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UNA SPECIE, DUE EMISFERI: POPOLAZIONI ANTARTICHE E ARTICHE DI PROTISTI CILIATI APPARTENGONO ALLA STESSA SPECIE BIOLOGICA

Abstract - *One species, two hemispheres: Antarctic and Arctic populations of ciliated protists belong to the same biological species* - Microbes dwelling in oceans are of vital importance to the planet health, and their taxonomic, biological and ecological characterization is essential to better understand how the oceans are changing and how the ocean life is adapting to changes. Among a vast collection of strains of the ciliated protist, *Euplotes,* isolated from sediments of coastal waters in Terra Nova Bay (Antarctica), Tierra del Fuego, Greenland and Svalbard Islands, we first identified a complex of twenty strains morphologically and genetically representative of *E. nobilii*. These strains were then analyzed for their mating and breeding interactions and found to represent also the same biological species. Mating pairs were isolated from Antarctic and Arctic strain combinations and shown to be fully capable of completing inter-partner gene exchange and generating viable offspring. From the determination of nuclear gene sequences coding for 18S ribosomal RNA it was lastly possible to identify two Arctic strains characterized by sequence motifs specific of the Antarctic strains, thus implying that the *E. nobilii* populations rely on an effective inter-polar dispersion through the cold Oceanic deep currents to remain genetically connected.

Key words - Ciliated protists, microbial biogeography, natural hybrids, ribosomal genes.

Riassunto - I microorganismi che proliferano negli oceani sono di vitale importanza per la salute del nostro pianeta e la loro caratterizzazione tassonomica, biologica e ecologica è essenziale per sviluppare modelli sperimentali di uso più immediato e relativamente più semplice di quelli multicellulari nello studio di come gli ecosistemi marini reagiscono ai sempre più rapidi e drammatici cambiamenti ambientali che caratterizzano la nostra epoca. In questo contesto, ci siamo interessati alla caratterizzazione bio- e filogeografica di alcune decine di ceppi del ciliato *Euplotes* raccolti da sedimenti di acque costiere di Baia Terra Nova in Antartide, Terra del Fuoco, Groenlandia e Isole Svalbard. Dopo aver identificato 20 di questi ceppi come appartenenti alla stessa specie morfologica e genetica, *E. nobilii*, abbiamo proceduto a verificarne l'appartenenza anche alla stessa specie biologica mediante analisi delle loro interazioni sessuali legate al fenomeno della coniugazione. Queste interazioni hanno portato alla formazione di coppie i cui partner hanno mostrato di condividere lo stesso pool genico, in quanto pienamente capaci di completare un mutuo scambio genico e generare progenie vitale. Dalla determinazione delle sequenze geniche nucleari codificanti l'RNA ribosomale 18S è stato infine possibile identificare due ceppi di origine artica caratterizzati da motivi di sequenze geniche specifici dei ceppi antartici e, di conseguenza, concludere che le fredde (< 5 °C) correnti oceaniche di profondità giocano un ruolo chiave nella dispersione delle popolazioni antartiche di *E. nobilii* verso i mari artici.

Parole chiave - Protisti ciliati, biogeografia microbica, ibridi naturali, geni ribosomali.

INTRODUCTION

Microorganisms represent the smallest (in terms of the cell body size), but certainly most important component of the ocean life because of their direct involvement in all nutrient cycles and their basal position in every marine food web. Therefore, understanding how and where they live is essential to better understand the reactions of the marine life to the chemo-physical alterations that global warming is imposing to oceans. In this context, polar microorganisms are attracting increasing interest because of the centrality of their role in global-scale biogeochemical cycles, first of all the exchange of carbon dioxide with the atmosphere (Falkowski *et al.*, 2008).

A relevant result of this interest in the study of polar microbiology was the finding of microbial species that, like a diverse range of plant and animal species (Lindberg, 1991; Crame, 1993), warrant the definition as «bipolar» (or «anti-tropical»), i.e., species embracing high-latitude populations physically separated in distribution across the tropics (Darling *et al.*, 2000; Montresor *et al.*, 2003; Brandt *et al.*, 2007; Pawlowski *et al.*, 2007). This concept of species bipolarity has inherently raised an intriguing question on whether co-specific Antarctic and Arctic microbial populations evolved independently since the effective separation (around 10 million years ago) between the Arctic and Antarctic cold-water provinces or, instead, a trans-tropical gene flow still ensures that these polar populations maintain a genetic continuity (Darling *et al.*, 2000).

To address this question, the most commonly adopted morphological approach is clearly equivocal because of recurrent phenomena of parallel, or convergent, morphological evolution that take place in response to similar environmental stimuli. Much more effective is the criterion of «biological species», so as in animals and plants it is deduced from studying the ability of organisms to interbreed and generate viable offspring. However, this criterion has only seldom been applied in microbial ecology and biogeography because not only the prokaryotic (bacterial) component but also

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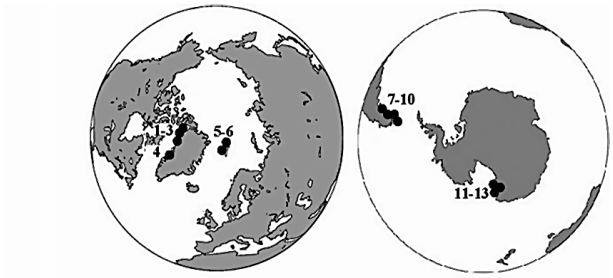
the eukaryotic (protistan) component are in large part asexual. Furthermore, most of the protist species that manifest sex are unable to survive and reproduce in the laboratory for the relatively long times that are usually necessary to carry out breeding analyses.
An exception to this general situation is represented by the vast protistan group of the ciliates. In addition to being easily collected from any natural habitat in which there is availability of liquid water and readily expanded into permanent laboratory clonal cultures, ciliates can effectively be studied sexually in laboratory because many species rely on the genetically controlled phenomenon of conjugation (in which cells of different and mutually compatible mating types unite temporarily in pairs) to exchange nuclear genes and reshuffle their gene pool (Dini & Nyberg, 1993). Of a numerous collection of Antarctic, Fuegian and Arctic strains representative of the most cosmopolitan and ubiquitously distributed ciliate genus, *Euplotes*, we studied the mating interactions of a group of 20 strains that were initially assigned to the nominal species *E. nobilii* on morphological and genetic grounds, and obtained direct evidence that these strains represent interbreeding and interfertile populations of the same biological species characterized by a bipolar distribution.



	Collection sites	Latitude/Longitude	Strains collected
Arctic	**1,** Qaanaaq	N 77 32'/W 69 20'	5QAA15; 2QAA; 1QAA
	2, Thule	N 76 32'/W 68 50'	2QAN1; 3QAN7
	3, Siorapalaluk	N 77 46'/W 70 48'	4QN9
	4, Ilulissat	N 69 13'/W 51 06'	4ILa4
	5, Pyramiden	N 78 39'/E 16 22'	4Pyrm4
	6, Longyearbyen	N 78 13'/E 15 38'	LGY4-4
Tierra del Fuego	**7,** Hoste Island	S 55 15'/W 69 00'	IsH3-1
	8, Punta Arenas	S 53 08'/W 70 54'	PA1-1
	9, Puerto Natales	S 51 43'/W 72 31'	PNA-1h
	10, Pinguinera Seno Otway	S 52 59'/W 71 14'	PUN1
Antarctic	**11,** Edmonson Point	S 74 19'/E 165 07'	AC-4; AC-5; Ed.Po-6
	12, Observatory Forecast Campus	S 74 44'/E 164 00'	AC-3
	13, Icaro Faraglione Campus	S 74 41'/E 163 59'	AC-1; Far; Sop

Fig. 1 - Map of the principal sites that have been visited to collect *E. nobilii* strains.

Materials and methods

Collection sites and strain cultivation

The complex of 20 wild-type strains of *E. nobilii* used in this study were each obtained starting from a single specimen isolated from sediments of shallow coastal waters of thirteen Arctic, Fuegian and Antarctic sites: four sites in Western Greenland (seven strains), two in the Svalbard Islands (two strains), four in Tierra del Fuego (four strains), and three in Terra Nova Bay (Ross Sea) (seven strains) (Fig. 1). Strains were grown either in natural or artificial seawater (32‰ salinity, pH 8.0), and maintained as stable laboratory cultures in cold rooms at 2-4 °C. Nutrients were provided by the green algae *Dunaliella tertiolecta* grown in natural sea water supplemented with Walne medium.

Morphological analysis

For each of the 20 strains the taxonomic status as morpho-species was established by measuring or counting all the phenotypic traits (Fig. 2) that are commonly used in the diagnosis of the nearly one hundred species that have been described in the genus *Euplotes* (Gates, 1977; Borror & Hill, 1995; Wilbert & Song, 2008; Jiang *et al.*, 2010; Pan *et al.*, 2012). The determination of phenotypic traits was carried out on ten selected specimens of each preparation at 1000 × magnification on a Leica DMR microscope, using the dedicated Leica IM1000 version 1.0 software STATISTICA (StatSoft, Inc.).

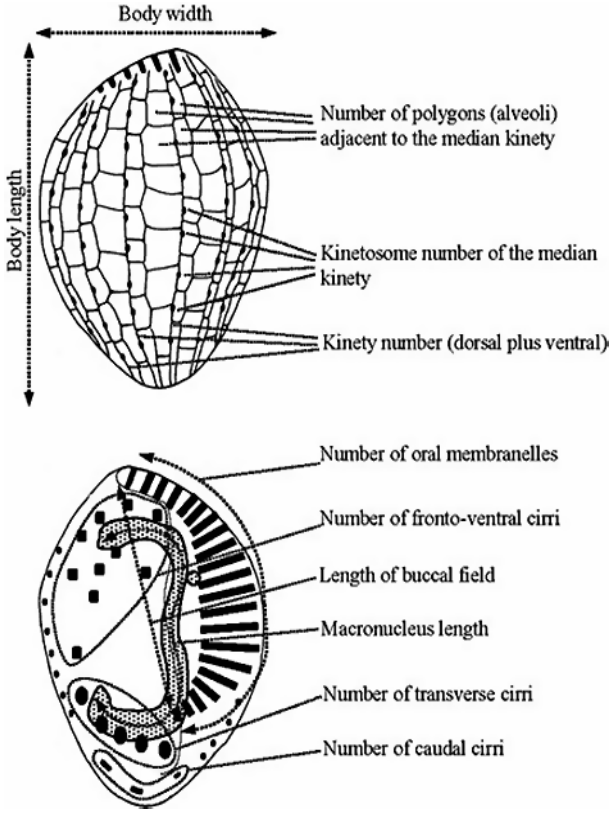


Fig. 2 - Illustration of the 11 phenotypic traits of taxonomic value used in the morphologic analysis of *E. nobilii*. (Upper) Cell dorsal surface. (Lower) Cell ventral surface.

DNA purification, PCR amplification, and gene sequencing

Purified DNA preparations were obtained, following an optimized protocol (La Terza *et al.*, 2009) and PCR-amplified for cloning the 18S nuclear SSU-rRNA gene using universal eukaryotic 18S primers. The PCR amplifications were run in a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, U.S.A.), following a standard program (30 sec of denaturation at 94 °C, 30 sec of annealing at 55 °C, 2 min of extension at 72 °C, for 35 cycles), with an initial denaturation step of 5 min at 94 °C and a final extension step of 5 min at 72 °C. The PCR products were purified using Quantum Prep PCR Kleen Spin columns (Bio-Rad, Hercules, CA, U.S.A.), and sequenced in both directions with an ABI Prism 310 automated DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Phylogenetic tree

The 18S nuclear SSU-rRNA gene sequences of *E. nobilii* strains used in this study were aligned using the program ClustalX (Thompson *et al.*, 1997), and the alignment was adjusted manually with the program BioEdit Sequence Alignment Editor (Hall, 1999) to optimize the base-pairing scheme of the rRNA gene molecule secondary structure (Van de Peer *et al.*, 2000). The phylogenetic tree was inferred by the Maximum Likelihood method applied according to the program Tree-puzzle version 5.0 (Schmidt *et al.*, 2002), and the most appropriate model of substitution warranting a reliable application was selected using the Modeltest 3.6 software (Posada & Crandall, 1998). The reliability of the internal branches of the phylogenetic tree was assessed using the bootstrap method (Felsenstein, 1988), with 1,000 replicates.

Mating interactions

Mating mixtures were prepared from cultures resuspended in fresh sea water for 1-2 days at an adjusted concentration of about 3×10^3 cells/ml. The intensity of mating reactions was assessed 2-3 days after cell mixing as ratio of paired to single cells. Mating pairs were individually isolated in a few drops of filtrate of their original mixtures, and left to separate into the two ex-conjugant cells and generate offspring clones. Ten pairs of sister progeny clones were expanded in stable cultures and used to determine the type of parental mating pairs.

Results and discussion

The complex of 20 Antarctic, Fuegian and Arctic *Euplotes* strains were first analyzed morphologically to verify their fitting with the original morphological description of *E. nobilii* (Valbonesi & Luporini, 1990), and genetically to establish their phylogenetic relationships on the basis of comparisons among 18S small-subunit(SSU)-rRNA nuclear gene sequences (Fig. 3). Complete SSU-rRNA gene sequence identity was revealed by the Antarctic and Fuegian strains, while the Arctic strains diverged up to a maximum of four nucleotide mutations and one to three mutations were found to separate the Arctic strains from the Antarctic and Fuegian strains. Although a single nucleotide mutation in the SSU-rRNA gene sequence has been proposed in the genus *Stylonychia* to represent a significant inter-species divergence (Bernhard *et al.*, 2001), in the light of the finding (Vallesi *et al.*, 2008) that more than 60 nucleotide mutations in SSU-rRNA gene sequence separate *E. nobilii* from its "sister" species of temperate waters, *E. raikovi*, we decided to regard these one- to four-nucleotide substitutions in the SSU-rRNA gene sequences of the *E. nobilii* strains as a case of evolutionarily not-significant intra-species sequence variability.

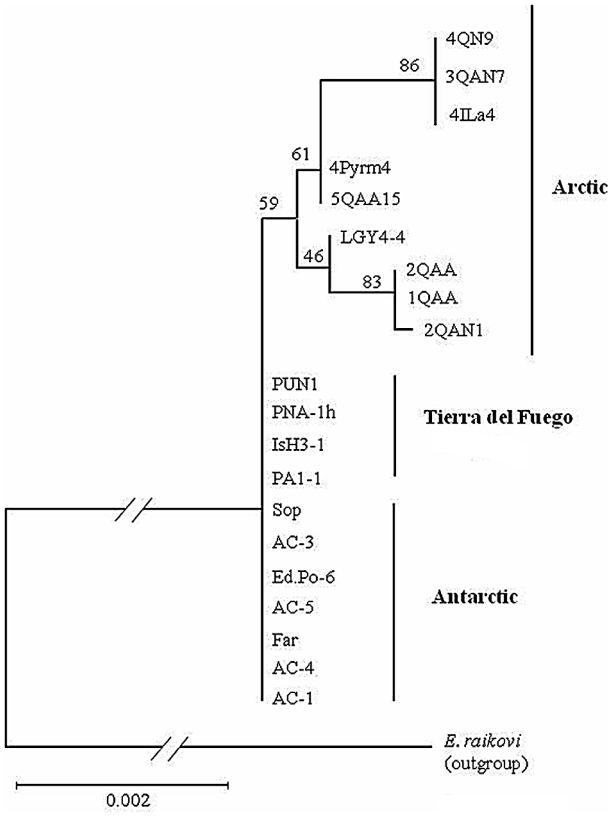


Fig. 3 - Phylogenetic correlations among Antarctic, Fuegian, and Arctic strains derived from multiple alignment of SSU-rRNA nuclear (18S) gene sequences. The numbers at nodes of the phylogenetic tree are values estimated as percentages from 1,000 bootstrap replicates. The 18S gene sequence of *E. raikovi*, regarded as the species most closely allied to *E. nobilii* (Vallesi *et al.*, 2008), was chosen as outgroup. The scale bar corresponds to a distance of substitutions per 1,000 nucleotide positions.

Following this preliminary morphological and genetic taxonomic identification of each strain with (the nominal species) *E. nobilii*, most attention was focused on the strain mating interactions by mixing strains in every pairwise combination. Mating pair formation (conjugation) was regularly observed to occur at varying levels of intensity in each combination of the three Arctic strains, 5QAA15, 2QAN1, and 4Pyrm4 with the three Antarctic strains, AC-1, AC-3, and AC-4, thus implying that these six strains shared genetic homogeneity and mating compatibility (Fig. 4).

However, a crucial aspect of mating interactions of ciliates, and *Euplotes* species in particular, is that mixing of two strains of different and compatible mating types (e.g., I and II) does not necessarily result in the formation of only heterotypic pairs (I-II) destined to complete cross-fertilization between mutually exchanged gametic nuclei. Homotypic pairs (I-I and II-II), that are obviously forced to perform self-fertilization (or autogamy in pairs), may equally be formed in addition and/or complete substitution of the heterotypic ones (Dini & Nyberg, 1993). The composition in hetero- and homotypic pairs of a mating mixture (pairs that all are morphologically alike) may thus be revealed only a posteriori by analyzing offspring clones for their patterns of inheritance of the mating-type trait. Unfortunately, this procedure is hardly applicable to polar ciliates, which are characterized by cell cycles that are more than four-fold longer than in ciliates of temperate waters, and by life cycles with immaturity (or adolescence) periods (during which cells are unable to mate) lasting months (Valbonesi & Luporini, 1993).

The difficulty to obtain an unequivocal distinction between homo- and heterotypic pairs, as well as between heterotypic pairs with cross-fertilization and heterotypic pairs without cross-fertilization, was circumvented by utilizing the SSU-rRNA gene sequences (which had previously been determined in relation to the phylogenetic analysis) as strain-specific and bi-parentally inheritable nuclear markers. All the possible types of mating pairs (i.e., homotypic and heterotypic with or without cross-fertilization) were detected in the nine possible pair-wise mating combinations between the three Antarctic and three Arctic strains, and each type of pair was found capable of generating viable offspring albeit with varying rates of survival. Of decisive importance to prove gene flow between Antarctic and Arctic *E. nobilii* populations was the finding that the mixtures between the Arctic strain 5QAA15 and each one of the three Antarctic strains all formed heterotypic pairs capable of completing cross-fertilization between mutually exchanged gametic nuclei. The offspring clones generated by mating pairs isolated from these three mixtures all appeared to have inherited both the parental SSU-rRNA gene sequences. On the other hand, homotypic pairs and heterotypic pairs with self-fertilization were observed in the mixtures involving each one of the three Antarctic strains with the other two Arctic strains, 4Pyrm4 and 2QAN1, thus implying that inter-strain mating compatibility is a necessary but not sufficient condition to ensure the occurrence of an effective gene exchange. The homotypic pairs were revealed by finding that the SSU-rRNA gene sequences of the offspring clones were all identical with one another and with the sequence of one of the two parental strains, and the heterotypic mating pairs with self-fertilization by finding that the two parental SSU-rRNA gene sequences were inherited in a roughly 1:1 ratio (Fig. 4).

In the light of the successful utilization of SSU-rRNA gene sequence profiles to detect effective gene exchange in mating pairs raised in laboratory between Antarctic and Arctic strains, preliminary attempts have been carried out to obtain genetic evidence for cross-breeding events between *E. nobilii* Antarctic and Arctic cells also in nature. In this perspective, we extended the analysis of SSU-rRNA gene sequences to new *E. nobilii* strains with the specific aim of detecting "double peaks" in the profiles of these sequences; hence, peaks identifying strains characterized by heterozygosity between distinct genotypes. Two strains, one (2QAA) representative of the *E. nobilii* Western Greenland population and one (LGY4-4) representative of the Svalbard population, have in effect been found to contain SSU-rRNA gene sequences characterized by the presence of double peaks.

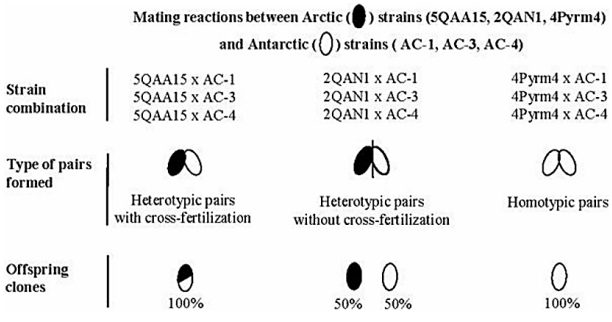


Fig. 4 - Mating interactions between Antarctic and Arctic *E. nobilii* strains.

Conclusions

Results from cross-breeding analyses between Antarctic and Arctic strains of *E. nobilii* have significantly reinforced previous evidence from morphological and phylogenetic observations that these strains represent genetically homogeneous antipodal populations which are largely sharing a common gene pool. The survival rates of F_1 offspring from crosses between Antarctic and Arctic strains were found to be in general relatively low (in the range from 20 to 63%) (Di Giuseppe *et al.*, 2011), and we were unsuccessful in raising long-term viable F_2

offspring clones from backcrossing F_1 clones to either one of the two parental strains (data not shown). These results (yet reflecting also practical difficulties in managing breeding analyses of a strictly cold-adapted organism) clearly imply the existence of some degrees of isolation and/or post-zygotic barriers to the gene flow between the Antarctic and Arctic *E. nobilii* populations. However, the strong mating compatibility and the effective cross-fertilization that we showed to take place between the Antarctic and Arctic populations represent compelling evidence that the separation between these populations has not yet passed the point of no return and, hence, validate the concept that *E. nobilii* is a biological species characterized by a bipolar distribution. How antipodal populations of *E. nobilii*, and in general of microorganisms that are similarly unable to form resting cysts for passive environmental dispersal, may maintain genetic continuity in spite of their apparent ecological discontinuity is an intriguing question that can hardly be verified directly. This continuity implies that trophic individuals of these populations are able to swarm and, hence, to ensure a gene flow from one to the opposite extremity of the globe by dwelling and multiplying in the stably cold currents that cross the equatorial ocean depths. Support to this implication derives from the identification of *E. nobilii* Arctic strains containing SSU-rRNA nuclear gene sequences characterized by heterozygous sites, that likely reflect phenomena of natural hybridization between genetically distinct natural populations (Di Giuseppe *et al.*, manuscript in preparation). These phenomena have for long been extensively studied only in animals and plants (Arnold, 1997; Barton, 2001), but their driving role in speciation and evolution has now been documented also in microorganisms, in diatoms in particular (Casteleyn *et al.*, 2009; D'Alelio *et al.*, 2009).

The activity of swarmers is probably not the only force underlying the genetic continuity between the bipolar *E. nobilii* populations. A synergistic force is arguably represented also by the capacity of these populations to maintain a chemical continuity via diffusible protein signals, known as pheromones, which *E. nobilii*, like other *Euplotes* species (Luporini *et al.*, 2005), constitutively synthesizes in relation to the mating-type mechanism which is responsible for the cell mating interactions (Vallesi *et al.*, 2012). These pheromones have been isolated from both the Antarctic and Arctic *E. nobilii* populations and shown to be structurally homologous, mutually cross-reactive, and characterized by structural specificities that secure unmatched long-lasting activity and wide-range dispersal range in any marine environment (Di Giuseppe *et al.*, 2011).

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