figure 4 *Protocruzia granulosa* from life (**A–G**) and after protargol impregnation (**H, I**). (A, B) Shape variants in the same individual. (C, D) Shape variants in the same individual, note the food vacuole (arrowhead in C) and the anterior projection (arrowhead in J). (E) Left lateral view, to show the cilia. (F) Posterior cell portion to show the extrusomes (arrowheads). (G) Cortical granules. (H, I) Ventrolateral (H) and dorsolateral (I) views of the same specimen. Ma, macronucleus. Scale bars = 30 μm (A–D), 20 μm (H, I), 10 μm (G).

individuals of the same population described in our present work, and the organism's name has been updated in the GenBank.

The topologies of the ML and BI trees were basically accordant; thus, only the BI tree is shown here with support values from both algorithms indicated on the branches (Fig. 6). Two subphyla, the Postciliodesmatophora and Intramacronucleata, are recovered in our phylogenetic trees, which also show the two superclades within the Intramacronucleata, i.e. one composed of the classes Spirotrichea, Armophorea, and Litostomatea (SAL) and the other composed of the classes Colpodea, Oligohymenophorea, Nassophorea, Phyllopharyngea, Plagiopylea, and Prostomatea (CONthreeP). All *Protocruzia* species form a fully supported monophyletic group, with an early diverging position in the trees. In both trees, *P. tuzeti* forms together with the clade containing *Protocruzia adherens* AY217727 and *Protocruzia contrax* DQ190467 a fully supported cluster, which is sister to *P. granulosa* (70% ML, 0.98 BI).

DISCUSSION

Comparison of *Protocruzia tuzeti* with original description and congeners

In many cases, the identification of *Protocruzia* species is still difficult though seven valid species of *Protocruzia*

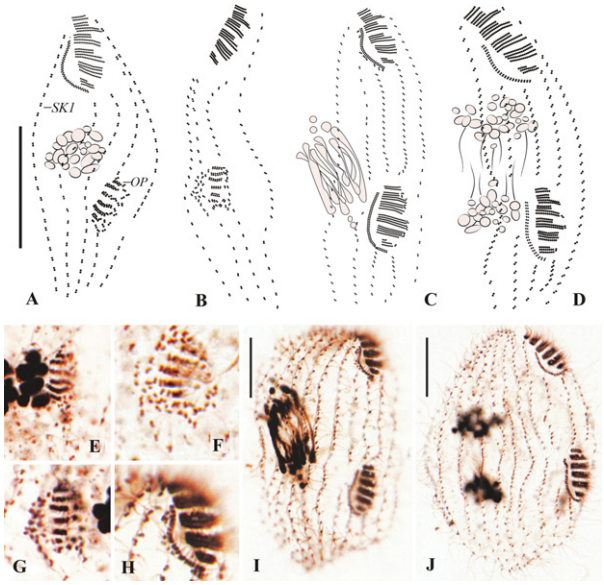

Figure 5 Morphogenesis of *Protocruzia granulosa* after protargol impregnation. (**A, E**) Ventral views of the early dividers to show the oral primordium. (**B, F, G**) Ventral views of two later early dividers to show the development of the oral primordium (B and G are of the same specimen). (**C, I**) Left lateral views of a middle divider. (**D, J**) Left lateral views of a late divider; note the splitting of the nuclear apparatus. (**H**) Parental oral apparatus of same specimen as shown in C and I. SK1, somatic kinety 1; OP, oral primordium. Scale bars = 20 μm.

have been described so far. There are several reasons for this: (i) the species passes few diagnostic characteristics (ii) the ranges of body size usually overlap between species, and (iii) the previous reports are mostly insufficient as descriptions of the ciliary patterns lack (Kahl 1931, 1932, 1933).

The synonymization of *P. tuzeti* and *Protocruzia depressa* with *P. contrax* by Song and Wilbert (1997) without knowledge about the ciliature seems to be premature because of the difficulties in accurate species identification and an unexpected diversity that had recently been revealed by SSU rDNA sequence difference (Gao et al. 2016; Gentekaki et al. 2014).

Protocruzia tuzeti was first reported succinctly by Villeneuve-Brachon (1940) and redescribed in detail from Black Sea material by Grolière et al. (1980). The population studied here corresponds well with the type population which was characterized by a cell size of 30–35 μm × 17–19 μm, a rounded rear end, and eight or nine somatic kineties (Fig. 7B). The Black Sea population is also similar to the type population in body size and shape. The former has, however, more somatic kineties (10–11 vs. 8 or 9) which might be attributed to the intraspecific variability (Grolière et al. 1980). In the right lateral aspect of the ciliary pattern in a morphostatic specimen by Grolière et al. (1980), no postoral dikinetid was shown (Fig. 7A), while all dividers possess one. Thus, we conclude that the Black Sea specimens also have a postoral dikinetid as our specimens. In addition, they also match well the Shenzhen form in the habitat, body size and shape, and ciliary pattern (especially in the large gap between kineties 1 and n) (Fig. 7A), though the former have slightly more somatic kineties (10–11 vs. 8).

Ammermann (1968) described *P. depressa* (Fig. 7G) from the North Sea. This species is similar to *P. tuzeti* in body size (40–50 μm × 15–30 μm). Its oral apparatus was described as "front two, then a single, then five groups of three membranelles", which is different from *P. tuzeti.* In addition, it has a conspicuously pointed anterior end, a dominant concavity, fewer kineties (5 vs. 8–11), and extrusomes restricted to the anterior cell portion.

Protocruzia contrax (Mansfeld, 1923) Kahl, 1932 was originally described as highly contractile, having 11 membranelles and two kineties based on observation of live cells (Fig. 7C). Though the number of membranelles was also confirmed by the following observation of Kahl (1932), it is unusual for such a tiny cell (15- to 25-μm long) to possess more than 10 membranelles. Considering that the adoral membranelles sometimes split in living cells, it is probable that Mansfeld and Kahl overestimated the number of membranelles. Although *P. contrax* needs a redescription, we can still distinguish it from *P. tuzeti* by the highly contractile body (vs. slightly), the smaller size (15–25 μm vs. 30–60 μm), the absence of cortical granules or extrusomes (vs. presence), and fewer kineties (2 vs. 8–11) (Kahl 1932; Mansfeld 1923).

Song and Wilbert (1997) described a form from Qingdao under the name of *P. contrax*, which has a slight contractile body and 9–12 somatic kineties and lacks a contractile vacuole (Fig. 7D, E). We suppose, therefore, that their specimens were misidentified. They also differ from *P. tuzeti* in the absence of cortical granules and extrusomes (vs. presence), and the presence of a postoral kinety (vs. 1–3 postorally positioned dikinetids) between kineties 1 and n.

Compared with *Protocruzia adherens* (Mansfeld, 1923) Kahl, 1932 (Fig. 7I, J), *P. tuzeti* has fewer adoral membranelles (6 vs. 9–10), more somatic kineties (8–11 vs. 5 or 6), and kineties on the left cell side (vs. absent; Kahl 1932; Mansfeld 1923).

Protocruzia labiata Kahl, 1932 was briefly reported from Germany, but has not been redescribed. Many important characteristics, e.g. the extrusomes and the ciliary pattern, are still unknown. However, Kahl (1932) emphasized that the adoral membranelles of this species form two groups (vs. no specialty). In his drawing, the sparsely arranged cortical granules form rows (Fig. 7F) (Kahl 1932). Prudently, reinvestigation is needed to confirm the validity of this species.

The type species of the genus *Protocruzia*, *P. pigerrima* (Cohn, 1866) Faria, da Cunha & Pinto, 1922 is also one of the species whose ciliary pattern is unclear (Fig. 7J, K). As it has a pointed (vs. rounded) rear end and a terminal contractile (?) vacuole (vs. absent), it should be distinct from *P. tuzeti* (Cohn 1866; Kahl 1933).

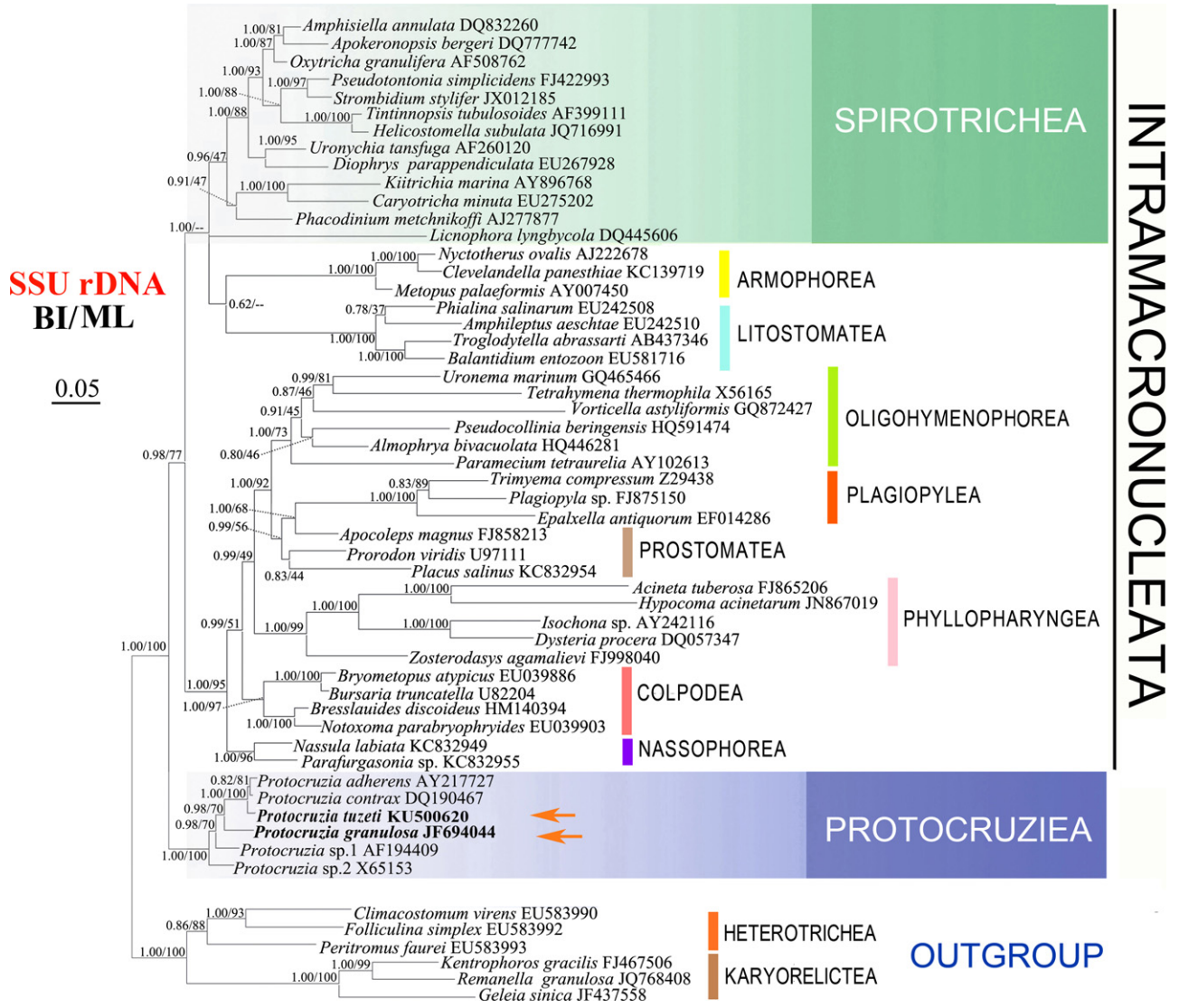

Figure 6 Bayesian inference (BI) tree based on the small subunit ribosomal RNA gene (SSU rDNA) showing the positions of *Protocruzia tuzeti* and *Protocruzia granulosa* (in bold). Numbers at the nodes represent the posterior probability of the BI and the bootstrap values of the maximum likelihood (ML) out of 1,000 replicates. Clades with a different topology in the ML tree are indicated by "--". The scale bar corresponds to 5 substitutions per 100 nucleotide positions.

Comparison of *Protocruzia granulosa* with the original description and congeners

This species was first discovered by Kahl (1933) on Heligoland, an island in the North Sea. It was superficially described and has not since been reinvestigated, using silver staining (Fig. 7M). As a consequence, we identify our population from Hong Kong mainly based on the body size, shape and extensibility, the habitat, and the cortical granules, only the color of the cortical granules differs (colorless vs. brownish). As this feature might depend on the environmental conditions and the observers, we believe that both are conspecific.

Protocruzia pigerrima is the only species that also has a pointed rear end as *P. granulosa*. Both differ in the body flexibility (slightly vs. highly), cell length (40–60 μm vs. 60–180 μm), and the cortical granules (absence vs. presence; Cohn 1866; Kahl 1933).

Concerning morphogenesis, *P. tuzeti* is the only species of the genus that has been studied, using protargol staining methods (Grolière et al. 1980). The morphogenetic events in *P. granulosa* and *P. tuzeti* have many features in common, with the exception of the stomatogenesis. Foissner (1996) characterized the stomatogenesis of *P. tuzeti* as mixokinetal, i.e. he assumed that both the parental somatic and oral ciliature are involved in the formation of the oral primordium based on the work of Grolière et al. (1980). However, in the latter paper, the parental oral structures are not demonstrated as being involved; according to the good line drawings provided, only one

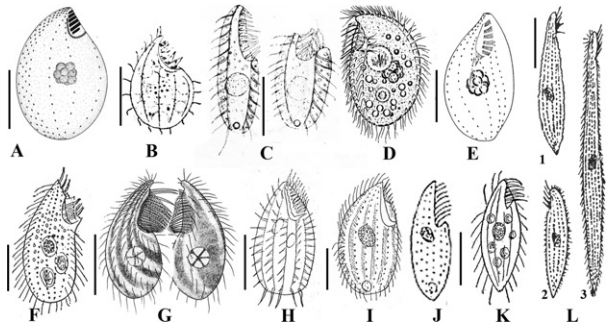

Figure 7 Species of *Protocruzia* in vivo (**B–D, F–N**) and after protargol impregnation (**A, E**). (A, B) *Protocruzia tuzeti* (A, from Grolière et al. 1980; B, from Villeneuve-Brachon 1940). (C) *Protocruzia contrax* (from Mansfeld 1923; originally reported as *Diplogmus contrax*), the same cell in typical form (left) and in contracted state (right). (D, E) *Protocruzia contrax* sensu Song and Wilbert 1997 (from Song and Wilbert 1997). (F) *Protocruzia labiata* (from Kahl 1932). (G) *Protocruzia depressa* (from Ammermann 1968). (H, I) *Protocruzia adherens* (H, from Mansfeld; I, from Kahl 1932). (J, K) *Protocruzia pigerrima* (J, from Cohn 1866; K, from Kahl 1933). (L) *Protocruzia granulosa* (from Kahl 1933), different cells from the same population (1. Typical form; 2. Not-extendable form; 3. Extended form). Scale bars = 15 μm (A, D–F, I–K), 10 μm (B, G), 30 μm (C, H, L).

postoral kinety participates in the formation of the oral primordium, i.e. the species displays the monoparakinetal type of stomatogenesis. In contrast, the stomatogenesis of *P. granulosa* belongs to the polyparakinetal type. For both species, the parental paroral membrane is reorganized in middle dividers. Yet, details are unknown because of the limited number of stages.

Phylogenetic position of *Protocruzia*

Due to its special morphological and ultrastructural features, *Protocruzia* has a highly ambiguous taxonomic history, and was placed either in the class Karyorelictea or Heterotrichea (Ammermann 1968; Foissner 1996; de Puytorac 1994; Ruthmann and Hauser 1974; Small and Lynn 1981; Song and Wilbert 1997), whereas previous phylogenetic studies based on SSU rDNA sequences revealed a close relationship with the Spirotrichea (Hammerschmidt et al. 1996; Lynn 1996; Shin et al. 2000). Therefore, Lynn (2008) classified this taxon as subclass Protocruziidia within the class Spirotrichea. However, with an increasing number of genes and taxa sampled as well as an improved performance of phylogenomic analyses, *Protocruzia* was excluded from the Spirotrichea and placed incertae sedis in the phylum Ciliophora owning to its deeper and earlier diverging position in the molecular genealogies (Gentekaki et al. 2014). Consistent with previous studies, all species of *Protocruzia* form a fully supported monophyletic group and occupy a basal position within the Ciliophora in the present study. Considering that the macronuclear division in *Protocruzia* exhibits some mitosis-like features (Ruthmann and Hauser 1974), which is unique within the phylum, we agree with the suggestion that *Protocruzia* represents a separate lineage at class level (Gao et al. 2016; Li et al. 2010).

TAXONOMIC SUMMARY

Order Protocruziida Jankowski, 1980
Family Protocruziidae Jankowski, 1980
Genus *Protocruzia* Faria, da Cunha & Pinto, 1922

Protocruzia tuzeti Villeneuve-Brachon, 1940

Improved diagnosis. Marine *Protocruzia*, cell size about 30–60 μm × 12–25 μm in vivo; body slightly contractible, anterior end pointed, posterior end rounded; extrusomes often clustered; cortical granules colorless, densely arranged around and between somatic kineties on right side but irregularly scattered on left side; 8–11 somatic kineties, conspicuous gap between kineties 1 and n; 1–3 postoral dikinetids; six adoral membranelles; one macronuclear complex with 13–19 globular nodules.

Voucher slide. Slides containing the protargol-impregnated specimens are deposited in the Natural History Museum, London (No. NHMUK 2016.6.27.1), as well as in the Laboratory of Protozoology, Ocean University of China (No. JJM2009042205-1).

Protocruzia granulosa Kahl, 1933

Improved diagnosis. Marine *Protocruzia,* cell size about 60–180 × 10–15 μm in vivo; body slender, extremely contractile, both ends sharply pointed; cortical granules brownish or colorless, sparsely arranged around and between somatic kineties; 13 or 14 somatic kineties with 1–3 postoral kineties; six adoral membranelles; one macronuclear complex with 17–28 globular nodules.

Voucher slide. Slides containing the protargol-impregnated specimens are deposited in the Natural History Museum, London (No. NHMUK 2016.6.27.2), as well as in the Laboratory of Protozoology, Ocean University of China (No. JJM2010010703-1).

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (project numbers: 51279112, 41576134, 31522051), the Fundamental Research Funds for the Central Universities (201562029, 201564022), and the Deanship of Scientific Research at King Saud University (PRG-1436-24). We thank Dr. Feng Gao for her suggestions in molecular analysis.

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SUPPORTING INFORMATION

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Figure S1. Sampling sites.