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A Morphometric Study of Euryhalinity in Marine Populations of the Ciliate Genus *Euplotes*

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ABSTRACT. Twenty different clonal strains of marine and brackish *Euplotes*, representing four morphotypes, were tested for hyposalinity tolerance by a method which gradually acclimated the cells to lower salinity medium. The lowest salinities in which the strains could thrive ranged from 60% of normal seawater to complete freshwater. The morphological effects of culture medium salinity were also examined for two strains of a small "*Euplotes charon*" morphotype, as well as for two mating compatible "*Euplotes vannus*" strains and several of their exconjugates. There were no differences between the euryhaline strains grown in fresh or saltwater, except for a slight increase in overall cell size in one strain when cultured in freshwater medium. *E. vannus* strains increased in overall cell size with decreased salinity; also, the dorsal surface of the cells can become disorganized when the cells are cultured in 30% normal seawater.

Supplementary key words. Hyposalinity tolerance, morphometrics, species problem, systematics.

CILIATES occur in a wide range of moist environments, from purely aquatic ones to soil microhabitats and a variety of parasite niches [5]. Many ciliate species are commonly found in both fresh and marine waters. While some forms are stenohaline, many are quite euryhaline, with some strains of certain species able to thrive equally well in freshwater or seawater habitats [10]. Euryhalinity has a profound effect on an organism's ecology, enabling it to exploit a wide range of stable habitats, as well as variable habitats, such as tidal rivers or salt lakes where seasonal runoff can produce extreme salinity changes.

Euryhalinity also has implications for ciliate systematics. Within the genus *Euplotes*, for example, most species descriptions simply report each new strain as being either marine or freshwater, with no attempt to acclimate the cells to different salinities [8]. There are, in fact, several strikingly similar species descriptions differing only in their reported habitats, such as *E. crenosus* Tuffrau, 1960 (freshwater) and *E. octocirratus* Agamaliev, 1967 (marine).

Ciliates respond to the osmotic stress of their environment via several strategies. Contractile vacuole complexes are commonly present for balancing water influx [for a complete review, see 24], whereas intracellular solute concentrations in some cases are adjusted to prevent water efflux [6, 22, 23, 26].

This study determines the range of salinities in which several different strains of marine and brackish *Euplotes* can thrive. Such tests of salinity tolerance can be conducted in two general ways: by a gradual change of salinity, or by a direct transfer of cells to a new salinity. These methods test different attributes of the cells. The gradual change method tests the ability of the cells to acclimate to a new osmotic environment, while the direct transfer method tests the ability of the cells to withstand a sudden osmotic shock. Cells which are found to be euryhaline by the gradual change method may not be able to tolerate a direct transfer from marine to freshwater media. A further issue in testing the salinity tolerance of strains is how long the strains can survive in the new medium. To be considered truly euryhaline, cells not only would have to survive the change in salinity, but also would have to continue to grow and divide in the new environment. Both direct transfer and a rather complicated gradual change method were employed by Finley [11] to test the salinity tolerances of many freshwater species of protists (including species he identified as *E. charon* and *E. patella*). In recent studies, Gianni & Piras [19, 20] used direct transfer methods to determine the extremes of hyposalinity and hypersalinity tolerances in the *vannus-crassus-minuta* group of *Euplotes*.

Gause [17] was the first to examine some of the morphological effects of salinity changes on two species of *Euplotes*, using a gradual transfer procedure. Gause subjected freshwater clonal strains he identified as *E. patella* to a culture medium of 1% salinity, as calculated by the salts to water mass ratio (normal seawater is 3.7%). Gause maintained the cells at this salinity for 18 days. He then compared the cell lengths and widths of strains grown in freshwater to those grown in 1% salinity, finding that the latter conditions yielded significantly smaller cells. Gause also noted that in strains he identified as *E. vannus*, a change in salinity from 2.5–5% caused a significant decrease in cell size. To examine the nature of this change, Gause took compatible mating types of *E. vannus* grown in both 2.5 and 5% salinity and mixed them; he then measured the subsequently cultured exconjugates. The 5% exconjugates were significantly smaller than the average of the 5% parental strains; the 2.5% exconjugates, however, were not different from the 2.5% parental average. When the 5% exconjugates were brought gradually to 2.5% salinity, they remained significantly smaller than the 2.5% control exconjugates, suggesting to Gause that selection had taken place. When the cells were taken to 7% salinity, no such adaptations were observed (it was in fact lethal to the strains). Similarly, no such adaptations were observed when the cells were brought to 1% salinity.

Along with testing the salinity tolerance of several strains of *Euplotes*, this study examines the morphological effects of salinity, partially repeating Gause's [17] work with greater detail. Strains of two different morphotypes of *Euplotes* were used for

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Table 1. List of strain designations, sampling locations and dates, sampling habitat and range of salinity tolerance, arranged by morphotype.

Strain	Location	Date habitat	Salinity range[a]
Cirrotype 10, double dargyrome (*E. charon* morphotype)			
Ant3	Antigua, W.I.	7/88 Marine	100–10
Cal4	San Simeon, CA	4/84 Brackish	100–0
Can3	Cancun, Mexico	3/84 Marine	100–20
Cay2	Grand Cayman, W.I.	11/81 Marine	100–50
CC1	Plymouth, MA	3/90 Marine	100–5
CC12	Rocky Neck, CT	3/90 Marine	100–10
CC31	Plymouth, MA	3/90 Marine	100–5
Galv1	Galveston, TX	9/82 Brackish	100–0
Jfla5	Fort Lauderdale, FL	7/87 Marine	100–20
Kli2a	Emerald Island, NC	3/90 Marine	100–30
KK8	Knights Key, FL	3/90 Marine	100–2.5
Perd1	Perdido Key, FL	3/90 Marine	100–10
Mia1	Miami Beach, FL	7/86 Marine	100–20
SI4	Chatham, MA	8/90 Marine	100–30
SI10	Chatham, MA	8/90 Marine	100–10
Cirrotype 9, double dargyrome (*E. affinis* morphotype)			
Kli2b	Emerald Island, NC	3/90 Marine	100–0
Cirrotype 7, double dargyrome (*E. raikovi* morphotype)			
Mor1	Casablanca, Morocco	7/90 Marine	100–10
Cirrotype 10, single dargyrome (*E. vannus* morphotype)			
Ost3	Osteende, Belgium	9/90 Marine	100–60
Elba	Elba, Italy	< 79 Marine	100–10
LL1	San Terenzio, Italy	6/77 Marine	100–<30[b]

[a] The salinity ranges are given as percentage dilutions of artificial seawater.

[b] The strain LL1 was brought to 30% SWM by the direct transfer method and was not tested below 30% salinity.

this morphometric study, including strains of *E. vannus* (cirrotype 10, single dargyrome) and two geographically distant strains of a smaller, cirrotype 10, double dargyrome *Euplotes* isolated from brackish water, belonging to a yet undescribed species.

MATERIALS AND METHODS

Cultivation. Culture medium for the marine ciliates was marine Cerophyl® (MCER) based on an artificial seawater medium (SWM) enriched with a cereal grass extract [14], while the freshwater medium (FWM) was based on a nutrient-enriched soil extract [14]. The various salinities used in the euryhalinity experiments were expressed as percent dilutions of MCER. To produce these intermediate salinities, MCER was diluted with CER: FWM enriched with cereal grass extract [14]. *Euplotes* from raw samples (collected from various geographic locations described in Table 1) were isolated as single cells to create clonal cultures.

Morphometric data collection. Cells were stained by a modification of the Chatton-Lwoff silver staining technique [4, 12]. Cell measurements were taken using a Leitz Dialux microscope equipped with bright field planapochromatic optics and a Leitz tracing device (Ernst Leitz Wetzlar GMBH, Wetzlar, Germany). Specimens were viewed at 500× magnification for simple length/width measurements, and at 1,250× under oil immersion for more detailed measurements. All specimens selected for measurement were well impregnated with silver stain and had either their dorsal or their ventral surfaces oriented perpendicular to the focal axis. Cells to be measured were centered in the field of view, and the crosshairs of a Bitpad One electronic digitizer (Summagraphics, Fairfield, CT), as seen through the drawing tube, were superimposed on the various points of interest and these points were digitized.

For each strain used in the morphometric analyses, samples (at least 100 cells) of cell lengths and widths were collected. More detailed measurements (Fig. 1, Table 2) were taken for a morphometric comparison of two small, cirrotype 10, double dargyrome strains, and of *E. vannus* strains grown at selected salinities. These detailed measurements of 25 ventral surfaces included the end points of the length and width axes (and the point where the peristomial shelf crosses the width axis), the positions of the individual frontoventral, transverse and caudal cirri, the positions of the inner edges of the adoral zone of membranelles and the endpoints of the paroral membrane (along the length and width axes). The measurements of 25 dorsal specimens (necessarily different specimens from the ventral measurements) included the number and position of the kineties, and the number and position of the kinetosomes within each kinety. Also on the dorsal surface, the central point of each sub-pellicular vacuole in the central three rows (the right-most row being determined by the anterior end of the AZM) was digitized.

Statistical methods. Unless indicated otherwise, all data were analyzed using the S statistical package [2] on an AT&T 3B15 super-minicomputer. The general univariate statistics used (means, variances, etc.) are described in Sokal & Rohlf [25]. The sums of squares simultaneous test procedures were done on an IBM PC using the SSSTP program given in Sokal & Rohlf [25]. This method tests all possible comparisons among means and, based on a given confidence level, produces groups of means which are significantly different from one another.

Boxplots (box-and-whisker plots) [see 29] were used to graphically present some data because they give a clear picture of both the central tendencies and the spread of the data. The "box" of the boxplot encapsulates the central 50% of the data (defined by the upper and lower quartiles), with the centerline denoting the median. The "whiskers" encompass the standard range of the data, with very extreme values plotted as asterisks.

Multivariate analysis was conducted using principal components analysis (PCA) [see 27]. In this study PCA was done in two different ways. One way was by taking all the variables (including the counts), standardizing them, and creating the correlation matrix. The other method excluded the counts from the data, thereby creating a matrix of linear measurements with the same units (micrometers). This method employs the covariance matrix and gives a truer sense of the variational structure of linear data [27].

Gates [13, 14] described a reliable and useful method of quantifying the patterns of the ventral cirri using relative intercirral distance distributions. When 17 subclonal strains of *E. harpa* Stein, 1859 were grown under various temperature and feeding regimes [14], the largest Kolmogorov-Smirnov statistic (K-S) [see 14] obtained among the 17 genetically identical strains was 0.0693, a number which provides a conservative standard by which other strain comparisons can be judged (and will be in this study).

Test of salinity tolerance by gradual transfer. Cells for this experiment were grown to densities of 42–84 cells/ml in 100 × 20 mm polystyrene petri plates with double-strength MCER. Stock solutions were prepared of intermediate salinities (90%, 80%, . . . , 10%, 5% and 2.5% MCER). The volume of the dilution dish (DD), containing the cells growing in their original culture medium, was adjusted to 50 ml. Changes in salinity of the DD were made by successively removing a specified volume of the existing medium and replacing it with CER, such that the salinity of the medium in the DD was decreased by 5% at each step. Cells were allowed to accommodate for 30 min be-

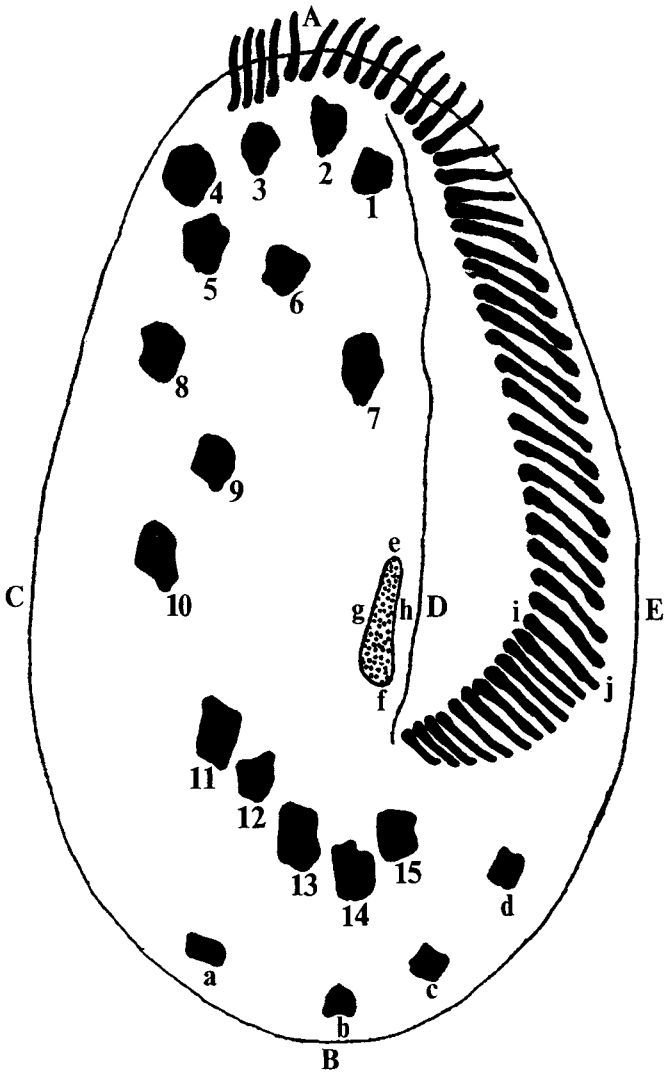

Fig. 1. Diagram of the ventral surface of a cirrotype 10 *Euplotes* with the digitized points identified. Figure abbreviations: A, anterior cell margin; B, posterior cell margin; C, right cell margin; D, point where the peristomal shelf crosses the width axis; E, left cell margin; 1–10, frontoventral cirri; 11–15, transverse cirri; a–d, caudal cirri; e–h, anterior, posterior, right and left (respectively) margins of the paroral membranelle; i & j, endpoints of the longest adoral membranelle.

Table 2. List and description of linear measurements and counts for the ventral surface of cirrotype 10 *Euplotes*; standard points refer to landmarks on Fig. 1B.

Measurement	Point	Point	Description
1	A	B	cell length
2	C	E	cell width
3	C	D	ventral plate width
4	2	7	streak I, top section
5	7	15	streak I, bottom section
6	3	6	streak II, top section
7	6	14	streak II, bottom section
8	4	5	streak III, top section
9	5	13	streak III, bottom section
10	8	9	streak IV, top section
11	9	12	streak IV, bottom section
12	10	11	streak V
13	1	15	buccal cirrus to transverse cirrus 1
14	2	14	frontoventral-transverse (FVT) field
15	7	8	width of FVT field
16	7	10	oblique mid-width of FVT field
17	4	7	oblique anterior FVT field width
18	11	15	width of transverse cirral (TC) field
19	11	14	lateral length of TC field
20	—	—	number of caudal cirri
21	—	—	mean spacing of caudal cirri
22	—	—	# of adoral zone membranelles (AZM)
23	—	—	mean spacing of AZM
24	i	j	maximum width of AZM
25	e	f	paroral membranelle (PM) length
26	g	h	PM width
27	A	14	anterior cell margin to TC
28	A	e	anterior cell margin to PM top
29	C	g	right cell margin to right PM margin

tween salinity changes. At specific salinities (those of the intermediate stock solutions), the removed medium was placed in one of the wells of a 6-well, flat-bottom, polystyrene tissue culture plate (Falcon 3046) as a growth dish (GD). To each GD, 1 ml of the appropriate medium was added at 30 min intervals, until the GD was brought to a premarked, 10 ml level. The wells were immediately checked to see that there were at least 20 cells. The GD were stored in plastic 24 × 18 × 8 cm sealed containers with dampened blotter paper to prevent evaporation, maintained for three weeks, and checked periodically for survival and evaporative loss (which was replaced slowly with double-distilled water).

Test of salinity tolerance by direct transfer. Cells for this experiment were grown to high densities in 100 × 20 mm polystyrene petri dishes, concentrated by centrifugation to 1 ml, and allowed to recover for about 30 min. Meanwhile, the wells of two 6-well polystyrene tissue culture plates (Falcon 3046) were filled to 10 ml with medium of the salinities described above. The cells were gently vortexed to distribute them evenly, and 75 μl of the concentrated cells (> 100 cells) was slowly injected into each of the wells. The cells were checked immediately, and periodically thereafter for three weeks, for survival. This method was used to produce the parental strains used in the mating experiments, and it was also used for strains which were found to be completely euryhaline by the gradual transfer method.

Standard mating procedure. Two clonal and mating compatible strains of *E. vannus*, LL1 and Elba (which were known to produce viable and fertile exconjugates at 100% salinity), were brought to and maintained in cultures from 100–30% media salinities (at 10% increments) via the direct transfer method. Mating tests only involved strains of identical culture medium salinity. The strains were induced to conjugate after mixing by three rounds of centrifugation and rinsing with sterile saltwater of the appropriate salinity. The final centrifugation yielded 1 ml of concentrated cells; after about 8 h, firm pairs usually had formed. Four pairs from each of the dilutions were isolated separately in the central wells (containing a few drops of the appropriate medium) of three-spot depression slides. Once the pairs had separated, the exconjugates were isolated into the outer two wells of the depression slides. If anlagen were seen and cell division did not commence for over 24 h, the cells were assumed to be true exconjugates. Exconjugate strains from three of these pairs were kept. The parental strains were designated by the first two letters of their original strain name, followed by a number corresponding to the salinity at which they were grown (e.g. Elba grown in 40% SWM is called EL40). The exconjugate strains were designated by first giving the salinity at which they were

Table 3. Summary table of parental and exconjugate strains grown in various salinities. SD, standard deviation; n, sample size.

	100% SWM strains						
	EL100	LL100	100x2a	100x2b	100x3a	100x3b	100xm[a]
Length (μm)							
Mean	83.44	81.86	90.34	90.08	92.83	89.26	90.61
SD	7.01	6.78	5.57	5.26	6.02	5.73	5.76
Width (μm)							
Mean	54.15	55.94	63.34	64.90	62.73	62.28	63.32
SD	6.57	6.55	5.67	5.06	7.09	5.99	6.02
n	105	102	51	51	49	51	202
	90% SWM strains						
	EL90	LL90	90x2a	90x2b	90x3a	90x3b	90xm[a]
Length (μm)							
Mean	90.66	97.84	92.64	89.81	89.46	88.58	90.13
SD	6.08	6.09	6.66	6.09	6.64	4.58	6.21
Width (μm)							
Mean	57.27	72.57	65.59	62.97	60.59	63.00	63.04
SD	5.51	6.12	6.52	7.64	6.67	6.31	7.00
n	52	55	52	53	52	51	208
	80% SWM strains						
	EL80	LL80	80x2a	80x2b	80x3a	80x3b	80xm[a]
Length (μm)							
Mean	92.69	95.06	94.82	91.03	92.21	95.23	93.32
SD	5.65	8.93	9.66	6.78	5.78	5.58	7.35
Width (μm)							
Mean	63.22	71.76	62.25	52.10	60.09	70.94	61.39
SD	5.04	8.53	6.14	4.42	6.35	6.72	8.93
n	51	51	54	52	52	50	208
	70% SWM strains						
	EL70	LL70	70x1a	70x1b	70x3a	70x3b	70xm[a]
Length (μm)							
Mean	86.70	94.90	92.27	89.06	96.35	102.21	94.92
SD	5.96	7.88	5.82	6.04	6.45	6.78	7.94
Width (μm)							
Mean	58.90	70.37	68.21	66.78	70.15	75.39	70.10
SD	6.54	6.84	5.89	6.49	6.63	8.35	7.58
n	52	50	52	51	50	50	203
	60% SWM strains						
	EL60	LL60	60x1a	60x1b	60x2a	60x2b	60xm[a]
Length (μm)							
Mean	92.09	81.06	97.90	99.34	96.59	94.35	97.00
SD	7.11	7.34	6.25	5.13	6.70	5.68	6.22
Width (μm)							
Mean	64.69	66.86	70.36	70.24	70.79	70.97	70.60
SD	6.25	9.14	7.34	5.54	7.89	8.40	7.36
n	51	50	51	52	55	55	213
	50% SWM strains						
	EL50	LL50	50x1a	50x1b	50x2a	50x2b	50xm[a]
Length (μm)							
Mean	95.78	98.61	92.93	92.61	105.67	101.07	98.17
SD	6.78	6.75	4.84	5.67	8.67	10.62	9.57
Width (μm)							
Mean	64.22	75.24	65.15	62.54	76.67	80.36	71.32
SD	6.35	8.55	5.95	5.49	7.78	8.78	10.35
n	51	55	52	52	54	54	212

Table 3. Cont.

	40% SWM strains						
	EL40	LL40	40x1a	40x1b	40x2a	40x2b	40xm[a]
Length (μm)							
Mean	96.23	85.80	102.86	96.09	94.34	96.88	97.58
SD	5.30	6.18	7.74	5.87	5.27	5.94	7.04
Width (μm)							
Mean	63.26	56.49	81.27	72.47	69.32	78.29	75.35
SD	4.52	6.33	10.25	8.43	6.33	9.94	10.01
n	50	52	54	52	53	51	210
	30% SWM strains						
	EL30	LL30	30x1a	30x1b	30x2a	30x2b	30xm[a]
Length (μm)							
Mean	91.52	95.25	108.84	98.46	106.03	98.90	103.07
SD	6.10	6.50	8.58	5.88	7.92	5.05	8.30
Width (μm)							
Mean	62.45	70.58	86.67	79.64	89.50	82.90	84.69
SD	6.69	7.77	10.16	7.90	8.72	7.51	9.36
n	103	106	53	54	54	52	213

[a] These are the exconjugate strains' means within the given salinities.

produced and grown, followed by an "x," followed by the number of the isolated pair, followed by a letter designating the specific exconjugate of a pair (e.g. exconjugate "a" of the third pair isolated from a mating at 60% SWM is called 60x3a).

Morphological effects of salinity on *E. vannus*. The parental strains and the exconjugate strains produced by the standard mating procedure were cultured at the appropriate salinities for at least three weeks before proceeding with fixation and staining. Six exconjugate cell lines were created by the above described technique for all salinities except 30% SWM. In this case, although conjugation was apparently successful in 30% salinity, cells became bloated and spherical during the reorganization period and died without dividing. As an alternative, six 30% SWM exconjugate strains were produced by directly transferring some of each of the 40% exconjugate strains, which were by then dividing regularly, to 30% SWM.

The parental and exconjugate strains were maintained on similar light and feeding schedules for three weeks before they were fixed, stained, digitized and statistically processed. Lengths and widths of 100 specimens of the parental strains at each salinity and 50 specimens each of the four exconjugate lines (produced from two conjugating pairs at each salinity) were tabulated. In addition, detailed ventral measurements (Table 2) were calculated from the coordinate data for 25 specimens of each of the parental and exconjugate strains grown at 100% and 30% SWM. From the latter strains, 25 dorsal specimens each (necessarily different individuals from the ventral specimens) were used to tabulate the number of kinetosomes, kineties and the number of alveoli in the central three rows. Also calculated were the average longitudinal spacing of kinetosomes over the entire cell and in the central kinety.

Morphological effects of salinity on two euryhaline strains of *Euplotes*. Two strains of cirrotype 10, double dargyrome *Euplotes*, which were cultured in 33% MCER from originally brackish water samples, were used in this experiment. These geographically separate strains, Cal4 (San Simeon, CA) and Galv1 (Galveston, TX), were found to be completely euryhaline by the gradual transfer method (see results section). Therefore, the strains were cultured in both 100% SWM and in FWM for over

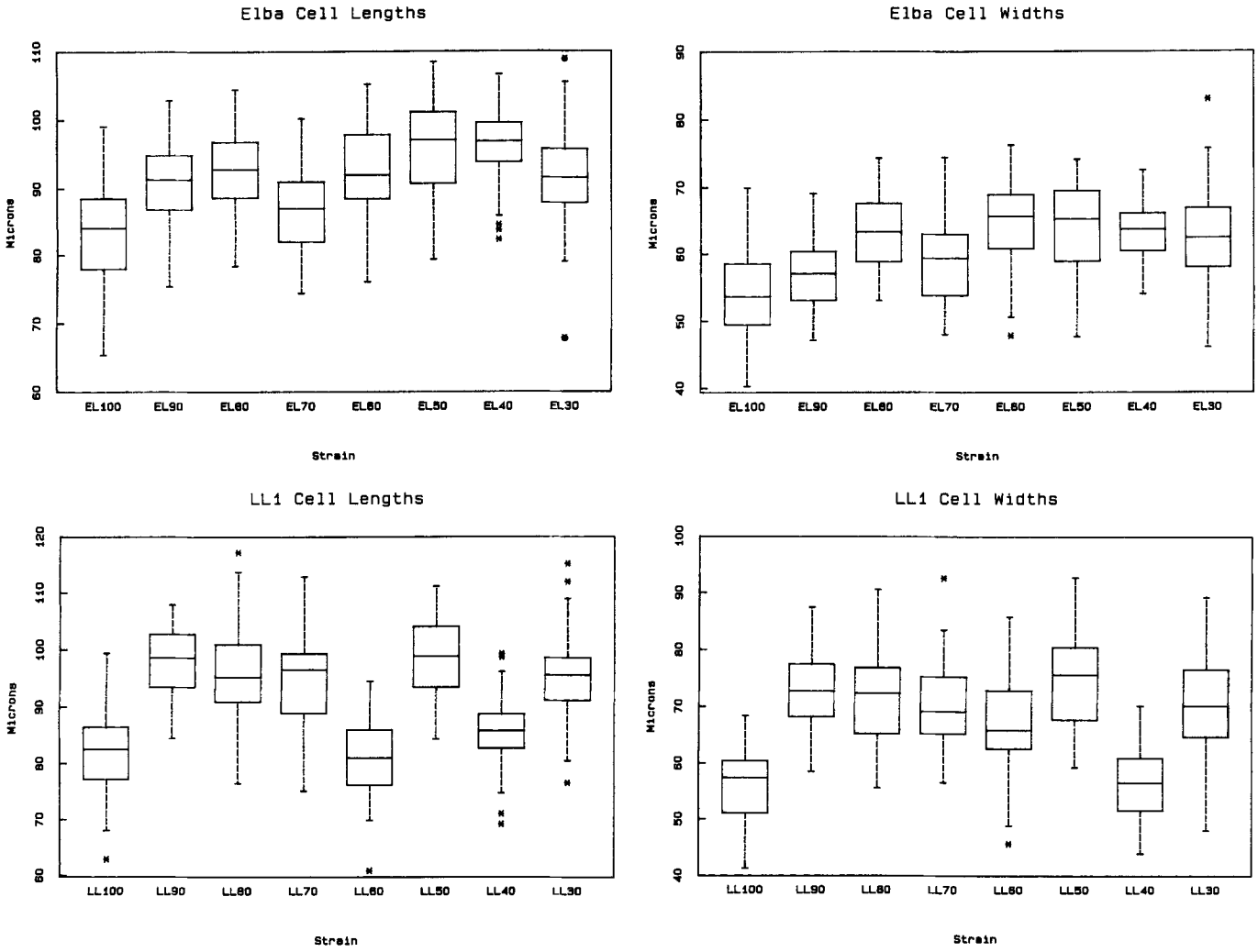

Fig. 2. Boxplots of cell lengths and widths of *E. vannus* strains Elba (EL) and LL1 (LL) cultured in various salinities. The culturing salinities are given as suffixes to the strain abbreviation, e.g. LL80 = LL1 cultured at 80% of normal saltwater medium. **a**. boxplots of Elba cell lengths. **b**. boxplots of Elba cell widths. c. boxplots of LL1 cell lengths. **d**. boxplots of LL1 cell widths.

a month before being fixed and stained for morphometric analysis.

The effects of the culture medium on the gross measurements of cell length and width were examined for 100 specimens of each strain. A more detailed analysis of morphological effects of salinity was conducted on strain Cal4, using all the ventral landmarks for 25 specimens from each culture medium.

RESULTS

Ranges of salinity tolerance among selected strains of *Euplotes*. There was considerable variation in hyposalinity tolerance among the various marine and brackish strains of *Euplotes*, ranging from complete euryhalinity, to only being able to thrive as low as 60% SWM (Table 1). The two cirrotype 10, double dargyrome strains which were cultured from brackish samples (Cal4 and Galv1) were among the three completely euryhaline strains. The third euryhaline strain (Kling2b) was of the cirrotype 9, double dargyrome morphotype (the *E. affinis* morphotype as described by Gates [15]) and was cultured from a marine sample taken from a North Carolina beach. The two *E. vannus* morphotypes (cirrotype 10, single dargyrome) had quite different salinity tolerances. The smaller *vannus* type (Ost3) had the least hyposalinity tolerance of all the strains tested, at 60% SWM. Elba is a larger *vannus* type that was able to thrive at salinities as low as 10% SWM. The one cirrotype 7, double dargyrome morphotype tested (Mor1) also could thrive as low as 10% SWM. The majority of the strains tested in this study were cirrotype 10, double dargyrome, and they ranged in hyposalinity tolerance from 50–0% SWM. Of all the strains tested in this study, most (15 strains out of 19) were able to thrive down to 20% SWM or lower.

Morphological effects of salinity on *E. vannus*. The parental strains were significantly larger (by t-tests at $P < 0.001$ confidence), in both length (Elba $t_{206} = 8.9$; LL1 $t_{206} = 14.54$) and width (Elba $t_{206} = 3.81$; LL1 $t_{206} = 14.66$) when cultured at 30% SWM compared with 100% SWM (Table 3). The Elba strain showed a continuous increase in both length and width (Fig. 2a, b) with decreasing salinity, with the interquartile ranges of the measurements at each salinity overlapping with those of the adjacent salinities. The LL1 parental strain's interquartile ranges were erratic and discontinuous with decreasing salinity (Fig. 2c, d). The LL1 cell lengths fell into two groups of overlapping interquartile ranges. The group of strains with larger cell widths consisted of the 90, 80, 70, 50 and 30% SWM cultures of LL1,

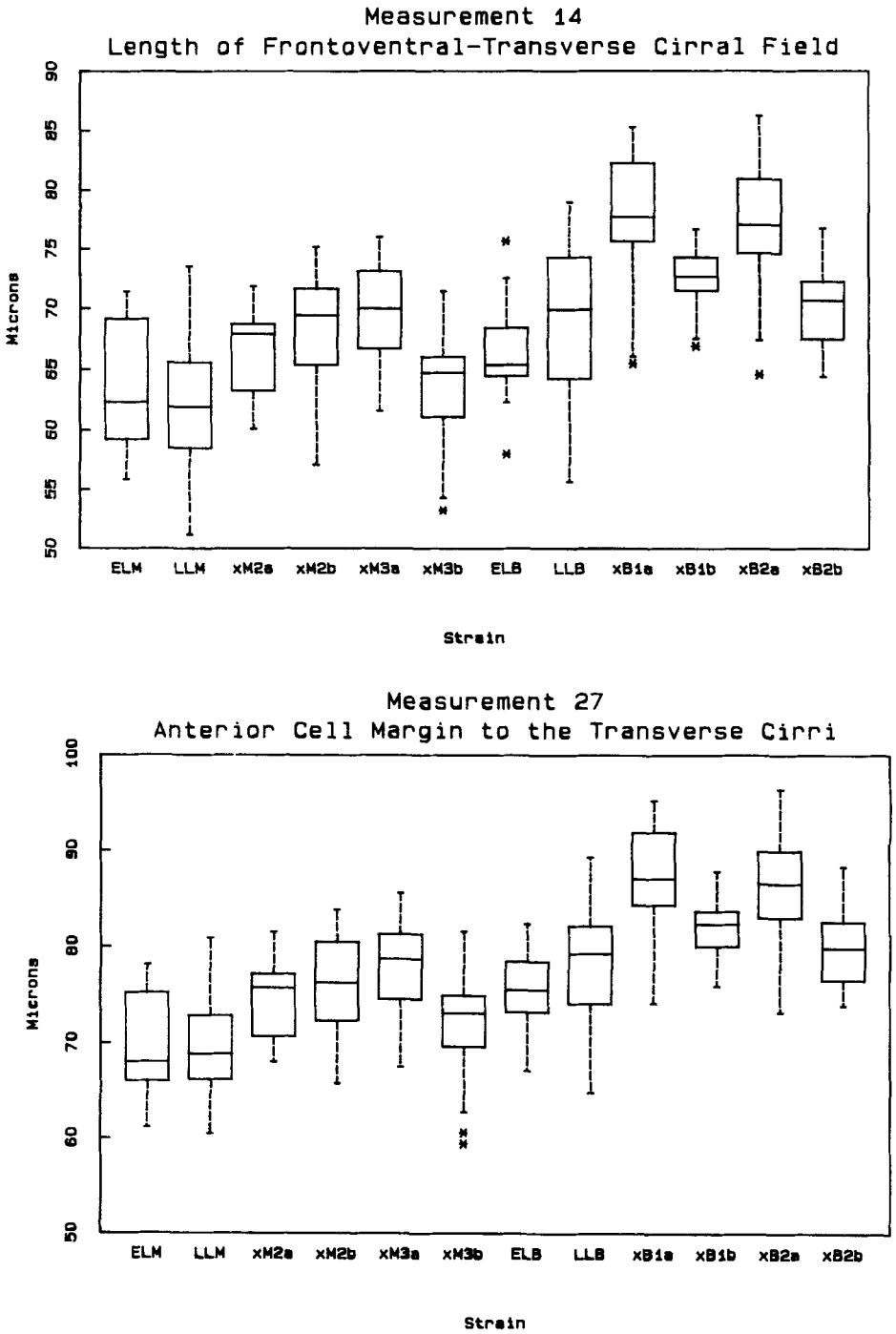

Fig. 3. Boxplots of selected ventral measurements of *E. vannus* strains Elba (EL), LL1 (LL) and exconjugate strains (x) cultured in normal saltwater medium (M) and in brackish medium (B, 30% of normal saltwater medium salinity). **a.** boxplots of the lengths of selected strains' frontoventral-transverse cirral field (measurement 14). **b.** boxplots of the distances from the anterior cell margin to the transverse cirri (measurement 27) of selected strains. **c.** boxplots of the distances from the right cell margin to the paroral membranelle (measurement 29) of selected strains.

in which the interquartiles ranged from about 90 to 104 μm. The strains with smaller cell lengths (100, 60 and 40% SWM) had interquartile ranges from about 76 to 90 μm. Similarly, the cell widths fell into two non-overlapping groups. The group with larger cell widths (90, 80, 70, 60, 50 and 30% SWM cultures) had overlapping interquartiles from about 62 to 80 μm, while the smaller group (100 and 40% cultures) ranged from about 51 to 61 μm.

When the four exconjugate cultures of each salinity were grouped together statistically, there was a general trend of increase in cell length and width with decreasing salinity (data not shown). There also tended to be more variation in these measurements as the salinity decreased. There were no general size trends in the individual exconjugate strains of a given salinity, either among themselves, or in relation to the parental strains. The only significant differences in cell length between the ex-

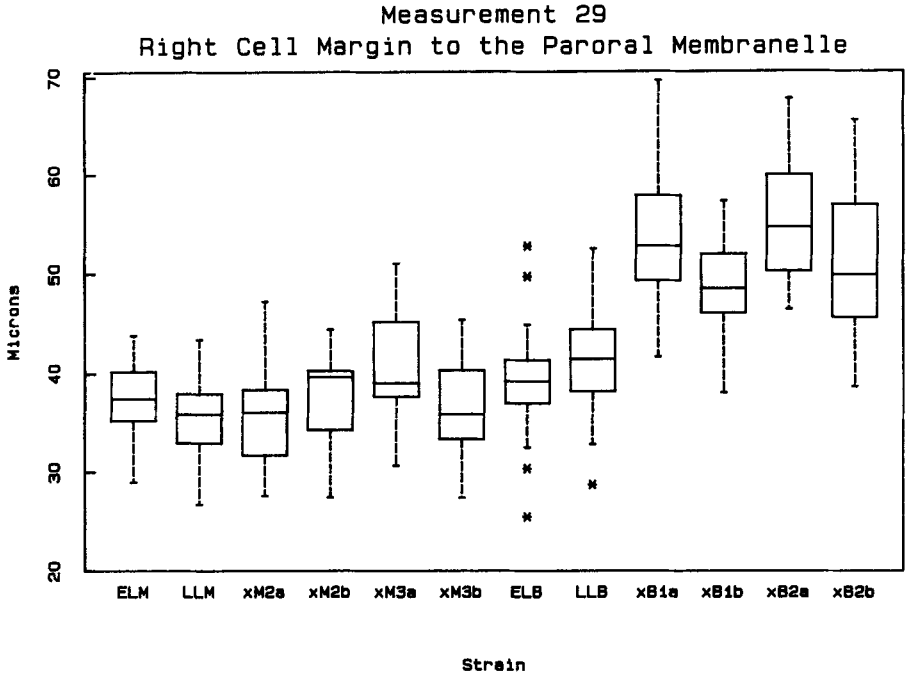

Fig. 3. Continued.

conjugate and parental strains occurred in the 100, 60 and 30% cultures, where the exconjugates as a group tended to be larger. The mean lengths and widths between the sibling exconjugate strains cultured at 100% SWM were similar (the only significant difference being in cell length between exconjugate strains 100x3a and 100x3b: $t_{98} = 3.04$, $P < 0.001$). The mean lengths and widths between the sibling exconjugate strains cultured at 30% SWM, however, were all significantly different (between 30x1a and 30x1b, length $t_{105} = 7.31$ [$P < 0.001$], width $t_{105} = 4.00$ [$P < 0.001$]; between 30x2a and 30x2b, length $t_{104} = 5.50$ [$P < 0.001$], width $t_{104} = 4.17$ [$P < 0.001$]).

The detailed ventral measurements of the 100 and 30% SWM strains were analyzed univariately. These measurements were compared statistically using the sums of squares simultaneous test procedure [25], for each of the parental and exconjugate strains, cultured at 100 and 30% SWM (data not shown). For each measurement, the strains are combined into groups which do not overlap significantly by this procedure. For the majority of measurements, there is overlap among all of the strains, such that they form only one continuous group. In nine of the measurements, however, there are two non-overlapping groups, with the group containing the strains with the larger means always including exconjugate strains 30x1a and 30x2a, and sometimes including exconjugate strains 30x1b and/or 30x2b. Measurements in which there are nonoverlapping groups include length and width, 15, 16, 27 and 29 (which are relative measurements along the length and width axes), 10 (the anterior cirrus to the mid-cirrus of streak IV, and relatively short when compared to the other measurements) and 24 (the width of the widest adoral zone membranelle). Measurement 24 correlated most highly with cell length ($r = 0.531$, $P < 0.01$) and width ($r = 0.532$, $P < 0.01$). The only exception, in which the smaller subgroup does not include 30% exconjugate strains, is measurement 3 (the width of the ventral plate, which correlates most highly with cell width [$r = 0.852$, $P < 0.01$]), in which case parental strain EL100 is significantly smaller than all the other strains, which form a continuous group.

Boxplot analyses of each of the measurements (data not shown) also revealed that the exconjugate strains 30x1a and 30x2a tended, as a group (with no consistent relation between the two), to be larger than the other strains and particularly their sibling exconjugate strains. The boxplots also showed that the exconjugate strains 100x2b and 100x3a also tended to be larger (with no consistent relation between them) than their sibling exconjugate strains. As examples of these trends, boxplots of measurements 14, 27 and 29 (which were found by PCA (see below) to be the three most important measurements other than length and width) are shown in Fig. 3a–c. Overall the exconjugate strains at 30% SWM tended to form the largest group, and the 30% SWM parental strains tended to be larger than the 100% SWM parental strains, with no consistent relationship between Elba and LL1 at either salinity. The 100% SWM exconjugate strains tended to be larger than the 100% SWM parental strains. Other than the trends described above, there were no outstanding patterns in the linear measurements or in the number of caudal cirri. The 30% SWM exconjugate strains, however, tended to have more adoral zone membranelles than the other strains (data not shown). The number of adoral zone membranelles positively correlated most highly with cell width ($r = 0.592$, $P < 0.01$), but negatively correlated with the spacing of the membranelles ($r = -0.642$, $P < 0.01$).

Multivariate analysis. The detailed ventral measurements of the strains were also analyzed multivariately using PCA (Fig. 4a, b). The plots of the first three principal components (PC I, II and III) (which account for 34.9, 8.8 and 6.5% of the total variation, respectively) showed overlapping ranges of variation when all 12 strains were considered together. Individual strains were distinguished from certain other strains, though no individual strains were separate from all other strains (data not shown). When the strains were plotted as 100 and 30% salinity groups (Fig. 4a) there was a definite trend of separation (despite overlap) along PC I, with the freshwater strains tending to have higher values. There were no such trends associated with PC II and III. The first three PC correlated highly with several trans-

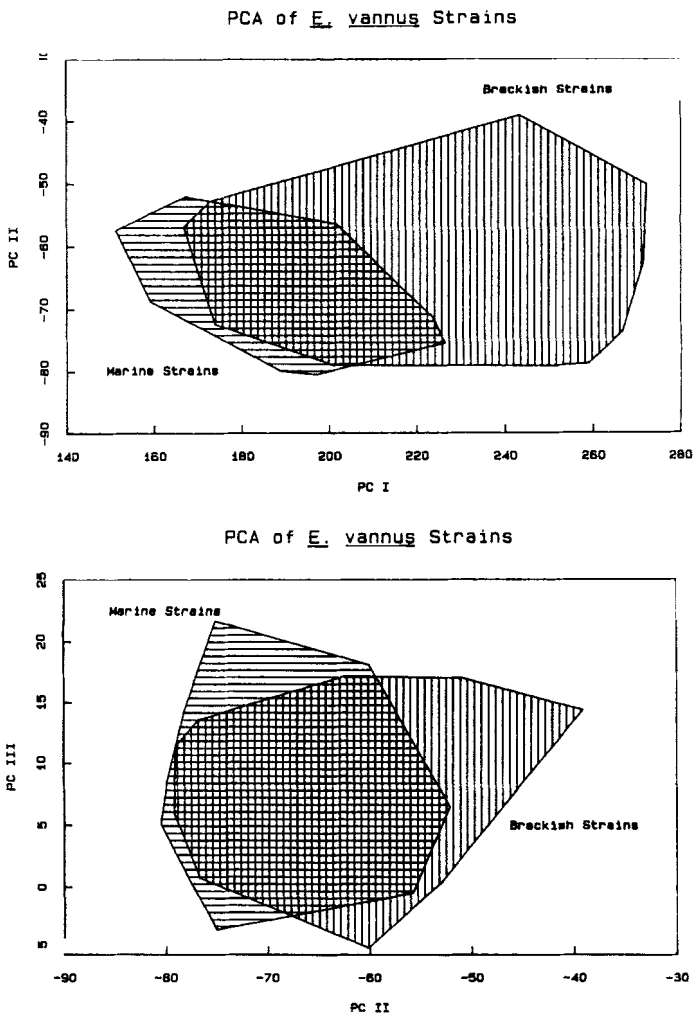

Fig. 4. Projections onto paired principal components axes of the ventral measurements for *E. vannus* parental and exconjugate strains cultured in normal saltwater medium (marine) and in 30% saltwater medium (brackish). The individual data points are grouped by culture medium and are enclosed by convex hulls; the hulls of the marine strains are shaded with horizontal lines and the hulls of the brackish strains are shaded with vertical lines. **a**. projection of the data onto principal components axes I and II. **b**. projection of the PCA data onto principal components axes II and III.

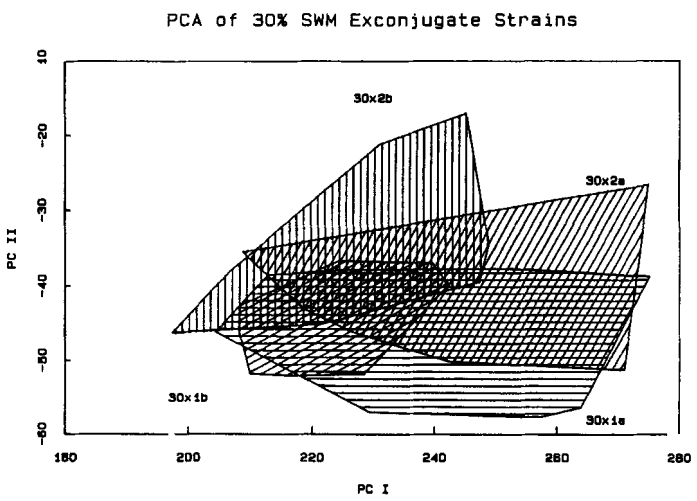

Fig. 5. Ventral measurement PCA of the four exconjugate *E. vannus* strains cultured in 30% saltwater medium, projected onto principal components axes I and II. The data are grouped by strain and surrounded by convex hulls.

verse, width-associated measurements (data not shown). Principal component I correlated most highly with cell width (r = 0.473, $P < 0.01$), followed by cell length (r = 0.371, $P < 0.01$).

Separate PCA were performed on several different subsets of the 30% SWM parental and exconjugate strains. One such PCA compared the four 30% SWM exconjugate strains: 30x1a, 30x1b, 30x2a and 30x2b. When PC I and PC II (28.0 and 10.6% of the variation, respectively) were plotted against each other, there was considerable overlap among all four strains (Fig. 5). There was, however, a trend for separation between two two-strain groups along PC I, with strains 30x1a and 30x2a extending into larger values. Strain 30x2b extended into higher values along PC II, but this extension was only supported by two individuals of the strain. PC I was overwhelmingly influenced by cell width and length (data not shown), followed by linear measurements 29 (from the left margin to the paroral membranelle), 27 (from the anterior margin to cirrus II/1) and 14 (from cirrus II/1 to the buccal cirrus).

Other PCA of the linear data were performed (data not shown). These included two separate PCA comparing the 30% SWM parental strains with the pairs of sibling exconjugates. In both cases there was considerable overlap among all the strains, but the exconjugate strains tended to separate from the parental strains along PC I, with the exconjugate strains generally occupying the higher values of the PC. Separate PCA of just 30% SWM sibling exconjugates revealed no previously unnoted separations. In all of these PCA, the most heavily loaded measurements included cell length and width, measurements 9 (cirrus III/1 to III/3), 14, 27 and 29. Scaled PCA (which included the counts of caudal cirri and adoral zone membranelles) also revealed no previously unnoted separations, and their most important measurements were similar to those above.

Cirral patterns. The distributions of relative intercirral distances were calculated for the parental strains at both salinities and for the eight exconjugate strains. The graphical depiction of all these distributions (Fig. 6) shows that all of the strains have similarly "shaped" distributions, with the greater variation among the strains occurring in the frequencies of the larger relative distances. Kolmogorov-Smirnov statistics (K-S) [25], which were calculated between each of the strains of *E. vannus* (data not shown), ranged from 0.0111 (between exconjugate strains 30x2a and 30x2b) to 0.0715 (between parental strain EL30 and exconjugate strain 30x1b). This latter comparison was the only K-S value to exceed the standard value of 0.0693, set by Gates [13] as a maximum of inherent clonal variability of cirral patterns of *Euplotes*. The average K-S value was 0.0343, with a standard deviation of 0.0123. The relative intercirral distances of the two most different strains, EL30 and 30x1b, were plotted together, as were those for the strains pooled into two groups by salinity (data not shown). The strains when grouped by salinity were less dissimilar both graphically and by K-S statistic (0.0198) than the two most dissimilar individual strains.

Dorsal surfaces. A striking feature was observed in the stained specimens of the 30% SWM strains, where the dorsal argentophilic network appeared disorganized relative to 100% SWM strains (Fig. 7a, b). In some individuals, the dorsal surfaces seemed to be of a haphazard "double" type; in an attempt to quantify these observations, detailed coordinate data were taken from the dorsal surfaces.

Except for the Elba parental strain, the cells cultured in 30%

SWM had significantly more dorsal kinetosomes (Table 4) than those cultured in 100% SWM. The strain with the smallest average kinetotype (113.19 dorsal kinetosomes) was exconjugate strain 100x3b, and the strain with the largest average kinetotype (176.74) was exconjugate strain 30x1a. The overall average kinetotype of the 100% SWM strains was 117.26 (SD 14.05), which was significantly lower ($t_{198} = 17.43$ [$P \ll 0.001$]) than the overall average kinetotype of the 30% strains (152.39, SD 20.46).

When only the central row of cilia was considered (Table 4), the 30% SWM strains all had more kinetosomes than the 100% SWM strains, though all the strains formed one continuous group by SSSTP. The lowest average number of cilia in the central row was found in the LL1 (100% SWM) parental strain (10.42) and the largest average was found in the exconjugate strain 30x1a strain (16.11).

There were no trends in corticotype associated with culture salinity. The only notable feature of the data on number of ciliary rows was for the Elba parental strain, which showed no variation (among the 25 measured specimens) in its corticotype of nine.

The qualitative observations of the subpellicular vacuolar disorganization associated with the change in culture salinity were supported by the quantitative results (Table 4). All of the 30% SWM strains (except exconjugate strain 30x2b) had significantly more vacuoles in the central three rows than the 100% strains by SSSTP, though there was great variation among the 30% strains. Exconjugate strain 100x2a has the lowest average number of vacuoles in the central three rows (37.33), while the highest average (69.95) is found in exconjugate strain 30x1a; the highest number of vacuoles found in the central three rows of an individual cell (88) also was found in this strain. The overall mean for the 100% strains, 39.51 (SD 3.85), which was significantly less ($t_{198} = 18.67$, $P \ll 0.001$) than the overall mean for the 30% strains was 55.89 (SD 10.08). The strains formed one continuous group by SSSTP when only the central row of alveoli was considered.

With respect to the total number of kinetosomes, the 100% SWM sibling exconjugates were not significantly different from one another, nor were the parental strains when cultured in 100% SWM. When cultured in 30% SWM, however, the kinetotype of 30x1a was greater than 30x1b ($t_{47} = 14.7$ [$P \ll 0.001$]), 30x2a was greater than 30x2b ($t_{45} = 4.49$, $P < 0.001$) and LL30 was greater than EL30 ($t_{49} = 5.61$, $P < 0.001$).

With respect to the number of alveoli in the central three rows (Table 4), there were significant differences within the 100% SWM sibling exconjugate strains, with 100x2b being greater than 100x2a ($t_{47} = 2.32$, $P < 0.05$) and 100x3a being greater than 100x3b ($t_{48} = 6.25$, $P < 0.001$). The parental strains at 100% SWM were significantly different, with EL100 having a greater average number of alveoli than LL100 ($t_{50} = 2.22$, $P < 0.05$). These same comparisons among the 30% SWM strains also were all significant, but the magnitudes of the differences were much greater than within the 100% SWM strains. With respect to the mean number of alveoli, exconjugate strain 30x1a was greater than 30x1b ($t_{47} = 9.65$, $P < 0.001$), 30x2a was greater than 30x2b ($t_{45} = 4.94$, $P < 0.001$) and the parental strain LL30 was greater than EL30 ($t_{49} = 3.01$, $P < 0.01$).

Morphological effects of salinity on two strains of euryhaline *Euplotes*. The culture medium (100% SWM or FWM) had no significant effects on the Cal4 gross measurements of cell length ($t_{207} = 0.47$, $P > 0.5$) and width ($t_{207} = 0.43$, $P > 0.5$, Table 5, Fig. 8). Strain Galv1 was significantly wider when cultured in freshwater ($t_{212} = 4.75$, $P < 0.001$), but not significantly longer ($t_{212} = 1.81$, $P > 0.05$). Strain Galv1, on the whole, was significantly larger in both length and width than Cal4.

When the Cal4 strain was examined more closely for any effects on the ventral morphology due to culture salinity, none were found. Principal components analysis of the linear measurements (data not shown) showed no separation when the strains were plotted in the first three PC (which accounted for 19.9, 10.5 and 7.6% of the total variation). PCA of scaled ventral measurements and counts (data not shown) also showed no separation when the strains were plotted in the first three PC (which contained 14.3, 7.7 and 7.1% of the total variation). Furthermore, the distributions of the relative intercirral distances were remarkably similar, with a K-S statistic between the two strains of 0.0084.

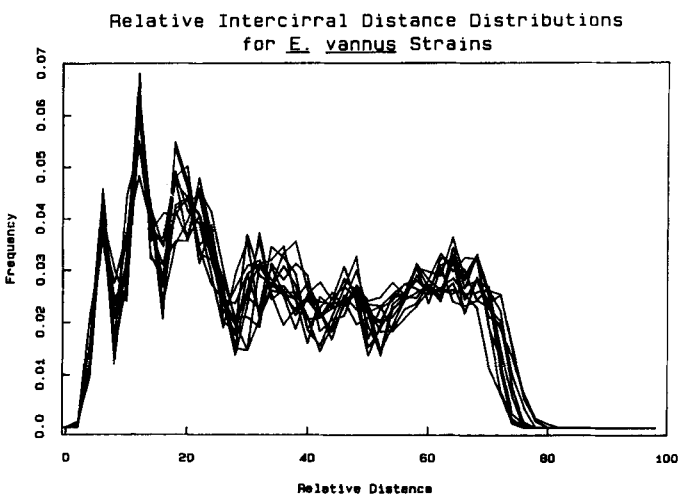

Fig. 6. Relative intercirral distance distributions of the parental and exconjugate *E. vannus* strains cultured in normal and 30% saltwater medium.

DISCUSSION

Of the over 50 described species of *Euplotes*, remarkably few have been described as euryhaline. This study found that three of the 19 strains tested for salinity tolerance were completely euryhaline, not only surviving, but thriving in both 100% SWM and in FWM. The most likely reason that so few "species" of *Euplotes* are reported to be euryhaline is that the describers almost never attempted to test their strains' salinity tolerance, but rather reported only the sampling habitat. This study demonstrates that *Euplotes*, in general, are quite euryhaline, in that all the strains tested could thrive in brackish water and several could survive and reproduce equally well in freshwater as in saltwater.

***E. affinis* morphotype.** One of the species which has been described in the literature as euryhaline is *E. parkei* [7, 8], which is a cirrotype eight (though occasional individuals within a clonal strain possess a ninth cirrus), double dargyrome species, roughly 40 μm in length and 30 μm in width. Strain Kling2b, which was tested for salinity tolerance in this study, fits the description of *E. parkei* (cirrotype nine) and also was found to be euryhaline. The strain used for the original description of *E. parkei* was sampled from a freshwater source in Vienna, Austria and was later transferred to and cultured in marine Erdschreiber solution (and also was maintained in freshwater Erdschreiber [7]). The method of transfer to the new medium was not reported, but it is known that it was a direct transfer (C. Curds, pers. commun.). Though not reported in the results of this study, Kling2b is also able to withstand a direct transfer from SWM to FWM.

***E. charon* morphotype.** There were 15 strains of cirrotype 10, double dargyrome *Euplotes* (*E. charon* type) [15] tested for sa-

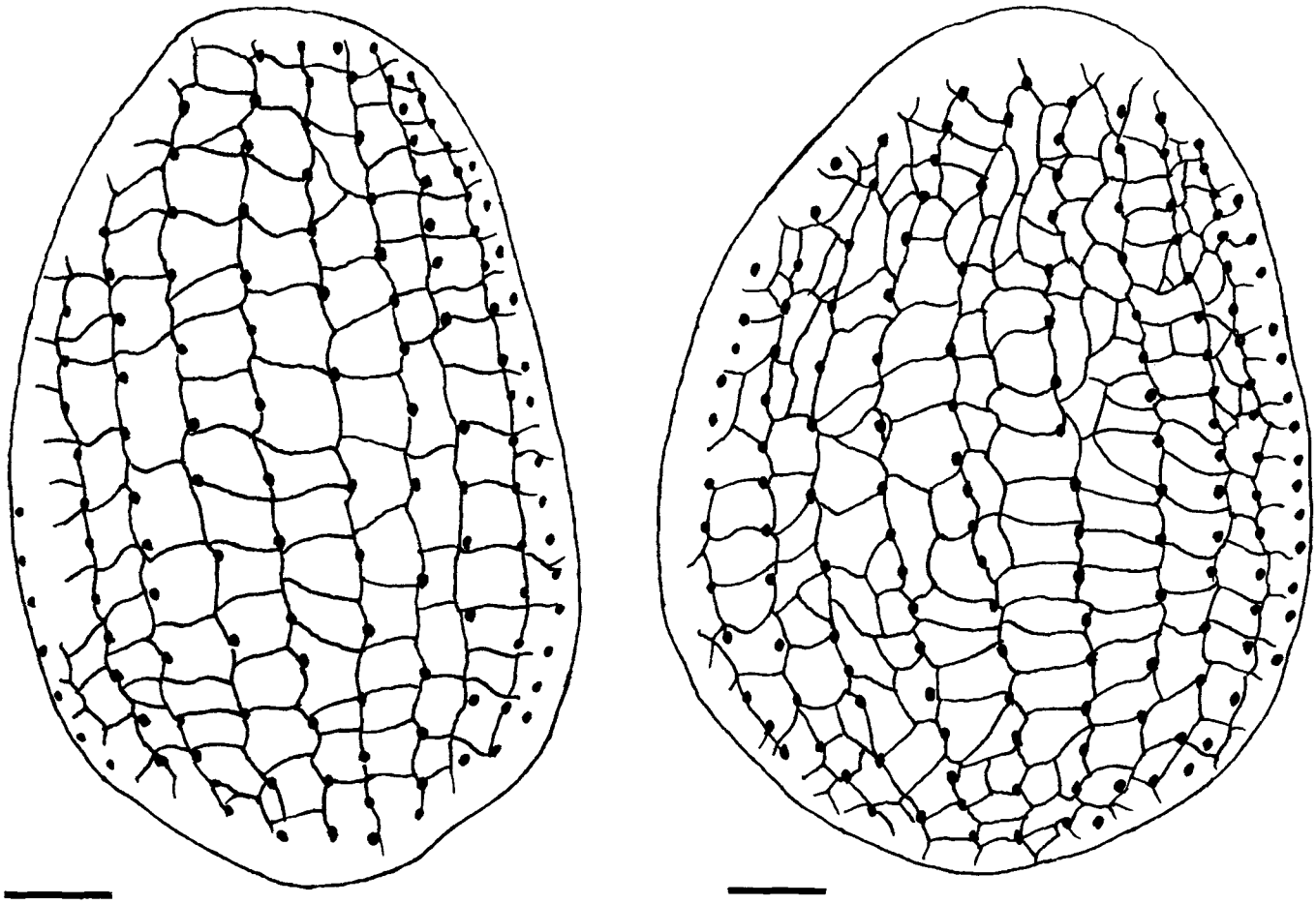

Fig. 7. Diagrams of representative *E. vannus* dorsal surfaces, drawn using a camera lucida at 1,250× magnification; the scale bar indicates 10 μm. **a.** diagram of a dorsal surface of a specimen of clonal *E. vannus* strain LL1 cultured in normal saltwater medium. **b.** diagram of a dorsal surface of a specimen of the same clonal strain of *E. vannus* cultured in 30% saltwater medium.

linity tolerance in this study, and there was considerable variation found among the strains. The strain with the least tolerance for hyposalinity was Cay2, which could not thrive at less than 50% SWM. This particular strain was also unusual in that it exhibited extreme variation in cell size (a few cells in a culture would be three to four times larger than the others), and showed variation in the dargyrome not commonly associated with clonal strains. It is possible that the low range of hyposalinity tolerance and the size variation are related as indicators of a genetically unstable clone, as all but one other of the strains tested (Ost3, which is of a different morphotype) were able to withstand at least as low as 30% SWM.

The two strains which were completely euryhaline (Cal4 and Galv1) were both sampled from brackish habitats, and initially cultured in 33% SWM. The actual salinity of the water from which they were sampled, however, was not recorded. The fact that both strains which were naturally thriving at midrange salinities were completely euryhaline, suggests that even the gradual transfer method of salinity change may be too rigorous for many strains sampled from fully marine environments.

Two of the strains (SI4 and SI10) were collected at the same time along the lee shore of South Island (currently South Point), near Cape Cod, MA. They were able to conjugate and produce viable exconjugates, and therefore definitely belong to the same biological species. They were not, however, found to have the same range of salinity tolerance (Table 1), demonstrating variation in physiological performance in different genotypes.

***E. vannus* morphotype.** The three strains of the *E. vannus* morphotype (cirrotype 10, single dargyrome) also had widely different ranges of salinity tolerance. These strains, however, were not sampled from the same location, and they, in fact, did not appear identical, in that Ost3 is considerably smaller, and fits classical descriptions of *E. minuta*, whereas Elba and LL1 fit descriptions of *E. vannus* [8]. Elba and LL1 were quite euryhaline, and preliminary data from another study (unpubl. observ.), in fact, suggest that the optimal salinity for *E. vannus* growth is somewhat less than 100% SWM.

Systematic implications. Recognizing that *Euplotes* are quite euryhaline and that the strains used in species descriptions, historically, were almost never tested for salinity tolerance, a cautious systematist must be skeptical of similar species descriptions, for which the sampled habitat is the only discriminating factor.

The two described freshwater *E. charon* morphotypes, *E. crenosus* Tuffrau, 1960 and *E. inkystans* Chatton in Tuffrau, 1960 [28], are each quite similar to several described marine species [8]. *E. crenosus* is described [28] as a 50–70 μm long, corticotype eight, freshwater species (though the original sample location in France was not reported). The general morphological description is quite similar to the descriptions of *E. octocirratus* Agamaliev, 1967 [1, 8] and *E. magnicirratus* Carter, 1972 [3]. *E. octocirratus* was originally sampled from the Caspian Sea and was described as a small (55–60 μm in length), corticotype seven species [1, 8]. *E. magnicirratus* was originally sampled from a

Table 4. Summary table of *E. vannus* dorsal data. Med, median; Min, minimum value; Max, maximum value; SD, standard deviation and n, sample size.

Strain	Mean	Med	Min	Max	SD	n
Total number of kinetosomes						
EL100	114.85	114.5	82	148	14.56	26
LL100	116.31	112.5	90	154	15.67	26
100x2a	119.88	122.0	101	145	11.67	25
100x2b	125.67	127.5	99	153	14.71	24
100x3a	114.88	111.5	96	149	12.91	24
100x3b	113.19	112.0	81	134	12.52	26
EL30	129.85	32.5	88	153	14.17	26
LL30	151.96	51.0	128	185	13.94	25
30x1a	176.74	175.0	163	191	7.65	25
30x1b	143.63	144.0	129	156	8.12	24
30x2a	170.14	170.0	132	188	13.70	22
30x2b	153.16	153.0	131	183	12.22	25
Number of alveoli in the central three rows						
EL100	41.26	42.0	34	46	3.15	26
LL100	38.83	39.0	30	48	4.61	26
100x2a	37.33	37.0	31	44	3.75	25
100x2b	39.17	38.0	34	48	3.66	24
100x3a	42.50	42.5	38	48	2.69	24
100x3b	37.77	38.0	31	43	2.66	26
EL30	56.19	57.0	44	68	5.78	26
LL30	61.84	63.0	40	71	7.55	25
30x1a	69.95	68.0	54	88	9.65	25
30x1b	49.77	50.5	42	55	3.52	24
30x2a	52.91	52.0	46	65	4.28	22
30x2b	45.32	44.0	37	59	5.99	25

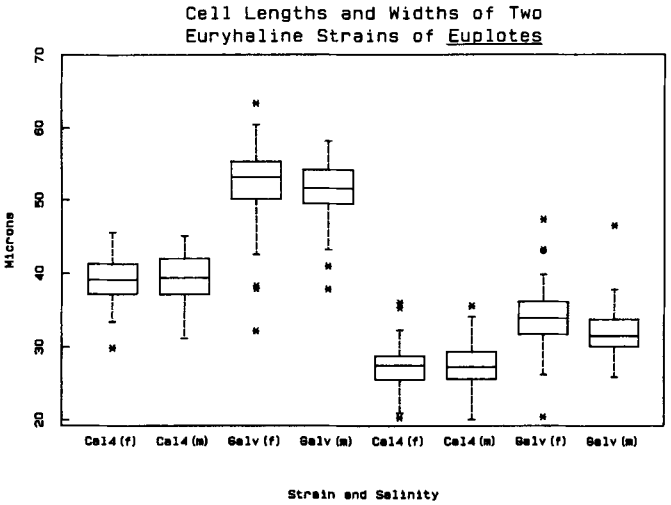

Fig. 8. Boxplots of cell lengths (first four boxes) and widths (last four boxes) of two small, euryhaline, *E. charon* morphotype strains, Cal4 and Galv1 (Galv). The strains were cultured in both freshwater (f) and saltwater medium (m).

tidal pool in Nassau, Bahamas; it was described [3] as 51.0–64.5 (mean 54.0) μm in length and 36.0–43.5 (mean 40.0) μm in width (based on 100 measured specimens). It was described further as having unusually large frontoventral and transverse cirri, and eight dorsolateral kineties [3, 8].

The strain Galv1 (in either fresh or saltwater), of this study, very closely fits the original description of *E. crenosus.* In freshwater, Galv1 ranges in cell length from 32.2 to 63.4 μm (mean 52.6), and has a median corticotype of eight, and the strain is not significantly different from this in size in saltwater (Table 5).

E. octocirratus and *E. magnicirratus* are quite similar in their morphological descriptions, and, when habitat is not considered a discriminating factor, *E. crenosus* is quite similar to both of them. The fact that none of these species were tested for range of salinity tolerance, and the discovery of a morphologically similar, euryhaline strain, strongly suggests redundancy among the published descriptions. Conservatively, there is not sufficient systematic evidence to warrant separate binomial designations. We propose that the taxa *E. octocirratus* and *E. magnicirratus* be submerged as junior synonyms of the taxon *E. crenosus,* which holds nomenclatural superiority.

E. inkystans Tuffrau, 1960 was described [28] as a 70–80 μm long, cirrotype 10, freshwater species, with 10 dorsolateral kineties (again, the original sample site was not reported); this is quite similar to the description of the classic species *E. charon* Müller 1786, which was described as a 70–76 μm long, marine species, with 12 dorsolateral kineties [8]. Strain Kling2a, when cultured in SWM, ranges from 67.9–90.4 μm (mean 77.1) in length, from 47.8–70.8 μm (mean 55.3) in width, and has a median corticotype of 10. Kling2a was able to be cultured in as low as 30% SWM (Table 1). Due to the morphological similarity of *E. charon* and *E. inkystans,* and the broad euryhalinity of Kling2a, which is morphologically similar to both species, we propose submerging the taxon *E. inkystans* into the taxon *E. charon* (which has nomenclatural priority), thus maintaining a conservative systematic scheme.

The other euryhaline strain in this study which is of the *E. charon* morphotype, Cal4, is most similar to the marine species *E. trisulcatus* Kahl, 1932, which was described as 40 μm long, 30 μm wide and as having seven dorsolateral kineties [8, 21]. Strain Cal4 cultured in SWM ranges from 34.2–46.2 μm (mean 41.5) in length, and in width from 21.4–32.7 μm (mean 28.3), and has a median corticotype of seven. There were no significant changes in Cal4 when it was cultured in FWM (Table 5). There are no cirrotype 10, double dargyrome, freshwater species described in this size range. Rather than describe a new species, however, it is more prudent to redescribe *E. trisulcatus* as a euryhaline species, or at least extend the description to say that a strain of this morphotype has been found to be euryhaline. We prefer the latter redescription, because there are extant strains of this morphotype in our laboratory, which cannot be distinguished morphometrically from each other (unpublished data), and which show a range of hyposalinity tolerance.

There are sets of species of other morphotypes described in the literature which are similar in diagnostic characteristics except for the reported habitat. A cirrotype nine, double dargyrome example of this is the freshwater species *E. daidaleos* Diller & Kounaris, 1966 [9] and the marine species *E. octocarinatus* Carter, 1972 [3]. Without testing species as similar as these for range of salinity tolerance, and without possessing euryhaline forms which tie the similar descriptions together, we cannot as confidently lump them together. However, as we have shown that habitat is not a reliable criterion in the diagnoses of certain species, we justifiably can be suspicious of other descriptions where it is the primary difference between two species.

Morphometrics. The effects of hyposalinity on morphology were different between the two morphotypes examined. There were several significant effects observed in *E. vannus,* whereas neither of the two *E. charon* euryhaline strains, Cal4 and Galv1, showed any significant changes in the gross measures of length and width between marine and freshwater medium (except for a slight increase in cell size of Galv1 in freshwater medium). There were also no significant differences in any of the detailed ventral measurements between Cal4 grown in marine and freshwater medium. These euryhaline strains had no difficulty being

Table 5. Summary table of euryhaline strains Cal4 and Galv1, cultured in marine (mar) and freshwater (fre) medium. SD, standard deviation; Min, minimum value; Max, maximum value and n, sample size.

	Cell length					Cell width			
	Mean	SD	Min	Max	n	Mean	SD	Min	Max
Cal4 (fre)	39.12	2.89	29.8	45.5	104	27.19	2.83	20.3	35.9
Cal4 (mar)	39.32	3.21	31.1	45.1	103	27.36	2.95	20.1	35.6
Galv1 (fre)	52.57	4.58	32.1	63.5	110	33.82	3.44	20.4	47.4
Galv1 (mar)	51.53	3.74	37.8	58.2	102	31.76	2.85	25.9	46.5

transferred from marine to freshwater medium (they could in fact withstand the shock of direct transfer between the two), and they grew equally well in both media. It is not surprising, therefore, that these strains showed no morphological changes which could be attributed to osmotic stress.

In contrast, this study has shown that the overall size and the morphologies of both the ventral and the dorsal surfaces of *E. vannus* were affected by hyposalinity. The data suggest three different factors affecting the gross measurements of cell length and width. One was the direct effect of lowered salinity, which increased the size of both the parental strains and their resulting exconjugate strains.

Another size effect was that the average of exconjugate strains tended to be larger than the average of their parental strains at many of the salinities tested, including 100 and 30% SWM (Table 3). This, perhaps, represents a rejuvenation of the cell cycle and a hybrid vigor resulting from the cross of aging parental clones.

The third size difference occurs between sibling exconjugate clones cultured at 30% SWM, in which one of the sibs was generally larger in the gross measurements of length and width, as well as several of the ventral measurements, than the other. Though only two pairs of exconjugates were tested, this phenomenon is suggestive of maternal cytoplasmic inheritance. And though the relationship between the parental strains in magnitude of the ventral measurements was inconsistent, LL30 was significantly larger than EL30 in cell length and in cell width, whereas the parental strains were not significantly different in these measurements at 100% SWM. If this is a case of maternal inheritance, the exconjugate strains 30x1a and 30x2a are likely to be the cytoplasmic descendants of the LL1 partner of the isolated conjugating pair.

Principal components analysis of the *E. vannus* ventral measurements revealed separation of the 30% SWM from the 100% SWM strains, and between sibling exconjugate strains, along PC I (Fig. 4a, b). This axis was predominantly influenced by cell length and width, as well as several of the larger cirral position measurements. This suggests that the separation seen in these PCA was due to a general size difference in the strains, rather than a change in cell shape or cirral positioning.

The assertion that there were no significant changes in the cirral pattern is supported by the analysis of the relative intercirral distances (Fig. 6), with all but one of the K-S statistics being smaller than the largest found between clonal strains of *E. harpa* by Gates [14]. Furthermore, the sibling exconjugate strains 30x2a and 30x2b, which were significantly different in cell length and width, and in several ventral measurements, had the lowest K-S value found in this study (0.0111). The one K-S value (0.0715) found in this study to be larger than Gates' standard (0.0693) was between a parental strain and an exconjugate strain (EL30 and 30x1b), and therefore is not a new measure of clonal inherent variability. It is, however, a measure of inherent variability within a species (as one strain is the progeny of the other), and therefore would provide a more conservative criterion by which to assess the similarities of cirral patterns in other *Euplotes* samples.

Hyposalinity tolerance. The disorganization of the dorsal surface in *E. vannus* in decreased salinity was the most notable direct observation in this study. It was shown that the 30% SWM strains, as a group, had significantly more kinetosomes and alveoli than did the 100% SWM strains as a group. Another notable result was the relationship between the 30% SWM sibling exconjugate strains, in which strains 30x1a and 30x2a (which were relatively larger than their siblings) had a significantly greater number of kinetosomes and number of alveoli than their respective sibling strains. Also at 30% SWM, the parental strain LL30 had greater numbers of kinetosomes and of alveoli than EL30, which further suggests that 30x1a and 30x2a are cytoplasmic descendants of LL1. Overall, it seems that LL1 and its descendants have a unique response to the hyposaline environment, and therefore exhibit greater morphological changes, possibly due to osmotic stress. The trend toward increased numbers of alveoli with decreasing salinity may also be seen in the taxonomic literature, in that all of the described freshwater species are at least double dargyrome, and often multiple or complex; single dargyrome specimens, on the other hand, have only been found in marine habitats. It could be that a greater number of subpellicular alveolar compartments are necessary to handle the increased osmotic stress of freshwater.

The dorsal argyrome has long been thought to be one of the most stable and diagnostic criteria in *Euplotes* [28, 8, 15, 16]. The dorsal surface has often been the basis of new species descriptions, although Gates & Curds [16] demonstrated the invalidity of distinctions within the double dargyrome category. The dorsal surfaces of *E. vannus* strains in this study, when grown in 30% SWM, bear a striking resemblance to those of *E. mutabilis* Tuffrau, 1960 [28]. *E. mutabilis* is not recognized as a valid species [15, 18], but rather as a mutant strain of *E. vannus*. This study shows that such a disorganized state can be induced by culture conditions in otherwise normally appearing strains. Unfortunately, we did not return the 30% SWM strains to 100% SWM to see if the disorganized condition persisted.

In Gause's [17] experiments, it was shown that *E. vannus* cells cultured in 5% salinity medium were significantly smaller than those cultured in 2.5% salinity. Gause called this change adaptive, stating that a "parallelism is observed of adaptive modifications with hereditary distinctions between species." The distinction between species referred to was a trend of a regular decrease in body size among *Euplotes* as their habitats increase in salinity, which he based on three species he identified as *E. patella, E. vannus* and *E. elegans*. The taxonomic literature, however, does not support this conclusion, in that the reported lengths and widths of freshwater species are not, as a group, larger than those reported for saltwater species (data summarized in [8]).

It is possible that the inverse relationship between cell size and salinity that Gause observed experimentally within strains of *Euplotes*, could have been due simply to osmotic stress. When

cells are shifted into higher salt concentrations, the water in the cytoplasm tends to flow out of the cell, causing a temporary diminution of cell size unless the cells are able to physiologically cope with the stress and control the flux through contractile vacuole activity, or altering the cytoplasmic solute concentration (for review see [10]).

Gause performed inheritance studies with *E. vannus* which suggested that there was in fact selection for smaller cell size in higher salinities, and selection for larger cell size (though apparently less strongly so) in lower salinities, suggesting that the somatic changes in size may in fact be adaptive rather than simply the result of osmotic stress. Strangely, Gause did not observe a somatic change in size when he transferred *E. vannus* from 2.5 to 1.0% salinity (1.0% salinity is the equivalent of 26% SWM), whereas we found significantly larger cell lengths and widths in both of our parental strains at 30% SWM. This difference could be accounted for by the fact that Gause's strains were originally cultured in the equivalent of 66% SWM, and thus, the net change in salinity was not as great as ours. Gause did not state whether or not his strains of *E. vannus* could be cultured at salinities lower than 1%.

Gianni & Piras [19, 20] found variation in salinity tolerances (using direct transfer methods and checking for survival after 24 h) among groups of strains they identified as *E. vannus, E. crassus* and *E. minuta* [19], and also between two groups of strains identified as *E. crassus* [20]. The range of hyposalinity tolerance they found for their *E. crassus* strains was similar to the values found in this study for the strains Elba and LL1 (which were identified as *E. crassus* and given to our lab by Fernando Dini and Pierangelo Luporini, respectively). Gianni & Piras [19] found that their strains of *E. minuta* could tolerate considerably lower salinities than could the similar strain (Ost1) in this study. Because those studies [19, 20] used direct transfer methods, and particularly because the cells were checked for survival after only one day, the results do not (nor did the authors claim to) provide a range of salinities in which the cells likely may be found in nature.

Conclusion. In this study we have shown that *Euplotes* strains, in general, are quite euryhaline. We have also shown that the habitat of the cells can affect the morphology of what was thought to be one of the most stable of characteristics. As *E. vannus* strains have been shown to thrive and even conjugate at brackish salinities, and they have also been collected from estuarine habitats, the disorganized dargyrome may be a naturally occurring condition. Furthermore, the range of habitat in *Euplotes* is important systematically and, therefore, it should be examined thoroughly rather than just reporting the sampling habitat. Through applications of these principles, we have submerged several redundant species descriptions.

In protistology, species descriptions are still based mainly on cell morphology, as genetic recombination is too rare among forms to use the biological species concept and molecular phylogenies remain sparse. It is important, therefore, that criteria for species descriptions be quite conservative, lest the literature become cluttered with redundant descriptions. In this study, despite occasionally extensive cellular disorganization upon hyposalinity stress in originally marine strains, the cirral patterns of the strains remained remarkably constant, supporting the assertion [15] that cirral patterns and dargyromes should be the primary taxonomic criteria used in *Euplotes* descriptions based on morphology alone.

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Quantitative Analyses of Interbreeding in Populations of *vannus*-Morphotype *Euplotes*, with Special Attention to the Nominal Species *E. vannus* and *E. crassus*

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ABSTRACT. Classical genetic techniques were applied to clonal cultures of the *Euplotes vannus-crassus-minuta* sibling species complex in an effort to provide some resolution to the species problem among these hypotrichs. Complex mating interactions were observed among clonal stock cultures derived from samples collected from sympatric and allopatric populations in a wide geographic survey. These results suggested that the classical model for the mating type inheritance and determination in these *Euplotes* is necessary but not sufficient to describe the mating mating interactions among populations of these ciliates. Successful conjugation between the nominal species *E. crassus* and *E. vannus* was observed routinely, and crosses between these two nominal species did not differ significantly from those among the other clonal stock cultures with respect to mating intensity and exconjugant survival. Data from backcrosses suggests that *E. vannus* and *E. crassus* can and do exchange genes. Based upon these data, we conclude that *E. vannus* and *E. crassus* comprise a single, highly polymorphic species with countless small populations, among which incomplete genetic exchange takes place.

Supplementary key words Multiple mating type systems, species concepts, systematics.

TAXONOMIC classification at the species level should be both parsimonious and unambiguous. The task of species identification and description in protists is especially problematic, since many protists possess similar morphologies but may differ greatly with respect to other criteria [31, 32, 34, 43]. In ciliates, many of which may exchange genes through the process of conjugation, interbreeding criteria may be used to distinguish among species that possess uniform or nearly uniform morphologies [2, 24, 31, 32, 34, 43]. Indeed, interbreeding criteria have been used extensively in the genera *Paramecium* and *Tetrahymena* [24, 31, 34, 42–44].

The nominal species *Euplotes vannus*, *E. crassus* and *E. minuta* are in the midst of a taxonomical controversy in which two mutually exclusive classification schemes are opposed [5, 9, 11, 12, 17, 18, 27, 28, 37, 40, 46–48]. The first, based largely upon quantitative analyses of morphological criteria [9, 11, 12] and supported by the results of some mating studies [16, 35, 36] holds that these three species should be classified as one and the same species. The second, based upon eclectic approaches combining general morphology and isozyme analyses with heavy weighting on the results of mating tests [17, 18, 40, 47], supports the view that these three species are evolutionarily distinct entities and should maintain their separate status. One recent publication [18] argues for the splitting of the nominal species *E. crassus* into a sibling species complex, based upon an autecological study of a few strains.

The *vannus*-type *Euplotes* have an obligate outbreeding genetic economy with multiple mating types [5, 6, 20, 21, 37, 38]. These mating types are inherited in Mendelian fashion as different alleles at a single locus, and they are expressed with serial, peck-order dominance [20, 21, 36]. Exceptions to this occur in some strains that have been observed to follow one of two apparent inbreeding strategies, either autogamy or intraclonal conjugation (selfing) [3–5, 7, 14, 22, 38, 39]. This classical model for the mating system in these *Euplotes* has been challenged recently [48], based upon the results of mating tests performed on strains of *Euplotes* assigned to the species *E. crassus*. We set out to confirm those results using an extensive geographic collection of strains belonging to the *vannus* morphotype (cirrotype 10, single dargyrome), many of which were given no a priori species assignations. Furthermore, we sought to resolve the species problem within these ciliates using a quantitative analysis of the results of classical crossing experiments.

MATERIALS AND METHODS

Samples. Information pertinent to the collection of the strains used in this study is presented in Table 1. Samples labelled SPt1 through SPt5 were all collected from the same tidal pool on the same day and thus represent a sympatric contemporaneous population. All the remaining samples used in these experiments were collected, or received from other laboratories, over a period of many years. All strains used in this study were confirmed to belong to the *vannus* morphotype by silver staining [10, 11], but no classical species assignments were made by us. Nominal *E. crassus* strains MN1, MN4, MN5, Elba, G11 and H7*1C, kindly provided by Dr. Fernando Dini, and nominal *E. crassus* strains LL1, EC.LIV2, EC.POR3, EC.POR7 and EC.TM3 and nominal *E. vannus* strains EV.AL2, EV.SB2 and EV.TM1, kindly provided by Dr. Pierangelo Luporini, were assigned to those species by their providers (Table 1).

Cultivation. Except where stated otherwise, culturing and experiments were performed at room temperature (25 ± 5° C). *Euplotes* were grown in marine Cerophyl® medium (MCER). The preparation of this, and all other media used in these experiments are the same as in [15]. Clonal stock cultures V1 through V48 were grown on monoxenic MCER with *Klebsiella pneumoniae* as the food organism. Clonal stock cultures V49

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