Research article

# Analysis of autapomorphic point mutations provides a key for the tangled taxonomic distinction of the closely related species, *Euplotes crassus*, *E. minuta* and *E. vannus* (Ciliophora, Euplotida)

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# Abstract

A well-defined clade of the *Euplotes* phylogenetic tree is represented by marine species characterized by a single-type dargyrome and ten fronto-ventral cirri. Three of them, namely *Euplotes crassus*, *E. minuta* and *E. vannus*, form a complex of closely related species of large use in experimental ciliatology. Despite morphometric and genetic analyses having substantiated their taxonomic separation, ambiguities still persist in strain assignments to one or another species. In addition to objective reasons intrinsic to significant overlapping of most morphological parameters, ambiguities also result from divergences (inherited from past literature) in deciding which of the two morphotypes, *E. crassus* or *E. vannus*, is characterized by a larger or a medium cell body size (*E. minuta* being clearly distinct by a smaller morphotype). By analysing nuclear SSU-rRNA gene and ITS region sequences from 37 strains, previously assigned to *E. crassus*, *E. minuta* and *E. vannus* based on conventional taxonomic parameters, we identified and used ITS autapomorphic point mutations to design three species-specific primers. In combination with an *Euplotes*-generic primer, they proved to be very effective in running polymerase chain reactions that produce amplicons of species-specific size that reliably resolve ambiguities in assigning strains to *E. crassus*, *E. minuta* or *E. vannus*.



***Keywords***: Ciliate taxonomy; Diagnostic PCR; Euplotida; Sister species

# Introduction

*Euplotes* Ehrenberg, 1830 is an extraordinary speciose genus represented by nearly 150 nominal species, colonizer of every marine, brackish and freshwater habitat, besides wet soil, leaf litter and mosses (Berger 2001; Borror and Hill 1995; Curds 1975; Syberg-Olsen et al. 2016; Tuffrau 1960). Easily collected and cultivated, they rival *Paramecium* and *Tetrahymena* species in experimental ciliatology (Nanney 1980). Three closely related *Euplotes* species, namely *E. crassus* (Dujardin, 1841) Diesing, 1850, *E. minuta* Yocom, 1930 and *E. vannus* (Müller, 1786) Diesing, 1850 have stood at forefront of the research interest since the sixties, when they were for the first time stably cultivated and studied by Heckmann (1963, 1964) and Nobili (1965a) for the Mendelian genetics of the multiple mating type systems, earlier discovered and shown by Kimball (1942) to control mating in *E. patella* (Müller, 1773) Ehrenberg, 1838. They dwell in sympatry in the sandy interstices of the eulittoral zone, and share the same two basic diagnostic traits of the *Euplotes* taxonomy, i. e. a single-type dargyrome and 10 fronto-ventral cirri (cirrotype-10). Over the years, the experimental utilization of *E. crassus*, *E. minuta* and *E. vannus* has rapidly been widened: first, to various aspects of their breeding patterns and reproductive biology involving autogamy (self-fertilization) in addition to mating (Dini 1984; Dini and Luporini 1980; Luporini 1970; Siegel and Heckmann 1966); then, to a broad spectrum of research fields including molecular genetics (Alimenti et al. 2011; Klobutcher and Farabaugh 2002; Klobutcher et al. 1981; Roth and Prescott 1985; Tang et al. 2020; Vallesi et al. 2014), evolution of the genetic code (Lozupone et al. 2001; Turanov et al. 2009), chemistry of secondary metabolites (Guella et al. 2010) and, most recently, organization of the nuclear and mitochondrial genomes (Chen et al. 2019; de Graaf et al. 2009; Huang et al. 2021; Lobanov et al. 2017).

This flurry of research activity, however, has not always been associated with a univocal taxonomic distinction of *E. crassus*, *E. minuta* and *E. vannus*, as well as two other (candidate) sister species, *E. balticus* (Kahl, 1932) Dragesco, 1966, and *E. cristatus* Kahl, 1932, which have

so far received very secondary experimental relevance.

The fixation of genetic boundaries delimiting *E. crassus*, *E. minuta* and *E. vannus* faces difficulties with a common capacity of various strains representative of each species to cross-mate, as well as form (albeit under laboratory conditions) hybrid (yet sterile) mating pairs (Nobili 1964, 1965b; Nobili et al. 1978), as also happens between other *Euplotes* sister species such as *E. patella* and *E. octocarinatus* Carter, 1972 (Kuhlmann and Sato 1993). In coherence with this capacity, *E. crassus*, *E. minuta* and *E. vannus* have been regarded, first, to form an 'Artenkreis' by Nobili (1964) and, then (along with *E. mutabilis*, a no longer valid species), a complex of sibling species within the monophyletic "*vannus* group of genus *Euplotes*" by Génermont et al. (1976, 1985), in analogy with the sibling-species complexes proposed in *Paramecium aurelia* by Sonneborn (1975) and in *Tetrahymena pyriformis* by Nanney and McCoy (1976).

At the same time, the fixation of the morphological boundaries is affected, in addition to extensive intra-clonal morphometric variations often magnified between cell cultures of different ages and grown under different dietary conditions, by an age old problem specific to the distinction between the closely comparable *E. crassus* and *E. vannus* morphotypes, both nearly double in size compared to the ('minute') *E. minuta* morphotype originally reported by Yocom (1930) to be on average 50 x 29 µm. This problem originates from a substantial incongruence in the *E. crassus* and *E. vannus* descriptions between the original key by Kahl (1932) to the species of *Euplotes* and the "Revision du genre *Euplotes* …" by Tuffrau (1960). In Kahl's key, *E. crassus* is described with a body length of 70–100 µm and an oval ("ovoid") shape, and *E. vannus* with a body length of 130–200 µm and a reniform shape ("Die Zone biegt nach gestrecktem Verlaufe neben l. Korperrande scharf zum Pharynx um (wie bei *extensus*)"). In Tuffrau's revision, instead, *E. crassus* and *E. vannus* are described with precisely upturned body sizes, i. e. 100–127 and 75–97 µm, respectively, and unchanged body shapes ("Ovale allongé presque parfait" in *E. crassus*; "Irrégulièrment ovalisé, un peu étranglé sur la droite, au contraire faiblement élargi sur la gauche"

in *E. vannus*), with the addition of new information on the presence of 10 (eight dorsal and two latero-ventral) kineties in *E. crassus* and nine (seven dorsal and two latero-ventral) kineties in *E. vannus*. Although the *E. crassus* and *E. vannus* body sizes and shapes reported by Kahl (1932) were fully validated by Heckmann (1963, 1964) and Nobili (1964, 1965b) in their genetic works on these species, the *E. crassus* and *E. vannus* descriptions by Tuffrau (1960) have earned much more reference since their tout court reiteration in the "Guide to *Euplotes* species" by Curds (1985). Not only has this reiteration consolidated cell-body sizes overturned between the *E. crassus* and *E. vannus* morphotypes. It has also contributed to crediting the notion that the *E. crassus* dorsal surface is "strongly sculptured by 8 longitudinal ridges" (Curds, 1975), after "dorsalment percourue par 8 longues cretes meridiennes" (Tuffrau, 1960). Instead, it is absolutely smooth, as in *E. minuta* and *E. vannus*, as better documented by Dini and Nyberg (1999) with scanning electron microscopy (Fig. 1).

These taxonomic ambiguities in distinguishing in particular between *E. crassus* and *E. vannus*, and the difficulties that are generally met in delimiting species within the so-called '*E. vannus* complex', were mostly tackled and discussed in the eighties by French authors (Génermont et al. 1985; Machelon 1982; Machelon et al. 1984) and Italian authors (Dini and Giannì 1985; Valbonesi et al. 1988). More to the point, it was the application by Valbonesi et al. (1988) of multivariate morphometrics criteria to a collection of 19 wild-type strains from the Mediterranean area (a few analyzed also in common with Génermont et al. 1985), which were preliminarily assigned to *E. crassus*, *E. minuta* or *E. vannus* on the basis of mating assays and isozyme patterns. This application defined a 'theoretical median organism' (TMO) distinctive of each species, and the most immediate parameter of this distinction was just provided (as recalled in Fig. 1) by the combination of 'body size and shape' which maintains that *E. minuta* is the smallest and oval-shaped species, *E. crassus* is the intermediate and oval-shaped species, and *E. vannus* is the largest and nearly rectangular/reniform species. Jointly with this TMO definition, neotype slides of silver-stained specimens of the strain closest to the TMO of each species were deposited at the Natural History Museum of London (*E. crassus*-neotype-1987:8:17:1, strain $LL_1$; *E. minuta*-neotype-

1987:8:17:2, strain $Lb_9$; and *E. vannus*-neotype-1987:8:17:3, strain $TM_2$) (Valbonesi et al. 1988).

A further effort to expedite a reliable *Euplotes* strain assignment to *E. crassus*, *E. minuta* or *E. vannus* has now been prompted by an analysis of the point mutations (nucleotide substitutions and insertions/deletions) lying in the sequences of the nuclear small subunit (SSU) rRNA gene (18S henceforth), and of the internal transcribed spacer (ITS) region (ITS1+5.8S+ITS2+28S partial), which have been determined from a new collection of 37 long-lived wild-type strains of worldwide geographic origin. Among the overall 226 point-mutations detected in these sequences, nearly a half were revealed to be species-specific autapomorphic characters of straightforward value to design species-specific oligonucleotides. Three of these oligonucleotides were selected and proved to be very useful for setting up a diagnostic polymerase chain reaction (PCR), which reliably and rapidly resolves the taxonomic strain assignment to one or another of the three species.

## Materials and methods

### Strains and morphometric analysis

The 37 strains used in this study are listed in Table 1, along with the site and year of collection. Each strain was expanded starting from a single wild isolate and grown, at 20-22 °C, as monoclonal cultures. The green alga *Dunaliella tertiolecta* was used as food source, cultivated in natural, or artificial seawater (salinity 33‰) previously filtered, heated twice to 85 °C, and enriched with the Walne medium (Walne, 1970).

Cortical structures and the nuclear apparatus were stained with the Chatton-Lwoff silver nitrate technique applied according to Corliss (1953), except for cell fixation with 2.5% (v/v) glutaraldehyde in sea water for 60 min, at 4 °C, or with the Feulgen reaction, respectively. Measurements were taken with the Leica IM 1000 software on 10 properly oriented silver-stained specimens, recorded with a Leica DMR light microscope equipped with a Leica DC 200 digital

camera (Leica Microsystems). The SPSS for Windows and SYSTAT Version 10 software (SPSS Inc.; Chicago, USA) were used to assess the multivariate technique of discriminant functions. Graphs were produced by the Statistical Version 5.1H for Windows (Statsoft; Tulsa, USA).

**Phylogenetic analyses**

The nuclear 18S and ITS region sequences were obtained by running routine PCR amplifications with the primers listed in Table S1, and are deposited at GenBank/EMBL databases with the accession numbers listed in Table 2. Their multiple alignments were performed with the CLUSTALX program (version 1.81) (Thompson et al. 1997), edited with the BioEdit program (version 7.0.0) (Hall 1999), and the homogeneity of the signal from the nuclear 18S and ITS region sequences was ascertained with a partition homogeneity test (Farris et al. 1995) on 100 replicates. The application of the Maximum Likelihood (ML), Maximum Parsimony (MP), and Bayesian Inference (BI) methods was performed with the programs Tree-Puzzle (version 5.0) (Schmidt et al. 2002), PAUP (version 4.b10) (Swofford 2002) and MrBayes (version 3.1) (Ronquist and Huelsenbeck 2012), respectively.

In the ML and BI analyses, the substitution model 'HKY + G' (Hasegawa et al. 1985) was selected as the most appropriate (-lnL = 7721.6787), using the Akaike Information Criterion (AIC) by the Modeltest program (version 3.7) (Posada and Crandall 1998). The model was applied to the nuclear 18S and ITS region dataset with the following settings: base frequencies of 0.2849 (A), 0.1902 (C), 0.2326 (G), 0.2923 (T); a transition/transversion (Ti/Tv) ratio of 1.4192; and a gamma distribution shape parameter (α) of 0.331.

In the BI analysis, two sets of Markov Chain Monte Carlo (MCMC) simulations were run, each set had four chains, and the generation number in the MCMC simulations was 1,110,000 with the first 250,000 discarded as burn-in.

The reliability of the internal branches in the ML and MP phylogenetic trees was evaluated with the bootstrap method and 1,000 replicates (Felsenstein 1988), while in the BI tree it was

evaluated by calculating posterior probabilities.

### Diagnostic PCR amplifications

Aliquots (100 ng) of total DNA preparations from each strain were added to 50 μL reaction mixtures containing 2 mM $MgCl_2$, 250 mM of dNTP, one unit of Taq DNA polymerase (Polymed, Florence, Italy) plus 0.2 mM of each primer, and run in a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) adopting the following amplification profile. After a denaturation step at 94 °C for 3 min, 35 amplification cycles were carried out, each consisting of one 94 °C denaturation step for 30 sec, one 60 °C annealing step for 30 sec, and one 72 °C elongation step for 60 sec. A final incubation step, at 72 °C for 5 min, was added to the last cycle. Amplicons were separated on 1% agarose gels containing ethidium bromide for visualization.

**Zoobank registration:** urn:lsid:zoobank.org:pub:59220706-D20C-4A99-9A49-FF4C305B5BF1

## Results and discussion

### Morphological identification

All 37 strains invariably showed a single-type dargyrome, 10 fronto-ventral plus five transverse cirri, and a very similar hook-shaped macronucleus posteriorly endowed with a foot-like knob and anteriorly associated with one, or occasionally a few perfectly spherical micronuclei. They were initially assigned to the *E. crassus, E. minuta,* or *E. vannus* morphotype based on measurements of the more variable diagnostic traits used in the *Euplotes* taxonomy, i.e., body length and width, buccal field length, numbers of (dorsal plus ventrolateral) kineties, dikinetids of the mid-dorsal kinety, argentophilic polygons adjacent to the mid-dorsal kinety, adoral membranelles and caudal cirri. The average values of recorded morphometric data are reported in Table 3 along with the (theoretical) measurements earlier computed by Valbonesi et al. (1988) for

the *E. crassus*, *E. minuta*, and *E. vannus* TMO's. The composition and the plot of the two linear discriminant functions (1 and 2), accounting for 55.2% and 44.8% of the variance of the measured or counted characters, are reported in Fig. 2. In the bidimensional space, the measured specimens separated into three clearly distinct species-specific clusters, with the character 'body length' providing more weight to function 1 in separating the *E. minuta* cluster from the two other clusters, and the character 'kinety number" providing more weight to function 2 in separating the *E. crassus* and *E. vannus* clusters.

**Phylogenetic relationships**

The morpho-taxonomic assignment of the 37 strains to one or the other species was next validated genetically by primarily determining the nuclear 18S and ITS sequences of each strain and, using these sequences, combined into a single data set that was not significantly incongruent (P = 0.83) on the basis of the partition homogeneity test (Farris et al. 1995), to build the phylogenetic tree shown in Fig. 3. Two closely equivalent additional trees, generated from the sequences determined also from the mitochondrial SSU rRNA gene and the nuclear translation elongation factor 1 alpha 1 gene are reported in Figs S1 and S2.

It appears that the 37 strains associate into three clearly distinct *E. crassus*, *E. minuta* and *E. vannus* groups, which are supported by high bootstrap and posterior probability values. Quantified as percent of nucleotide mutations on the whole dataset of the nuclear 18S and ITS region sequences, the inter-species divergences (preponderant in the more variable ITS region) increase from 2.8–3.7% between the *E. crassus* and *E. vannus* strains, to 5.0–5.2% between *E. minuta* and *E. vannus* strains, and to 5.9–6.5% between *E. minuta* and *E. crassus* strains. They greatly exceed the intra-specific divergences (Table S3) calculated to be minimal (0–0.1%) among *E. minuta* strains, intermediate (0–0.6%) among *E. vannus* strains, and maximal (0–1.3%) among the *E. crassus* strains which, consistent with their larger number and wider distribution, likely represent various,

partially separated genetic sub-groups or populations, as earlier reported in this species in relation to auto-ecological and breeding analyses (Giannì and Piras 1990; Luporini and Valbonesi 1992).

**Autapomorphic point mutations and diagnostic PCR amplifications**

Among 226 polymorphic sites overall detected by comparing the nuclear 18S and ITS region sequences determined from the 37 strains (Fig. S3), 101 were identified as autapomorphic point mutations: 44 located in the 18S sequences (mostly in the hypervariable V4 region), and 57 in the ITS region sequences (Fig. 4A). Nearly half of these 57 autapomorphic ITS sites localized to adjacent or very close positions, thus prompting a keen interest to design species-specific oligonucleotides. Three sequence stretches, each including at least five autapomorphic point mutations, proved to be very effective regions to design primers of practical use to run diagnostic PCR amplifications of *E. crassus*, *E. minuta* and *E. vannus* strains. They are: (i) the oligonucleotide 5'-AGAGTGCGAATAAATGCAACAAGCAA-3', tagged 'Ec-27mer' and designed on the 2,296-2,324 nucleotide stretch of the ITS-2 spacer region, specific to *E. crassus*; (ii) the oligonucleotide 5'-TCAGTGTATCTTTAACAACAAATAAAGA-3', tagged 'Em-28mer' and designed on the 2,151-2,178 nucleotide segment lying between the 5.8S gene and ITS-2 spacer region, specific to *E. minuta*; and (iii) the oligonucleotide 5'- TTTGATTAGTAGACTTGACATCATAAG-3', tagged 'Ev-27mer' and designed on the 1,977-2,003 nucleotide stretch/sequence of the ITS-1 spacer region, specific to *E. vannus* (Fig. 4B).

Used on DNA preparations from each of the 37 strains as forward primers in combination with the *Euplotes*-generic reverse primer 'ITSR' designed on the 28S gene sequence (Di Giuseppe et al. 2013), the three oligonucleotides, Ec-27mer, Em-28mer, and Ev-27mer, generated products of markedly different, species-specific lengths (Fig. 4B). The Ec-27mer + ITSR combination generated a 669-bp amplicon only from *E. crassus* strains; the Em-28mer + ITSR combination generated an 810-bp amplicon only from the *E. minuta* strains; and the Ev-27mer + ITSR

combination generated a 985-bp amplicon only from the *E. vannus* strains (Fig. 4C). The species-specificity of these PCR-based assays was further validated by mixing the three species-specific oligonucleotides and using this mixture in combination with the *Euplotes*-generic reverse primer ITSR to amplify DNA preparations from *E. crassus*, *E. minuta* and *E. vannus* strains. A single amplicon of the expected species-specific length was obtained from each amplification (Fig. 4C).

Assayed in PCR amplifications of DNA preparations from strains (Table S4) representative of *Euplotes* species showing closer (*E. euryhalinus* Valbonesi and Luporini, 1990, *E. focardii* Valbonesi and Luporini, 1990, and *E. rariseta* Curds, West and Dorathy, 1974), or more distant (*E. octocarinatus* Carter, 1972, *E. nobilii* Valbonesi and Luporini, 1990, and *E. raikovi* Agamaliev, 1966) phylogenetic relationships with *E. crassus*, *E. minuta* and *E. vannus,* the three oligonucleotides Ec-27mer, Em-28mer and Ev-27mer generated no products (data not shown).

# Conclusions

In ciliates, and protists in general, the taxonomic boundaries of the morphological variations observed in laboratory samples of natural populations can be inferred with reference to a narrow set of structural traits. Among these, only the cirrotype and dargyrome patterns are in *Euplotes* credited with a solid diagnostic value, both showing stable variations between genetically distinct conspecific populations. Having in common the same single-type dargyrome and the same cirrotype-10, the identification of the *E. crassus*, *E. minuta* and *E. vannus* morphotypes has necessarily been based on comparisons of other, less distinctive traits that morphometric analyses have attested, in *Euplotes* species in general (Gates 1978), to be taxonomically much less reliable, suffering substantial intra-clonal variations. As a result, only *E. minuta* has generally been accepted as being immediately recognized by a smaller/oval morphotype, while the distinction between *E. crassus* and *E. vannus* has persistently caused puzzlement due to contrasting views (inheritance of discrepancies in dated literature) in recognizing which of the two morphotypes is characterized by a

medium/oval, or a larger/reniform cell body.

Although biochemical, ecological, and phylogenetic approaches have in general supported the morphological separation of *E. crassus*, *E. minuta* and *E. vannus*, mating assays and breeding analyses between cultures delegated to represent in particular *E. crassus* and *E. vannus* natural populations generated contrasting views. Some studies pointed to the individuality of the two species (Dini and Nyberg 1999; Giannì and Piras 1990; Nobili et al. 1978; Schlegel et al. 1988; Valbonesi et al. 1988) and others pointed to inextricable genetic mixing consequent to lack of effective barriers to interspecific gene flow (Caprette and Gates 1994; Génermont et al. 1985; Machelon 1982). The mating type systems of the 'high-multiple' (virtually 'open') type, on which *Euplotes* species in general rely to control the gene exchanges through the sexual phenomenon of conjugation, well account for these contrasting conclusions (Valbonesi et al. 1992). The mating-type systems of the 'closed' ('binary', and 'low-multiple') type, such as those evolved in *Paramecium* and *Tetrahymena* (Orias et al. 2017), make it possible to reliably establish the species boundaries by analyzing mating and breeding compatibilities versus incompatibilities between sets of strains representing all the mating types of the species. Instead, the 'high-multiple' systems make it inescapable to set up experimental strain collections that can cover only a scanty pool of the real natural intra-specific genetic polymorphism. In addition, most collections are destined to vary markedly in composition from one to another laboratory in relation to the areas that are sampled and, except for a few reported cases (Génermont et al. 1985; Valbonesi et al. 1988), their usually short life spans do not facilitate inter-laboratory strain exchanges useful for carrying out taxonomic cross-validations.

The reliable resolution of chronical ambiguities in the identification of an *Euplotes* strain with *E. crassus*, *E. minuta* or *E. vannus* is clearly the main virtue of the diagnostic PCR protocol described in this work. Requiring small samples of cells as starting experimental material, it is also quite an immediate and affordable application, and this avoids investing time and resources

in stabilizing massive cultures necessary for an integrated morpho-genetic taxonomic approach.

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# Figure legends

**Fig. 1. A–D.** Photomicrographs of *E. crassus*, *E. minuta,* and *E. vannus* unfed resting cells. **A**. Brightfield image showing inter-species differences in body shape and size. **B–D**. Scanning electron microscopy images of the dorsal (on the left) and ventral (on the right) cell body surfaces of *E. vannus* (B), *E. crassus* (C), and *E. minuta* (D). On the dorsal surface of the *E. crassus* cell, the single-type dargyrome is locally highlighted by re-tracking the alveolar boundaries. Bars: 20 µm. (A, modified from Guella et al. 2010; B–D, modified from Dini and Nyberg 1999).

**Fig. 2.** Composition and plot of the first two discriminant functions (1 and 2) resulting from measurements of the eight recorded diagnostic characters of *Euplotes* taxonomy. Symbols in the clusters refer to total specimens analyzed for each species. Ellipses outline 95% confidence areas.

**Fig. 3**. Phylogenetic relationships among the 37 *E. crassus*, *E. minuta* and *E. vannus* strains, based on the nuclear 18S and ITS region sequence dataset. Bootstrap values for Maximum Likelihood (ML), Maximum Parsimony (MP), and posterior probability values for Bayesian Inference (BI) are provided for each node (ML/MP/BI). The scale bar corresponds to five substitutions per 1,000 nucleotide positions. The species *E. focardii* was used as outgroup, according to Di Giuseppe et al. (2014) and Syberg-Olsen et al. (2016). For strain designation, see Table 1.

**Fig. 4. A–C.** Autapomorphic point mutations, species-specific primers and diagnostic PCR amplifications. **A**. Autapomorphic sites identified in the 18S and ITS region sequences of the *E. crassus*, *E. vannus* and *E. minuta* strains. The indicated nucleotide positions are those reported in the nucleotide sequence alignment of Supplementary Fig. S3. The ITS autapomorphic sites included in the species-specific primers are boxed. **B**. Upper panel: schematic representation of the relative positions and orientations of the three species-specific primers and the *Euplotes*-generic

primer used in the diagnostic PCR amplifications. Lower panel: expected sizes of the amplicons from each *Euplotes* species. **C**. Electrophoretic separation of the amplicons obtained from the diagnostic PCR amplifications, run with the indicated primer combinations. The results shown are representative of one strain for each species.