Chapter 4
Pole-to-Pole Gene Flow in Protozoan Ciliates

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4.1 Backgrounds

Microorganisms represent the smallest but arguably most important component of the ocean life. They are essential to all nutrient cycles because they form the bottom of the marine food chain and outnumber all other marine species by orders of magnitude. Sampling of remote and inaccessible habitats and large-scale genomic analysis have shown how little we know about the microbial life in the oceans, and how our poor knowledge of the marine chemistry and biology is preventing us from foreseeing the detrimental effects that a too rapidly changing world has on the oceans' ecosystems. In this context, polar microorganisms are attracting particular interest because of their role in global-scale biogeochemical cycles, in particular the carbon dioxide exchange with the atmosphere (Falkowski et al. 2008).

Considering this interest, planktonic and benthic microbial communities from Arctic and Antarctic areas have become the focus of more systematic sampling

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and rigorous analyses for their taxonomic, prokaryotic (bacterial) and eukaryotic (protist), biodiversity. A relevant result of these analyses was the finding of microbial species that, like a diverse range of plant and animal species (Lindberg 1991; Crame 1993), warrant the definition "bipolar" (or "anti-tropical"), i.e. species represented by 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 the intriguing question whether co-specific Antarctic and Arctic populations evolved independently since the effective separation (approximately 10–15 million years ago) between the Arctic and Antarctic cold-water provinces, or whether a trans-tropical gene flow ensures that these polar populations maintain genetic continuity (Darling et al. 2000).

Morphological studies alone are clearly insufficient to address this question, due to recurrent phenomena of parallel or convergent morphological evolution that take place under similar environmental forces. Therefore, more solid grounds supporting the concept of species bipolarity have been obtained in some species of foraminifera and dinozoans from analysis of genetic variation in sequences of the small subunit (SSU) rRNA nuclear gene (Darling et al. 2000; Montresor et al. 2003; Brandt et al. 2007; Pawlowski et al. 2007). Nevertheless, unless the calibration of a molecular clock is supported by abundance in fossil records, as is the case in foraminifera (Pawlowski et al. 1997), also this genetic approach is impaired by the fact that the same DNA regions may evolve at different rates among closely related organisms.

A solution to overcoming this challenge is provided by the well-defined, monophyletic group of ciliates which are ideal organisms for the analysis of the breeding structure of natural microbial populations and, therefore, for obtaining data which satisfy the interbreeding criterion on which the biological (Darwinian) concept of species is founded. Ciliates govern their gene exchanges through a unique sexual phenomenon, known as conjugation (or mating), in which two functionally hermaphroditic individuals unite temporarily in pairs for a mutual exchange of a "migratory" ("male") gametic nucleus destined to fuse into a synkaryon with a "resident" ("female") gametic nucleus. In addition, ciliates more than any other group of polar microorganisms can readily provide living laboratory material in virtually unlimited amounts, since every aquatic polar habitat hosts a large variety of ciliate species that are relatively easy to collect and expand into permanent clonal cultures (i.e., descendants from single wild-type specimens).
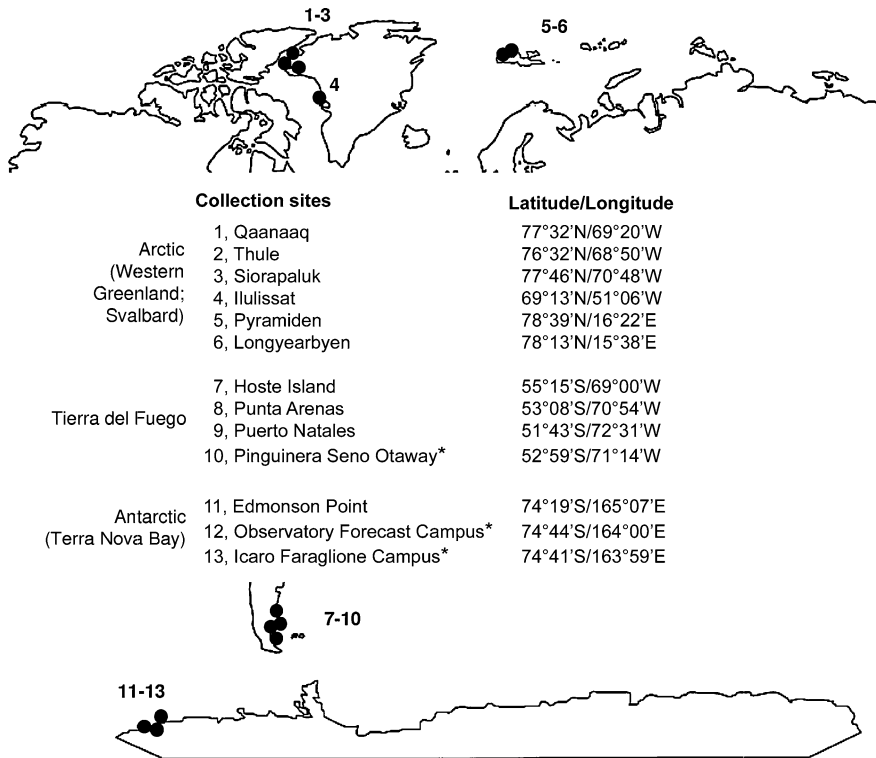
Here we review results that have been obtained from breeding analyses of Antarctic, Fuegian and Arctic populations of the ciliate *Euplotes nobilii*. These results have provided compelling evidence that these populations are genetically interconnected by gene flow and form a unique interbreeding biological species. The description of these results is preceded by an outline of our current knowledge of ciliate biodiversity in the polar regions, and by a rationalization of the genetic mechanism of the mating types that represents the major driving force of ciliate breeding strategies.

4.2 Ciliate Biodiversity at the Poles

Diversity of ciliate species in Arctic and Antarctic ecosystems has been studied inconsistently, and most studies have essentially described new morphological species from polar habitats (e.g. Corliss and Snyder 1986; Valbonesi and Luporini 1990a, b; Agatha et al. 1990, 1993; Petz et al. 1995; Kepner et al. 1999; Petz 2005; Wilbert and Song 2005, 2008). One exception is an extensive taxonomic survey of Antarctic and Arctic limnetic ciliates (collected from numerous lakes, ponds, and streams of Livingston Island in South Shetlands, Terra Nova Bay in Victoria Land, Brøgger Peninsula and Ossian Sarsfjellet in the Svalbard Archipelago), that has deliberately been carried out to assess whether ciliate polar biogeography is characterized by endemism, or global distribution of species (Petz 2004; Petz et al. 2007). Nearly 400 distinct morphospecies were overall identified. Although the majority resulted to be species already known from non-polar environments, 20 % were identified as new to science and 13 % appeared to be equally represented in Antarctic and Arctic waters. This finding represents the first (morphological) evidence that ciliates may include species represented by populations that live physically separated at the opposite ends of the globe.

4.3 Ciliate Mating Systems

As mentioned above, conjugation is the basic sexual phenomenon on which ciliates rely to perform gene exchange and regulate the genetic distance among populations. In many species, this phenomenon involves the differentiation of genetically and physiologically distinct cell classes that have been designated as "mating types" in relation to their capacity to interact mutually, either through physic cell–cell contacts or diffusible signaling pheromones, for mating-pair formation (Dini and Nyberg 1993). These mating types may only be two as shown by most species of *Paramecium*, or tens within each species as is the case in *Euplotes* and other hypotrichous ciliates (Phadke and Zufall 2009). While the binary mating systems have functionally been associated with the duality of sex, the functions of the multiple mating systems have generally been considered to be much more comparable, for example, with the devices that are intrinsic to the self/non-self recognition mechanisms evolved in flowering plants to ensure cross pollination (Luporini et al. 2005). Ciliate species with multiple mating types are in fact usually viewed as distinguishable by eco-genetic strategies strongly committed to outbreeding, because the mating options and the chances to mate with a stranger will, at least in principle, increase for every member of a species along with an increase in the mating type number.

| Collection sites	Latitude/Longitude
Arctic (Western Greenland; Svalbard)	
1, Qaanaaq	77°32'N/69°20'W
2, Thule	76°32'N/68°50'W
3, Siorapaluk	77°46'N/70°48'W
4, Ilulissat	69°13'N/51°06'W
5, Pyramiden	78°39'N/16°22'E
6, Longyearbyen	78°13'N/15°38'E
Tierra del Fuego	
7, Hoste Island	55°15'S/69°00'W
8, Punta Arenas	53°08'S/70°54'W
9, Puerto Natales	51°43'S/72°31'W
10, Pinguinera Seno Otaway*	52°59'S/71°14'W
Antarctic (Terra Nova Bay)	
11, Edmonson Point	74°19'S/165°07'E
12, Observatory Forecast Campus*	74°44'S/164°00'E
13, Icaro Faraglione Campus*	74°41'S/163°59'E

Fig. 4.1 Map of the principal sites that have been visited since 1988 to collect strains of polar species of *Euplotes*. Asterisks indicate informal names

4.4 Collection Sites and Polar *Euplotes* Species

During an interval of approximately 20 years, it has been possible to accumulate some hundreds of *Euplotes* strains as stable laboratory cultures starting from specimens isolated from various seashore sites of Antarctica, Tierra del Fuego and the Arctic (Fig. 4.1). These strains were initially analyzed morphologically to assign their species status, by measuring and counting common diagnostic parameters such as cell body dimensions, number and positional patterns of ciliary organelles, and number and geometry of cortical alveoli. Only a minority of strains have been found to represent known *Euplotes* species, such as *E. rariseta* and *E. kahli* (Valbonesi and Luporini 1990a; unpublished data), whereas the large majority appeared to represent new *Euplotes* species of which, however, only few have received official taxonomic designations so far (Valbonesi and Luporini 1990a, b).

One of the new species, *E. focardii*, is represented uniquely by Antarctic strains, and this is likely endemic to the Antarctic waters. In contrast, two other new species, i.e. *E. euryhalinus* and *E. nobilii* (Fig. 4.2), which were originally

Fig. 4.2 Scanning electron micrograph of *E. nobilii* showing the cell left site with the ciliary membranelles surrounding the oral groove. On the ventral site other compound ciliary organelles (cirri) are visible whose number and positional patterns have maximal diagnostic relevance for *Euplotes* species identification
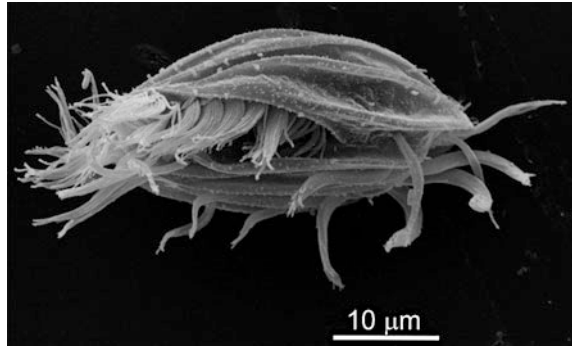
described with reference only to Antarctic strains, have subsequently been collected also from Fuegian and Arctic sites.

Because of their wider geographic representativeness and apparent incapacity to form passive dispersal stages (cysts) in response to variations in the cultivation conditions (unpublished observations), the overall complex of Antarctic, Fuegian and Arctic strains of *E. nobilii* have been chosen as experimental material to investigate, on phylogenetic and breeding grounds, whether they represented populations that are genetically discontinuous as a consequence of their spatial separation, or populations that are genetically continuous despite their spatial separation.

4.5 Phylogenetic Relationships

The phylogenetic correlations among the Antarctic, Fuegian and Arctic *E. nobilii* strains have been deduced by comparing the SSU-rRNA nuclear gene sequences determined for each of a set of 18 strains (seven Antarctic, four Fuegian and seven Arctic). As shown in Fig. 4.3, the Antarctic and Fuegian strains were found to possess completely identical sequences and, consequently, grouped altogether into the same cluster. Instead, sequence variations for one- to maximum four-nucleotide substitutions were found to be distinctive of the Arctic strains, that were thus resolved into multiple clusters distinct from the cluster including the Antarctic and Fuegian strains.

Although a single nucleotide variation between SSU-rRNA gene sequences has been reputed to be sufficient for an inter-species discrimination in other ciliates such as *Stylonychia* (Bernhard et al. 2001), more than 60 sequence variations have been found to mark the phylogenetic divergence of *E. nobilii* from its closest allied species, *E. raikovi* (Vallesi et al. 2008). In light of this degree of inter-species divergence in *Euplotes*, the one- to four-nucleotide mutations accumulated by the SSU-rRNA gene sequences of the *E. nobilii* Arctic strains have been regarded as reflecting sequence variations that have a merely intra-specific value, and not to be indicative of true genetic and evolutionary separation.
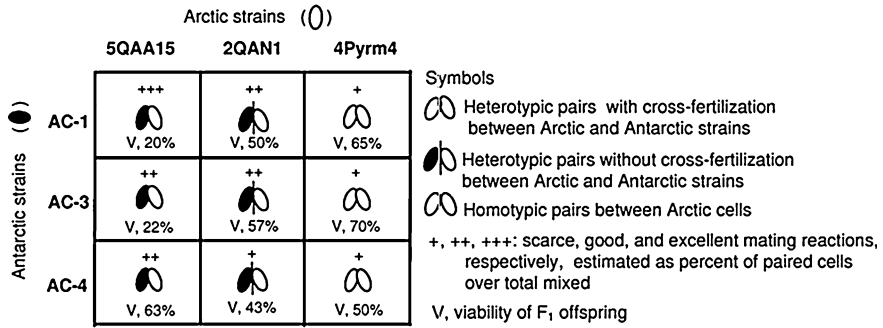
Fig. 4.3 Phylogenetic correlations among Antarctic, Fuegian, and Arctic strains derived from multiple alignment of SSU-rRNA nuclear gene sequences. The numbers at nodes of the phylogenetic tree are values estimated as percentages from 1,000 bootstrap replicates. The numbers in parentheses indicate the collection site of the strain (see Fig. 4.1). *E. raikovi*, used as outgroup, is the species most closely allied to *E. nobilii*
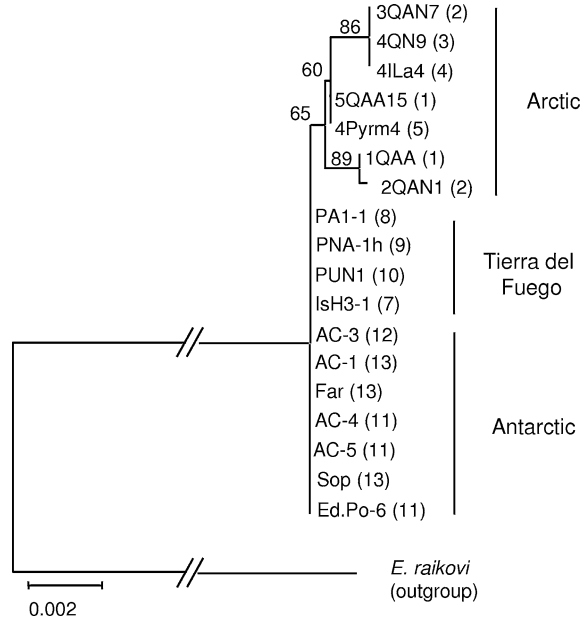
4.6 Mating and Breeding Interactions

Analyses of mating interactions, initially restricted to the Antarctic strains which were the first to be collected and morphologically diagnosed as representative of *E. nobilii* (Valbonesi and Luporini 1990a), provided evidence that, like other *Euplotes* species (Dini and Nyberg 1993), *E. nobilii* is represented in nature by multiple mating types that mediate their activity through diffusible, cell type-specific signaling pheromones (Felici et al. 1999). With the extension of the study of mating interactions to Fuegian and Arctic strains it was first shown that some pheromones structurally characterized from Antarctic and Arctic strains were represented by helical proteins in all members of the same homologous family (Alimenti et al. 2009; Di Giuseppe et al. 2011). Subsequently, it was observed that some Antarctic strains were fully able to form stable mating pairs not only in mixtures with Fuegian strains (with which there was sharing of identical SSU-rRNA nuclear gene sequences), but also in mixtures with Arctic strains (from which there were sequence divergences), thus implying a genetic continuity among the Antarctic, Fuegian and Arctic *E. nobilii* populations represented by these strains. This implication was verified through detailed genetic analysis of offspring clones raised from mating pairs formed in pair-wise mixtures of a selected set of three Antarctic strains (i.e., AC-1, AC-3 and AC-4 collected from Terra Nova Bay) and a selected set of three Arctic strains (i.e. 5QAA15 and 2QAN1 collected from Western Greenland, and 4Pyrm4 collected from Svalbard).

A crucial aspect of ciliate mating interactions, and *Euplotes* species in particular, is that mixing of two strains of different and compatible mating types (e.g. A

Arctic strains (◯)

	5QAA15	2QAN1	4Pyrm4
AC-1	+++ / V, 20%	++ / V, 50%	+ / V, 65%
AC-3	++ / V, 22%	++ / V, 57%	+ / V, 70%
AC-4	++ / V, 63%	+ / V, 43%	+ / V, 50%

Symbols

Heterotypic pairs with cross-fertilization between Arctic and Antarctic strains

Heterotypic pairs without cross-fertilization between Arctic and Antarctic strains

Homotypic pairs between Arctic cells

+, ++, +++: scarce, good, and excellent mating reactions, respectively, estimated as percent of paired cells over total mixed

V, viability of F_1 offspring

Fig. 4.4 Mating interactions between Antarctic and Arctic *E. nobilii* strains. For each pair-wise strain combination the corresponding box indicates the intensity of mating interactions given on a three-step scale, the type of cell mating pairs formed, and the viability rate computed as percentage of ex-conjugant cells that were able to develop a new nuclear apparatus and expand into fully viable progeny clones. Adapted from Di Giuseppe et al. (2011)

and B) does not necessarily result in the formation of only heterotypic pairs (AB) destined to complete cross-fertilization between mutually exchanged gametic nuclei. Homotypic pairs (AA and BB), 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 and Nyberg 1993). The composition in hetero- and homotypic pairs of a mating mixture (pairs that are all morphologically alike) may thus be revealed only a posteriori by analyzing off-spring clones for their patterns of inheritance of the mating-type trait. However, this procedure is hardly applicable to polar ciliates, which are characterized by cell cycles that are more than four times 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 and Luporini 1993). The difficulty to obtain a clear distinction between homo- and heterotypic pairs, as well as between heterotypic pairs with cross-fertilization and heterotypic pairs without cross-fertilization, has been avoided by utilizing the SSU-rRNA gene sequences (which had previously been determined in relation to the phylogenetic analyses) as strain-specific and bi-parentally inheritable nuclear markers.

All 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 survival rates (Fig. 4.4). 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 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 of the parental SSU-rRNA gene sequences, which were distinguished from one another by the presence of different nucleotides (C or T) in the sequence position 660 (Fig. 4.5). On the other hand,
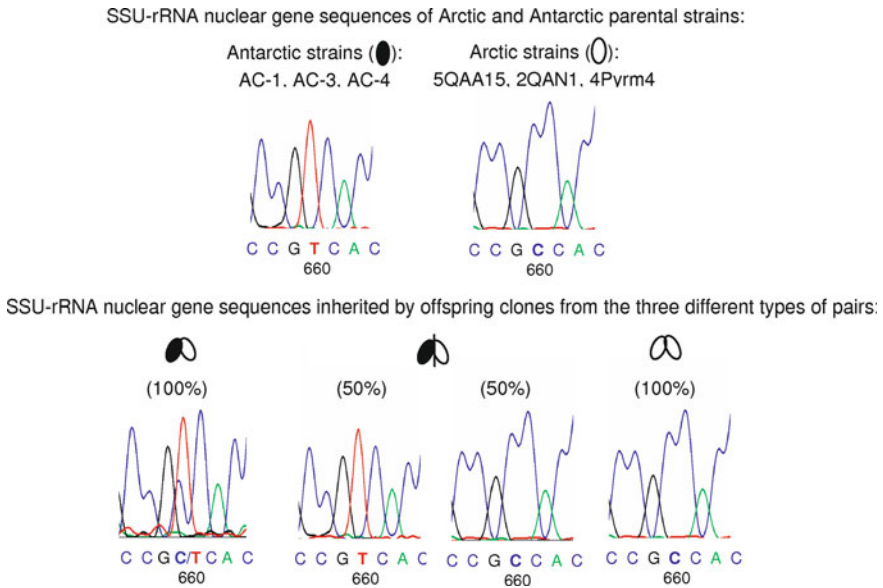
Fig. 4.5 Schematic presentation of the utilization of the strain-specific SSU-rRNA gene sequences to distinguish the different types of mating pairs (represented by symbols as in Fig. 4.4) formed by mating mixtures between Antarctic and Arctic strains. The number below the sequences indicates the position characterized by the nucleotide variation that is distinctive between the Antarctic and Arctic strains. The heterotypic pairs with cross-fertilization are unique in generating offspring clones with "hybrid" sequences characterized by a double C/T peak at the position 660, whereas the heterotypic pairs without cross-fertilization and the homotypic pairs generate offspring clones with unchanged sequences with respect to the parental strains

homotypic pairs and heterotypic pairs with self-fertilization were observed in the mixtures involving each 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 through the observation that the SSU-rRNA gene sequences of the offspring clones were all identical not only to each other but also to the sequence of one of the two parental strains; the heterotypic mating pairs with self-fertilization through the observation that the two parental SSU-rRNA gene sequences were inherited in a 1:1 ratio (Fig. 4.5).

4.7 Preliminary Evidence of Pole-to-Pole Gene Flow in Nature

In light of the successful utilization of SSU-rRNA gene sequence profiles in detecting effective gene exchange in mating pairs of Antarctic and Arctic strains raised in the laboratory, preliminary attempts were carried out to obtain genetic
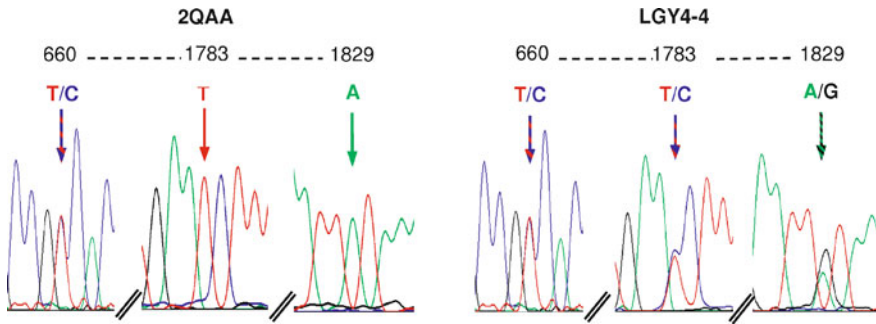
Fig. 4.6 Segments of SSU-rRNA nuclear gene sequences of the Arctic strains 2QAA and LGY4-4 showing the "hybrid" positions characterized by double T/C and A/G peaks

evidence of cross-breeding events between *E. nobilii* Antarctic and Arctic cells also in nature. These attempts were based on analyses of new Arctic *E. nobilii* strains to detect SSU-rRNA gene sequences characterized by the presence of double peaks denoting strains characterized by heterozygosity between distinct genotypes. As shown in Fig. 4.6, two strains, one (2QAA) collected from Western Greenland and one (LGY4-4) collected from Svalbard, have in fact been found (unpublished results) to contain SSU-rRNA gene sequences characterized by the presence of double peaks: only one in position 660 in strain 2QAA, and three in positions 660, 1783 and 1829 in strain LGY4-4. The double peaks in positions 1783 and 1829 appeared to be due to C/T and A/G combinations, and did not discriminate between a heterozygous condition generated by Arctic–Arctic cell cross-breeding and a heterozygous condition generated by Arctic-Antarctic/Fuegian cell cross-breeding. However, the double C/T peak in position 660 appeared to be compatible only with a heterozygous condition generated by Arctic-Antarctic/Fuegian cell cross-breeding, because every Antarctic/Fuegian SSU-rRNA gene sequence analyzed so far carries C in position 660 whereas every Arctic sequence carries T.

4.8 Concluding Remarks

Research on polar biogeography of eukaryotic microorganisms has to date largely neglected ciliates and privileged other taxa such as foraminifera and dinozoans, notwithstanding unique advantages we may derive from ciliates for assessing, directly in the laboratory, breeding performances and reproductive isolation of microbial natural populations. By crossing Antarctic, Fuegian and Arctic strains of *E. nobilii* it was possible to obtain compelling evidence that the populations represented by these strains are mating compatible and breeding fertile with one another and, therefore, share the same gene pool. How bipolar populations of *E. nobilii*, and in general microorganisms that are similarly unable to form resting

cysts for passive environmental dispersal, may maintain genetic continuity despite their apparent ecological discontinuity is an intriguing question that can hardly be verified directly. It implies that trophic individuals of these populations are able to swarm, and hence to ensure a pole-to-pole gene flow from one to the opposite extremity of the globe by dwelling (and multiplying) in the permanently cold currents that cross the equatorial ocean depths. To credit this implication with experimental data, *E. nobilii* strains are being analyzed for identifying both nuclear (bi-parentally inherited) and mitochondrial (uni-parentally inherited) ribosomal gene sequences characterized by single nucleotide polymorphisms, which represent the most common type of sequence variation in genomes and have rapidly emerged as powerful genetic markers of the evolutionary history of natural populations (Brookes 1999; Sannucks 2000; Nielsen 2000; Brumfield et al. 2003). As anticipated in Sect. 4.7, two Arctic strains characterized by nucleotide polymorphisms in their SSU-rRNA nuclear gene sequences have already been identified and the interpretation of these polymorphisms includes the possibility that they reflect phenomena of natural hybridization between Arctic and Antarctic populations. After having been extensively studied in animals and plants for their driving role in speciation and evolution (Arnold 1997; Barton 2001), these phenomena have now been genetically documented also between sympatric populations of diatoms (D'Alelio et al. 2009; Casteleyn et al. 2009).

The activity of swarmers is probably not the only force underlying the genetic continuity of the bipolar *E. nobilii* populations. A synergistic force is arguably represented also by the capacity of these populations to communicate and interact, like other *Euplotes* populations, via diffusible signaling pheromones that each cell synthesizes to promote not only mating but also vegetative growth (Luporini et al. 2005). Because of their structural homology, these pheromones are mutually cross-reactive and their structural specificities are such to secure unmatched long-lasting activity and wide-range dispersal in any marine environment (Di Giuseppe et al. 2011).

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