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Comparison and Redefinition of Four Marine, Coloured *Pseudokeronopsis* spp. (Ciliophora: Hypotrichida), with Emphasis on Their Living Morphology

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Summary. Species separation among some brightly coloured marine *Pseudokeronopsis* spp. is difficult because of the accumulation of inaccurate descriptions or misinterpretations in previous studies. Based on isolates collected from Chinese coastal waters, four "well-known" yellow, brown or reddish species, i.e. *P. carnea*, *P. flava*, *P. flavicans*, and *P. rubra*, are re-examined, particularly with respect to their cortical granules, cell colour, infraciliature and other morphological features *in vivo*. Improved diagnoses for these organisms are given, synonyms are listed and a key to their identification is supplied. A recently reported marine form, *Pseudokeronopsis pararubra* Hu, Warren *et* Suzuki, 2004, is believed to be a junior synonym of *P. carnea* and is synonymized with the latter.

Key words: coloured *Pseudokeronopsis*, marine ciliates, species separation.

INTRODUCTION

Species separation among the brightly coloured *Pseudokeronopsis* spp. is difficult (Cohn 1866; Entz 1884; Kahl 1932; Jerka-Dziadosz and Janus 1972; Borror and Wicklow 1983; Song *et al*. 2004a, b). This is mainly because many morphogenetic and morphological characters, including the ciliary pattern, either overlap or are very similar while others, such as the colour and arrangement of the pigment granules, are usually superficially and/or incorrectly described or overlooked (Borror 1972; Ruthmann 1972; Foissner 1984; Wirnsberger *et al.* 1987; Hu and Song 2000, 2001; Hu *et al.* 2004; Song *et al.* 2004a, b; Sun and Song 2005). Marine *Pseudokeronopsis* spp. with brown, red, orange or yellow colour are commonly reported yet there is much confusion concerning their identification and circumscription (Hu and Song 2000, 2001; Song *et al.* 2002, 2004a, b). Numerous ambiguities have therefore accumulated in the literature pertaining to these organisms.

Wirnsberger *et al.* (1987) reviewed in detail the morphometric and infraciliature data for three species, i.e. *P. carnea*, *P. flava* and *P. rubra*. That study, however, was based primarily on silver-impregnated specimens so the living features remained inadequately documented. At least five more nominal morphotypes

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from marine biotopes have since been described thus adding to the confusion for species identification and circumscription (Hu and Song 2000, 2001; Song *et al.* 2002, 2004b; Shi and Xu 2003; Hu *et al.* 2004; Wanick and Silva-Neto 2004).

In recent years four brightly coloured marine *Pseudokeronopsis* spp., i.e. *P. carnea, P. flava, P. flavicans* and *P. rubra*, have been isolated from coastal waters of China, mostly from the north China sea near Qingdao, but also from the south China sea near Zhanjiang. This provided the opportunity to make a detailed examination of these "well-known" organisms. Previous studies have demonstrated that these forms are morphologically and morphometrically similar and that their infraciliature is generally less useful than expected for species identification (Song *et al.* 2002). Consequently greater attention is here given to the living morphology. Random amplified polymorphic DNA fingerprinting (RAPD) was also carried out since this technique is known to have potential for separating morphologically similar taxa (Chen *et al.* 2000).

The aims of the present study were: to evaluate the characters for species identification and separation; to redefine the four species and provide an improved diagnosis for each; to supply a key to their identification.

MATERIALS AND METHODS

Population origins, cultivation and morphological observations. Populations of four species were examined during the present study (Table 1). Among these, one population of *Pseudokeronopsis flava* (Cohn, 1866) was collected in July 2001 from the littoral water near Zhanjiang, Guangdong Province, south China. The water salinity was ~33‰, water temperature was about 28°C. All other populations and species were isolated during the period 1997-2003 from coastal waters of Jiaozhou Bay near Qingdao, north China. The water salinity was ~30‰, water temperature was 10-24°C.

After isolation pure (uniprotistan) cultures were established with rice grains as food source for bacteria. Investigations were carried out both on newly sampled and on cultivated specimens in order to observe variations in cell morphology including pigment colour. For observing cell and pigment colour, bright field microscopy only was used. Protargol staining (Wilbert 1975) was performed to reveal the infraciliature. Measurements were made at a magnification of 1250×. Drawings were made with the help of a camera lucida. Terminology is mainly according to Foissner (1984) and Borror and Wicklow (1983).

DNA extraction and RAPD reaction. The following species were analyzed by RAPD: *Pseudokeronopsis rubra, P. carnea* and *P. flava* (two populations). Each species was isolated from two separate cultures. Briefly, the nucleotide extraction protocols were: cells were rinsed three times with sterile artificial marine water after being starved overnight and were then pelleted by centrifugation. Genomic DNA extraction, amplification by PCR with primers S2001, S2006, S2007 and S2010 (defined in Chen *et al.* 2000) and RAPD reactions were carried out according to Chen *et al.* (2000).

RESULTS

Comparative description of four similar *Pseudokeronopsis* spp.

In view of the fact that previous reports might contain inaccurate or inconsistently interpreted data, the descriptions provided here are based only on the populations isolated from the China seas by the research group in the Laboratory of Protozoology, OUC. The improved diagnoses, however, also take into account previously reported data.

Body shape, size, flexibility or rigidity

Although the body shape *in vivo* of all four species is basically similar (i.e. slender or belt-like) there are consistent, albeit comparatively small, differences between them (Fig. 9). Clearly the cell size is variable even within the same population, especially after a long-term culture. All morphotypes are flexible but seldom or never contractile. Locomotion is generally similar in all four species and so is not regarded as a useful character for species separation.

Pseudokeronopsis carnea. This species is the most plump of the four; ratio of length to width of about 3-4:1; body often flattened and folded but seldom twisted (Figs 2C, F, H). Cells in culture mostly about 200-250 µm long *in vivo* although some individuals might be 300 µm or more in length. Anterior end bluntly rounded, which is unlike the other three species, while the posterior end is inconspicuously narrowed (Figs 1A, D, E; 2C). The buccal field is wide and conspicuous, its ratio to body length is about 1:3 which is the highest of the four congeners.

Pseudokeronopsis flava. Specimens in freshly collected samples mostly about 200-250 µm long with ratio of length to width about 4:1 (Figs 1H, L). In culture the body size and shape are extremely variable, i.e. from 120 to >400 µm long, and usually conspicuously more slender than freshly collected forms with a ratio of length to width up to 6-9:1 though with some "abnormal" forms that are worm- or band-like (Figs 1I; 4A, F). The ratio of buccal field to body length is also the most variable of the four species ranging from 1:4 up to 1:6 in the worm-like forms (Fig. 4A).

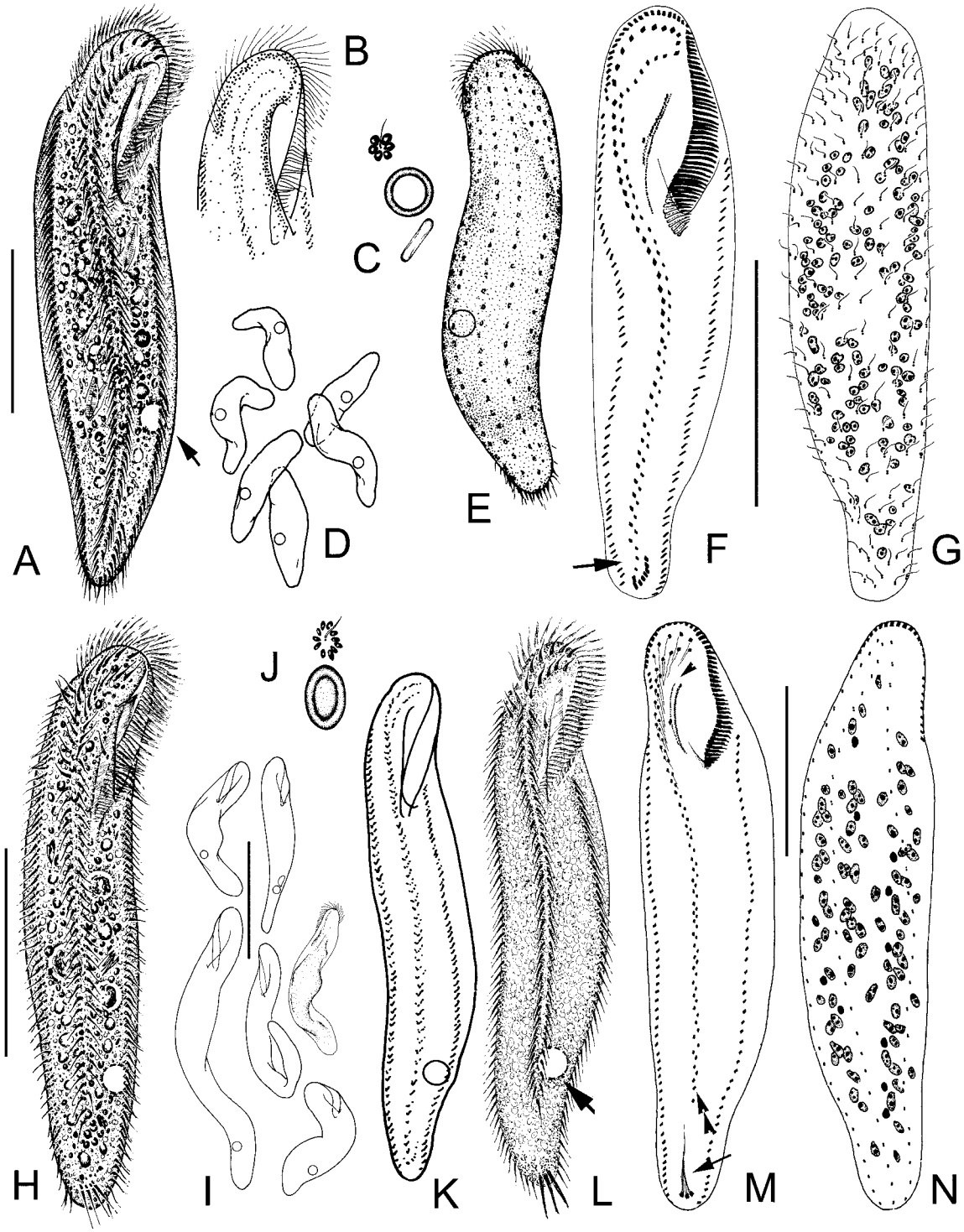
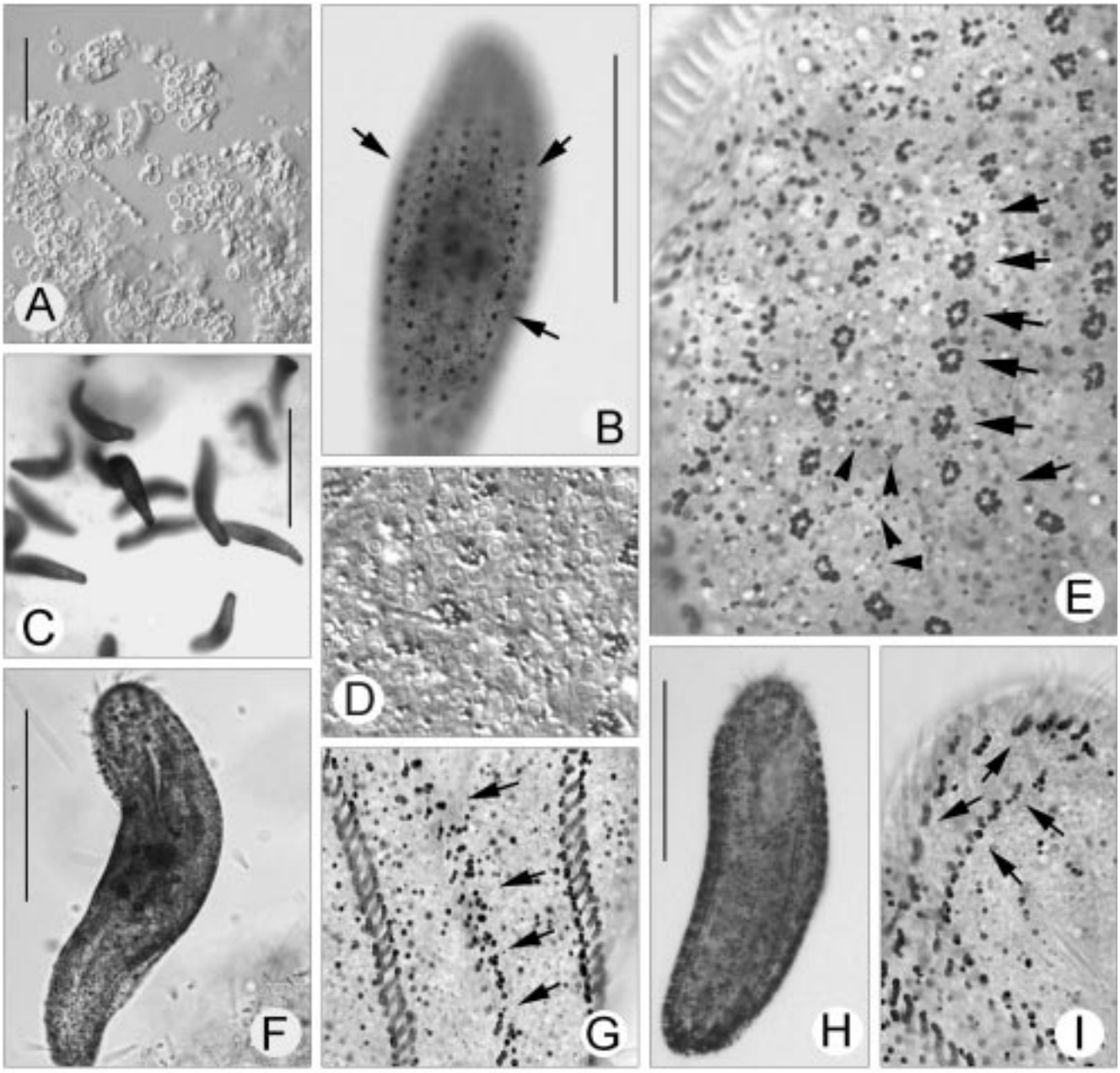
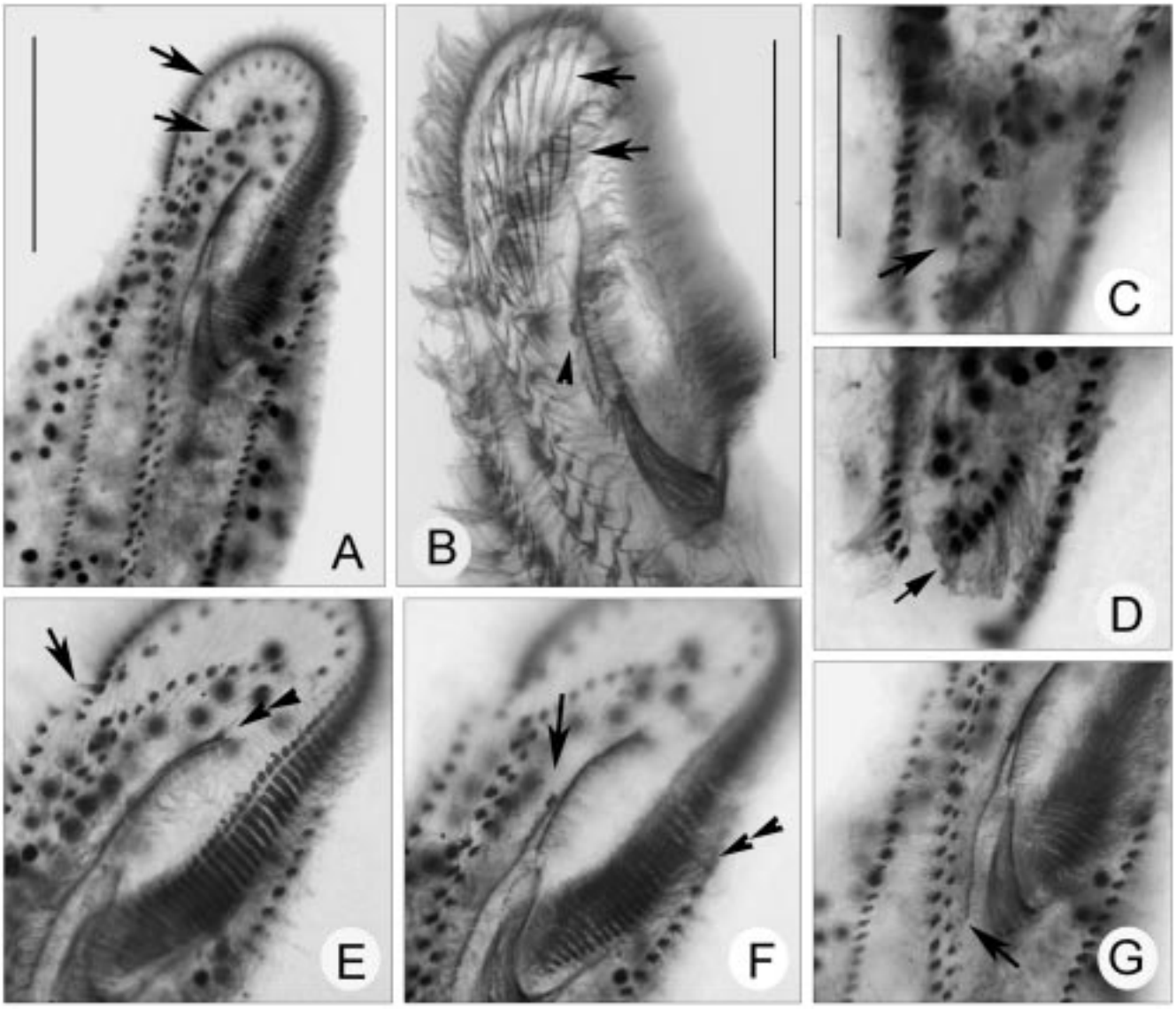


Fig. 1. *Pseudokeronopsis carnea* (A-G) and *Pseudokeronopsis flava* (H-N) from life (A-E, H-L) and after protargol impregnation (F, G, M, N) (I, L-N, from Song *et al.* 2004a, the rest are original). **A** - ventral view of a typical individual, arrow marks the contractile vacuole; **B** - anterior portion, to show the distribution of the pigment granules; **C, J** - to show the "blood-cell-shaped" granules (BCS-granules) and the pigment granules which are dark red in bright field microscopy; **D** - various body shapes; **E** - dorsal view at lower magnification, note the pattern of distribution of the pigment granules (marked as rows of dots); **F, G, -** ventral (F) and dorsal (G) views of the same specimen, arrow in F marks the last cirri of the midventral rows; **H** - ventral view of a typical individual; **I** - to show the various body shapes; **J, K, L** - ventral view, to demonstrate the distribution of pigment granules and the location of the contractile vacuole (arrow); **M, N** - ventral (M) and dorsal (N) views of the same specimen; arrowhead shows the undulating membranes; double arrowheads in M mark the last cirri of the midventral rows; arrow indicates the fibers of the transverse cirri. Scale bars: 60 µm (A, F, H); 50 µm (M); 100 µm (I).



Figs 2A-I. Photomicrographs of *Pseudokeronopsis carnea in vivo* (G, I, from Hu *et al.* 2004, called *P. pararubra*; the rest are original). **A** - to show the blood-cell-shaped granules (BCS-granules); **B** - dorsal view, to show the grouped pigment granules along the dorsal kineties (arrows); **C** - cells in natural state (without cover glass) at low magnification; **D** - dorsal view, to demonstrate both the grouped pigment granules and the BCS-granules; **E** - detailed portion of the dorsal side; arrowheads mark the sparsely distributed pigment granules; arrows indicate the grouped pigment granules which are arranged along the dorsal kineties; **F, H** - two individuals, to show the dark colour when observed at low to medium magnifications (100-200×); **G, I** - ventral view, to show the arrangement of the pigment granules; arrows mark the pigment granules along the midventral rows (G) and the frontal cirri (I). Scale bars: 10 μm (A); 80 μm (B, F, H); 200 μm (C).

Pseudokeronopsis flavicans. This form is less flexible than the other three species, often somewhat snake-like, distorted or ridged in middle portion, slightly uneven and irregularly bulged on the dorsal side (Fig. 6B). The body is slender with the posterior portion distinctly narrowed and tail-like (Fig. 6A). Body length *in vivo*



Figs 3A-G. Photomicrographs of *Pseudokeronopsis carnea* after protargol impregnation. **A, B** - ventral view, to show the general ciliature of the anterior portion; arrows mark the bicorona; **C, D** - ventral view of the posterior portion of the body; arrows mark the posterior end of the midventral rows (C) and the transverse cirri (D); **E** - ventral view; arrow marks the frontoterminal cirri; double-arrowheads mark the anterior ends of the undulating membranes; **F** - the same specimen as E; arrow indicates the buccal cirrus, double-arrowheads mark the left marginal row; **G** - ventral view of the buccal area, arrow marks the posterior end of the paroral membrane. Scale bars: 40 µm.

about 200-300 µm, cell length to width ratio about 5:1 (Figs 6A, B). The ratio of buccal field to body length is *ca* 1:4.

Pseudokeronopsis rubra. Body folded and flexible, shape relatively constant but often becomes plumper in culture (Figs 6G, 7A). Body length *in vivo* about 160-200 µm, less variable than the other three congeners. Both ends are conspicuously narrowed, especially the anterior end in freshly collected specimens. The ratio of buccal field to body length is about 1:4.

Cortical granules, pigments and cell colour

All four species possess two types of cortical granules: one is the typical pigment granule that renders the cell brightly coloured while the other is colourless. The pigment granules are about 1 µm in size and are invari-

ably arranged in a '*rubra*-pattern' (Song *et al.* 2004a), i.e. on the ventral side they are always densely arranged in short rows near the ciliary organelles and hence form belts along the cirral rows (Figs 1K, 2G, 6K), while on the dorsal side they are grouped in rosette-patterns around the dorsal cilia (Figs 1C, J; 2B, E, arrows; 4E; 6I). In addition to these grouped pigment granules there are also some that are sparsely distributed throughout the body giving the cell a weak colour when observed under higher magnifications (Fig. 2E, arrowheads). These granules disappear after fixation and/or impregnation but are detectable using epifluorescence microscopy (Fig. 7E).

The second type of cortical granule is colourless, larger than the pigment granules (about 1.5 μm in size), oval or circular in shape and flattened like that of a red blood cell, i.e. the margin is thicker than the central area (Figs 1C, J; 2A; 4C; 5A; 6I; 7B, C). These blood-cell-shaped granules (BCS-granules) are positioned beneath the pigment granules and are densely arranged on both ventral and dorsal sides without forming any discernible pattern.

The cell colour is rendered mainly by the pigment granules hence it can fade when the pigments lose the strength of their colour, which can happen during long-term (several months) culture. The food contents might be another cause of variability in cell colour since this usually makes the cell appear darker. In addition, it is recommended that cell colour is recorded at both lower (<100×) and higher (>200×) magnifications since there can be significant differences between these in the same specimen.

Pseudokeronopsis carnea. Pigment granules are more conspicuous than in the other three congeners, about 1 mm in size, always dark red or brown-reddish, arranged in typical *rubra*-pattern (Figs 2E, G, I), which can be clearly detected even at low magnification, e.g. 40× (Fig. 2B). The BCS-granules are larger (about 2 μm across) in this species than in the other three, completely circular in shape and densely packed (Figs 1C, 2A).

The cell colour is usually uneven, always dark-reddish or brown-reddish at lower magnification but less brightly coloured at higher magnifications, i.e. more or less brown-greyish except for the grouped pigment granules (Fig. 2B). No change in colour was observed during long-term (several months) culture.

Pseudokeronopsis flava. Pigment granules are smaller than those of *P. carnea*, about 0.5 μm across, bright yellow to yellow-brownish, and arranged in a typical *rubra*-pattern (Fig. 4E). No conspicuous change in the strength or brightness of the colour was observed during long-term culture, even after three years. BCS-granules about 1.5 μm long, always ellipsoidal in shape (Figs 4C, D; 5A).

The cell colour is generally constant (especially compared with that of *P. rubra*, for example), always yellow to yellow-brownish at lower magnification (Figs 4A, F, H) while less yellowish at higher magnifications (Figs 4C, E). It is clear that the colour is conspicuously stronger than that in *P. flavicans* (see below).

Pseudokeronopsis flavicans. Pigment granules yellow-brownish, ellipsoidal in shape and about 1 mm long, i.e. relatively larger than that in *P. flava* (*vs. ca* 0.5 μm). Since the colour of these pigment granules is weak, they are difficult to detect at lower magnifications. BCS-granules about 1.5 μm long, oval to ellipsoidal in shape, densely packed.

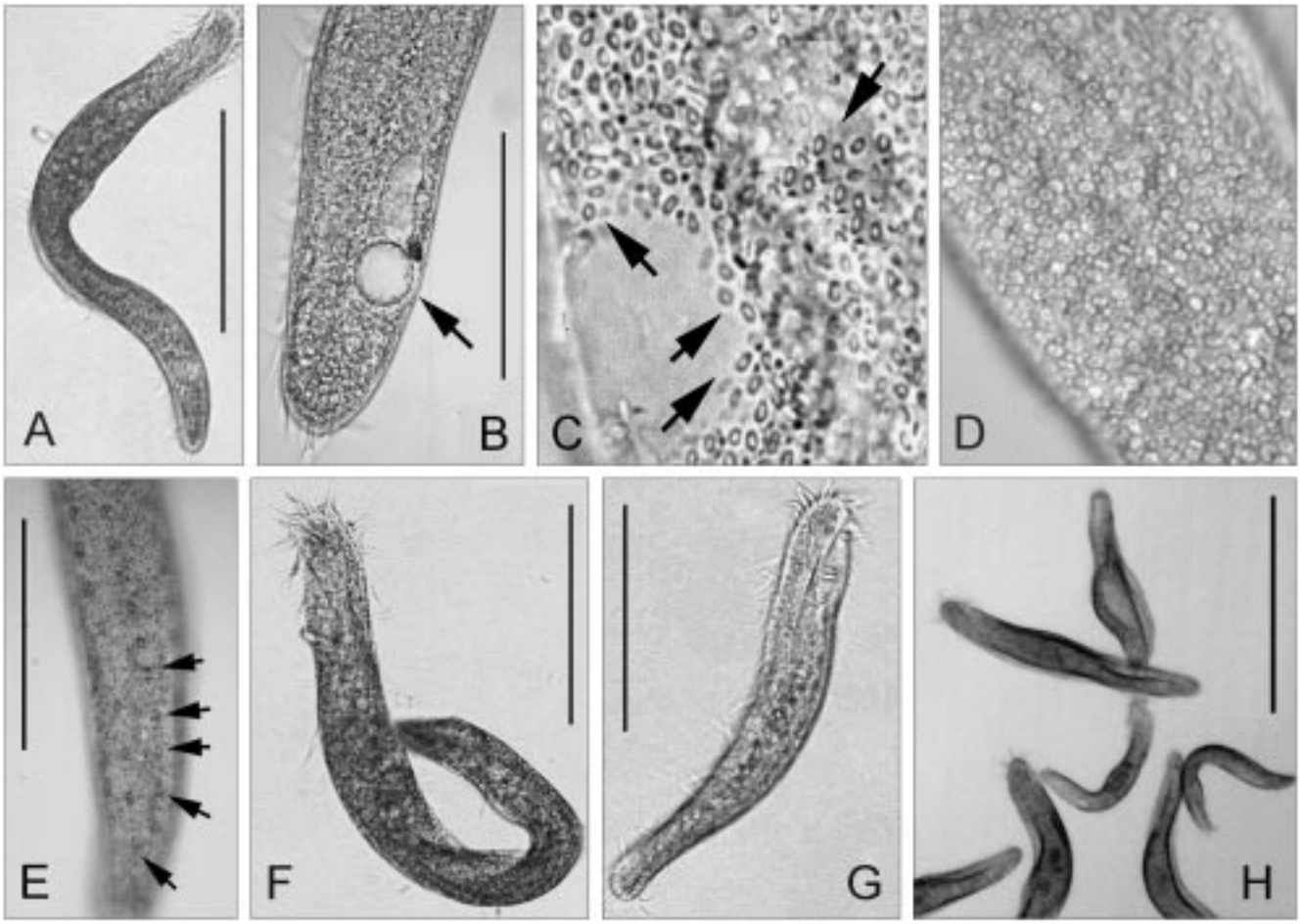
The general cell colour of this species appears much less bright than that of *P. flava*. It is yellowish to yellow-brownish at lower magnifications and yellow-greyish at higher magnifications.

Pseudokeronopsis rubra. As a unique character, the colour both of the pigment granules and of the whole cell may change and/or fade during long-term culture. The pigment granules are small (<0.5 μm in diameter) and generally dark brick-reddish in colour in freshly collected specimens (Figs 7D, F), but this may change while in long-term culture to orange-brownish or even brown-yellowish, similar to *P. flava* and *P. flavicans*. One isolate became completely yellow-brownish after six years in culture. The BCS-granules are about the same size as those in other congeners, oval in shape, densely packed (Figs 7B, C).

Cell colour in freshly collected samples is dark brick-reddish (Fig. 7A) at low magnification but light brick-reddish at higher magnifications.

Contractile vacuole

In some cases, the contractile vacuole is difficult to detect or even undetectable, especially in freshly collected specimens or cells kept in high salinity (>30‰) media. The rate of pulsation is slow, i.e. at several-minute intervals in our populations. The position of the contractile vacuole is constant as in most other hypotrichs (*s.l.*) and is here considered to be one of the most reliable characters for species identification. The appearance and position of the contractile vacuole within the cell are as follows:



Figs 4A-E. Photomicrographs of *Pseudokeronopsis flava in vivo* (A-C, E-G, from Song *et al.* 2004a, the rest are original). **A, F-H** - to show different body shapes and sizes; **B** - caudal portion, arrow indicates the contractile vacuole; **C, D** - detail of cell to demonstrate the BCS-granules (arrows in C); **E** - to show the grouped pigment granules arranged along the dorsal kineties (arrows). Scale bars: 100 µm (A, B, F-H); 50 µm (E).

Pseudokeronopsis carnea. Contractile vacuole in posterior half of cell, usually in posterior 2/5-1/3, easily recognizable (Figs 1A, D, E).

Pseudokeronopsis flava. Contractile vacuole in posterior half of cell, usually in posterior 1/4-1/6, large and detectable in most cases (Figs 1H, K, L; 4B).

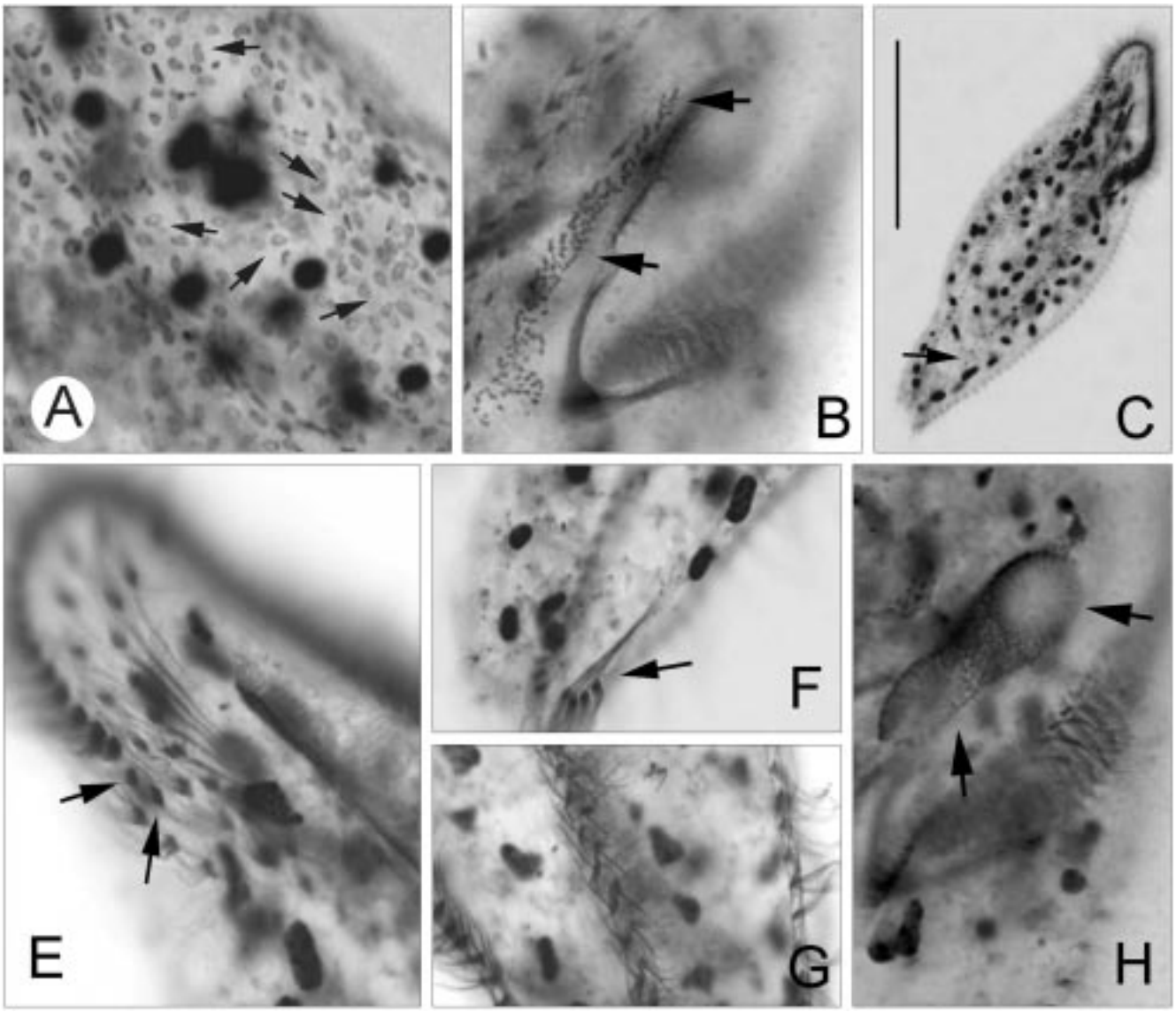
Pseudokeronopsis flavicans. Contractile vacuole in anterior half of cell, usually in anterior 1/3 (Fig. 6A); sometimes undetectable.

***Pseudokeronopsis rubra*.** Contractile vacuole in posterior half of cell, usually in posterior 1/3 (Figs 6G, J, K), often undetectable.

Infraciliature

The infraciliature of three forms, i.e. *P. carnea*, *P. rubra* and *P. flava*, originating from a variety of locations in South Africa, the Indopacific region and several European countries, were described in detail by Wirnsberger *et al*. (1987). These data demonstrated a great deal of inter- and intraspecific diversity (Wirnsberger *et al*. 1987). Consequently consideration of the ciliary features of these taxa, plus those of *P. flavicans*, are here based exclusively on the Chinese populations in order to maintain the consistency of the data.

As Table 1 shows, most aspects of the infraciliature show a high degree of variability between populations of the same species and there is a significant degree of overlap among the four species. Even characters that are very reliable in most other hypotrichs (*s.l.*), e.g. the number of dorsal kineties, tend to be rather variable in these species. Therefore, most aspects of the infraciliature are of limited value for species separation and identifica-



Figs 5A-H. Photomicrographs of *Pseudokeronopsis flava* after protargol impregnation (E-G, from Song *et al.* 2004a, the rest are original). **A** - detailed portion, to show the BCS-granules (arrows); **B** - ventral view, to show the cirral anlagen in the proter (arrows); **C** - ventral view; arrow marks the posterior end of the midventral rows; **E** - ventral view of anterior portion of cell, arrows mark the frontoterminal cirri; **F** - to show the short transverse row (arrow); **G** - ventral view, to show the closely arranged midventral rows; **H** - buccal area in proter, arrows indicate the oral primordium which is located beneath the buccal cavity. Scale bar: 80 µm.

Figs 6A-M. *Pseudokeronopsis flavicans* (A-F) and *Pseudokeronopsis rubra* (G-M) from life (A, B, G-K) and after protargol impregnation (C-F, L, M); (A, C, D, from Song *et al.* 2002; H, from Hu and Song, 2001, the rest are original). **A, B** - ventral view of two typical individuals; note the position of the contractile vacuole; **C, D** - ventral (C) and dorsal (D) views of the same specimen, to show the general infraciliature, arrow in **C** marks the last cirral pair of the midventral rows; **E, F** - ventral (E) and dorsal (F) views of the same specimen; arrows in E mark the posterior portion of the midventral rows; **G, H** - ventral view of two individuals, to demonstrate the different body shapes; **I** - to show the pigment granules, which are grouped around the dorsal cilia, and the BCS-granules; **J** - dorsal view, to show the distribution pattern of the pigment granules and the location of the contractile vacuole (arrow); **K** - ventral view, to demonstrate the distribution of pigment granules; **L, M** - ventral and dorsal views of an atypical specimen with strongly shortened midventral rows; arrow in L indicates the last cirral pair of the midventral rows. Scale bars: 80 µm (A, B, G, H); 50 µm (C-F, L, M).

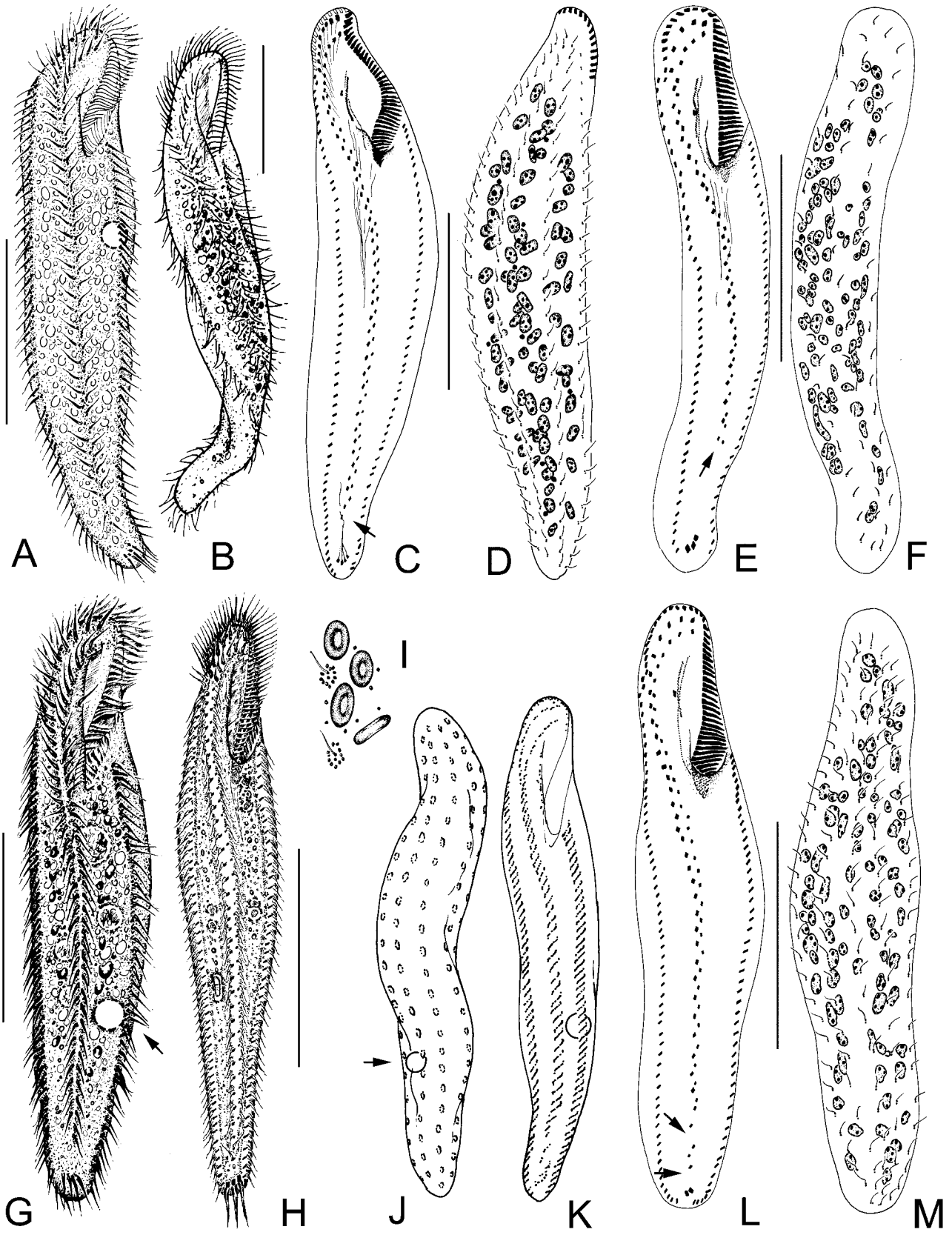

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Table 1. Morphometrical characterization of *Pseudokeronopsis carnea* (1st line), *P. flava* (2nd line), *P. rubra* (1999-population 3rd line; 2000-population 4th line), and *P. flavicans* (5th line). All data based on protargol impregnated specimens of China-populations. CV - coefficient of variation in %, Max - maximum, Min - minimum, n - number of cells measured, SD - standard deviation, SE - standard error of mean.

Character	Min	Max	Mean	SD	SE	CV	n
Body length in µm	208	288	249.6	24.8	6.2	9.9	16
	144	246	175.5	27.1	6.8	15.5	16
	124	264	180.7	37.5	8.2	20.8	21
	152	248	180.3	23.7	5.4	13.1	19
	183	238	209.1	21.2	8.0	8.1	14
Body width in µm	56	96	76.3	10.3	2.6	13.4	16
	48	72	51.75	8.7	2.2	16.8	16
	32	64	50.8	8.2	1.9	16.0	19
	32	56	39.3	6.9	1.6	17.6	19
	40	65	60.1	4.7	1.8	6.2	14
Number of membranelles	69	79	73.1	3.0	0.8	4.1	16
	43	51	45.4	2.6	0.6	5.6	16
	46	60	51.6	3.7	0.8	7.2	20
	40	53	46.9	3.7	0.9	7.9	19
	50	66	56.1	5.5	2.1	9.8	7
Number of cirri in left marginal row	58	79	66.4	5.7	1.4	8.6	16
	41	57	47.8	5.0	1.3	10.4	16
	45	62	52.7	5.2	1.3	9.9	17
	40	64	48.1	6.6	1.5	13.7	19
	40	57	52.4	3.1	1.4	5.8	8
Number of cirri in right marginal row	63	72	67.5	2.8	0.7	4.2	16
	43	60	52.63	5.6	1.4	10.7	16
	48	67	57.9	4.6	1.1	8.0	17
	40	60	49.7	5.6	1.3	11.3	18
	44	65	60.2	3.2	1.3	5.3	8
Number of transverse cirri	8	9	8.6	0.5	0.2	6.0	12
	3	4	3.7	0.5	0.1	12.6	14
	2	4	3.1	0.6	0.2	19.8	16
	3	4	3.7	0.5	0.1	12.6	18
	3	6	3.6	0.9	0.3	26.8	9
Number of frontoterminal cirri	2	2	2	0	0	0	16
	2	3	2.3	0.5	0.1	20.1	12
	2	2	2	0	0	0	20
	2	2	2	0	0	0	18
	2	2	2	0	0	0	11
Number of buccal cirri	1	1	1	0	0	0	14
	1	1	1	0	0	0	7
	1	1	1	0	0	0	21
	1	1	1	0	0	0	18
	1	2	1.1	0.3	0.1	30.9	9
Number of dorsal kineties	7	8	7.5	1.8	0.5	24.1	15
	3	3	3	0	0	0	16
	4	7*	5.1	0.7	0.2	14.0	16
	3	6*	3.8	0.4	0.1	10.6	16
	4	5	4.1	0.4	0.1	8.8	14
Number of cirral pairs in midventral rows	38	43	40.3	1.7	0.4	4.2	16
	24	36	29.6	3.7	0.9	12.3	16
	25	38	30.8	4.2	1.1	13.1	16
	23	35	27.1	3.7	1.0	13.8	13
	25	40	33.4	4.4	1.5	13.1	9
Number of cirral pairs in bicorona**	9	11	10.1	0.8	0.2	7.6	16
	4	6	5.6	0.7	0.2	13.2	13
	5	7	6.3	1.1	0.2	8.4	19
	5	6	5.1	0.4	0.1	6.9	15
	5	9	7.4	1.0	0.3	13.1	10

Table 1. (contd)

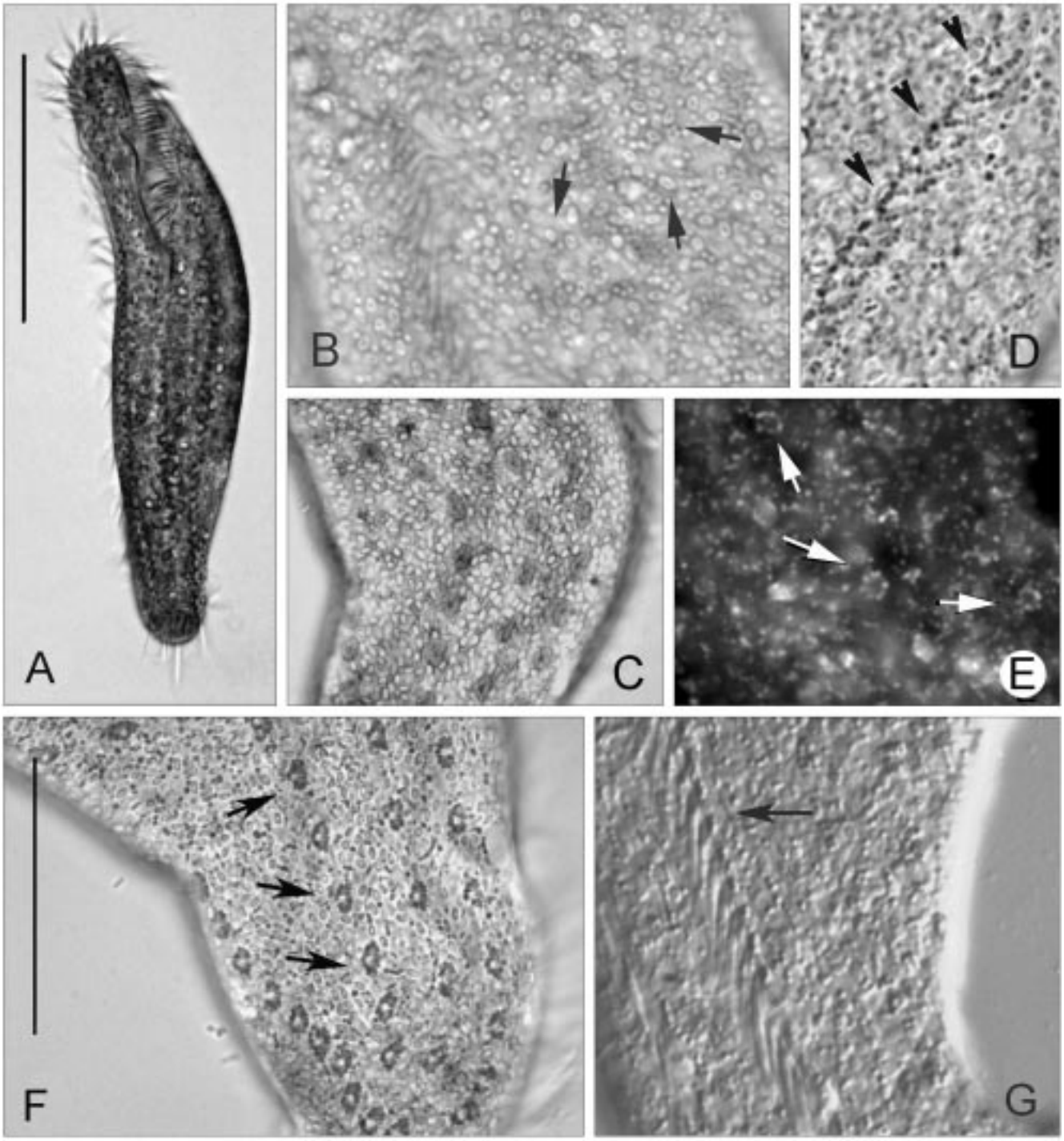
Length of buccal field in µm	80	94	87.6	3.3	0.8	3.8	16
	46	65	55.8	5.3	1.3	9.6	16
	51	76	62.2	7.2	1.6	11.6	20
	52	73	59.8	5.7	1.3	9.5	19
	55	72	65.1	7.4	2.8	9.0	14

* This data include the highly shortened, fragment-like kineties (usually one, seldom two); ** Bicorona: anteriormost frontal cirri, which are positioned in two widely spaced rows.

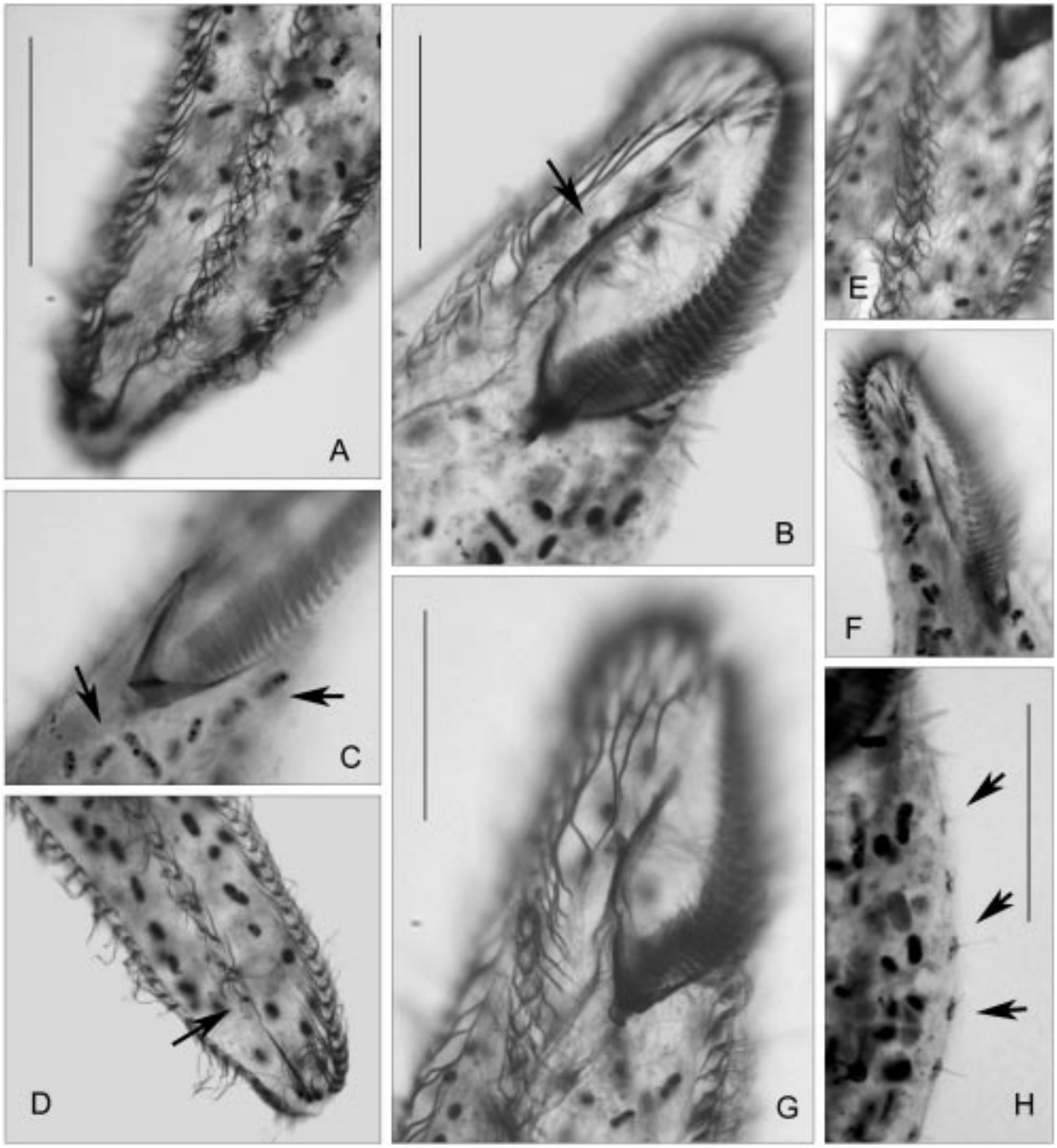
Table 2. Comparison of living morphology of four *Pseudokeronopsis* spp. All data based on the China-populations.

Character	*P. flavicans*	*P. carnea*	*P. flava*	*P. rubra*
Cell length *in vivo* in µm	200-300	180-350	140-350*	140-250
Main features of body shape	slender and often twisted, both cell ends narrowed	plump, frontal area wide, seldom twisted	band-like, cell size extremely variable	slender, both cell ends narrowed
General cell colour at low magnification (<100×)**	yellowish	dark brown- or brick-reddish	yellowish	brick-reddish to orange, yellowish***
Cell colour at mid- to high magnifications****	yellowish	reddish	yellowish	yellow-brownish to reddish***
Pigments on dorsal side at low magnification	undetected	grouped in rows	undetected	undetected
Size of pigments in µm	*ca* 1	*ca* 1	*ca* 0.5	*ca* 0.5
Colour of pigments	yellow-brown	dark, red-brown	yellow-brown	yellow-brown, orange to brick reddish
Shape of blood-cell-shaped granules	ellipsoid*****	completely circular	ellipsoid	ellipsoid*****
Size or length of blood-cell-shaped granules in µm	*ca* 1.2	*ca* 1.5	*ca* 1.2	*ca* 1.2
Ratio of buccal field to the body length	*ca* 1:4	*ca* 1:3	*ca* 1:4-5******	*ca* 1:4
Position of contractile vacuole	anterior 1/3	posterior 2/5-1/3	posterior 1/4-1/6	posterior 1/3
Data sources	Song *et al.* (2002)	present work; Hu *et al.* (2004)	Song *et al.* (2004a)	Hu and Song (2001)

* Some "abnormal", long band-shaped forms occasionally <400 µm long (see Fig. 4A); ** Observed under the stereo-microscope; *** Colour both of pigment granules and of the whole cell clearly fading during long-term culture, i.e. conspicuously variable from dark reddish when freshly collected to yellowish or orange after several weeks/months in culture; **** Cell colour here concerns the general appearance of the cytoplasm instead of the pigment granules (with bright field microscopy at 200× to 600× magnification); ***** Incorrectly depicted/described as round in shape according to Hu and Song (2001) and Song *et al.* (2002); ****** Not including the "abnormal" forms which have extreme length: width ratios (see text).



Figs 7A-F. Photomicrographs of *Pseudokeronopsis rubra* from life. **A -** to show a typical individual; **B, C** - to show the BCS-granules (arrows); **D, G** - ventral view, arrowheads (in D) and arrow (in G) indicate the pigment granules along the marginal row; **E** - with epifluorescence microscopy, arrows mark the grouped pigment granules; **F** - dorsal view, to show the pigment granules (arrows). Scale bars: 100 µm (A); 50 µm (F).



Figs 8A-H. Photomicrographs of *Pseudokeronopsis rubra* after protargol impregnation. **A, B, E-G** - ventral view of different portions of the cell, to show the general infraciliature, arrow in B marks the buccal cirrus; **C** - ventral view, to show the macronuclear segments (arrows); **D** - ventral view of posterior portion, arrow marks the posterior ends of the midventral rows; **H** - to show the dorsal cilia (arrows). Scale bars: 50 µm.

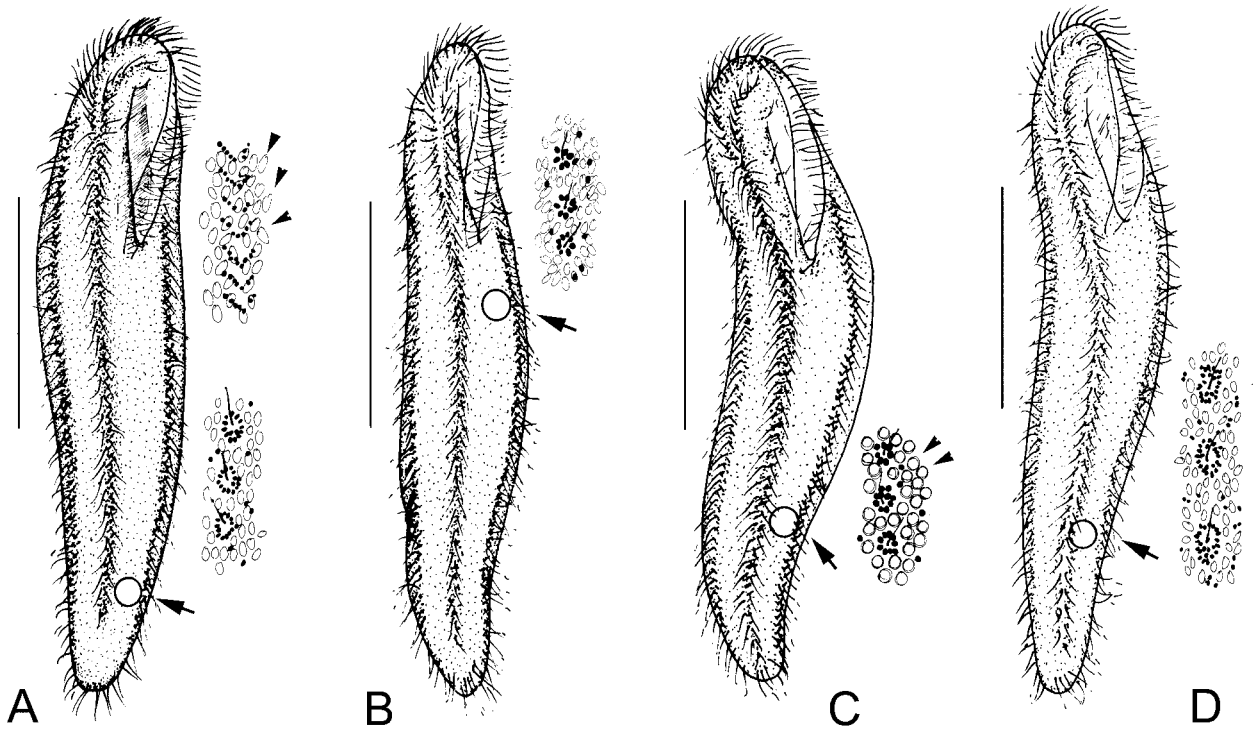


Fig. 9. Schema of four *Pseudokeronopsis* spp., (**A**) *P. flava*, (**B**) *P. flavicans*, (**C**) *P. carnea*, and (**D**) *P. rubra*, to show the general appearance of the living cells and arrangement of the pigment- and BCS-granules (arrowheads in insets). Arrows mark the contractile vacuole. Scale bars: 100 µm.

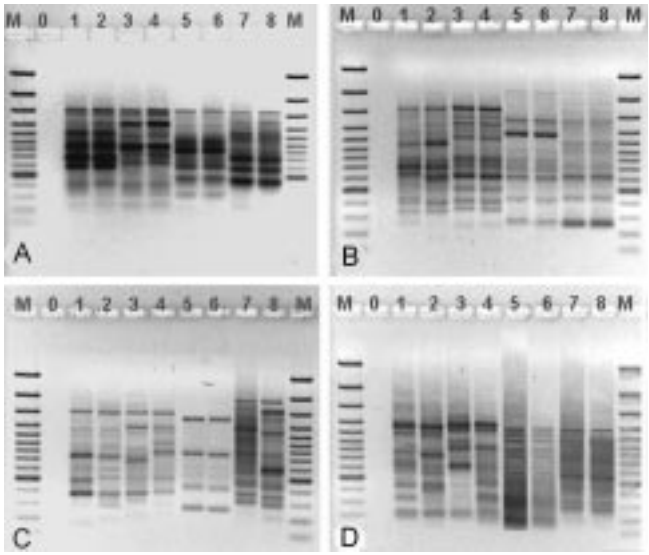
Table 3. Morphometric comparison of four *Pseudokeronopsis* spp, which are most informative in species separation at infraciliature level. All data are based on China-populations only. Distances are in µm.

Characters	*P. flavicans*	*P. carnea*	*P. flava*	*P. rubra*
Number of dorsal kineties	4-5	7-8 (5-8)	constantly 3	mostly 5 (3-7)
Number of cirral pairs in bicorona	~7 (5-9)	9-11 (8-12)	~ 5 (4-6)	~6 (5-7)
Number of transverse cirri	3-6	8-9 (7-11)	3-4	2-4
Number of membranelles	~55	~70 (70-120)	~45	~50
Distance (gap) from the posterior end of midventral rows to the transverse cirri	~15	no gap	~30	0*~15
Source of data	Song *et al.* (2002)	present work; Hu *et al.* (2004)	present work; Song *et al.* (2004a)	present work; Hu and Song (2001)

* This gap is usually so small as to be essentially absent in most specimens.

tion. Since the details of the infraciliature of each of the four species were reported comparatively recently (Hu and Song 2000, 2001; Song *et al.* 2002, 2004a; Shi and Xu 2003; Hu *et al.* 2004), only those of diagnostic value are highlighted here:

Pseudokeronopsis carnea. This species can be separated from the other three species by having: more cirral pairs in both the bicorona (9-11 *vs.* <7) and the midventral rows (*ca* 40 *vs. ca* 30); more transverse cirri (8-9 *vs.* <6); more dorsal kineties (7-8 *vs.* <7). The number of



Figs 10 A-D. RAPD banding patterns of three *Pseudokeronopsis* spp. (six strains) using the following oligonucleotide random primers: (**A**) S2001, (**B**) S2006, (**C**) S2007, and (**D**) S2010. M - 100 bp molecular markers; 0 - control without DNA; 1, 2 - *Pseudokeronopsis flava* pop I; 3, 4 - *P. flava* pop II; 5, 6 - *P. rubra*; 7, 8 - *P. carnea.*

adoral membranelles in this organism is also conspicuously higher than in the other three (*ca* 70 *vs.* <60) (Table 3). In addition, the AZM is relatively long compared to the body length (ratio 1:3), and there is almost no gap between the midventral rows and the transverse cirri (Fig. 1F, arrow).

Pseudokeronopsis flava. The most characteristic features in this species are: the large gap between the midventral rows and the transverse cirri, this being the largest in relation to the body length of the four congeners (Figs 1L, M); the comparatively low number of pairs of cirri in the bicorona (4-6); it constantly has three dorsal kineties. In addition, the AZM is relatively less dominant, the bases of the membranelles being comparatively shorter than those of other three species (Figs 1M, N; 5C).

Pseudokeronopsis flavicans. The general pattern of the infraciliature of this species is very similar to that of *P. flava* although the gap between the midventral rows and the transverse cirri is relatively smaller and there are often more cirral pairs in the bicorona (Fig. 6C). Unlike *P. flava*, *P. flavicans* has four (occasionally 5) dorsal kineties.

Pseudokeronopsis rubra. The infraciliature of this species is similar to *P. flava* and *P. flavicans* (Figs 6E, F). Its most distinctive feature is the number of dorsal kineties which varies from 3 (rarely) to 7 (also very rare, including some shortened fragments of kineties), but is normally in the range 4-6 (Table 1). Also noteworthy is the gap between the midventral rows and the transverse cirri which is so small as to be almost absent in most cases (Figs 6E; 8A, D) whereas it can be conspicuously larger in some specimens (Fig. 6L, arrow).

DNA fingerprinting patterns

RAPD band patterns for three species, *P. flava*, *P. rubra* and *P. carnea* (we failed to extract the genomic DNA for *P. flavicans*), are shown in figure 10. The two strains of the same morphospecies are clearly aligned and distinct from the other strains (1 and 2; 3 and 4; 5 and 6; and 7 and 8). Although some bands differ in brightness, analysis of bands showed that the two populations of *P. flava* have >80% bands in common whereas they have only approximately 30% common bands with the strains of *P. rubra* and *P. carnea*.

Synonyms and improved diagnoses

The diagnoses of all four species have been presented in separate publications in recent years (Wirnsberger *et al.* 1987; Hu and Song 2000, 2001; Song *et al.* 2002, 2004a; Hu *et al.* 2004). Wirnsberger *et al.* (1987) listed the synonyms and re-diagnosed three of the four species discussed here, i.e. *Pseudokeronopsis rubra, P. flava* and *P. carnea*, and we completely agree with their findings. Nevertheless, we consider it necessary to provide updated diagnoses based upon a standard set of criteria. Additional information and revised lists of synonyms are also supplied.

Pseudokeronopsis carnea (Cohn, 1866) Wirnsberger, Larsen *et* Uhlig, 1987

Oxytricha flava var. *carnea* Cohn, 1866

Pseudokeronopsis pulchra Borror *et* Wicklow, 1983

Pseudokeronopsis pararubra Hu, Warren *et* Suzuki, 2004

Pseudokeronopsis rubra sensu Shi *et* Xu, 2003

Improved diagnosis. Marine, dark reddish *Pseudokeronopsis*, usually 150-350 µm long *in vivo* with buccal field about 1/3 of body length; pigment granules dark brown-reddish or brick-reddish, arranged in typical *rubra*-pattern; BCS-granules completely circular in shape bicorona comprises 8-12 pairs of frontal cirri. 6-11 transverse cirri and 5-8 dorsal kineties; contractile vacuole positioned in posterior 2/5 to 1/3 of body; almost no gap between midventral rows and transverse cirri.

***Pseudokeronopsis flava* (Cohn, 1866) Wirnsberger, Larsen *et* Uhlig, 1987**

Oxytricha flava Cohn, 1866
Holosticha flavorubra var. *flava* Entz, 1884
Keronopsis flava (Cohn, 1866) Kahl, 1932

Improved diagnosis. Marine yellowish *Pseudokeronopsis*, usually 140-350 µm long *in vivo*; pigment granules dark brown-yellow, arranged in typical *rubra*-pattern; BCS-granules oval in shape; bicorona comprises 5 pairs of frontal cirri. 2-4 transverse cirri; midventral rows terminate conspicuously above the transverse cirri; 3 dorsal kineties; contractile vacuole positioned in posterior 1/4 to 1/6 of body; conspicuous gap between midventral rows and transverse cirri.

***Pseudokeronopsis flavicans* (Kahl, 1932) Borror *et* Wicklow, 1983**

Keronopsis flavicans Kahl, 1932

Improved diagnosis. Yellowish-coloured marine *Pseudokeronopsis*, about 200-300 µm long *in vivo*; pigment granules dark yellow-brown, arranged in typical *rubra*-pattern; BCS-granules oval in shape; bicorona comprising 5-9 pairs of frontal cirri; 3-6 transverse cirri; midventral rows terminate conspicuously above the transverse cirri; 4-5 dorsal kineties; contractile vacuole in anterior 1/3 of body; gap between midventral rows and transverse cirri inconspicuous.

***Pseudokeronopsis rubra* (Ehrenberg, 1838) Borror *et* Wicklow, 1983**

Oxytricha rubra Ehrenberg, 1835
Holosticha rubra Wallengren, 1900
Keronopsis rubra Kahl, 1932

Improved diagnosis. Brown-reddish to orange-yellowish marine *Pseudokeronopsis*, 140-250 µm in length *in vivo*; pigment granules brick-reddish to dark yellow-brown; BCS-granules oval in shape; bicorona with 5-7 pairs of frontal cirri; 2-4 transverse cirri; midventral rows usually terminate conspicuously above the transverse cirri; normally 4-6 dorsal kineties; contractile vacuole in posterior 1/3 of body; gap between midventral rows and transverse cirri variable, very small to conspicuous.

Deposition of the neotypes and voucher material

Neotype specimens of three forms, i.e. *P. flava*, *P. rubra* and *P. carnea*, have been deposited by Wirnsberger *et al.* (1987) in the Upper Austrian Museum in Linz, Austria, while the neotype slide of *P. flavicans* is deposited in the Laboratory of Protozoology, OUC, China (see Song *et al.* 2002). Voucher slides with protargol-impregnated specimens of China-isolates of all four species are deposited in the Natural History Museum, London, UK with the following registration numbers: *Pseudokeronopsis carnea* - one slide was deposited as the holotype of *Pseudokeronopsis pararubra* (misidentification - see Hu *et al.* 2004) - 2004:6:2:1, and a second as a voucher slide - 2005:3:24:10. *Pseudokeronopsis flava* - 2005:3:24:9. *Pseudokeronopsis flavicans* - 2001:12:28:3. *Pseudokeronopsis rubra* - 2005:3:24:11.

Key to the four morphologically similar *Pseudokeronopsis* spp.

1 Contractile vacuole positioned in anterior part of the cell.....*Pseudokeronopsis flavicans*
1' Contractile vacuole positioned in posterior part of the cell.....2
2 Blood-cell-shaped cortical granules completely round in shape; 8-9 transverse cirri; 8-12 pairs of cirri in bicorona.....*Pseudokeronopsis carnea*
2' Blood-cell-shaped cortical granules oval in shape; 5 transverse cirri; <8 pairs of cirri in bicorona.....3
3 Cell brown- to brick-reddish; 4-6 dorsal kineties; midventral rows not conspicuously shortened posteriad.. .....*Pseudokeronopsis rubra*
3' Cell yellowish; constantly 3 dorsal kineties; midventral rows conspicuously shortened posteriad..... .....*Pseudokeronopsis flava*

Conclusions

With reference to the data relating to the infraciliature, the four marine, coloured, *Pseudokeronopsis* spp. discussed here are difficult to identify. Consequently, a revised list of reliable and informative characters for species circumscription and separation is supplied, which are mostly obtained from live observations. These are: (1) position of the contractile vacuole; (2) shape of BCS-granules; (3) colour of cell and pigments; (4) number of dorsal kineties; (5) number of transverse cirri; (6) the number of pairs of frontal cirri in the bicorona, and (7) the gap between the midventral rows and the transverse cirri.

It is noteworthy that in their excellent work, Wirnsberger *et al.* (1987) reached similar conclusions and could distinguish three species, i.e. *Pseudokeronopsis rubra*, *P. flava* and *P. carnea*, by chromatography of the pigments. Likewise, as shown

here, the RAPD banding patterns demonstrate that the species separated by morphology are also clearly distinct genomically. By contrast, as revealed by both previous and the present studies, the infraciliature in all these four morphotypes is generally stable within populations but may show significant differences between populations, especially among those that are geographically well separated. Therefore care should be taken when using such characters for species identification.

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