RESEARCH ARTICLE

Seasonal dynamics of free-living tintinnid ciliate communities revealed by environmental sequences from the North-West Mediterranean Sea

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Abstract

The species-rich order *Tintinnida* is a group of planktonic ciliates ubiquitous in coastal marine waters, which can be well described using molecular estimates of diversity. We studied temporal changes of tintinnid diversity over 1 year in a coastal Mediterranean Sea location (Villefranche-sur-Mer, France) at five different depths (5, 25, 50, 100, and 160 m) and one additional year at 50 m depth by combining denaturing gel gradient electrophoresis (DGGE) community fingerprinting with direct PCR amplification, cloning and sequencing of small subunit (SSU), 5.8S, and large-subunit (LSU) rRNA genes together with the corresponding internal transcribed spacers (ITS). We amplified tintinnid sequences in all samples. All identified phylotypes were closely related to described species, showing that there is a good phylogenetic reference sequence data set allowing accurate estimation of tintinnid diversity in these waters. Tintinnid community composition exhibited marked seasonal changes. Surprisingly, the tintinnid SSU rDNA-based species composition did not show any clear relationship to measured environmental parameters (temperature, salinity, light, phytoplankton biomass). Nonetheless, the comparison of tintinnid community composition using UniFrac revealed three significant clusters of sequences grouping, respectively, samples collected in winter, autumn, and summer, leading to the hypothesis that seasonal effects on tintinnid community composition might be related to biotic parameters. In addition, phylogenetic trees based on the concatenated SSU + LSU rDNA and ITS sequences showed a better resolution than SSU rDNA alone to discriminate closely related species.

Introduction

The use of culture-independent molecular approaches based on sequencing to describe the communities of microbial eukaryotes, or protists, present in natural environments has led to the discovery of a huge diversity of these organisms (López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Epstein & López-García, 2008; Massana & Pedrós-Alió, 2008). Most of these studies are based on the PCR amplification of the nuclear-encoded small-subunit rRNA gene (SSU rDNA). Additional genetic markers such as the internal transcribed spacers (ITS) or the large-subunit rRNA gene (LSU rDNA) have also, although more rarely, been used to improve the phylogenetic resolution of SSU rDNAs (e.g. Marande *et al.*, 2009). Because many protist species cannot currently be identified through microscopic examination, culture-independent approaches are used to identify distributional patterns to study mechanisms driving biodiversity and biogeography. Several studies employing these methods have revealed that microbial eukaryotic community composition varies across environmental gradients related to depth (Countway *et al.*, 2007; surface vs. 2500 m), salinity (Estrada *et al.*, 2004; 4% vs. 37%), or oxygen

content (Orsi *et al.*, 2012; anoxic vs. oxic). However, the seasonality of aquatic protists has been insufficiently addressed, even if we know that temporal changes in community composition may be remarkable as revealed in marine surface waters (Massana *et al.*, 2004; Romari & Vaulot, 2004), continental lakes (Lepère *et al.*, 2006; Nolte *et al.*, 2010; Mangot *et al.*, 2013), and oligotrophic aquatic environments such as peatlands (Lara *et al.*, 2011).

If studies on spatial and temporal variation of protist communities using environmental surveys are limited, those of specific protist taxa are even rarer. Nonetheless, the study of temporal variation of particular protist lineages provides an increased level of resolution that may be useful to assess specific ecological questions. The control of dinoflagellate populations over time by specific parasites is such an example (Chambouvet *et al.*, 2008). Environmental molecular surveys using specific primers to amplify the SSU rDNA of certain protist lineages allow accurate assessment of the diversity of clades which are often overlooked using eukaryotic universal primers. For example, studies targeting specifically Cercozoa (Bass & Cavalier-Smith, 2004) or Haptophyta (Liu *et al.*, 2009; Simon *et al.*, 2013) showed an unsuspected genetic diversity within these groups. A few of these taxon-specific approaches aimed at revealing spatial patterns in marine ecosystems, showed, for instance, a depth-dependent community structure of diplonemids (Euglenozoa) in the water column (Lara *et al.*, 2009) or a variation of kinetoplastid (Euglenozoa) composition with distance in the deep-sea floor (Salani *et al.*, 2012). Marine ciliates (choreotrichs and oligotrichs) have been relatively well studied, and strong differences in community composition across environmental and depth gradients have been reported (Doherty *et al.*, 2007, 2010; Tamura *et al.*, 2011). But, so far, temporal variation of marine ciliate taxa has not been explored.

Tintinnid ciliates are in many ways an ideal model group to study how assemblages of microbial eukaryotes vary seasonally (Dolan & Pierce, 2013). Among ciliates, they are recognizable by the elaboration of a shell or lorica. As part of the microzooplankton, tintinnids are a category of planktonic heterotrophs responsible for grazing most of the algal production in marine systems. Accordingly, there is a long tradition of studying their seasonal dynamics in marine coastal waters, notably in the Mediterranean Sea based on morphological surveys, which usually revealed a strong seasonal variation (e.g. Modigh & Castaldo, 2002; Dolan *et al.*, 2006; Sitran *et al.*, 2007). In addition, the determination of SSU rDNA sequences from single Mediterranean tintinnid cells has recently enriched the genetic database for these ciliates and provided a strong phylogenetic framework for further environmental molecular analyses in this marine region (Bachy *et al.*, 2012). Furthermore, the distinctive morphology of tintinnids has made it possible to carry a recent comparative study of molecular (SSU rDNA sequences) and morphological estimates of species richness in Mediterranean plankton samples. The study showed that the number and identity of species observed morphologically was roughly similar to the number and identity of operational taxonomic units (OTUs; defined as groups of sequences with $\geq$ 99% identity) identified in SSU rDNA libraries from the same samples (Bachy *et al.*, 2013). The correspondence between diversity of sequences in gene libraries and morphology-based estimates permits conclusions to be made regarding diversity changes in tintinnid ciliate communities across samples using SSU rDNA, as well as allowing linkage between past morphology-based studies and recent sequence-based observations.

We sought to determine whether spatial and temporal changes in tintinnid community composition, classically assessed by microscopic observations, are also detected by molecular approaches as well as establish at what level of taxonomic resolution detectable changes occur. To do so, we monitored the genetic diversity of tintinnids over a 1-year period in a coastal Mediterranean Sea location (Villefranche-sur-Mer, France). We sampled at five different depths and combined community fingerprinting analyses using denaturing gel gradient electrophoresis (DGGE) with the direct PCR amplification and sequencing of SSU, 5.8S, and LSU rRNA genes together with the corresponding internal transcribed spacers (ITS).

Materials and methods

Sampling site and environmental measures

We obtained samples from a station named 'Point B+' (43°41′00″N, 7°18′44″E) near the entrance to the Bay of Villefranche, which permits sampling relatively deep waters for a coastal site (180 m depth). Sampling was conducted monthly from February 2009 to February 2010 at five different depths in the water column: 5, 25, 50, 100, and 160 m depth (Supporting Information, Table S1). In addition, three samples from 50 m depth were collected in May, September, and December 2010, representing different seasons of a second year. Plankton samples were collected using 12-L Niskin bottles. The entire volume was immediately prefiltered through 200-μm Nitex mesh and then filtered through 5 μm pore diameter TMTP (Millipore) filters to concentrate biomass in the 5–200 μm fraction. Filters were conserved in absolute ethanol at −20 °C until further processing. Systematically, the day before sampling, temperature, salinity, chlorophyll a, and photosynthetically available radiation (PAR) were

measured by the SOMLIT (Service d'Observation en Milieu LITtoral, http://somlit.epoc.u-bordeaux1.fr/fr/). Typical temperature-induced stratification occurred in the water column from April to October in 2009 and 2010.

DNA purification and denaturing gradient gel electrophoresis

Genomic DNA was purified from the biomass retained in filters using the Ultraclean MoBio Soil DNA kit (MoBio, Solana Beach, CA). Nucleic acids were resuspended in 100 μL 10 mM Tris-HCl, pH 8. From the February 2009 to January 2010 samples, ITS region fragments of *c.* 250–260 bp were amplified with the specific tintinnid forward primer Tin454-ITSFw-GCclamp (5′- CGCGCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCAATYTGTTGCAGRGCGMAAGC-3′) and the reverse tintinnid primer Tin454-ITSRev (5′-AGCAATAGAAGGGCATCTA-3′). These group-specific primers were especially designed to amplify the 5.8S rDNA and partial adjacent ITS 1 and 2, which present variable GC content (Bachy *et al.*, 2013). PCRs were carried out in 25 μL of reaction buffer containing 2 μL of the total DNA, 1.5 mM $MgCl_2$, dNTPs (10 nmol each), 20 pmol of each primer, and 0.2 U Taq Platinum DNA polymerase (Invitrogen, Carlsbad, CA). PCR amplifications were performed with the following conditions: an initial denaturation step at 94 °C for 3 min, 30 cycles consisting of a denaturation step at 94 °C for 15 s, an annealing step of 30 s (a touch-down procedure with a decreasing annealing temperature from 60 to 50 °C for the 10 first cycles was applied followed by an annealing temperature of 50 °C for the following 20 cycles), a polymerization step at 72 °C for 1.5 min, and a final step of 1-h extension at 72 °C. Migration of PCR products was carried out in a denaturing gradient gel using the CBS (CA) electrophoresis system. A solution of urea (7 M) and 40% formamide was used as denaturing agent with a concentration gradient from 20% to 40% in a polyacrylamide gel (8%). Twenty-five microlitre 25 μL of PCR product was loaded for each sample in parallel with 50-bp ladder DNA markers (Promega, Lyon, France) every four lanes for next-step computational normalization. The gel was run at 150 V for 6 h at 60 °C in 0.5× Tris–acetate–EDTA (1×: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8,0) buffer and then stained with SYBR Gold (Invitrogen) for 1 h and photographed under UV light. Characteristic bands of various migration levels were cut and purified for sequencing. Photographed gels were normalized using the BioNumerics® 5 software (AppliedMaths, Saint-Martens-Latem, Belgium) based on the position of ladder standards. Band positions were assigned manually. Clustering analysis of all the DGGE patterns was carried out using UPGMA (unweighted pair group method with arithmetic mean) clustering applying a Dice coefficient.

Small-subunit rRNA gene, ITS region, and partial large-subunit rRNA gene library construction

We amplified and cloned a region encompassing the SSU rDNA, the ITS region, and partial LSU rDNA (amplicon size *c.* 1900 bp) using specific primers for tintinnids from seven selected samples: five samples from five different depths of the Villefranche water column collected in February 2009, VilleFr-1 (5 m), VilleFr-2 (25 m), VilleFr-3 (50 m), VilleFr-4 (100 m), VilleFr-5 (160 m), and two samples collected at 50 m depth in June 2009 (VilleFr-23) and February 2010 (VilleFr-58). To amplify the SSU rDNA–ITS–partial LSU rDNA fragment, the set of tintinnid-specific primers 18S-Tin3F (5′-GCGGTATTTATTAGATAWCAGCC-3′) and 28S-TinR1 (5′-TGGTGCACTAGTATCAAAGT-3′) was used (Bachy *et al.*, 2012). PCR was carried out under the following conditions: 35 cycles (denaturation at 94 °C for 15 s, annealing at 52 °C for 30 s, extension at 72 °C for 2 min) preceded by 3-min denaturation at 94 °C, and followed by 15-min extension at 72 °C. The corresponding amplicon libraries were constructed using the Topo TA cloning system (Invitrogen) following the instructions provided by the manufacturer. Positive inserts of expected size were selected from each library and completely sequenced with the forward and reverse primers, and also the internal eukaryotic-specific primer 1498R (5′CACCTACGCAAACCTTGTTA; López-García *et al.*, 2003). We determined between 20 (VilleFr-5) and 36 sequences (VilleFr-23) for each library. We applied the same approach to 16 additional samples collected in Villefranche (VilleFr-8, -18, -22, -24, -25, -28, -31, -32, -33, -34, -44, -48, -53, -63, -68, -73; Table S1) for which we generated fewer sequences (between 1 and 11). Long amplicon libraries had previously been constructed for the VilleFr-43 (Nov 2009, Villefranche Bay, 50 m depth) and Ioni-7 (Oct 2010, Ionian Sea, 77 m depth) samples (Bachy *et al.*, 2013). We searched for recombination events to detect potential chimeras within our clone sequences, which may be especially difficult when working on phylogenetically closely related sequences. The Recombination Detection Program, RDP version 3.42 (Martin *et al.*, 2005), was used with settings (a Bonferroni corrected *P*-value cutoff of 0.01) for the different detection methods, including GENECONV (Padidam *et al.*, 1999), Chimaera (Posada & Crandall, 2001), and Maxchi (Smith, 1992) implemented in RDP3.42. With a reference data set of 25 tintinnid sequences covering the same region (Bachy *et al.*, 2012), a total of eight potential PCR-derived recombined

sequences (three in VilleFr-3, four in VilleFr-4, one in VilleFr-5) were removed from further investigations. A total of 195 complete high-quality sequences generated from the Villefranche Bay samples remained for clustering and phylogenetic analyses. Sequences newly reported in this paper have been deposited in the GenBank database under accession numbers KF662488-KF662721.

Clustering of tintinnid sequences and phylogenetic analyses

Sequences were aligned using MAFFT (Katoh *et al.*, 2002). Then, two distance matrices were generated in Phylip format based either on the SSU rDNA alone, or on the complete SSU rDNA+ITS+LSU sequences. The resulting matrix was used as input for Mothur (Schloss *et al.*, 2009) to group sequences in operational taxonomic units (OTUs) by average linkage clustering. In this study, an OTU is defined as a cluster of SSU rDNA sequences sharing $\geq$ 99% identity. This level of similarity is considered a good proxy for ciliate species identification, particularly for tintinnids (Bachy *et al.*, 2013; Santoferrara *et al.*, 2013). Coverage values were calculated using the Good estimator (Good, 1953) following the equation $C = (1 - n/N) \times 100$, where C is the percentage of coverage of the library, n the number of singletons, and N the total number of clones examined. A subsequent clustering step of the complete sequences sharing $\geq$ 99% of similarity was then carried out.

For in-depth phylogenetic analyses, we selected a representative sequence for each OTU. Together with their most similar sequences (known/described species) identified by BLAST (Altschul *et al.*, 1997) in GenBank and some typical Mediterranean tintinnid sequences (Bachy *et al.*, 2012), they were aligned using MAFFT. A phylogenetic tree was constructed using maximum likelihood (ML) with the software TREEFINDER (Jobb *et al.*, 2004) under the general time reversible (GTR) model of sequence evolution with four substitution rate categories and a Gamma law to accommodate for among-site variation. Nonparametric bootstrap analyses were inferred using 1000 replicates. To test whether the general topology of the tree remained congruent, we performed a Bayesian analysis on the same data set with the GTR + Γ + I model of sequence evolution, using the software MRBAYES (Huelsenbeck & Ronquist, 2001). Bayesian inference was also conducted using MrBayes, starting with a random tree, ran for one million generations in four chains and excluding the first 35 000 generations as a burn-in. The ML and Bayesian analyses produced congruent topologies.

We then conducted phylogenetic analyses with all the sequences and positions available from the fully sequenced SSU rDNA+ITS+LSU rDNA regions, to determine whether working with more additional molecular markers (implying more positions) could improve the phylogenetic resolution within the tintinnid order. We obtained a data set containing 291 sequences of *c.* 1900 bp from the 24 gene libraries (see above; Table 1) from Villefranche samples and from the gene library generated from the Ionian sample Ioni-7 (Bachy *et al.*, 2013). Individual clone sequences were identified to the species level based on the SSU rDNA similarity, as for this marker, we have a more extensive phylogenetic data set of sequences from described single cells. We selected a representative complete sequence for each OTU identified and constructed an ML tree together including 20 reference tintinnid sequences covering the full-length SSU+5.8S+ partial LSU and ITS.

Table 1. Summary of SSU rRNA gene sequences analyzed from the eight selected tintinnid gene libraries and the associated number of clones, OTUs, and coverage indices

Sample	Depth (m)	Date	No. of clones	No. of OTUs	Coverage (%)
VilleFr-1	5	Feb 09	21	3	95
VilleFr-2	25	Feb 09	22	4	95
VilleFr-3	50	Feb 09	23	6	91
VilleFr-4	100	Feb 09	22	7	82
VilleFr-5	160	Feb 09	20	6	100
VilleFr-23	50	Jun 09	36	9	94
VilleFr-43	50	Nov 09	30	9	83
VilleFr-58	50	Feb 10	24	5	92

Multivariate analyses

To compare tintinnid diversity between the eight studied samples of our seasonal sampling and the distant Ioni-7 sample, we performed a hierarchical clustering in UniFrac with an ML tree built as mentioned above with representative sequences of each OTU labeled according to the sample it comes from. Using the weighted and unweighted UniFrac, we assessed how our tintinnid communities were related to one another considering both sampling depth and season. The principal coordinates describe how much the variation in the data set is explained by the first two axes. The weighted and unweighted analyses gave very similar results. We also assessed the ordination of tintinnid communities with the associated environmental variables by analyzing the same data set that included the OTUs from the coastal site of Villefranche. For this, we performed a constrained ordination (redundancy analysis, RDA) on this community data (OTU absolute frequency data) with all the environmental variables available. To test whether there was an effect of environmental variables on the tintinnid community compositions, we applied a Monte Carlo permutation test, using

unconstrained permutations (null hypothesis: there is no effect of the environmental variables on species/OTU representation, i.e. the effect of variables is zero). We also performed a principal component analysis (PCA) with the same data set including the community data (OTU absolute frequency data) to visualize how OTU occurrences correlated with the ordination of samples. The RDA, Monte Carlo permutation test, and PCA were performed using the software CANOCO version 3.1 (Braak & Smilauer, 2002).

Results

Fingerprinting analysis of planktonic tintinnid communities in the water column along 1 year of survey

As a first approximation to characterizing the composition of the tintinnid communities in the Bay of Villefranche, we generated tintinnid-specific fingerprints of a relatively variable genomic area covering the ITS region using denaturing gel gradient electrophoresis (DGGE) for all the samples collected monthly from February 2009 to January 2010 at five different depths (5, 25, 50, 100, 160 m; see Table S1). We observed bands corresponding to tintinnid ITS regions in all the DGGE profiles (Supporting Information, Fig. S1), ranging from 2 bands for the less-diverse samples (VilleFr-12 and -19) to 12 bands for the richer ones (VilleFr-36 and -37). This indicates that tintinnid ciliates were present throughout the year at the different depths sampled. A cluster analysis of DGGE profiles divided the samples in two major groups seemingly according to their depth in the water column. One cluster corresponded to surface and intermediate depth samples (5–50 m) obtained on different dates, including five 100-m samples and one deep sample (VilleFr-5, 160 m), and the second cluster included the other deep samples (100–160 m), the surface waters (5–25 m) of June and July 2009 (VilleFr-21, -22, -26 and -27), and the 25-m sample of September 2009 (VilleFr-32). There were also nine samples (VilleFr-2, -6, -7, -11, -12, -13, -17, -19, and -56) which did not group with the two major clades. All the bands identified in DGGE patterns were cut, and their corresponding DNA fragments were purified and sequenced, which showed that all of them did indeed affiliate with tintinnid sequences (Table S2).

Spatio-temporal patterns of tintinnid diversity based on SSU rDNA sequences

For further in-depth diversity analyses of tintinnids, we selected the five samples VilleFr-1 (5 m), VilleFr-2 (25 m), VilleFr-3 (50 m), VilleFr-4 (100 m), VilleFr-5 (160 m) corresponding to the different sampling depths of the Villefranche water column in February 2009 plus three additional samples at a same depth (50 m) collected during the other three seasons VilleFr-23, VilleFr-58, and VilleFr-43. They overall displayed rich band profiles in DGGE gels and grouped in different subgroups within the first major cluster (Fig. S1). The sample VilleFr-43 was the subject of a recent study comparing tintinnid diversity assessed based on morphology with diversity assessed by SSU rDNA and ITS amplification, cloning and Sanger sequencing and pyrosequencing; a good agreement between the morphospecies and the OTUs identified was shown (Bachy *et al.*, 2013).

For each selected sample, we characterized the tintinnid diversity by cloning and (Sanger) sequencing of SSU, 5.8S, and partial LSU rRNA genes and internal transcribed spacers (ITS) using tintinnid-specific primers. The analysis of several markers from the ribosomal RNA cluster, including the ITS which is generally more variable than the coding regions, frequently allows description of diversity at a finer taxonomic level compared with that derived from SSU rDNA sequences alone. However, because we have robust information on how the tintinnid genetic diversity relates to the morphological diversity at the species level only for the SSU rRNA gene, we first measured the composition of tintinnid OTUs using a 99% sequence similarity threshold for this marker. A total of 19 OTUs were detected for all the eight samples. Species richness varied between 3 in VilleFr-1 and 9 in VilleFr-23 and VilleFr-43 (Table 1). Despite a relative low number of clones analyzed per library, we obtained a good coverage for them, in most cases well above 90%, and in all cases higher than 82%, which suggested that we had described most of the tintinnid-specific diversity present in our samples (Table 1).

For subsequent phylogenetic analyses, we selected one representative of our SSU rDNA sequences per OTU and aligned these sequences along with a representative set of sequences of well-described Mediterranean tintinnids (Fig. 1). Even if the relationships between many tintinnid families and some lineages *incertae sedis* (*Tintinnopsis* spp. and *Climacocylis* sp.) were unresolved, as shown in a previous taxonomic study (Bachy *et al.*, 2012), the sequences within families grouped together with strong support (ML bootstrap values -BV- $\geq$ 92%). The OTUs identified in this study were distributed across nine families of the order *Tintinnida*, with the richest diversity found within the *Tintinnidae* with 5 OTUs related with *Amphorides quadrilineata*, *Steenstrupiella steenstrupi,* and *Salpingella acuminata*. Some OTUs were detected in low amounts in only one sample, suggesting that they are less abundant, at least at the depths sampled throughout the year. This was the case of the OTU17 related with *Tintinnidium mucicola*

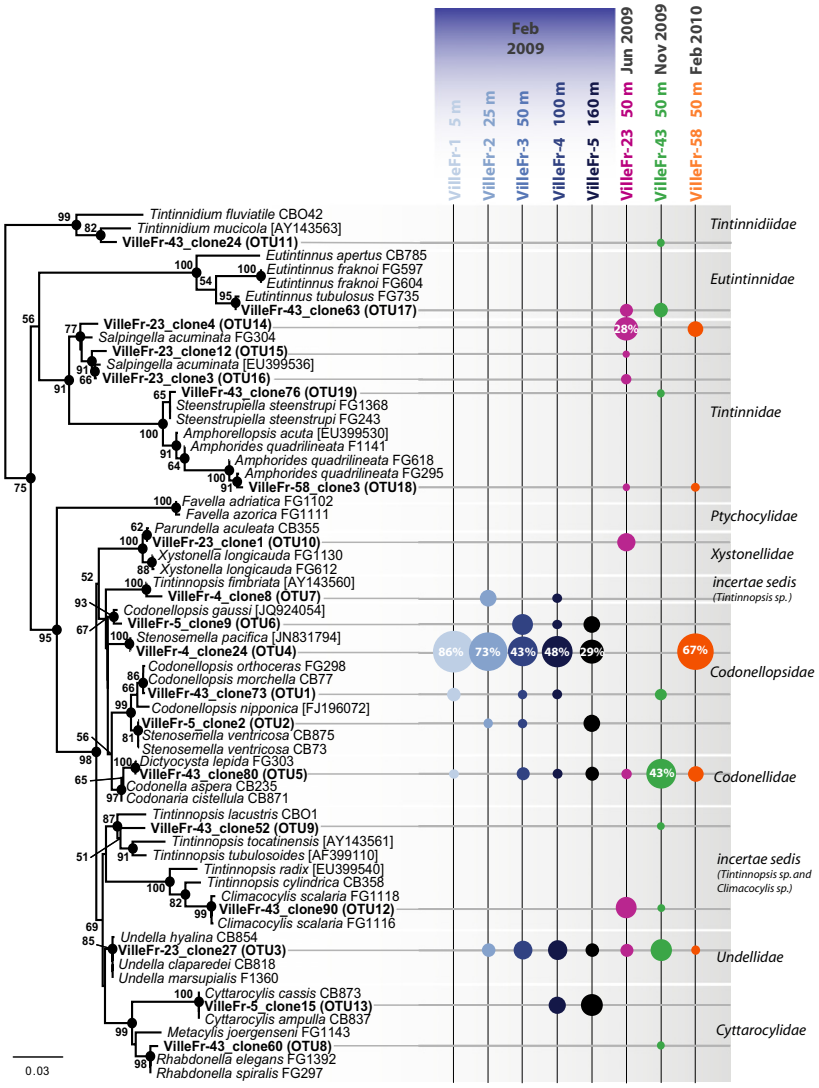

Fig. 1. Maximum likelihood (ML) phylogenetic tree of SSU rDNA of tintinnids identified at different depths and seasons in the Villefranche Bay. The tree was constructed using 1146 unambiguously aligned positions. Sequences determined in this study are indicated in gray. Relative proportions of the different OTUs in each sample are indicated by circles of proportional size on the right. The percentage of sequences for the most abundant OTUs is indicated within the circles. The scale bar indicates the number of substitutions per site for a unit branch length. Maximum likelihood bootstrap values above 50% are indicated at nodes, and Bayesian posterior probabilities higher than 0.90 are indicated by filled circles.

only found in November 2009 (VilleFr-43) or the OTU10 related with *Parundella aculeata* only found in June 2009 (VilleFr-23; Fig. 1). For more frequently represented OTUs, we observed differences both in space and time.

Regarding the tintinnid community composition throughout the water column analyzed in February 2009, some OTUs appeared more abundant in deeper waters (OTU13 related to *Cyttarocylis* spp.), others at intermediate depths (OTU3 related to *Undella* spp.), and others in surface waters (OTU4 related to *Stenosemella pacifica*). At the same time, other OTUs were detected in low amounts at various depths from surface to deeper waters, suggesting that they were present all throughout the water column (OTU5, related to *Dictyocysta* spp. or OTU1 related to *Codonellopsis* spp.; Fig. 1).

Regarding the tintinnid community composition throughout the different seasons, we also observed marked patterns. One of the most notable differences observed was the absence of tintinnids belonging to the families *Tintinniidae*, *Eutintinnidae*, *Tintinnidae*, and *Xystonellidae* in February 2009, members of which were detected in other seasons. Only members of the related families *Codonellidae*, *Codonellopsidae*, *Undellidae*, and *Cyttarocylidae* were detected in winter in diverse relative abundances (Fig. 1). If we compare samples collected at the same depth, 50 m, but from different seasons, some OTUs were detected only in winter (the OTU2 related to *Codonellopsis gaussi* and the OTU6) and others only in other seasons (e.g. the OTU12 related to *Climacocylis* and members of the *Tintinnidiidae*, *Eutintinnidae*, *Tintinnidae*, and *Xystonellidae*), whereas some OTUs were detected throughout all the seasons. The latter case was that of the OTU3 – *Undella* spp. – which was remarkably present both throughout the year and the water column (Fig. 1). The

OTU4, closely related to *Stenosemella pacifica,* was numerically dominant in all winter samples, found at all depths in February 2009 and in the 50 m deep February 2010 but not the rest of the year (Fig. 1). The prevalence of this OTU in gene libraries from February 2009 decreased with the depth, ranging from 86% of all tintinnid sequences at the surface sample (VilleFr-1, 5 m depth) to 29% at the deepest sample (VilleFr-5, 160 m depth).

Tintinnid community comparison and abiotic environmental factors

To determine whether the tintinnid community composition was relatable to bulk physicochemical environmental parameters, we measured temperature, salinity, light, and fluorescence at the time of sampling. Although temperature and light (photosynthetic active radiation, PAR) varied with depth and seasons, fluorescence and salinity remained relatively constant in the Villefranche water column (Table S3). Furthermore, temperature changes were relatively limited; temperature varied from 13.4 °C (February 2010, 50 m) to 18 °C (June 2009, 50 m) over all seasons and was constant in February 2009 in all samples (13.7 °C), reflecting the mixed water column conditions typical of the winter. This remarkable constancy of parameters (with the exception of light) did not seem to correlate, at first sight, with the observed changes in the tintinnid-specific richness or assemblages.

To test whether there was a correlation between physicochemical parameters and tintinnid community composition, we carried out a Monte Carlo permutation test, using unconstrained permutations in redundancy analyses (RDA). The results showed no significant effect of the measured physicochemical parameters on tintinnid assemblages (P-value = 0.806). Nonetheless, the phylogenetic method weighted UniFrac for comparing compositions of tintinnid communities showed grouping between samples depending on the period of sampling (Fig. 2), which suggests that the causes of such clustering may be not related directly to the abiotic parameters considered. We applied principal coordinate analysis (PCoA) of the pairwise weighted UniFrac distances to the tintinnid communities detected in our eight selected samples from Villefranche plus one additional sample that we had previously studied from the Ionian Sea (Ioni-7) as well as cluster analysis using UPGMA. They showed that the Villefranche and Ionian Sea samples fell into three well supported and distant groups (Fig. 2). The first two principal coordinates which explained, respectively, 68.4% and 16.7% of the variation showed that the winter samples VilleFr-1 to -5 (Feb 2009) and VilleFr-58 (Feb 2010) grouped together, autumn samples VilleFr-43 (Nov 2009) and Ioni-7 (Oct 2010) grouped together, and the remaining, summer sample VilleFr-23 (Jun 2009) was placed apart (Fig. 2a). Jacknife bootstraps supported (> 90%) these basically seasonal sample groupings (Fig. 2b). To see how the different OTUs influenced community changes, we carried out an ordination analysis based on a linear ordination method (PCA, Fig. S2). It indicated that the main gradient of species composition change was

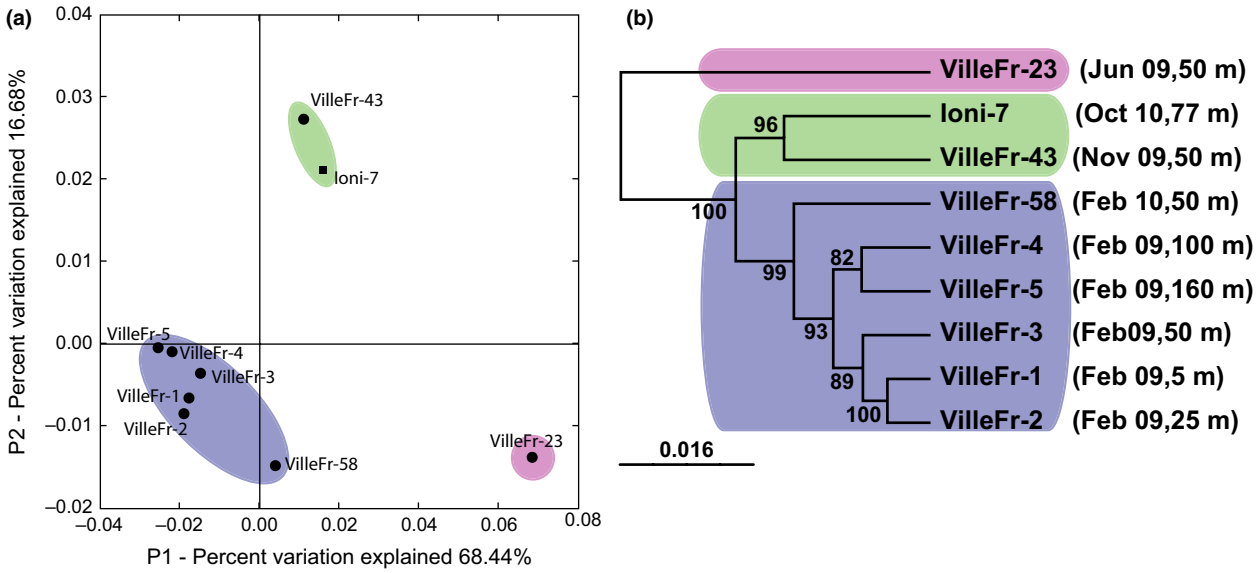

Fig. 2. (a) Hierarchical clustering of samples using principal coordinate analysis (PCoA) (b) Hierarchical clustering of samples using UPGMA. Numbers at nodes indicate jackknife support values. The scale bar shows the distance between clusters in UniFrac units: a distance of 0 means that two environments are identical, and a distance of 1 means that two environments contain mutually exclusive lineages.

related to the abundance of the OTU4 (*Stenosemella* related) and that the divergent position of the sample VilleFr-43 was explained by the more frequent occurrence of the OTU5 (*Dictyocysta* related) and the OTU3 (*Undella* related).

Intraspecific tintinnid diversity

To study the diversity of tintinnids at a finer taxonomic scale, we estimated the number of OTUs by applying a threshold of ≥ 99% sequence identity at the full-length sequences amplified (SSU + ITS1 + 5.8S + ITS2 + partial LSU rDNA). For the eight samples studied, we detected a total of 26 OTUs according to this finer criterion, seven more than when we considered the SSU rDNA alone. This increase in the number of OTUs was not surprising as the ITS region (ITS1+5.8S rDNA+ITS2) and the beginning of the LSU rDNA are more divergent than the SSU rDNA (Bachy *et al.*, 2012; Santoferrara *et al.*, 2013). For example, the sequences of OTU3, OTU4, and OTU6 related with, respectively, *Undella*, *Stenosemella,* and *Codonellopsis* (Fig. 1) were split in the new clustering analysis into four, three, and three OTUs, respectively (data not shown). To know whether this richer diversity correlated with different morphological features, we built an ML tree with the full-length sequences that composed the perennial OTU3 (*Undella* sp.) based on the SSU rDNA, together with the two sequences of *Undella* present in GenBank, *Undella claparedei* isolate CB818 and *Undella hyalina* isolate CB854. The Fig. 3a shows different subclusters of sequences that are either closely related with known species or composed exclusively of environmental sequences. A pool of 11 sequences detected in VilleFr-2 to 5 and VilleFr-23 were phylogenetically associated with *Undella claparedei* (subclade 3-I). Two distant clades were exclusively composed of four and two sequences detected in VilleFr-43 (subclade 3-II and 3-III), among which the latter ones appeared to be associated with *Undella hyalina* with strong support (BV 100%). The six remaining sequences (VilleFr-4_clone18 and VilleFr-58_clone19, VilleFr-3_clone18 and VilleFr-43_clone45, VilleFr-5_clone21 and VilleFr-2_clone4) were more distantly related, clustering in three clades (subclade 3-IV, 3-V, 3-VI, respectively). This may suggest that part of the morphologic diversity existing within the genus *Undella* remains to be related with these SSU rDNA and ITS sequences.

The case of the OTU5 related with *Dictyocysta* illustrates the recurrent problem of lack of resolution of the SSU rDNA to discriminate closely related species in some tintinnid families. The sequences clustering as OTU5 based on SSU rDNA sequences alone were still part of the same OTU when considering also the ITS, 5.8S and partial LSU sequences. However, an ML tree inferred with the full-length sequences of this OTU, together with the sequences of *Codonella aspera* isolate CB235 and *Codonaria* spp. isolates CB25, CB82, and FG42, revealed that sequences attributed to *Dictyocysta* based on the SSU rDNA sequences are composed of three subclades moderately to strongly supported (BV 75% to 100%; Fig. 3b). Surprisingly, these three subclades were related with distinct tintinnid species. Sequences from VilleFr-4, -5, -23, -43, and -58 clustered with *Codonella* and *Codonaria* (subclade 5-I). Whereas sequences unambiguously identified as *Dictyocysta* (based on the similarity between clone sequences and *Dictyocysta lepida* isolate FG303 (JQ408188) on the SSU rDNA ≥ 99.8%) from VilleFr-1, -3, -23, -43, and -58 clone libraries clustered together in two closely related subclades (subclade 5-II and 5-III) with no described relatives having the ITS region sequenced.

Concatenated SSU rDNA+ITS+5.8S rDNA+LSU rDNA phylogeny

To improve the resolution of the current tintinnid phylogeny, we carried out a molecular phylogenetic analysis of concatenated gene markers. A total of 291 long environmental tintinnid sequences (*c.* 1900 bp) encompassing the SSU rDNA and up to the beginning of the LSU rDNA were retrieved from the eight studied samples and 17 other samples from Villefranche analyzed in this work (Table S1) and the Ionian Sea. Sequences were classified based on the SSU rDNA for which there is a more extensive phylogenetic data set of sequences from described tintinnids. We aligned these environmental sequences to 20 taxonomically defined sequences publicly available covering the same genomic region. After molecular phylogenetic analyses (Fig. S3), the environmental sequences could be classified into clusters of tintinnids corresponding to family *sensu* Bachy *et al.* (2012): *Tintinnidiidae* (one sequence), *Tintinnidae* (41 sequences), *Eutintinnidae* (nine sequences), *Cyttarocylididae* (12 sequences), *Xystonellidae* (12 sequences), *Undellidae* (46 sequences), *Codonellopsidae* (117 sequences), *Codonellidae* (33 sequences), *incertae sedis* (corresponding to the tintinnid genera of uncertain affiliation *Tintinnopsis* spp. and *Climacocylis* spp., 6 and 14 sequences, respectively).

In addition to the eight Villefranche samples explored in greater detail, we also constructed gene libraries from other Villefranche samples corresponding to other depths and/or seasons, although we produced a limited amount of sequences. We obtained in this way a total of 65 additional sequences (Table S1), whose analysis revealed some interesting features. For example, sequences grouping with the abundant OTU4 were also retrieved in March

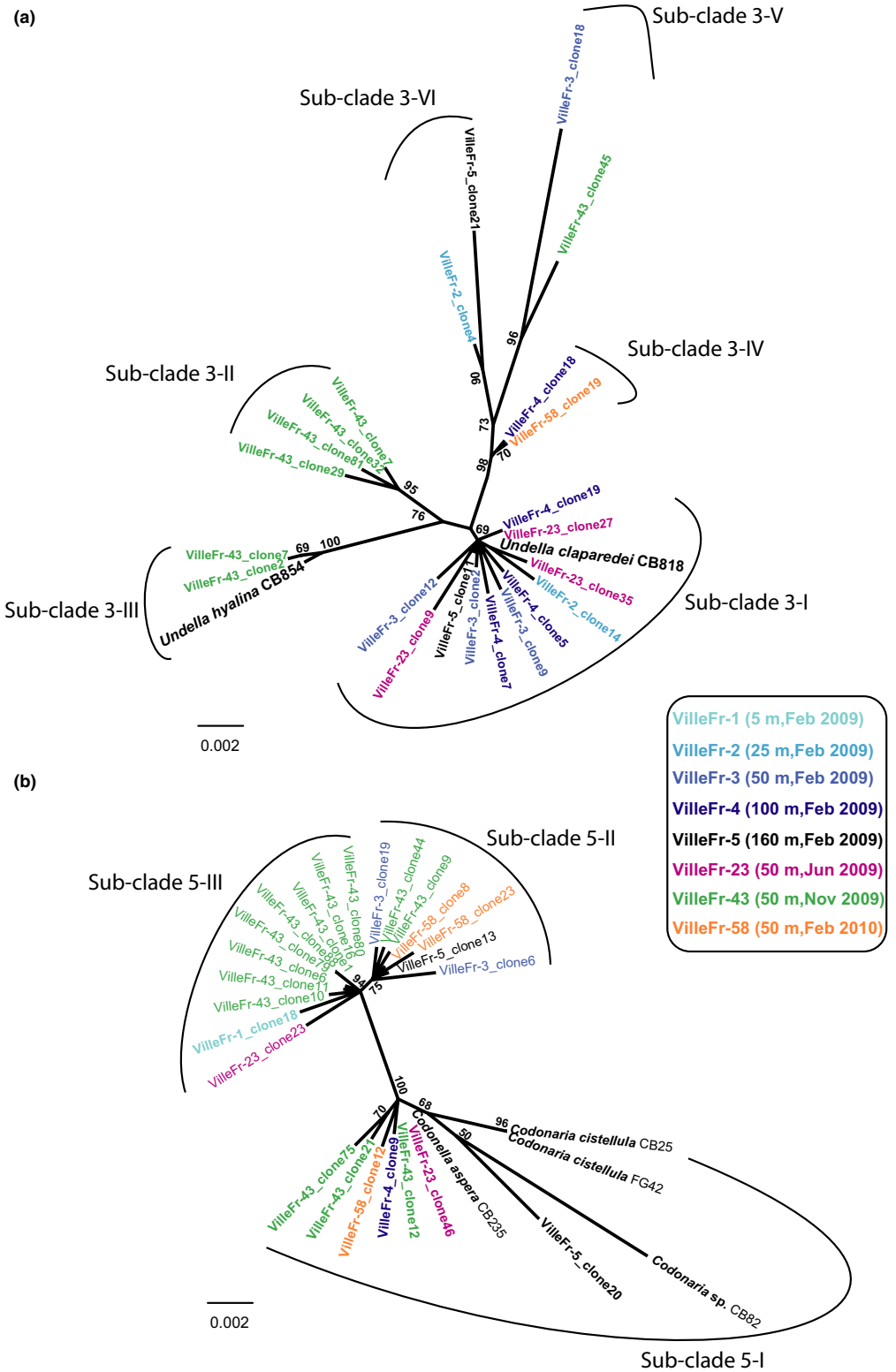

Fig. 3. Maximum likelihood (ML) phylogenetic tree of the SSU+5.8S+LSU rDNA and ITS region showing the position of the tintinnid OTUs 3 and 5. The tree was constructed using 1681 and 1790 unambiguously aligned positions, respectively. Numbers at nodes indicate bootstrap values. Sequences from this study are colored depending on their origin and date of sampling. Numbers of subclades cited in the text are indicated on the molecular phylogenies.

2009 at 50 m depth, in June 2009 at 100 and 160 m depths, and in November 2009 at 100 m depth. So, this clade was not exclusively present in winter samples from February 2009 and 2010, but also in samples from June and November 2009, but at depths deeper than 50 m (Fig. S3). This was in agreement with the results reported from the DGGE analysis, where the bands we cut, purified, and sequenced showed that OTU4 representatives were present during the whole temporal survey and at all the sampling depths (Fig. S1 and Table S2).

Discussion

Our molecular survey of tintinnid community composition revealed a relatively wide and rich phylogenetic diversity. A total of 19 OTUs based on SSU rDNA sequence similarity were detected (Fig. 1 and Fig. S1). This number of OTUs was in good agreement with the number of DGGE bands identified (22). The OTUs were distributed in nine different families of *Tintinnida*, from the most basal family *Tintinnidiidae* to the most distal families *Codonellopsidae* and *Codonellidae*. Currently, the numerous tintinnid morphospecies with published SSU rDNA sequences allow reliable phylogenetic identification at the genus/species level within this order (Bachy *et al.*, 2012). Remarkably, all the SSU rDNA sequences retrieved from the eight samples studied in more detail from the Villefranche Bay were $\geq 99\%$ identical to tintinnid sequences publicly available. In contrast, in other ciliate groups such as the *Oligotrichia*, a sister clade of the *Tintinnida*, molecular data for morphospecies are relatively sparse. Consequently, sampling by molecular methods suggests considerable phylogenetic diversity difficult to relate to described species (Doherty *et al.*, 2007; Tamura *et al.*, 2011). For tintinnids, establishing the link between morphology and SSU rDNA sequences has been facilitated by the distinctive morphology of tintinnid loricas and their relatively easy identification in planktonic samples using an inverted microscope as compared with other ciliate species for which identification requires more complex treatments such as histological staining (Montagnes & Lynn, 1987). It validates *a posteriori* the extensive work of single-cell isolation, identification, and subsequent SSU rRNA and ITS sequencing in general and at our sampling site of Villefranche (Bachy *et al.*, 2012), encouraging this kind of single-cell approach in other protist groups to try to relate environmental sequences with described species.

The analysis of SSU rDNA libraries revealed distinct spatial and seasonal patterns in the occurrences of tintinnid OTUs detected. A limited number of OTUs were found in two or more seasons. These temporal shifts in communities are characteristic of tintinnid assemblages described using microscopic analysis in most coastal ecosystems, and specifically in our well-studied site of Villefranche Bay (Balech, 1959; Rassoulzadegan, 1979; Dolan *et al.*, 2006). We did not observe a clear relationship between the OTU diversity within our samples and several typical variables (depth, salinity, temperature, PAR, and fluorescence). The lack of correlation between abiotic parameters and tintinnid community composition suggests that biotic factors may be a more important factor shaping the tintinnid community in coastal waters. In fact, several ecological studies based on morphological specificities of tintinnids, mainly the oral diameter, which has been related to the optimum prey size, showed that communities are determined by resource partitioning among different species in coastal waters (Dolan *et al.*, 2013). In addition, tintinnid communities could also be influenced by competition with other microplankton grazers and by mortality due to metazoan grazers (Pierce & Turner, 1992) or parasites (Bachvaroff *et al.*, 2012). Consequently, to understand the driving forces shaping tintinnid communities, the study of their diversity by molecular approaches, which efficiently describes their morphological diversity (Bachy *et al.*, 2013), should be combined with both the study of abiotic and, most importantly, biotic (resources, predation, competition) environmental factors.

As mentioned, although the causes are not well established, we observed a strong seasonal effect on tintinnid community composition. Although the tintinnid composition varied across samples, some samples were more similar to each other and grouped together in the PCoA analyses (Figure 2). A cluster grouped the five February 2009 samples from all the depths (5–160 m). During this season, the water column experiences mixing from the surface to the bottom, which would explain a homogeneous tintinnid distribution through the water column, even if subtle differences exist between samples. These variations of the tintinnid composition with depth could be explained by local-scale variability as evidenced in the Villefranche Bay at very small geographic scale based on tintinnid morphology (Dolan & Stoeck, 2011). They could also be explained by a stochastic sampling effect in the case of OTUs with relative low abundances. However, stochastic effects are unlikely to explain the decrease in the dominant OTU4 with depth (Figure 1). In this case, we cannot discard that the apparent indirect effect of biotic factors is related, for instance, with the availability of prey whose distribution is stratified, for instance photosynthetic organisms adapted to particular light intensities. This cluster also contained the other winter sample collected 1 year later in February at 50 m depth. Therefore, the tintinnid community composition appears reproducible in winter from 1 year to the next, mainly

because of dominance of the OTU4 phylotype. Seasonal changes in tintinnid community composition are well known; typically species with agglutinated loricas dominate in winter, and those with hyaline lorica are more common in summer (Dolan & Pierce, 2013). It may well be that species with agglutinated lorica (such as *Stenosomella*) rely on the presence of small mineral particles in the water column to form their lorica, corresponding with the mixed, turbulent water column conditions of winter (Dolan & Pierce, 2013). Similarly, a second 'seasonal' cluster contained the two samples collected during autumn 2009 and 2010 from distant locations (coastal Ligurian Sea vs. offshore Ionian Sea). A distinctive cluster represented the only sample collected in late spring, which may be potentially characterized by tintinnid community living in a recently stratified water column environment.

Study of the tintinnid community using SSU rDNA+ 5.8S rDNA+ LSU rDNA and ITS revealed a greater specific diversity than that detected from the clustering of SSU rDNA sequences alone. For example, the perennial *Undella*-related OTU3 defined on the strict criterion of $\geq$ 99% SSU rDNA sequence identity was more diverse when considering ITS and LSU rDNA positions. In this case, the detailed phylogenetic approach showed that, at least, a part of this diversity is linked with morphological variations within the genus. The case of the *Dictyocysta*-related OTU5 is more difficult to interpret. Considering SSU rDNA alone or SSU rDNA+ITS+LSU rDNA positions, the clustering based on sequence identity defined only one OTU. However, the ML tree revealed that this OTU was composed of different well-supported phylogenetic groups related with particular morphological traits. In environmental molecular surveys, to discriminate species choosing appropriate sequence similarity threshold is a crucial prerequisite for a fair assessment of diversity. For practical reasons, the cutoff level for species is based on SSU rDNA sequence similarity (frequently established at 99% for protists). However, this criterion does not accommodate the whole complexity of eukaryotic (Caron *et al.*, 2009), ciliate (Nebel *et al.*, 2011), or tintinnid diversity (Santoferrara *et al.*, 2013), underlining the fact that it should be specifically adapted to each eukaryotic taxon whenever molecular and morphological data are available. We previously showed that even partial sequences of SSU rDNA can discriminate tintinnid species diversity with reasonable accuracy (Bachy *et al.*, 2013). However, the sequencing of ITS region and partial LSU rDNA together with the SSU rDNA has revealed more extensive genetic diversity. Whether this genetic diversity detected within and among OTUs correlates with inter- or intraspecific diversity, or even with intraindividual variability is unclear. To unravel these different low taxonomic levels of diversity within tintinnids, finer population genetic studies based on clearly defined species and targeting molecular markers other than the SSU rDNA are required.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Clustering of DGGE profiles of tintinnid assemblages obtained at the Villefranche Bay at monthly intervals (February 2009 to February 2010) and at different depths (5–160 m).

Fig. S2. Principal component analysis (PCA) of tintinnid OTUs and the eight selected samples from DGGE analysis for which the diversity was studied in greater depth.

Fig. S3. ML phylogenetic tree of the SSU+5.8S+LSU rDNA + ITS region including environmental tintinnid representative sequences retrieved in this study and a wide taxonomic representation of known tintinnid species.

Table S1. List of samples collected in the Villefranche Bay during the temporal monitoring.

Table S2. Closest tintinnid OTUs to sequences of ITS region fragments amplified from DGGE bands.

Table S3. Environmental parameters measured on the eight studied samples collected in Villefranche-sur-mer at point B+.