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Ciliate communities and hidden biodiversity in freshwater biotopes of the Pistoia province (Tuscany, Italy)

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Abstract

Ciliates are essential components of aquatic environments, playing a pivotal role in microbial loops. Thus, the composition and dynamics of ciliate communities have been subjected to intense studying. Morphological methods have been traditionally employed, until the development of next-generation sequencing recently allowed to explore the topic with exclusively molecular techniques. However, the results of the two approaches are hardly comparable, and the pictures they offer can be quite different. This may be due, among other reasons, to two factors: (1) morphological descriptions may miss a large portion of "hidden biodiversity" (including rare species and resistance forms) that is detected instead by molecular methods; (2) identification errors may arise due to difficulties in recognizing microbial taxa without in-depth analyses. In this survey of freshwater systems of the Pistoia province (Tuscany, Italy) we address both issues, trying to quantify the hidden diversity through prolonged observations of differentially treated sample aliquots, combining morphological identification with Sanger sequencing. We provide the first insights into the ciliate fauna of this area presenting results that are suitable for future comparisons thanks to their multidisciplinary origin, and supply the first molecular data on well-known taxa such as *Linostomella* and *Disematostoma*.

Keywords: Ciliate ecology; Late-blooming taxa; Morphology; NMDS; Sanger sequencing; 18S rRNA gene

Introduction

Unicellular protists of the phylum Ciliophora (ciliates) are important components of aquatic environments for several reasons, mainly because of their "intermediate" position in food webs linking nutrient flow between smaller microorganisms and larger metazoans (Azam et al. 1983; Beaver and Crisman 1989; Finlay and Esteban 1998; Pomeroy 1974; Segovia et al. 2015). Thus, the study of composition and dynamics of free-living ciliate communities is essential for a deep understanding of ecological relationships in these ecosystems. However, several issues hinder this endeavor. To cite some, ciliates are too inconspicuous to be observed without a microscope; their correct identification at the species level may be difficult and time-consuming (especially since many described taxa are not valid biological units, as proved by taxonomic investigations); and a large part of the biodiversity is usually "hidden" (Dunthorn et al. 2014; Fenchel et al. 1997; Finlay and Esteban, 1998).

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Here the term "hidden biodiversity" is intended to describe those taxa belonging to the investigated group and actually present in the community, but not detected by a given approach (Fenchel et al. 1997; Forster et al. 2015; Sogin et al., 2006). The efficiency of survey methods grows when this fraction of diversity wears thin. Organisms may be undetectable for several reasons, but resistance forms likely account for most of the "hidden" portion of free-living ciliates (Chao et al. 2006; Foissner 1987). Inconspicuous and less represented morphotypes may also evade detection.

For the sake of completeness, it is worth noticing that in other contexts "hidden" or "cryptic" biodiversity is used to highlight the fact that molecular methods may reveal a high degree of diversity within a single morphotype (Bickford et al. 2007; a classic example in ciliates was reported by Sonneborn (1975).

Another obstacle to a thorough analysis of ciliate communities present in natural environments is that it is difficult to "standardize" the sampling procedure. This occurs because investigators may be interested in different subenvironments, like the sediment/water interface or a certain layer in the water column, that harbor unlike communities and are sampled in different ways. This also entails that some components of aquatic ecosystems receive greater attention: in freshwater environments, lakes and ponds (e.g. Gaedke and Wickham 2004; Gong et al. 2005; Madoni and Sartore 2003; Mieczan 2007; Mironova et al. 2011; Pfister et al. 2002; Xu and Cronberg 2010) are scrutinized more often than lotic systems (e.g. Dias et al. 2008; Kiss et al. 2009; Madoni and Braghiroli 2007; Pauleto et al. 2009), and planktonic communities far more than their benthonic and psammonic counterparts (counterexamples include Madoni and Braghiroli 2007; Madoni and Sartore, 2003; Dias et al. 2008).

Finally, the recent shift to an extensive use of molecular methods in ecological surveys of microorganisms produces an additional difficulty in comparing works, since most recent papers exclusively provide either morphological or molecular results (Dunthorn et al. 2014; Weisse 2014). The modern employment of high-throughput next-generation sequencing methods is certainly improving the classical cloning protocols, but it cannot explain by itself the discrepancies between "culture-independent" and traditional methods, nor provide a key to compare different types of data (Bachy et al. 2013; Medinger et al. 2010; Stoeck et al. 2014).

In this work, we attempted a "combined" approach in order to perform a preliminary survey of freshwater biotopes in the Pistoia province (Tuscany, Italy). We observed the composition of free-living ciliate communities of various ecosystems, focusing on the sediment/water interface layer. We compared morphological identification of taxa with 18S rRNA gene sequencing in order to estimate potential errors and incongruities. To specifically address the issue of hidden biodiversity, we performed a long-term observation of differentially treated aliquots from each sample, following a methodology similar to those proposed by Chao et al. (2006) (for soil habitats) and Fenchel et al. (1997), only seldom applied in more recent investigation of freshwaters. It was our expectation to find significant differences among aliquots, and to uncover hidden elements of the community during prolonged inspection. Being able to quantify these factors would enable other studies to take into account the bias introduced by considering only the taxa detectable during a single examination.

Material and Methods

Sampling strategy

The Pistoia province in Tuscany (Italy) is geographically separated into a northern mountainous area (part of the Tuscan-Emilian Apennines) and a southern flatland. Ten sites representing lentic and lotic freshwater systems from plain and highland territories (respectively less than 200 and more than 1.000 meters above sea level) were selected. Lentic sites were further subdivided in artificial and natural (see Fig. 1), encompassing in the latter category the different aquatic biotopes of the province, including small mountain lakes, seasonal streams and the naturalistically important Fucecchio Marshland. One to four stations per site were chosen, and one sample of sediments and water (approximately in 1:5 ratio; total volume: 100 mL) was collected at each station at a shallow depth (10-50 cm). Half of the volume (50 mL) was kept for *in vivo* observations in a Falcon tube; the rest was subdivided in two parts immediately fixed in ethanol 70% (to be stored at −20 °C) and preserved in Bouin's fluid 10% (to be stored at room temperature) (Montagnes and Lynn 1993), respectively kept for future studies. Water temperature, pH, and O_2 concentration at sampling sites were measured with a SensoDirect 150 probe (Lovibond, Dortmund, Germany). The process was repeated in three different seasons: Autumn 2012 (a), Spring 2013 (b), Autumn 2013 (c).

Checklist compilation

The first (t_0) checklist was compiled on the sampling day. Falcon tubes were gently shaken, and about 25 mL of the original sample poured in two Petri dishes (aliquots I and II). The remaining volume was kept in the tube (aliquot III) to promote establishment of oxygen-depleted conditions. Both Petri dishes were observed at the dissecting microscope at 10–40× magnification, noting down ciliate morphotypes as shown in Supplementary Table S1. Each row of the checklist table constitutes an "observation". Morphotypes were assigned to broad abundance categories (rare, 1–3 organisms; some, 4–30 organisms; many, >30 organisms) and identified at the lowest taxonomic level allowed by a cursory observation, relying on specialized manuals (Curds 1982; Foissner et al. 1999; Kreutz and Foissner 2006; Lee et al. 2000). Whenever possible, organisms were observed in more detail under