The morphology and phylogeny of two euplotid ciliates, *Diophrys blakeneyensis* spec. nov. and *Diophrys oligothrix* Borror, 1965 (Protozoa, Ciliophora, Euplotida)

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The morphology, infraciliature and molecular phylogeny of two marine ciliated protozoans, *Diophrys blakeneyensis* spec. nov. and *Diophrys oligothrix* described here, were investigated using microscopic observations of live and protargol-impregnated specimens, and by small subunit (SSU) rRNA gene sequence analysis. *Diophrys blakeneyensis* spec. nov. is characterized as follows: cell oval to rectangular in outline; size variable, approximately 60–180 x 30–80 μm in vivo; adoral zone comprising about 45 membranelles; usually five frontal, two ventral, five transverse, two left marginal and three caudal cirri; five dorsal kineties with more than 10 dikinetids each; 7–23 spherical to ellipsoid macronuclear nodules in a ring-like pattern; marine biotope. The population of *Diophrys oligothrix* described here corresponds well with previous populations in terms of its general morphology and ciliary pattern, in particular the continuous ciliary rows on the dorsal side with loosely arranged cilia. The main differences between the present and previously reported populations are the broader buccal field and greater number of dorsal kineties in the present population, both of which are regarded as population-dependent features. Phylogenetic analyses based on SSU rRNA gene sequence data demonstrate that *Diophrys blakeneyensis* is most closely related to *Diophrys oligothrix*, and both organisms cluster with two congeners with high bootstrap support within a larger group that contain the core species of the *Diophrys*-complex. Cladistic analysis based on morphological and morphogenetic data broadly agree with the SSU rRNA gene sequence phylogeny. Both analyses suggest that the genus *Diophrys* may be polyphyletic.

INTRODUCTION

*Diophrys*-like spirotrich ciliates are commonly found worldwide in marine and estuarine biotopes, especially sandy beaches and salt marshes (Agamaliev, 1971; Alekperov, 1984; Alekperov & Asadullayeva, 1999; Aliyev, 1990; Borror, 1963, 1965a, 1965b, 1972; Budenbrock, 1920; Carey, 1992; Curds & Wu, 1983; Czapik, 1981; Dragesco, 1963; Dragesco & Dragesco-Kernéis, 1986; Fauré-Fremiet, 1964; Fernández-Leborans & Novillo, 1994; Ganapati & Rao, 1958; Hartwig, 1973, 1974; Hill, 1981; Hu, 2008; Kahl 1932; Kattar 1970; Mansfeld 1923; Petz et al., 1995; Raikov & Kovaleva, 1968; Ruinen, 1938; Shen et al., 2011; Song & Packroff, 1993, 1997; Song & Wilbert, 1994, 2002; Song et al., 2007, 2009a, 2009b). The morphologies of *Diophrys*-like spirotrich ciliates share a prominent right posterior-lateral concavity where the sickle-shaped caudal cirri are located. In the past 30 years there have been two taxonomic revisions of the genus *Diophrys* (Curds & Wu, 1983; Song et al., 2007). Until 2010, eleven nominal species of *Diophrys* were recognized, six of which show highly divergent patterns of infraciliature, especially with respect to the numbers and distribution of frontoventral and marginal cirri. Jankowski (1979) erected a separate genus, *Paradiophrys*, for these divergent species. Subsequently Hill & Borror (1992) erected a third genus, *Diophryopsis*, and divided the *Diophrys*-like species among these three genera. This classification was accepted by Jankowski (2007) who erected the subfamily *Diophryinae* to include these three genera, a decision that was supported by Shao et al. (2010). Recently, three more *Diophrys*-like genera have been established, namely *Apodiophrys*, *Heterodiophrys* and *Pseudodiophrys* (Jiang & Song, 2010; Jiang et al., 2011).

Members of the genus *Diophrys* are characterized by having a prominent right posterior-lateral concavity where the sickle-shaped caudal cirri are located. In the past 30 years there have been two taxonomic revisions of the genus *Diophrys* (Curds & Wu, 1983; Song et al., 2007). Until 2010, eleven nominal species of *Diophrys* were recognized, six of which show highly divergent patterns of infraciliature, especially with respect to the numbers and distribution of frontoventral and marginal cirri. Jankowski (1979) erected a separate genus, *Paradiophrys*, for these divergent species. Subsequently Hill & Borror (1992) erected a third genus, *Diophryopsis*, and divided the *Diophrys*-like species among these three genera. This classification was accepted by Jankowski (2007) who erected the subfamily *Diophryinae* to include these three genera, a decision that was supported by Shao et al. (2010). Recently, three more *Diophrys*-like genera have been established, namely *Apodiophrys*, *Heterodiophrys* and *Pseudodiophrys* (Jiang & Song, 2010; Jiang et al., 2011).

**Abbreviations:** BI, Bayesian inference; SSU rRNA, small subunit rRNA. The GenBank/EMBL/DBJ accession numbers for the SSU rRNA gene sequences of *Diophrys blakeneyensis* and *Diophrys oligothrix* are JN172996 and JN172995, respectively.
These studies, however, did not support the monophyly of the subfamily Diophryinae. Furthermore, the validity of the genera Apodiophrys and Paradiophrys, which are mainly based on morphological characters, was challenged by the molecular data (Jiang et al., 2011). These conflicts between the morphological and molecular data cannot be resolved for a variety of reasons including the well-known limitations of single gene genealogies and a lack of data for a sufficient number of species from a sufficient range of geographical locations.

During a faunistic survey of ciliates in salt marshes along the eastern and southern coasts of the UK, several species of Diophrys were isolated and studied using light microscopy. In addition the small-subunit RNA (SSU rRNA) gene of some isolates was successfully sequenced. In this paper we document the morphology and SSU rRNA gene sequences of two Diophrys species, Diophrys blakeneyensis spec. nov., and Diophrys oligothrix. The molecular phylogeny of each was analysed based on SSU rRNA gene sequence data. Evolutionary relationships among the Diophrys-like ciliates are discussed in the light of morphological, morphogenetic and molecular data.

**METHODS**

**Morphological studies.** Populations of *D. oligothrix* Borror, 1965 and *D. blakeneyensis* spec. nov. were collected on 4 August 2010 from the intertidal region of a salt marsh near Blakeney village, Norfolk, UK (1° 1’ E; 52° 57’ N). This region of the salt marsh was dominated by the glasswort, *Salicornia europaea*. A second population of *D. blakeneyensis* spec. nov. was collected on 30 September 2010 from the intertidal region of a salt marsh at West Wittering, near Chichester Harbour, Hampshire, UK (0° 54’ W; 50° 46’ N) where sea purslane, *Atriplex portulacoides*, was dominant. Samples were collected at low tide and comprised small volumes of wet sediment covered with algal mat. Seawater from adjacent marsh ditches was also collected from which environmental data, i.e. water temperature, pH and salinity were recorded. The seawater samples were also used for the maintenance of ciliates in the laboratory.

In the laboratory, subsamples were diluted using seawater collected *in situ*. Specimens were isolated with a micropipette and examined *in vivo* using bright-field and Nomarski differential interference contrast microscopy (Xu et al., 2011). The protargol impregnation method of Wilbert (1975) was used in order to reveal the infraciliature. Counts and measurements of stained specimens were performed at a magnification of ×1250. Drawings were made with the help of a camera lucida. Terminology and systematics mainly follow Jankowski (2007) and Lynn (2008).

**DNA extraction and PCR amplification.** Specimens were individually isolated and washed three times in autoclaved filtered seawater. Two to four cells of each of the two species collected at Blakeney were applied to Indicating FTA Elute Cards (WB12041; Whatman) and allowed to dry for at least 3 h at room temperature prior to processing or storage. Elution of DNA from Indicating FTA Elute Cards was carried out according to the manufacturer’s instructions. Primer 82F (5’-GAAACTGGGAATGGGCTC-3’) and the universal eukaryotic primer Euk B (Medlin et al., 1988) were used to amplify the SSU rRNA gene using the cycling parameters: 5 min at 94 °C; 35 cycles of 30 s at 94 °C, 1 min at 60 °C and 2 min at 72 °C; and one cycle of 7 min at 72 °C. PCR products corresponding to the expected size were separated by agarose gel electrophoresis. Cleared DNA was cloned into the pMD18-T vector (Takara Biotechnology) and transformed into a competent *Escherichia coli* strain. Sequencing in both directions was carried out on an ABI 3730 automatic sequencer (Applied Biosystems).

**Phylogenetic analyses.** In order to analyse the molecular phylogeny of *D. blakeneyensis* spec. nov. and *D. oligothrix*, their SSU rRNA gene sequences were aligned with SSU rRNA gene sequences of 40 spirorich ciliates obtained from GenBank (for accession numbers see Fig. 7). Alignments were constructed, using MUSCLE v3.7 with default parameters and were manually refined in BioEdit 7.0.5.2 (Hall, 1999). Preliminary maximum-likelihood (ML) analyses performed online on the CIPRES Portal v.2.0 (URL: http://www.phylo.org/sub_sections/portal) with RAxML-HPC BlackBox 7.2.7 (Stamatakis, 2006; Stamatakis et al., 2008) showed that different selections of representative taxa (e.g. 42, 49, 58 and 67 species) resulted in trees with similar topologies. Based on these preliminary analyses, we selected a set of 42 SSU rRNA gene sequences for further phylogenetic analyses with *Protocruzia adherens* and *Protocruzia contrax* as the outgroup species. The resulting alignment included 1737 characters and is available from the authors upon request. Maximum-likelihood bootstrap analyses were carried out with 100 replicates using RAxML with the setting as described in Stamatakis (2006) and Stamatakis et al. (2008). Bayesian inference (BI) was performed with MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003) using the GTR+I+G model as selected by AIC in MrModeltest v2.0 (Nylander, 2004). Four simultaneous chains were run for 1,000,000 generations sampling every 100 generations. The first 25% of sampled trees were discarded as burn-in prior to constructing a 50% majority rule consensus tree.

**Cladistic analysis.** A cladistic analysis of 17 species, representing seven genera of uronychiids, was carried out based on morphological and morphogenetic data, both from the present investigation and from previously published studies. These include species of the six *Diophrys*-like genera plus members of the genus *Uronychia*. The phylogenetic relationships within the family Uronychiidae were elucidated by applying Hennig’s argumentation method (Hennig 1982; for details see Agatha, 2004). For discussions on the justification for determining the apomorph and plesiomorph, see Agatha (2004); Berger (2008); Borror & Hill (1995); Miao et al. (2007), and Shao et al. (2008).

**RESULTS**

*Diophrys blakeneyensis* spec. nov. (Figs 1, 2 and 3; Table 1)

Diagnosis. Medium- to large-sized *Diophrys*, approximately 60–180 × 50–150 μm *in vivo*; body elliptical in outline and slightly greyish to yellowish; pellicle flexible, with underlying granules closely spaced and arranged in lines or around membranelles, cirri and dorsal cilia; approximately 45 adoral membranelles; six to nine frontalveentral, two or three marginal, five transverse and three caudal cirri; five dorsal kinetics; macronuclear apparatus comprising 7–23 spherical to ellipsoidal nodules generally arranged in a ring; marine habitat.

Type locality. Intertidal region of a salt marsh near Blakeney village, Norfolk, UK (1° 1’ E; 52° 57’ N). Seawater collected from marsh ditches nearby was 18 °C, pH ca. 8.0 and salinity 29 %.

Type specimens. A protargol slide with the holotype specimen is deposited in the Natural History Museum, London, UK,
with registration number NHMUK 2011.10.28.1. Two para-
type slides are deposited in the Laboratory of Protozoology,
Ocean University of China, Qingdao, China with registration
numbers Bla20108452 and Chi20109301.

Etymology. Named after the village where the species was
first discovered.

Description. Cell size is highly variable, approximately 60–
180 × 50–150 μm in vivo; body shape oval to rectangular
(Figs 1a, 2a–e, h, i). Anterior end with a conspicuous, thin
collar (Figs 1a and 2e) accompanied by ridges; cilia of
apical membranelles emerge near the ridges. Posterior end
bluntly pointed with three strong caudal cirri emerging
from concave area on right dorsal side (Figs 1a, 2a, e, 2h
arrow, 2i double-arrowhead). Dorsoventrally flattened with
ventral side flat and dorsal side bulging evenly when viewed
from lateral aspect. In ventral view, two longitudinally
oriented ribs are visible, right rib starting near distal end of

Fig. 1. Morphology of Diophrys blakeneyensis spec. nov. from life (a–c) and after protargol impregnation (d–i). (a) Ventral view of
a typical individual. (b, c) Note the arrangement of cortical granules around the transverse cirri (b) and around the dorsal
bristles and among the dorsal kineties (c). (d) Ventral view of a specimen just after division, showing infraciliature and nuclear
apparatus (outlined); arrowhead indicates caudal cirri, double-arrowhead marks membranelle at distal end of adoral zone. (e)
Ventral view of a cell with 6 frontal cirri; arrows show micronuclei. (f) Ventral view of an individual with 4 frontal cirri and 3
marginal cirri (arrowheads); arrows show micronuclei, double-arrowhead marks membranelles at distal end of adoral zone. (g)
Ventral view of a cell with reduced ventral cirrus (arrow); arrowheads show micronuclei, double-arrowheads mark membranelles
on dorsal side. (h, i) Ventral and dorsal views of the same specimen, showing infraciliature and nuclear apparatus; arrow
indicates micronucleus. AZM, adoral zone of membranelles; CC, caudal cirri; DK1–5, dorsal kinety 1–5; EM, endoral
membrane; FC, frontal cirri; Ma, macronucleus; PM, paroral membrane; TC, transverse cirri, VC, ventral cirri. Bars, 40 μm.
Fig. 2. Photomicrographs of *Diophrys blakeneyensis* spec. nov. from life. (a) Ventral view, showing two ribs, right rib starting from distal end of adoral zone, left rib starting near posterior portion of buccal field (arrows). (b) Dorsal view, showing membranelles at distal end (arrows) and caudal cirri (arrowhead). (c, d, f) Ventral view of different specimens to show different body shapes and the arrangement of cirri; arrows in (c, d) and arrowheads in (f) mark marginal cirri; arrowheads in (c) show membranelles at distal end of adoral zone, arrows in (f) indicate frontal cirri. (e) Ventral view, showing dorsal cilia (arrows) and undulating membranes. (g) Ventral view of portion of a contracted cell, showing cortical granules around cirri and membranelles (arrows). (h) Dorsal view, showing membranelles at the apical end of adoral zone (arrowheads) and sickle-shaped caudal cirri derived from right posterior-lateral concavity (arrow). (i) Dorsal view, showing membranelles at distal end (arrow) and right posterior-lateral concavity (double-arrowhead). (j) Ventral view of portion of cell, showing macronuclear nodules (arrows). (k) Ventral view of a plumper body; arrows show dorsal cilia, arrowheads indicate marginal cirri, double-arrowhead marks ingested diatom. (l) Dorsal view of portion of cell to show densely aligned cortical granules. (m) Cortical granules (arrowheads) around dorsal bristles (arrows) making rosettes. UM, undulating membranes. Other abbreviations as in Fig. 1. Bars, 50 μm.
Fig. 3. Photomicrographs of *Diophrys blakeneyensis* spec. nov. after protargol impregnation. (a) Ventral view, showing the general ciliature and nuclear apparatus; arrow marks membranelles at distal end of adoral zone. (b) Ventral view of an individual with only one ventral cirrus (arrow). (c, f) Ventral view of specimens with seven and six frontal cirri, respectively. (d) Ventral view, showing fibres from the two rightmost transverse cirri (arrowhead) and two marginal cirri (arrows). (e) Left lateral view, noting two marginal cirri (arrows) and the leftmost dorsal kinety shortened posteriorly. (g, j, k) Dorsal (g) and ventral (j, k) views of the same specimen, showing infraciliature and dorsal kineties (arrows). (h, i) Ventral view of different cells, showing shape variance of micronuclei (arrows). (l) Ventral view of portion of cell, showing paroral and endoral membranes. (m) Details of macronuclear nodules and micronuclei (arrows). (n) Ventral view of proximal portion of cell, showing ventral, transverse and marginal cirri. (o) Ventral view of proximal portion of cell with three marginal cirri. (p–r) Ventral (p, q) and dorsal (r) views of the same specimen, showing infraciliature and nuclear apparatus; arrows in (p) and (r) indicate dorsal kineties while in (q) marks marginal cirri; arrowhead in (q) shows reduced ventral cirrus. (s) Ventral view, arrows show spherical micronuclei. MC, marginal cirri. Other abbreviations as in Fig. 1. Bars, 50 µm.
adoral zone, left rib starting near posterior portion of buccal field (Figs 1a, 2a, arrows). Wide depression in central region of cell between ribs. Five strong transverse cirri located in posterior half of cell within wide depression; several short ridges between transverse cirri (Figs 1a and 2c).

**Table 1.** Morphometric characterization of *Diophrys blakeneyensis* spec. nov. (upper row: Blakeney population; middle row: West Wittering population) and *Diophrys oligothrix* (lower row)

Data based on protargol-impregnated specimens. CV, Coefficient of variation; DK, dorsal kinety; Max, maximum; Min, minimum; n, number of specimens examined.

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<th>Characteristic</th>
<th>Min</th>
<th>Max</th>
<th>Mean</th>
<th>SD</th>
<th>SE</th>
<th>CV</th>
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<td>Body width (µm)</td>
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<td>Length of adoral zone of membranelles (µm)</td>
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<td>0</td>
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<td>25</td>
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<td>19</td>
<td>12.1</td>
<td>3.32</td>
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<td>15</td>
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<td>1.49</td>
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<td>0.91</td>
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Numerous tiny yellow–greenish cortical granules, about 0.5 μm across, beneath pellicle, often grouped around bases of membranelles, cirri and dorsal bristles (Figs 1b, c, 2g arrows, 2m arrowheads) or arranged in lines both on ventral and dorsal sides (Figs 1c and 2l) rendering the cell yellowish at low magnification. Beneath this layer of cortical granules are larger granules (possibly mitochondria), about 1.5 μm in size, densely arranged all over the body both on ventral and dorsal sides. Cytoplasm colourless or yellowish and transparent, although endoplasm contains numerous granules (1–8 μm in diameter) and several food vacuoles that typically contain diatoms. Contractile vacuole not recognized. 7–23 macronuclear nodules (Ma), globular to ellipsoid, 10–20 μm long, generally arranged in a ring (Figs 1d–g, i, 2j arrows; 3a–c, h, i, p–s); one to six spherical micronuclei closely associated with macronuclear nodules (Figs 1e, f, i, arrows, 1g arrowheads, 3h, i, m, s arrows). Locomotion by rapid crawling on substrate or by swimming.

Cilia of anterior and distal membranelles approximately 20–25 μm long in vivo. Frontal, ventral and left marginal cirri also ca 20–30 μm in length in vivo. Transverse and caudal cirri very strong with cilia 30–40 μm and 25–35 μm long in vivo, respectively. Dorsal cilia easily seen in live specimens, about 5–7 μm long in vivo (Figs 2e, k arrows).

Buccal field extending over 60% of body length and about 65% of body width. Adoral zone composed of 38–51 membranelles, only two or three of which at distal end reach right side of body (Figs 1d, f double-arrowhead, 2c arrowheads, 2i, 3a arrow); 10–14 apical membranelles are located on dorsal side (Figs 1g double-arrowheads, 2h arrowheads). Apical membranelles comprise three equal-length rows of kinetosomes, ventral membranelles 3- or 4-rowed with two or three long rows and one short row. Paroral membrane (PM) distinctly curved, anterior half composed of many oblique, short rows of 3 or 4 kinetosomes; endoral membrane (EM) about 60% length of PM and comprises two rows of densely arranged kinetosomes; PM and EM optically intersect near their posterior ends (Figs 1e, h, 3l). Four to seven (usually five) frontal cirri (FC) arranged in a group in anterior region of frontal area, one to three (usually two) ventral cirri (VC) located together as ‘pre-transverse cirri’ (Figs 1d–h, 2f arrows, 2c, k, 3b arrow, 3c arrows, 3j, o, q, s). Invariably five transverse cirri (TC; Figs 1d–g, 2c, d, f, k, 3a, c, j, o, q); two bundles of conspicuous fibres emerge from two rightmost TC and extend to cirrall III/II (Fig. 3a, n, 3d arrowhead). Usually two (rarely three) widely spaced left marginal cirri (MC), one near proximal portion of adoral zone, the other about level with transverse cirri (Figs 1d, e, g, h, 1f arrowheads, 2c, d, arrows, 2f, k arrowheads, 3c–e, q arrows, 3o). Three caudal cirri (CC) at right cell margin (Figs 1d arrowhead, e–h). Five dorso-lateral kineties (DK) each with about 10–17 dikinetids and conspicuously shortened anteriorly; leftmost and rightmost kineties also shortened posteriorly; three central kineties terminate near posterior pole (Figs 1d, h, i, 3g, k, p, r arrows).

Environmental data. For details of the Blakeney site, see ‘Type Locality’ above. Seawater was collected from marsh ditches near the sampling site at West Wittering. The water temperature was 22 °C, pH ca. 8.0, and salinity 35%.

**Diophrys oligothrix** Borror, 1965 (Figs 4 and 5; Table 1)

**Description.** Cell size variable, about 70–120 × 30–60 μm in vivo; body shape oval, ratio of length to width ca. 2 : 1 (Fig. 4a, b, d, e, g, h). Anterior end with a conspicuous, thin collar (Fig. 4f arrow) accompanied by ridges (Fig. 4c arrowheads); cilia of apical membranelles emerge near these ridges (Fig. 4h arrowheads). Posterior end bluntly pointed with three strong caudal cirri emerging from a concave area on right dorsal side (Figs 4c, e, h, n arrow). Dorsoventrally flattened with ventral side flat and dorsal side bulging evenly (Fig. 4i). In ventral view two ribs are visible, right rib starting from distal end of adoral zone, left rib starting near posterior portion of buccal field (Figs 4a, b, e, g arrow); wide depression in central region of cell between the two ribs. Row of five strong transverse cirri emerge from within wide depression in posterior half of cell (Fig. 4r); several short ridges between transverse cirri that are sometimes visible after protargol impregnation (Figs 4p arrows, 5b, f arrowheads).

Numerous tiny yellow–greenish cortical granules (approximately 0.5 μm across) beneath pellicle, often grouped around bases of membranelles (Fig. 4k arrowheads), cirri (Fig. 4j arrowheads) and dorsal bristles (Fig. 4o arrowheads) or arranged in lines on dorsal side (Fig. 4s) rendering cell yellowish at low magnification. Beneath this layer of cortical granules are larger granules (possibly mitochondria), approximately 1.5 μm in size, disc-like with a central depression, densely arranged all over body on both ventral and dorsal sides (Fig. 4r arrows). Cytoplasm colourless or yellowish and transparent, containing several food vacuoles and numerous granules 1.8 μm in diameter (Fig. 4l). Contractile vacuole not recognized. Usually two sausage-shaped macronuclear nodules, one located in anterior half of cell near right margin, the other positioned in posterior half near left margin (Figs 4l, 5a, f); anterior nodule occasionally with a constriction in middle portion (Fig. 5k arrow) or divided into two parts (Fig. 5g). Two to four spherical or ellipsoid micronuclei adjacent to macronuclear nodules (Fig. 5k arrowheads). Locomotion by rapid crawling on substrate or by swimming. Feeds on bacteria, flagellates and small ciliates, e.g. scuticociliates (Fig. 5d).

Cilia of anterior and distal membranelles approximately 20–25 μm long in vivo. Frontal, ventral and left marginal cirri also ca 20–30 μm in length. Transverse and caudal cirri very strong with cilia 30–40 μm and 25–35 μm long, respectively. Dorsal cilia readily visible in live specimens, approximately 5–7 μm long (Fig. 4q, s arrowheads).

Buccal field ca. 60% of body length and ca. 50% of body width (Fig. 4a, b, d–f). Adoral zone comprising 31–39 membranelles, of which only two or three distalmost ones reach right side of body (Fig. 5g arrowhead, 5j double-arrowhead); eight or nine apical membranelles located on dorsal side; apical membranelles comprise three equal-length...
Fig. 4. Photomicrographs of *Diophys oligothrix* from life. (a, b, d, e, g, m, n) Ventral view of different cells with variable body shape, showing cirral pattern and two ribs, one near right cell margin and the other near left cell margin (arrow in a, b, e and g), two marginal cirri (arrowheads in m and n), caudal cirri (arrow in n). (c) Ventral view, showing apical membranelles located near ridges (arrowheads). (f) Ventral view, showing thin collar (arrow) where apical membranelles (double-arrowhead) are located. Arrowheads show marginal cirri. (h) Ventral view, showing cirri-like apical membranelles (arrowheads). (i) Right ventro-lateral view. (j) Ventral view of portion of cell, showing small cortical granules around cirri (arrowheads). (k, p) Ventral view of anterior (k) and posterior (p) portion of cell, showing membranelles at distal end (arrow in k), short ridges between transverse cirri (arrows in p) and small cortical granules around cirri (arrowheads). (l) View of portion of cell, showing one sausage-like macronuclear nodule. (o) Dorsal view of portion of cell, showing small cortical granules arranged in lines and clustered around dorsal bristles. (q, s) Dorsal view of portion of cell, showing dorsal bristles and small cortical granules. (r) Ventral view of portion of cell, showing transverse cirri and large granules (arrows). MN, macronuclear nodule. Other abbreviations as in Fig. 1. Bars, 50 μm.
Fig. 5. Photomicrographs of *Diophrys oligothrix* after protargol impregnation. (a, e) Ventral view of two different specimens, showing the ciliature; arrows show marginal cirri. (b) Ventral view of portion of cell; arrows show fibres from two rightmost transverse cirri, which extend anteriorly to the frontal cirrus IV/2; arrowheads mark short ridges between transverse cirri. (c) Ventral view of an individual, arrow marks fragmented marginal cirrus. (d) View of portion of cell, showing ingested scuticociliates. (f) Ventral view of posterior portion of cell; dorsal kinety 1 terminates at level of transverse cirri; arrowheads point to short ridges between transverse cirri. (g) View of individual with three macronuclear nodules; arrowhead marks membranelles at distal end. (h, i) Ventral view of anterior portion of the same cell, showing membranelles at distal end (arrows) and frontal cirri (arrowheads). (j, m) Ventral (j) and dorsal (m) view of the same specimen, showing infraciliature; arrow shows the conspicuously shortened dorsal kinety 1; arrowhead marks right rib; double-arrowhead indicates membranelles at distal end of adoral zone; Ventral cirri are enclosed by broken line. (k) View of a specimen with two sausage-shaped macronuclear nodules and two micronuclei (arrowheads); arrow shows a constriction in the anterior macronuclear nodule; double-arrowhead marks dorsal kinety 5, which starts at anterior one-third of cell. (l) Ventral view of anterior portion of cell; arrow marks the reduced frontal cirrus; arrowhead indicates fibres projecting anteriad. MC, marginal cirri; MN, macronuclear nodule. Other abbreviations as in Fig. 1. Bars, 50 μm.
rows of kinetosomes (Fig. 5i arrows), ventral membranelles 3- or 4-rowed (i.e. two or three long rows and one short row). Paroral membrane distinctly curved, its anterior half composed of many oblique, short rows of three or four kinetosomes; endoral membrane approximately 65% length of paroral, with two rows of densely arranged kinetosomes; paroral and endoral optically intersect near their posterior ends (Fig. 5a, c, e, j, l). Five frontal cirri grouped in anterior

Fig. 6. Morphology of five closely related species from life (a, d, g, j, l) and after protargol impregnation (b, c, e, f, h, i, m, n). (a–c) *Diophrys appendiculata* (from Song & Packroff, 1997). (d–f) *Diophrys scutum* (from Song & Packroff, 1997). (g–i) *Diophrys apoligothrix* (from Song et al., 2009a). (j, k) *Paradiophrys multinucleata* (from Hartwig, 1973, originally named *Diophrys multinucleata*). (l–n) *Diophrys oligothrix* (from Song & Packroff, 1997). Bar, 25 μm.
region of frontal area (Fig. 5a, e, j, h, i arrowheads). Two ventral cirri located together as ‘pre-transverse cirri’ (Fig. 5e, f, j). Five transverse cirri (Fig. 5a–c, e, f, j) with two bundles of conspicuous fibres that emerge from rightmost two and extend to cirrus III/2 (Fig. 5b arrows). Two widely spaced left marginal cirri, one near proximal portion of adoral zone, the other approximately level with transverse cirri (Fig. 5a, e arrows, 5j); posterior marginal cirrus occasionally fragmented (Fig. 5c arrow). Five dorso-lateral kineties, each comprising seven or eight dikinetids on average and conspicuously shortened anteriorly; leftmost and rightmost kineties shortened posteriorly, three central kineties terminate at posterior pole of cell (Fig. 5f, m, j, arrow, k double-arrowhead). Three caudal cirri (Fig. 5e, j).

Environmental data. Seawater collected from marsh ditches near the sampling site was 18 °C, pH ca. 8.0, and salinity 29%.

Molecular data and phylogenetic analyses based on SSU rRNA gene sequences (Fig. 7)

The SSU rRNA gene sequences of D. blakeneyensis spec. nov. and D. oligothrix were deposited in GenBank with accession numbers JN172996 and JN172995, respectively. The length and DNA G+C content of the SSU rRNA genes were 1647 bp and 44.51% for D. blakeneyensis spec. nov. and 1574 bp and 44.66% for D. oligothrix.

The BI and ML trees inferred from SSU rRNA gene sequences had similar topologies for the Diophrys-complex group regardless of the number of taxa included in the preliminary analyses (i.e. 70, 60 or 24 taxa). As shown in Fig. 7, D. blakeneyensis spec. nov. grouped with D. oligothrix (Qingdao population) with low support (0.61 BI, 54 % ML), and then clustered with D. oligothrix (Blakeney population) with high support (1.00 BI, 99 % ML). The next closest

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Table 2. Morphological and morphogenetic characters on which the phylogenetic tree (Fig. 8) of representatives of uronychiids is based

For discussions on justification for determining apomorphic vs plesiomorphic character states, see Agatha (2004), Berger (2008), Borror & Hill (1995), Miao et al. (2007), and Shao et al. (2008).

<table>
<thead>
<tr>
<th>Character</th>
<th>Apomorph</th>
<th>Plesiomorph</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Left marginal cirri located:</td>
<td>Subcaudally</td>
<td>Post-buccally</td>
</tr>
<tr>
<td>2. Left marginal row</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>3. Caudal cirri from:</td>
<td>Single anlage</td>
<td>Two or more anlagen</td>
</tr>
<tr>
<td>4. Body</td>
<td>Rigid</td>
<td>Flexible</td>
</tr>
<tr>
<td>5. Marginal anlagen</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>6. Dorsal cilia</td>
<td>Long and rigid</td>
<td>Short and flexible</td>
</tr>
<tr>
<td>7. Cell surface</td>
<td>Sculptured</td>
<td>Not sculptured</td>
</tr>
<tr>
<td>8. Dorsal ciliary rows</td>
<td>Fragmented</td>
<td>Continuous</td>
</tr>
<tr>
<td>9. Frontal cirri distributed:</td>
<td>In a pattern</td>
<td>Randomly</td>
</tr>
<tr>
<td>10. Undulating membrane</td>
<td>One highly reduced</td>
<td>Two</td>
</tr>
<tr>
<td>11. Macronucleus</td>
<td>In more than two parts</td>
<td>In two parts</td>
</tr>
<tr>
<td>12. Adoral zone of membranelles</td>
<td>Bipartite</td>
<td>In one part</td>
</tr>
</tbody>
</table>

Fig. 8. Cladistic relationships of 17 species of the family Uronychiidae representing the six Diophrys-like genera plus Uronychia, based on morphological and morphogenetic information. See Table 2 for an explanation of the characters used. For discussions on characters used in this analysis see Agatha (2004), Berger (2008), Borror & Hill (1995), Miao et al. (2007), Shao et al. (2008). Abbreviations for taxa: Aova, Apodiophrys ovalis; Dapo, Diophrys apoligothrix; Dapp, Diophrys appendiculata; Dbla, Diophrys blakeneyensis; Dhys, Diophryopsis hystrix; Doli, Diophrys oligothrix; Dpar, Diophrys parappendiculata; Dscu, Diophrys scutum; Hzhu, Heterodiophrys zhui; Para, Paradiophrys; Pnig, Pseudodiophrys nigricans; Uro, Uronychia.
Table 3. Morphometrical and morphological comparison of eight related and well-described diophryid species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
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<tbody>
<tr>
<td>Adoral zone of membranelles</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>NB</td>
<td>B</td>
</tr>
<tr>
<td>Dorsal kinety</td>
<td>5</td>
<td>5</td>
<td>4–5</td>
<td>4</td>
<td>5</td>
<td>5–6</td>
<td>4–5</td>
<td>5</td>
<td>6–9</td>
<td>5</td>
</tr>
<tr>
<td>Left marginal cirri</td>
<td>2, postorally</td>
<td>2, postorally</td>
<td>2, postorally</td>
<td>2, postorally</td>
<td>2, postorally</td>
<td>2, postorally</td>
<td>2, postorally</td>
<td>1, postorally</td>
<td>2, postorally</td>
<td>1, postorally</td>
</tr>
<tr>
<td>Frontoventral cirri</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>7, grouped</td>
<td>9, mostly in 2 rows</td>
</tr>
<tr>
<td>Transverse cirri</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Macronucleus</td>
<td>7–23</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Body surface</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Dorsal ciliature</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows fragmented; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia short, flexible</td>
<td>DK rows continuous; cilia long, rigid</td>
</tr>
</tbody>
</table>

relatives were *Diophrys parappendiculata* and *Diophrys appendiculata*, the type species of *Diophrys*. This subclade was sister to a group composed of *Diophrys scutum*, *Diophrys apoligothrix*, *Diophryopsis hystrix* and *Pseudodiophrys nigricans*. *Heterodiophrya zhu*, the type species of the genus *Heterodiophrya*, was the next closest relative to this large clade which, along with another major clade consisting of other *Diophrys*-like species and four species of the genus *Uronychia*, represented the family *Uronychiidae*.

**Cladistic analysis based on morphological and morphogenetic characters (Table 2, Fig. 8)**

The morphological and morphogenetic characters used for the cladistic analysis are shown in Table 2. The tree based on this analysis showed that *D. blakeneyensis* spec. nov. is most closely related to *D. oligothrix* which together form a sister group to *D. appendiculata* and *D. parappendiculata* (Fig. 8). The genus *Diophrys* is polyphyletic when *D. apoligothrix* and *D. scutum* are included.

**DISCUSSION**

*Diophrys blakeneyensis* spec. nov. and its position in the SSU rRNA tree (Figs 6, 7 and 8; Table 3)

The two populations of *Diophrys blakeneyensis* spec. nov. correspond well in terms of their living morphology, buccal apparatus and somatic ciliature so they are very likely conspecific. Morphometric investigations showed that only three of 16 characters, i.e. the numbers of transverse cirri, caudal cirri and dorsal kineties, are invariant within populations of *D. blakeneyensis* spec. nov. which suggests that these characters can be used to distinguish among species of the genus *Diophrys* (Table 1). Three other traits, i.e. the numbers of adoral membranelles, left marginal cirri and frontal cirri, varied little between the two populations of *D. blakeneyensis* spec. nov. thus supporting their conspecificity. Of the remaining characters, the numbers of macronuclear nodules and dkinetids in each dorsal kinety are relatively highly variable within both populations but they overlap considerably, again supporting their conspecificity (Table 1).

The genus *Diophrys sensu* Hill & Borror (1992) is characterized by having two groups of frontoventral cirri, usually one or two left marginal cirri near the proximal portion of the adoral zone, two separate undulating membranes and three posterior-laterally located caudal cirri. Based on this definition, eight species have previously been assigned to *Diophrys*. Of these, only *Diophrys salina* lacks any infraciliature information; however, it clearly differs from *D. blakeneyensis* spec. nov. in its small body size (30–40 μm), well-developed anterior adoral membranelles, and short and fine frontoventral and transverse cirri (Ruinen, 1938).

Partial information is available on the infraciliature of *Diophrys peloetes* which may or may not be a separate species (Song et al., 2007). It resembles *D. blakeneyensis* spec. nov. in terms of body shape and size and the pattern of its ventral somatic infraciliature. However, the former has more distal adoral membranelles extending far onto the right ventral side (10 from illustration vs 2 or 3) and 8 dorsal kineties all of which are parallel to the main body axis (vs invariably 5 DK that curve posteriorly toward the right margin) (Borror, 1965b).

The remaining six species of the genus *Diophrys* are all well-described. Morphological data for five of these, plus *Pseudodiophrys nigricans* and *Diophryopsis hystrix* for which molecular data are also available, are summarized in Table 3 along with data for *D. blakeneyensis* spec. nov. Among the six *Diophrys* species, *D. oligothrix* and *D. blakeneyensis* spec. nov. are more closely related to one another than to any other congener in terms of morphological characters (Table 3; Figs 6 and 8). They have a similar body shape and cirral pattern (e.g. continuous dorsal kineties, numbers and arrangement of frontoventral and left marginal cirri) but differ in the number of macronuclear nodules (usually two vs usually more than ten). Their close relationship was also supported by the SSU rRNA gene sequence data with *D. blakeneyensis* spec. nov. having 99.1% and 99.2% similarities to each of two populations of *D. oligothrix*, i.e. differences of 14 nt from our new isolate of *D. oligothrix* JN172995, and 13 nt from *D. oligothrix* DQ353850.

Each of the five other species of *Diophrys* constantly has two macronuclear nodules and thus cannot be confused with *D. blakeneyensis* spec. nov. Additionally, the latter can be distinguished from its various congeners by a combination of the following features: (1) the number of adoral membranelles (ca. 45 vs 61–73 in *D. scutum*; Song & Packroff, 1997; Fig. 6d–f); (2) the number of marginal cirri (usually two vs invariably one in *D. apoligothrix*; Song et al., 2009a; Fig. 6h, i); (3) dorsal cilia distributed sparsely and evenly (vs dorsal cilia in groups in *D. appendiculata* and *D. parappendiculata*; dorsal cilia densely spaced in *D. scutum*) (Shen et al., 2011; Song & Packroff, 1997; Fig. 6a–c); (4) cell surface not sculpted (vs cell surface conspicuously sculptured in *D. apoligothrix*; Song et al., 2009a; Fig. 6g). The separation of *D. blakeneyensis* spec. nov. from *D. apoligothrix*, *D. scutum*, *D. parappendiculata* and *D. appendiculata* was also justified by their positions both in the SSU rRNA gene tree and the morphology/morphogenetic-based tree (Figs 7 and 8).

Although SSU rRNA gene sequence data are not available for *Diophrys japonica*, this species can be easily separated from *D. blakeneyensis* spec. nov. by the numbers of macronuclear nodules (2 vs 7–19), left marginal cirri (invariably 1 vs 2), and dorsal kineties (invariably 4 vs invariably 5) and in the presence (vs absence) of the fragment kinety (Hu, 2008).

*Paradiophrys multinucleata* resembles *D. blakeneyensis* spec. nov. in having multiple macronuclear nodules (Hartwig, 1973; Fig. 6j, k). The former, however, can easily be separated from the latter by the arrangement of the macronuclear nodules (packed together in the central region of the body vs in a ring-like pattern), body shape (broadly oval with truncated apical end vs oval to rectangular), the
number of transverse cirri (4 vs 5), and the number and position of the marginal cirri (2 postbuccal vs 3 subcaudal) (Hartwig, 1973).

The identity and phylogenetic position of *Diophrys oligothrix* (Figs 6, 7 and 8; Table 3)

*Diophrys oligothrix* was first reported from a marsh pool in New Hampshire, USA (Borror, 1965a). It was subsequently isolated and redescribed from coastal waters in Qingdao, China and seawater ponds on King George Island, Antarctica (Song & Packroff, 1997; Song & Wilbert, 1994, 2002). Our population corresponds well with previous populations in terms of body size and shape and its ciliary pattern, especially the continuous ciliary rows on the dorsal side with loosely arranged cilia. There are, nevertheless, three minor differences with the Blakeney population having a relatively longer buccal field (ca. 60% of body length vs about 50% of body length in other populations), 31–39 adoral membranelles (vs 26–37 in the Qingdao and Antarctic populations, 40–45 in the New Hampshire population), and invariably five dorsal kineties (vs four or five, usually four in other populations). Based on previous studies (Curds & Wu, 1983; Song et al., 2007) these variations can be considered as population-dependent, so these populations are very likely conspecific (Table 3, Fig. 8). Based on SSU rRNA gene sequence data, however, the two populations of *D. oligothrix* do not group together, the Qingdao isolate clustering first with *Diophrys apoligothrix* do not group together, the Qingdao isolate clustering first with *D. blakeneyensis* spec. nov. Possible explanations for this inconsistency include: (1) the phylogeny based on SSU rRNA gene sequence data may not reflect the true relationships between these two species, especially given the low support value for this node (54% ML, 0.61 BI); (2) the two sequenced isolates of *D. oligothrix* might represent cryptic species. Neither of these hypotheses can be tested presently because the type population of *D. oligothrix* has yet to be analysed genetically so its phylogenetic position on the SSU rRNA gene tree is not known. The morphological data do not justify the establishment of a novel species for the population isolated in the present study so we identify it as a population of *D. oligothrix*.

Note on the phylogenetic positions of the genera *Pseudodiophrys*, *Diophryopsis* and *Diophrys*

*Diophrys apoligothrix* is similar to *Pseudodiophrys nigricans* with the two species sharing a number of morphological characters including: (i) bipartite adoral zone of membranelles; (ii) sculpted cell surface; (iii) frontoventral cirri in two groups; (iv) continuous dorsal ciliary rows; (v) five transverse cirri; and (vi) two macronuclear nodules (Jiang et al., 2011; Song et al., 2009a; Table 3). However, the former differs from the latter in having: (1) two undulating membranes (vs a single, highly reduced undulating membrane), the latter character being an apomorphy according to Agatha (2004) and Berger (2008), and; (2) one (vs two) left marginal cirrus. Thus, the genus *Pseudodiophrys* is not congeneric with *Diophrys*. Interestingly, *Pseudodiophrys* groups very closely with *Diophrys apoligothrix* in both the morphological/morphogenetic tree and the SSU rRNA gene tree (Figs 7 and 8) so it is possible that *Pseudodiophrys* is a subgenus of *Diophrys*. However, such a decision should await further data, in particular SSU rRNA gene sequence data for other species of *Diophrys* and in particular *D. japonica*, which closely resembles *D. apoligothrix* in having one left marginal cirrus and five dorsal cirri. Such data should help to resolve the true position of *D. apoligothrix* and its relationship to *Pseudodiophrys*.

*Diophryopsis hystrix* resembles *Diophrys* in having two undulating membranes, three posterior-laterally located caudal cirri, and two groups of frontoventral cirri, so they are related. The former, however, distinctly differs from the latter by its asymmetrical (vs oval to rectangular) body shape, highly developed spine-like (vs short and flexible) dorsal cilia, anterior frontoventral cirri in two rows (vs non-rowed), highly reduced (vs highly developed) paroral membrane, and the origin of the left marginal cirrus which derive from the dorsal kinety (vs marginal anlage (Table 3)). Furthermore, *Diophryopsis* branches early within the clade of *Diophrys*-like unronychids in both the morphological and SSU rRNA gene trees (Figs 7 and 8). These data support the separation of *Diophryopsis* from *Diophrys* at the generic level (Song et al., 2007; Shao et al., 2010).

All available data indicate that the genus *Diophrys* is not monophyletic, but rather a moderately supported polyphyley (Figs 7 and 8). Furthermore the validity of the genera *Pseudodiophrys* and *Diophryopsis*, which were established mainly by morphological data, is still questionable since it has previously been reported that single-gene analyses of this group can suffer from phylogenetic artefacts (Yi et al., 2009). Therefore, more comprehensive taxon sampling, coupled with multi-gene analyses, will be required to resolve the phylogenetic relationships among the *Diophrys*-like hypotrichs.

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