The macronuclear genome of the Antarctic psychrophilic marine ciliate *Euplotes focardii* reveals new insights on molecular cold adaptation

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The macronuclear (MAC) genomes of ciliates belonging to the genus *Euplotes* species are comprised of numerous small DNA molecules, nanochromosomes, each typically encoding a single gene. These genomes are responsible for all gene expression during vegetative cell growth. Here, we report the analysis of the MAC genome from the Antarctic psychrophile *Euplotes focardii*. Nanochromosomes containing bacterial sequences were not found, suggesting that phenomena of horizontal gene transfer did not occur recently, even though this ciliate species has a substantial associated bacterial consortium. As in other euplotid species, *E. focardii* MAC genes are characterized by a high frequency of translational frameshifting. Furthermore, in order to characterize differences that may be consequent to cold adaptation and defense to oxidative stress, the main constraints of the Antarctic marine microorganisms, we compared *E. focardii* MAC genome with those available from mesophilic *Euplotes* species. We focussed mainly on the comparison of tubulin, antioxidant enzymes and heat shock protein (HSP) 70 families, molecules which possess peculiar characteristic correlated with cold adaptation in *E. focardii*. We found that α-tubulin genes and those encoding SODs and CATs antioxidant enzymes are more numerous than in the mesophilic *Euplotes* species. Furthermore, the phylogenetic trees showed that these molecules are divergent in the Antarctic species. In contrast, there are fewer hsp70 genes in *E. focardii* compared to mesophilic *Euplotes* and these genes do not respond to thermal stress but only to oxidative stress. Our results suggest that molecular adaptation to cold and oxidative stress in the Antarctic environment may not only be due to particular amino acid substitutions but also due to duplication and divergence of paralogous genes.

*Euplotes focardii* is an Antarctic ciliate classified as an obligate psychrophilic stenothermal organism1–4. As all ciliates, *E. focardii* is characterized by the presence of cilia on its surface and by nuclear dimorphism: a micronucleus (MIC) that represents the germ line, and a macronucleus (MAC) serving as the somatic line involved in the gene expression during the vegetative stage. The MAC derives from the MIC after extensive DNA rearrangements during conjugation, including the fragmentation of chromosomes and the elimination of non-protein-coding DNA segments5. As a consequence, the MAC genome is commonly composed by nanochromosomes, i.e., tiny chromosomes capped by telomeric sequences that, in general, contain a single coding sequence (CDS)6,7. The nanochromosomes are amplified to thousands of copies (~2000)5. The copy number may oscillate since it is probably unregulated through cell replication, as it has been described for *Stylonychia*8 and *Oxytricha*9.

As a psychrophilic unicellular organism directly exposed to environmental cues, *E. focardii* represents an excellent model for the study of cold adaptation, offering some potential advantages over psychrophilic multicellular models10. Low temperatures exert several physicochemical constraints on cold living organisms, including the process of microtubule polymerization11. Microtubule polymers are of fundamental importance in many eukaryotic cellular processes, including cell motility, maintenance of cytoskeletal architecture, intracellular transport, and mitosis. Microtubules assemble from α- and β-tubulin heterodimers with the help of γ-tubulins, a...
(the primary produced Reactive Oxygen Species, abbreviated as ROS), and the reduction of the produced H2O2 and glutathione S-transferases GST) may be necessary. Specifically, the dismutation by SOD of the superoxide and glutathione systems (glutathione synthetase GS, glutathione reductase GR, glutathione peroxidases GPx, by CAT and GPx (often in association with thiol-containing enzymes, PRX, TRXR and glutaredoxins) are of extreme importance for stress response in all cells. Likewise, GSTs are important to inactivate unsaturated aldehydes, epoxides, and hydroperoxides, secondary metabolites of the reactions described above.

E. focardii is also a good model for studying adaptation to oxidative stress. Like any Antarctic marine microorganism, this ciliate is constantly exposed to a high oxygen concentration and abundant reactive oxygen species (ROS), due to the higher O2 solubility in water at low temperature and to high UV radiation due to the ozone hole. Accordingly, strengthened defenses against oxidative stress, e.g., by increasing the antioxidant enzymes system, including superoxide dismutase (SOD), catalase (CAT), peroxiredoxins (PRX), thioredoxin reductase (TRXR) and glutathione systems (glutathione synthetase GS, glutathione reductase GR, glutathione peroxidases GPx, and glutathione S-transferases GST) may be necessary. Specifically, the dismutation by SOD of the superoxide (the primary produced Reactive Oxygen Species abbreviated as ROS), and the reduction of the produced H2O2 by CAT and GPx (often in association with thiol-containing enzymes, PRX, TRXR and glutaredoxins) are of extreme importance for stress response in all cells. Likewise, GSTs are important to inactivate unsaturated aldehydes, epoxides, and hydroperoxides, secondary metabolites of the reactions described above.

In mesophilic organisms, heat shock proteins (HSPs) are the main macromolecules involved in this mechanism and, acting as chaperones, provide the stabilization, the partial refolding, or the detection of proteins irreversibly damaged. In particular, the Hsp70 group is represented by proteins that are quickly induced under stress conditions by the activation of the hsp70 gene expression. However, hsp70 genes have been reported to have lost heat inducibility in several Antarctic marine organisms, including E. focardii.

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Results and discussion

A draft E. focardii macronuclear genome assembly. The E. focardii MAC genome assembly obtained using the SPAdes algorithm showed a significant improvement (25%) of the number of 2-telomere nanochromosomes with respect to the assembly previously produced by Newbler (stored in GenBank as version MJUV01000000) i.e., 17,798 sequences containing telomeres at both ends (Table 1) vs 12,922 previously reported. SPAdes assembly was performed on cleaned reads, e.g., after the clean-up of all algal or bacterial contaminants (see “Materials and methods” section). A summary of the SPAdes assembly data is shown in Table 1: these data were obtained after an extra clean-up of sequences without telomeres and with a high GC content.

| Assembly name and version | E. focardii | E. vannus | E. octocarinatus |
|---------------------------|-------------|-----------|-----------------|
| Assembly size (Mb)        | 49.33       | 85.1      | 88.9            |
| %GC                      | 31.51       | 37.0      | 28.2            |
| Contigs (n)               | 31,114      | 38,245    | 41,980          |
| Telomeres (n)             | 87,301      | 156,713   | 132,894         |
| N50 (bp)                  | 2047        | 2714      | 2947            |
| Mean contig length (bp)   | 1583        | 2225      | 2117            |
| Max contig length (bp)    | 50,409      | 40,045    | 53,269          |
| 2-telomeres contigs (n)   | 17,798      | 25,871    | 30,058          |
| Mean 2-telomere contig length (bp) | 1954 | 2369 | 2474 |
| 1-telomeres contigs (n)   | 6136        | 7637      | 4368            |
| Mean 1-telomere contig length (bp) | 1280 | 2039 | 2114 |
| 0-telomeres contigs (n)   | 7180        | 4737      | 7554            |
| Mean 0-telomeres contig length (bp) | 927  | 1736 | 790  |
| % Contigs (with telomere) | 76.9        | 87.2      | 81.9            |

Table 1. E. focardii macronuclear genome assembly statistics in comparison with E. vannus and E. octocarinatus.

The structural component of both centrioles and basal bodies. There are other components of the tubulin superfamily, such as the δ-tubulin and the ε-tubulin, that most likely interact longitudinally with α-tubulins at the minus ends and with β-tubulins to the plus ends of microtubules, respectively. Furthermore, δ-tubulin may be involved in the formation of the C triplet tubules in the basal bodies, while ε-tubulin may be located to the centrosome. Generally, in organisms that live in temperate environments, the assembly of microtubules from tubulin heterodimers requires physiological temperatures and these microtubules usually disassemble at temperatures below 4 °C. In chronically cold habitats, the microtubule dynamics in psychrophilic organisms, including the Antarctic ciliate E. focardii, most likely reflect adaptive modifications of tubulin heterodimers.

E. focardii is also a good model for studying adaptation to oxidative stress. Like any Antarctic marine microorganism, this ciliate is constantly exposed to a high oxygen concentration and abundant reactive oxygen species (ROS), due to the higher O2 solubility in water at low temperature and to high UV radiation due to the ozone hole. Accordingly, strengthened defenses against oxidative stress, e.g., by increasing the antioxidant enzymes system, including superoxide dismutase (SOD), catalase (CAT), peroxiredoxins (PRX), thioredoxin reductase (TRXR) and glutathione systems (glutathione synthetase GS, glutathione reductase GR, glutathione peroxidases GPx, and glutathione S-transferases GST) may be necessary. Specifically, the dismutation by SOD of the superoxide (the primary produced Reactive Oxygen Species, abbreviated as ROS), and the reduction of the produced H2O2 by CAT and GPx (often in association with thiol-containing enzymes, PRX, TRXR and glutaredoxins) are of extreme importance for stress response in all cells. Likewise, GSTs are important to inactivate unsaturated aldehydes, epoxides, and hydroperoxides, secondary metabolites of the reactions described above. In this regard, it was shown that the transcription regulation of SOD, CAT, GR and GPx is strongly affected by oxidative stress in the mesophilic ciliate Tetrahymena thermophila.

Thermal stress response could also be a further biomarker to understand the adaptation of these organisms. In mesophilic organisms, heat shock proteins (HSPs) are the main macromolecules involved in this mechanism and, acting as chaperones, provide the stabilization, the partial refolding, or the detection of proteins irreversibly damaged. In particular, the Hsp70 group is represented by proteins that are quickly induced under stress conditions by the activation of the hsp70 gene expression. However, hsp70 genes have been reported to have lost heat inducibility in several Antarctic marine organisms, including E. focardii.

E. focardii species are characterized by an unusual and pervasive mechanism of programmed translational frameshifting. A previous study reported an extensive analysis of this mechanism in the E. crassus genome and included some comparison with the E. focardii genome to gain some insights into the conservation and molecular basis of this frameshifting. Here, we report a deeper E. focardii MAC genome analysis based on reads that have been completely reassembled and annotated. Furthermore, we focused on characterizing Hsp70, tubulin and antioxidant enzymes gene families. Our results suggest that molecular adaptation to cold and oxidative stress defense in the Antarctic environment may be based on a variable number of paralogous genes.

Table 1. E. focardii macronuclear genome assembly statistics in comparison with E. vannus and E. octocarinatus.
Stylonychia and a database containing all ciliates available proteomes), the average of the CDSs’ length, of the introns’ length
tricha E. focardii genome would likely best be achieved by using long-read sequencing, such as that provided by Pacific Biosciences.
assembly contiguity; (ii) high genome homozygosity; (iii) fewer paralogs. In future further improvement of this,
E. crassus likely due to the presence of a substantial number of paralogous genes (Fig. S3). The MAC genome assemblies of
three species also show an order of magnitude more paralogs around the 40% peak than (Table 1; Table 2).
(ciliate even though it has a substantial associated bacterial consortium30. Found in the final assembly suggesting that no phenomena of horizontal gene transfer recently occurred in this
ciliate even though it has a substantial associated bacterial consortium30. The results of the gene prediction performed using the AUGUSTUS software are summarized in Table 2.
Table 2. E. focardii Gene prediction features in comparison with E. vannus79 and E. octocarinatus38.

| Nanochromosomes with only 1 gene (n) | E. focardii | E. vannus | E. octocarinatus |
|-------------------------------------|------------|-----------|-----------------|
| Total genes (n)                     | 23,224     | /         | /               |
| Nanochromosomes with more genes (n)| 2650       | /         | /               |
| Exons (n)                           | 81,715     | 175,735   | 96,843          |
| Introns (n)                         | 52,832     | /         | /               |
| Average intron length (bp)          | 69.02      | /         | /               |
| Average number of introns per gene  | 1.83       | /         | /               |
| Average CDS length (bp)             | 1085       | 1460      | 1178            |
| Number of genes with assigned function | 15,357 (53.17%) | / | / |
| Number of predicted enzymes         | 3306 (11.45%) | / | / |

Properties of E. focardii translation. Ciliates, including Euplotes, frequently use the standard stop codons in atypical ways compared to the usual eukaryote assignments35,36. As previously reported38,57,58, we

(Fig. S1). The final GC distribution shows a well-defined peak (with respect to the distribution before cleaning) centered on the 31.51% (Table 1, Fig. S2), a value consistent with those reported for other ciliates with a nanochromosomal MAC genome architecture (i.e., Stylonychia lemmiae, Oxytricha trifallax, and Euplotes crassus, 31.5%, 31.2% and 36.9%, respectively)39. This homogeneous normal GC distribution is consistent with negligible bacterial contamination. Although we cannot rule out the presence of sequences belonging to yet unreported endosymbionts with low GC content, it is unlikely their sequence base composition would perfectly match that of E. focardii and produce a unimodal distribution. Nanochromosomes containing bacterial sequences were not found in the final assembly suggesting that no phenomena of horizontal gene transfer recently occurred in this ciliate even though it has a substantial associated bacterial consortium30.

The genome size is in line with expectations based on the previous studies on other ciliates7, as well as for the ciliates reported in Table 2. This number of nanochromosomes was chosen as the main parameter for the selection of the best assembly among those produced by the different versions and configurations/parameters of SPAdes algorithm described in “Materials and methods”. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession MJUV00000000. The version described in this paper is version MJUV02000000.

This assembly does not show any alternative fragmentation on the basis of the clustering analysis used in analyses of the Oxytricha MAC genome and consistent with previous observations in E. crassus31. Moreover, from the pairwise sequence identity analysis of the assembly (described in the “Paralog prediction” section of “Materials and methods”), E. focardii, and E. octocarinatus, do not show a peak at high (>90%) sequence identity typical of heterozygous alleles, as instead E. crassus and E. vannus show, but only one peak of around 40% identity, likely due to the presence of a substantial number of paralogous genes (Fig. S3). The MAC genome assemblies of E. crassus, E. octocarinatus and E. vannus all have considerably more complete nanochromosomes than E. focardii (Table 1; E. crassus MAC genomes are unpublished but have in excess of 30,000 nanochromosomes). However, all three species also show an order of magnitude more paralogs around the 40% peak than E. focardii. The smaller number of complete nanochromosomes in E. focardii thus likely reflects a combination of three factors: (i) lower assembly contiguity; (ii) high genome homozygosity; (iii) fewer paralogs. In future further improvement of this genome would likely best be achieved by using long-read sequencing, such as that provided by Pacific Biosciences.

The results of the gene prediction performed using the AUGUSTUS software are summarized in Table 2. The number of genes (predicted in the nanochromosomes and in the contigs with no telomeres that blast with a database containing all ciliates available proteomes), the average of the CDSs’ length, of the introns’ length and of the number of introns per gene are consistent with those previously reported for Stylonychia and Oxy-
tricha31, and for E. crassus28,33. Nanochromosomes encoding a single CDS represent 74.58% of the total, and nanochromosomes with more than one CDS are 8.51% of the total; these percentages are comparable to those of Stylonychia and Oxytricha (75% encoding a single CDS and higher than 7% with more than one CDS)32,33.

To assess genome completeness, we compared the predicted proteins of the E. focardii macronuclear genome assembly with the CEG database: 93% of this database’s sequences (231) have homologs in the E. focardii assembly. This value may suggest a small amount of incompleteness of the genome assembly. However, 11 out of 17 of the sequences without matches are also absent from the Oxytricha and Stylonychia genomes (Table 3), consistently with greater evolutionary distances of ciliates from the eukaryotes included in CEG, as also proposed for Stylonychia32. Genome annotation revealed a total of 90 ribosomal proteins, of which 36 belong to the standard eukaryotic small 40S subunit and 54 to the large 60S subunit. These values strongly support the present genome analysis, considering that the total standard eukaryotic ribosomal protein set that contains 32 proteins in the 40S subunit and 48 in the 60S subunit. Rfam analysis of structural RNAs in E. focardii, in addition to confirm the annotated tRNAs described in the next section, revealed the presence in the assembly of a nanochromosome (NODE_589) encoding both 18S and 28S rRNA genes, as well as 10 additional snRNA genes, including 3 for 5S rRNA and one for 5.8S rRNA. These results agree with those previously reported for Oxytricha trifallax28 and Stylonychia lemmiae32. Taken together these results support the completeness of the E. focardii MAC genome assembly.

Table 2. E. focardii Gene prediction features in comparison with E. vannus79 and E. octocarinatus38.
detected abundant frameshifting motifs (AAA, AAT, ATA, AAC etc. codons followed by a stop codon, either TAA or TAG) in the *E. focardii* MAC genome (see "Materials and methods" section) consistent with pervasive +1 programmed translational frameshifting in *Euplotes* ciliates. The frameshifting analysis on the predicted genes revealed that at least 4.2% of total genes could be affected by this phenomenon. This value could be underestimated since it excludes genes that had no BLAST hits (about 47%) to our sequence database and does not consider other possible frameshifting motifs beside those already identified in *E. crassus*28. Even though the comparison of the frameshifting sites between *E. focardii* and *E. crassus* revealed that these are not conserved in the same genes28, the occurrence of predicted frameshifting (just under 10%) would be in line with the observations in *E. crassus*37 and in *E. octocarinatus* by Wang et al.38. Considering the transcript-level quantity of these specific genes, as described in the "Materials and methods" section, the +1 ribosomal frameshifting activity does not significantly affect the transcription in *E. focardii* on the basis of the statistical test used (*p*-value = 0.386).

61 tRNAs were predicted in the *E. focardii* MAC genome assembly. 55 unique tRNAs were encoded on nanochromosomes and appear to be sufficient for the translation of all codons. These tRNAs include one selenocysteine tRNA (encoded on NODE_51680), with the typical long variable arm characteristic of such tRNAs, and a putative paralogous pair of cysteine tRNA genes with TCA anticodons (tRNA-Cys(TCA)-1: NODE_55662; tRNA-Cys(TCA)-2: NODE_55665; 90% identical to each other), which resemble those previously reported for *E. crassus*39. The tRNA-Cys(TCA) paralogs are in turn paralogs of tRNA-Cys(GCA), as for *E. crassus*39.

Previously a potential stop-suppressor tRNA of UAA, suggested to play a role in the ribosomal frameshifting was reported in *Euplotes octocarinatus*38,40. Though we found evidence of potential translational readthrough of stop codons (see subsequent analysis), we could not detect a similar tRNA in *Euplotes focardii* using BLASTN searches with the putative *E. octocarinatus* frameshifting tRNA as a query. Furthermore, neither tRNAscan-SE nor Aragorn predicted a similar tRNA to the putative frameshifting *E. octocarinatus* tRNA in *E. focardii*. The tRNA secondary structure prediction software did however predict an unrelated tRNA with a potential stop cognate anticodon on contig NODE_32101 (Fig. 1A). NODE_32101 encodes an additional tRNA-Glu(TTC), which is 66.7% identical to the putative suppressor tRNA (CTA) (Fig. 1). A different tRNA-Glu(TTC) encoded on a separate nanochromosome (NODE_54875) is 77% identical to NODE_32101 tRNA-Glu(TTC). BLASTN

### Table 3. CEGs missing from alignment results using default CEGMA criteria.

| CEG name | Styloynchia | Oxytricha | E.focardii |
|----------|-------------|-----------|------------|
| DNA-directed RNA polymerase, subunit RPB10 | KOG3497 | KOG3497 | |
| Mitochondrial F1F0-ATP synthase, subunit delta/A TP16 | KOG1758 | KOG1758 | KOG1758 |
| 6-Phosphogluconate dehydrogenase | KOG2653 | KOG2653 | KOG2653* |
| Sugar (pentulose and hexulose) kinases | KOG2531 | KOG2531 | KOG2531 |
| Predicted snRNP core protein | KOG3448 | | |
| Glucose-6-phosphate 1-dehydrogenase | KOG0563 | KOG0563 | KOG0563 |
| 6TU (ovarian tumor)-like cysteine protease | KOG2606 | | |
| 6-phosphogluconolactonase- like protein | KOG3147 | KOG3147 | KOG3147 |
| Spindle assembly checkpoint protein | KOG2385 | KOG2385 | KOG2385 |
| RNA polymerase II transcription initiation/nucleotide excision repair factor TFIIH, subunit SSI1 | KOG2807 | | |
| UDP-glucose pyrophosphorylase | KOG2638 | KOG2638 | KOG2638 |
| Mitochondrial import inner membrane translocase, subunit TIM13 | KOG1733 | KOG1733 | KOG1733 |
| Translation initiation factor 3, subunit g (eIF-3 g) | KOG0122 | KOG0122 | |
| Uncharacterized conserved protein | KOG2967 | | |
| Mitochondrial import inner membrane translocase, subunit TIM9 | KOG3479 | KOG3479 | |
| Predicted translation initiation factor related to eIF-2B alpha/beta/delta subunits (CIG2/ID12) | KOG1468 | KOG1468 | KOG1468 |
| Ubiquitin fusion-degradation protein | KOG1816 | | |
| Uncharacterized conserved protein | KOG3237 | | |
| Molecular chaperone Prefoldin, subunit 4 | KOG1760 | | |
| Small nuclear ribonucleoprotein (snRNPs) SMF | KOG3482 | KOG3482* | |
| 60S ribosomal protein L38 | KOG3499 | KOG3499 | |
| 60S ribosomal protein L39 | KOG0002 | | |
| Multifunctional methyltransferase subunit TRM112-like protein isoform 1 | KOG1088 | | |
| Retention in endoplasmic reticulum 1 | KOG1688 | | |
| Translationally-controlled tumor protein | KOG1727 | | |
| Dolichyl-P-Glc:Man9GlcNac2-PP-dolichyl glucosyltransferase | KOG2575 | | |
| Ubiquitin-like protein 5 | KOG3493* | | |
| H/ACA ribonucleoprotein complex subunit 3 | KOG3503 | | |
| Total CEGs not found | 14 | 17 | 20 (17) |
searches with both of the candidate tRNAs versus the *E. octocarinatus* genome yield top hits to *E. octocarinatus* Contig33653 (for NODE_32101 tRNA(CTA)) and Contig33553 (NODE_32101 tRNA-Glu(TTC)). For both *E. octocarinatus* nanochromosomes tRNA-Glu(TTC) genes are predicted in the regions of the BLASTN matches. Thus it appears NODE_32101 tRNA(CTA) is a paralog of Glu(TTC) tRNAs.

We observed expression of mature tRNAs for both predicted tRNAs on NODE_32101 in the YAMAT-seq reads (Supplemental Table 1), at low levels, but well within the limits of other predicted *E. focardii* tRNAs. It can be seen that the secondary structure predicted by RNAfold (Fig. 1A) does not place the anticodon 5'-CTA-3' symmetrically in the anticodon loop, as is typically the case. However, this may be incorrect structure prediction, similar to the incorrect predictions by RNAfold we observed while attempting to predict the *E. focardii* tRNA-Cys(TCA) structures using this software. In the alignment of *E. focardii* and *E. octocarinatus* tRNAs, it can be seen...
that TCA aligns to the predicted TTC anticodons of the Glu-tRNAs (Fig. 1B). In future, to ascertain whether the candidate tRNA(CTA) on NODE_32101 is functional it would be necessary to search additional genomes from other Euplotes species, and observe whether there are similar putative tRNA genes with TCA anticodons and also whether there are co-varying substitutions that support structural conservation and anticodon position.

Other than frameshifting “stop” codons, there are a few reports of potential translation of in-frame “stops”. In E. focardii, based on multiple sequence alignments, a TAG codon in a beta-tubulin gene was hypothesized to be translated as tryptophan. Ricci et al. described the use of an in-frame TAG codon in two other Euplotes genes. Recently, Wang et al. reported the use of TAG to encode an amino acid, likely glutamine, in the cathepsin B gene of the closely related freshwater Euplotes octocarinatus. Given these observations and considerable plasticity in termination codon usage in ciliates, we wondered if the E. focardii stop codons may occasionally be translated in other genes.

In standard genetic code organisms, which do not possess tRNAs directly cognate to “stop” codons, translational readthrough, may use near-cognate pairing of tRNAs (i.e., possessing two of the three complementary anticodon-codon pairings between bases). TAA/TAG codons are most frequently translated as glutamate and TGA as tryptophan in eukaryotic translational readthrough. To investigate what amino acids TAA/TAG codons in E. focardii might encode, we examined the frequency of amino acids aligned to these codons in conserved alignments extracted from translated BLAST matches (Fig. 1C). We focused on matches with substantial sequence conservation up- and downstream of stops to exclude potential sites of translational frameshifting. As a baseline for comparison, we also examined the frequencies of amino acids aligned to TGA codons, which predominantly encode cysteine, and CAA/CAG codons, which encode glutamine. For both codon kinds, the most frequent aligned amino acids are the expected ones (Fig. 1C). TAA/TAG codons are most often frequently aligned to glutamine.

Whether TAA/TAG codons are translated by translational readthrough or conventionally by a tRNA such as the candidate tRNA(CTA) in E. focardii, remains to be determined. In other eukaryotes translational readthrough typically occurs at low levels, typically a small percentage of non-translational readthrough, and leads to short extensions of proteins. Consequently, in future it will be of interest to determine the translational efficiency of in-frame stops in Euplotes, particularly in genes like the beta-tubulin paralog with an in-frame TAG occurring close to the N-terminus, especially if it is a relatively highly translated protein like other beta-tubulins. Furthermore, it would also be of interest to determine what amino acid the candidate tRNA(CTA) may be charged with, and if there is transamidation of glutamate to glutamine, as observed in Bacillus subtilis and many other organisms.

The tubulin super families in E. focardii genome. The protein annotation procedure allowed the identification of 15,357 proteins (Table 2), 3306 of which are enzymes. Using CD-HIT, 5222 were grouped in clusters with at least 40% of identity. We focused on the analysis of the members of tubulins, antioxidant enzymes, and Hsp70 gene families to examine whether the number of paralogs and their evolution in each superfamily may be related to the E. focardii cold-adaptation.

In vitro polymerization studies performed with E. focardii purified tubulin heterodimers demonstrated their ability to form microtubules at temperatures close to the freezing point of the Antarctic marine habitat, as also reported for tubulin heterodimers purified from Antarctic fishes. In addition, the study of the tubulin superfamily in an Antarctic psychrophilic ciliate is even more interesting with respect to other psychrophilic organisms because it contributes not only to the understanding of the molecular basis of microtubule cold adaptation but also to microtubular structure complexity. Indeed, differently from other eukaryotic microorganisms, ciliates assemble 17 different types of microtubules throughout their life cycle, even though all microtubule functions are carried out in a single cell.

The macronuclear genome sequencing from several ciliates allowed the characterization of up to five alpha- and beta-tubulin isoatypes. Therefore, a multigenic tubulin family is a common characteristic in ciliates and these tubulin isoatypes may be responsible of the formation of functionally different microtubules and with different dynamics properties.

In previous papers, we reported the characterization of a single α-tubulin, four β-tubulins, and two γ-tubulin isoatypes from E. focardii. Previously, the presence of four β-tubulin isoatypes induced us to hypothesize that in E. focardii microtubules cold adaptation was based mainly on molecular modification of the β-tubulin subunit of the heterodimer rather than on the α-tubulin subunit. The comparison of these sequences with the homologs from non-cold adapted Euplotes species revealed the presence of unique amino acid substitutions in the E. focardii tubulin isoatypes that may be correlated with cold adaptation. Therefore, we further investigated this relevant class of proteins in the E. focardii genome.

All tubulin sequences that were detected in the final assembly were checked starting from those previously characterized. All the sequences already collected into the UniProt Knowledgebase (TBβ1—UniProt: Q9NN2N6; TBβ2—UniProt: COL7F0; TBβ3—UniProt: COL7F1; TBα1—UniProt: Q8WRT6; TBγ1—UniProt: A3F2R1; TBγ2—UniProt: A3F2R2) were identified and confirmed as reported in Table 4, with the exception of TBβ4 (the sequence collected as C0L7F2 in UniProt is included in the phylogenetic tree of Fig. 2B). However, new isoatypes were discovered after protein annotation. Specifically, a new β-tubulin isotype (named TBβ5) and six additional α-tubulin isoatypes. The relationship among these isoatypes and those for other Euplotes species are shown in the phylogenetic trees in Fig. 2. In the trees, we introduced homologs from Tetrahymana, Oxytricha and Stylonychia to better evidence the degree of E. focardii gene amplification and divergence. E. focardii α-tubulins branches (highlighted as bold in Fig. 2A) are scattered throughout the phylogeny of ciliate homologs: ATU1 and ATU2 isoatypes cluster with the homologs from E. crassus in the same group containing the “canonical” Tetrahymana α-tubulin, whereas ATU3 to ATU7 isoatypes form a separate clade with E. crassus ATU4 and ATU5, and...
one isotype from Oxytricha. This result suggests that the isotypes from ATU1 to ATU5 preceded the divergence of E. focardii from the other Euplotes species, and were maintained in the Antarctic ciliate, whereas ATU6 and ATU7 derived from an additional event of gene duplication in E. focardii that gave origin to new distinct isotypes.

An high number of isotypes may be considered as an additional strategy for tubulin cold adaptation beside the presence of unique residues substitutions in the primary structure of tubulin heterodimers.

By contrast, E. focardii β-tubulin family appears less amplified and divergent than alpha tubulin. The branches (highlighted as bold in Fig. 2B) are less scattered and cluster with the corresponding E. crassus and E. octocarinatus homologs, even though BTU5 forms a well separated clade. BTU1 and BTU2, and BTU3 and BTU4, may be originated by recent gene duplications in E. focardii.

With the high number of different α-tubulin isotypes in the Antarctic ciliate E. focardii, we reconsidered the importance of the α-tubulin subunit in microtubule cold adaptation.

Molecular flexibility is regarded as a hallmark of cold adapted molecules, in particular enzymes, to cope with the reduction of dynamics and activity at low temperature. We applied Molecular Dynamics (MD) simulation on each α-tubulin isotype of the ciliates under study. We found that only three E. focardii isotypes (2, 4 and 5; Fig. 3B) show a higher flexibility at 4 °C with respect to the E. crassus α-tubulin isotypes. The only exception in E. crassus is the isotype 2. This result can be considered as a further evidence of the E. focardii tubulin cold adaptation and suggests that not all isotypes must be flexible to function at low temperatures, as we reported also for the β-tubulin isotypes.

The similarity of E. focardii α-tubulin isotype 2 with that from E. crassus suggests a common origin of this α-isotype.

We also identified seven α-like and two β-like tubulins (e.g., more divergent isotypes; Table 4) and two δ and one ε isotypes. By contrast, the assembler was not able to produce two distinct complete contigs/nanochromosomes for γ-tubulin. In other words, γ-tubulin type 2 previously obtained from macronuclear DNA purified from E. focardii cells is present in the contig only in a short fragment form (see Table 4 legend). This is probably due to the high sequence similarity between the two γ-tubulin isotypes since raw reads specific for this second isotype

Table 4. Tubulins comparison between E. focardii, E. crassus and E. octocarinatus. *NODE_87922_length_309_cov_44.0984 (fragment of 309 nt without telomeres only present in the total non-cleaned assembly). Protein sequence already deposited in UniProtKB (the ID numbers of E. crassus proteins are: Q8MM87 for TBα1, Q8MU38 for TBα2, Q8MU39 for TBα3, P20365 for TBβ3, P54403 for TBγ1 and P54404 for TBγ2; the ID numbers of E. octocarinatus proteins are: Q08114 for TBα1, Q08115 for TBβ1, Q0GGY3 for TBβ2 and P90548 for TBγ2). Contig contains a further sequence portion (in comparison with that deposited in UniProtKB) most likely derived by an assembling or gene prediction error.
were obtained after sequencing but difficult to align with this assembler. In the phylogenetic tree, *E. focardii* GTU1 and GTU2 isotypes (highlighted as bold in Fig. 2C) appear to be more related to the single γ-tubulin isotype from *E. octocarinatus* (that is encoded by two similar genes) than the two distinct isotypes from *E. crassus*. In *E. focardii*, the presence of distinct γ-tubulin isotypes is associated to different roles in the nucleation of cellular microtubules (as previously reported⁵⁵) more than to cold-adaptation.

**The antioxidant enzymes system.** A major issue for Antarctic marine organisms is oxidative stress since they experience high dissolved oxygen, more soluble in cold seawaters, typical of Antarctic marine environment⁵⁹. Two key classes of enzymes involved in antioxidant defenses are the superoxide dismutases and catalases.

Superoxide dismutases (SOD, EC 1.15.1.1) are the ubiquitous metalloenzymes that catalyse the dismutation of superoxide anion into molecular oxygen and hydrogen peroxide (O$_2^-$ + O$_2^-$ + 2H$^+$ $\rightarrow$ O$_2$ + H$_2$O$_2$)⁶⁰.⁶¹. SODs are
grouped into three protein families, based on the metal cofactor they contain and on the protein fold. Copper, zinc SODs (Cu,Zn SODs) are found in the cytoplasm of eukaryotes, in the chloroplasts of some plants and in the periplasmic space of bacteria. This group of SODs is often referred to as SOD1, but if these Cu,Zn enzymes are present in the extracellular fluids of eukaryotes are referred as SOD3. Iron- and manganese-containing SODs (FeSOD and MnSOD, referred as SOD2) are considered the primitive forms of SODs. FeSODs are found in prokaryotes and chloroplasts, while MnSODs are present both in prokaryotes and in the mitochondrial matrix of eukaryotes. Differently from MnSOD, Cu,Zn SODs were believed to be absent in protists until whole genomes sequencing revealed Cu,Zn SOD encoding genes in a number of different protists.

Figure 3. (A) trajectories data as backbone RMSDs for *E. focardii* α-tubulin isotype 2, for the first 10 ns, at 27 °C (red) and at 4 °C (blue). (B) the bar plot shows ΔRMSD values for each α-tubulin of *E. focardii* (blue), *E. crassus* (green), *E. octocarinatus* (yellow) and *T. thermophila* (6U0H, orange; ATU1 in Fig. 2) calculated as the difference between the RMSD\textsubscript{max} at 27 °C and the RMSD\textsubscript{max} at 4 °C (see "Materials and methods" section).
In a previous paper, two E. focardii Cu,ZnSODs and one MnSOD were biochemically characterized. All three SODs are active at 4 °C but at the same time they retain high activity upon 20 min incubation up to 55/60 °C. This feature is unusual in cold active enzymes that are often heat sensitive and undergo inactivation and unfolding even at mild temperature. Nevertheless, thermo-tolerance or even thermostability of cold adapted enzymes was previously reported suggesting that cold activity and thermo-tolerance may coexist in a molecule.

The sequences of the enzymes previously studied and present in the UniProt Knowledgebase (SOD1a—UniProt: W0FZ77; SOD1b—UniProt: W0FUJ3; SOD2a—UniProt: MG575644) were confirmed by the analysis of the genes obtained in the E. focardii genome here described (Table 5): the SOD classification was done on the base of similarity with cytoplasmic SODs (type 1) and with mitochondrial SODs (type 2) and was confirmed by the presence of Cu/Zn and Fe/Mn pattern signature, respectively. In the same genome analysis, we could predict four additional E. focardii isoforms, the SOD1d and SOD1e and two SOD3 isoforms (Fig. 4A). According to this tree, the SOD3 isoforms appear to derive from gene duplication events probably happened before the

| Anti-oxidant enzyme | Protein assembly code | E. crassus | E. octocarinatus |
|---------------------|-----------------------|------------|-----------------|
| SOD1a               | Protein_25445 §       | Protein_49381 § | 2168.g10728.t1 § |
| SOD1b               | Protein_25349 §       | Protein_49686 § | 20.871.g10083.t1 § |
| SOD1c               |                       |            | 32.362.g21016.t1 § |
| SOD1d               | Protein_26998 §       | Protein_46068 § | 19.489.g8844.t1 § |
| SOD1e               | Protein_23236 §       | Protein_46028 § | 20.227.g9513.t1 § |
| SOD2a               | Protein_23056 §       | Protein_44659 § | 32.233.g20903.t1 |
| SOD3a               | Protein_23639 §       | Protein_44373 § | 32.233.g20903.t1 |
| SOD3b               | Protein_23528 §       | Protein_44207 § | 32.233.g20903.t1 |
| CAT1                | Protein_12515        | Protein_24239 | 7699.g26798.t1 |
| CAT2                | Protein_12995         | Protein_24753 | 32.233.g20904.t1 |
| CAT3                | Protein_25541         | Protein_24714 | 32.233.g20903.t1 |
| CAT4                | Protein_25374         |            |                |
| CAT5                | Protein_27502         |            |                |
| CAT6                | Protein_32805         |            |                |
| CAT7                | Protein_23587         |            |                |
| PRX1                | Protein_22541         | Protein_45540 | 7627.g26732.t1 |
| PRX2                | Protein_22695         | Protein_40156 | 7810.g26912.t1 |
| PRX3                | Protein_23342         | Protein_45912 | 8218.g27323.t1 |
| PRX4                | Protein_23707         | Protein_44467 | 6416.g25544.t1 |
| PRX5a               | Protein_28419         |            |                |
| PRX5b               | Protein_44989         |            |                |
| PRX6                | Protein_39913         |            |                |
| TRXR1               | Protein_09887         | Protein_21154* | 9 proteins |
| TRXR2               | Protein_20916*        |            |                |
| TRXR3               | Protein_10344         | Protein_19652 |                |
| GR1                 | Protein_14684         | Protein_27646* |                |
| GR2                 | Protein_14104         | Protein_25768 |                |
| GPx1                | Protein_21251         | Protein_44685* | 11.975.g1890.t1 |
| GPx2                | Protein_22198         | Protein_44915* | 13.463.g3154.t1 |
| GPx3                | Protein_23095         | Protein_44916* | 32.168.g20842.t1 |
| GPx4                | Protein_25138         | Protein_43343 |                |
| GPx5                | Protein_22265         | Protein_44948 | 8754.g27852.t1 |
| GPx6                | Protein_48111         |            |                |
| GPx7                | Protein_43209         |            |                |
| GS1                 | Protein_14094         | Protein_25486 | 9010.g28082.t1 |
| GS2                 | Protein_14269         | Protein_35727 |                |
| GST                 | Protein_14270         | Protein_27010 |                |
| GST                 | 69 proteins           | 60 proteins  | 63 proteins    |

Table 5. Antioxidant enzyme comparison between E. focardii, E. crassus and E. octocarinatus. *Protein sequence already deposited in UniProtKB (the ID numbers of E.crassus proteins are: B8XTW3 for TRXR1, B8XTW4 for TRXR2, J9SMC8 for GR1, B8XTW9 for GPx1, B8XTX0 for GPx2 and J9T5F4 for GPx3). § Protein having Cu/Zn superoxide dismutase signature (Prosite pattern ID: PS00087 and PS00332). # Fe/Mn superoxide dismutase signature (Prosite pattern ID: PS00088). + Protein annotated as Cu/Zn superoxide dismutase but not have any known signature.
The divergence of *E. focardii* from other *Euplotes* species and some isotypes were then lost in *E. crassus*. As a general result, the *E. focardii* SOD encoding gene family appeared composed by a higher number of genes with respect to the mesophilic *E. crassus*, probably due to a repeated event of gene duplication.

A similar situation is evident for the catalase (CAT) genes (Fig. 4B). CAT (EC 1.11.1.6), that inhibits the DNA damage by decomposing the H_2O_2 into oxygen and water induced by nitrofurazone, was previously considered a good biomarker for detecting oxidative stress and, consequently, ecotoxicity in aquatic ecosystems. The number of *E. focardii* CATs genes is higher compared to the mesophilic *E. crassus*, probably due to a repeated event of gene duplication. As shown in Fig. 4B, CAT branches are scattered throughout the phylogeny of ciliate homologs. The low bootstrap values at these branches suggest that in *E. focardii* the CAT gene family underwent several events of recent gene duplications with potential adaptive outcomes that imply high divergence and consequently less supported phylogeny.

In general, the antioxidant enzymes system appears amplified in *E. focardii* (Table 6) suggesting that gene amplification may have contributed to combating the effects of increased oxygen concentration in the Antarctic seawaters. On the other hand, *E. focardii* possesses few gene encoding Thioredoxin NADPH Reductase (TrxR).
and Glutathione Reductase (GR) isoforms. With the exception of E. octocarinatus, the genes for these enzymes are present in small number in the ciliate genomes known so far (see Table 6): Tetrahymena, as an exception in ciliates, has no TRXR or GR genes but 6 isoforms of thioredoxin–glutathione reductase (TGR) genes, that are composed by a fusion of the sequences of TRXR and glutaredoxin domains and are capable of transporting electrons from NADPH to both Trx and GSH systems. E. octocarinatus has only 3 isoforms of TGR. In conclusion, the E. focardii antioxidant system appears to be based mainly on numerous SODs and CATs enzymes.

The E. focardii heat-shock protein 70 gene family. The E. focardii macronuclear genome possesses seven distinct nanochromosomes that encode Hsp70 isoforms. According to their predicted C-terminal domain sequences, we identified the respective Hsp70 subfamilies defined by the putative subcellular localization of the sequences containing telomeres at both ends vs 12,922 previously reported. Even though some assembly parameters remain lower in comparison to the other ciliates reported, complete nanochromosomes are now closer, as an indication that in E. focardii there is no amplification of the hsp70 encoding genes.

La Terza et al. demonstrated that the canonical cytoplasmatic E. focardii hsp70 gene (GenBank acc. no. AAP51165.1). Protein, 06381 in this paper) is not inducible by acute thermal stress, in contrast to the orthologous gene in E. nobili also found in Antarctica. Unlike in the Antarctic fish Trematomus bernacchii, the hsp70 gene does respond to several oxidative stressors, such as hydrogen peroxide in E. focardii. In order to understand whether the Euplotes Hsp70 isoforms newly described here respond to thermal and/or oxidative stressors, we tested the inducibility of these genes in cultures subjected to heat shock (18 °C) or oxidative stress (H_2O_2). Figure 6 shows that thermal stress did not induce transcription of the any of the seven Hsp70 genes relative to the control temperature (4 °C). In contrast, six of the seven genes (excluding isoform named Protein_05367) were induced by oxidative stress, strikingly so in the case of Protein_07400 isoform. We suggest that E. focardii is an optimal model for studying genome adaptation to cold environments. In this paper, we reported the analysis of the E. focardii MAC genome after a significant improvement of the assembly that were also cleaned-up of algal or bacterial contaminants. In particular, we obtained 17,798 sequences containing telomeres at both ends vs 12,922 previously reported. Even though some assembly parameters remain lower in comparison to the other ciliates reported, complete nanochromosomes are now closer, as a percentage of the total assembly, to those from other Euplotes species. Further -

| MAC genome will provide additional information to investigate translation mechanisms in organisms with alternative genetic codes associated with the evolution of novel tRNA variants, including a putative suppressor tRNA, and to investigate how cold adaptation may have evolved.

The analysis of this improved E. focardii genome assembly allowed a better characterization of gene families, in particular that of the tubulins, that were previously only partially identified by single gene cloning approaches. We identified a new β-tubulin isoform (TBβ5) and six additional divergent α-tubulin isoforms. In combination with the β-tubulin diversity, the role of the high number of different α-tubulins in microtubules cold adaptation should be reconsidered. Furthermore, we found that SODs and CATs families are composed by a higher number of genes with respect to the mesophilic Euplotes. The opposite trend was observed for the hsp70 genes: in this case isoform's diversification appears reduced with respect to homologs from other Euplotes species. Furthermore, expression of these genes was not induced by heat stress (18 °C for 30 min vs. a physiological temperature of 4 °C). On the other hand, hsp70 expression was raised during oxidative stress. We can conclude from these results that as for other Antarctic organisms, it is more important for E. focardii to cope with cold denaturation of proteins and oxidative stress than to respond to thermal stress. Consequently, the Hsp70 gene family did not expand like SODs and CATs families, that are involved in the antioxidant responses.

Table 6. Number of antioxidant enzymes predicted in ciliate organisms phylogenetically close to E. focardii. These values were obtained consulting the annotated proteins files.
All these results suggest potential roles for paralogy in environmental adaptation, warranting future experimental investigation. Genomic expansions of specific protein gene families contributing to physiological fitness in freezing polar conditions have previously been reported for Antarctic notothenioids (80). Gene diversification has been proved to produce a differential gene expression in specific adaptive conditions, as reported for the cold acclimation of the tea plant *Camellia sinensis* (81) and also for the *E. focardii* βT3-tubulin during cilia regeneration (14) and the *E. focardii* SOD 1b during cold stress (73). In future, RNA-seq analyses of *E. focardii* transcriptome in different environmental conditions coupled to detailed molecular analyses will provide deeper insights into the role of duplicated genes.

In conclusion, we propose that the molecular basis of cold adaptation that enabled *E. focardii* to thrive in the Antarctic Ocean may not be solely due to particular amino acid substitutions that enable these molecules to function at low temperatures but may have also arisen via gene duplications that increased protein functional diversity.

**Materials and methods**

**SPAdes assembly.** The Illumina HiSeq 2000 PE (paired-end, 100 bp, with BioProject ID SRX1959352) reads obtained after sequencing of *Euplotes focardii* macronuclear genome, previously trimmed using the Trimomatic software (version 0.36) (82) and checked using the FastQC software (version 0.11.5) (83), were assembled using the SPAdes algorithm (version 3.10.1) (84) with the “careful” option and the BayesHammer error correction algorithm (85). Other parameters were set to default values. Additionally, SPAdes version 3.9, version 3.11.1 and a different set of k-mer lengths were also used to check and identify the best version and configuration of the assembler for these reads.

Possible redundant “chaff” contigs were removed from the assembly, as previously reported (82), by mapping contigs shorter than 500 bp that had matches to the other contigs with greater than 80% coverage and 90% sequence identity.
Assembly clean-up and properties. To perform a quality assessment of the obtained assembly from SPAdes, avoiding bacterial contamination, the assembly was further analyzed checking the GC content by the QUAST software (version 4.5)\(^8\). The contigs having GC content higher than 45%, and a coverage lower than 10, were removed from the assembly using a custom algorithm written in Perl. This GC content percentage threshold was also set on the base of the minimum GC content of the most abundant bacteria in the consortium associated to *E. focardii* (data unpublished). Further bacterial contaminations were analyzed by using BLAST of the assembly versus the Genbank nr database setting bacteria as taxonomy filter, with 80% of hit coverage and 95% sequence identity of the matches. To check the goodness of the assembly, the SPAdes procedure was further repeated after this decontamination step. Currently, Genbank nr database also includes data of the last reported *Euplotes* endosymbiotic bacteria\(^8\),\(^9\).

Moreover, to remove possible contaminant algal sequences, the *Dunaliella salina* genome (used to feed *E. focardii*) was compared to the *E. focardii* macronuclear genome assembly, using blastn and cd-hit-2d software with a threshold of 0.95 (see “Paralog prediction” section for details).

After all the steps of assembling and cleaning, the assembly was evaluated using another custom Perl script providing information about the size, the number of contigs, the number of telomeres, the number of 2, 1, and 0 telomere contigs and the mean length of contigs.

Gene prediction and frameshifting analysis. The gene prediction procedure on the *E. focardii* macro-nuclear genome assembly was performed using the AUGUSTUS software (version 3.3.3)\(^8\) previously trained and tested on a manually curated data set, with no cases of translation frameshifting, from *Euplotes crassus* (data unpublished). The software was run using the following parameters: “–species = euplotes_crassus –UTR = on –alternatives-from-evidence = true –genemodel = complete –codingseq = on”. The results of this prediction were assessed/processed with another Perl script.

Genes predicted were additionally analyzed checking 12 potential frameshifting sites (5'-AAA TAA -3', 5'-AAT TAA-3', 5'-TTTAA-3', 5'-TTATAA-3', 5'-AACTAA-3', 5'-ATATAA-3', 5'-GAGTAA-3', 5'-AAATAG-3', 5'-GTA TAA-3', 5'-TTATAG-3', 5'-ATATAG-3' and 5'-TCCTAA-3', ranked by abundance), previously detected in *E. crassus*\(^2\), located at the end of these sequences and comparing them with the *E. focardii* proteome obtained after the Protein Annotation step. Sequences having these potential sites (7023) were checked and selected on the base of sequence length (lower than the related blast top hit length) and BLAST coverage (higher than 80%) versus the best hit obtained from nr reference database. Moreover, the transcript-level quantification of these genes, in comparison with all the others, was estimated using pair-end Illumina transcriptome reads of *E. focardii* and RSEM software package\(^9\), which allocates the multi-mapping reads on the base of an expectation maximization approach. A two-sample independent *t*-test was used to perform this comparison (*p*-value < 0.05 were considered to be significant).
Protein annotation. The proteins predicted from the *E. focardii* macronuclear genome assembly were searched and annotated using the OmicsBox software (version 1.4.11)\(^{91,92}\). The parameter settings used for the procedure were: blastp as blast program, nr as blastdb, 1.0e\(^{-3}\) as E-value and 20 as number of BLAST hits. Other settings for the annotation were set to the default values.

Assessment of genome completeness. The assessment of genome completeness was firstly conducted analyzing the percentage of conserved core eukaryotic genes (CEGs)\(^{93}\) searching the number of protein sequences contained in the CEG database (composed by 248 proteins) that were likely homologs with those of the *E. focardii* macronuclear genome assembly (i.e., with blastp E-values lower than 1e-10 and a match coverage higher than 70% of the length of the CEG proteins).

The tRNAs were initially predicted with the Aragorn algorithm (version 1.2.4)\(^94\). Ribosomal proteins were counted after the protein annotation, as previously described, and structural RNAs were identified by BLAST searches of the assembly against the Rfam database. Secondary and tertiary structures of a potential stop-suppressor tRNA were determined using the RNAfold web server\(^{95}\) and the RNAComposer automated RNA structure modeling server\(^96\), respectively.

Paralog prediction. The first step of this analysis was to cluster the *E. focardii* macronuclear proteome using the cd-hit software (version 4.7)\(^{97,98}\) with a sequence identity threshold of 0.95 to merge alleles (26,680 clusters). Therefore, the clustering was performed using a threshold of 0.4 to identify the largest and most represented protein families in the proteome (21,850 clusters of which 2312 with at least two elements). In this work, we have focused our interest on the Hsp70, tubulins and antioxidant enzymes family. Moreover, CD-HIT (and CD-HIT-EST) software was also used with a threshold of 0.95 to identify possible alternative fragmentation in the whole genome.

Pairwise sequence identity searches were performed on the *E. focardii* MAC genome assembly, in comparison with the *E. crassus*, *E. octocarinatus* and *E. vannus* assemblies, to estimate the distribution of alleles and paralogous sequences. By a custom Perl script, an alignment of all contigs against each other was performed into the assembly invoking the blastn algorithm and extracting the best non-self BLAST hits; then, MAFFT algorithm (–clustalout –maxiterate 1000 –globalpair / –localpair) was invoked to align the two sequences of each pair obtained; finally, its sequence identity was calculated.

tRNA sequencing, mapping and quantification. Total RNA was extracted using TRI Reagent (Sigma) according to the manufacturer’s protocol, deacylated at 37 °C for 40 min in 20 mM Tris–HCl (pH 9.0) and precipitated with 5 M ammonium acetate in 75% ethanol. This RNA was used to produce a library of mature tRNAs according to the manufacturer’s protocol, deacylated at 37 °C for 40 min in 20 mM Tris–HCl (pH 9.0) and pre-
and the protein root mean square fluctuation (RMSF) were determined using the GROMACS tools’ “gmx rms” and “gmx rmsf”, respectively, while the maximum RMSD value (RMSD$_{max}$) for each dynamic was calculated fitting the trajectory with the equation RMSD = t * RMSD$_{max}$ / ($t^a + \text{const}$) (Fig. 3A).

**Hsp70 gene transcription by *E. focardii* under stress.** We evaluated the inducibility of *E. focardii* Hsp70 genes in cultures subjected to thermal or oxidative stress. Cells entering stationary phase after ~1 week of vegetative proliferation in the presence of food (the green alga *Dunaliella tertiolecta*) were pelleted by low-speed centrifugation (500g, 3 min), and pellets were resuspended in seawater at cell density of ~5 × 10^7 cells/mL. Heat-shock was performed by warming cells from 4 to 18 °C over 30 min. Control cells were incubated at 4 °C for 30 min. Oxidative stress was produced by incubating cells at 4 °C in the presence of 100 µM H$_2$O$_2$ for 30 min. Total RNA from control or stressed cells was extracted with Trizol reagent (GIBCO BRL), and cDNA was synthesized from each template using the StrataScript Reverse Transcriptase (Stratagene).

Transcript levels corresponding to the seven *E. focardii* Hsp70 genes were measured in control and stressed DNA samples by comparative-threshold qPCR using the SYBR Green DNA-binding method and the primer pairs given in Supplemental Tables 2 and 3; the *Euplotes* SsSRNA gene (GenBank ID: EF094961) was used for normalization. To 100 ng of *E. focardii* cDNA were added 12.5 µl of 2× SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, Milan), 5 pg each of gene-specific forward and reverse primers, and water to a final volume of 25 µl. Amplification reactions were performed in triplicate in a Multicolor qPCR MX3000P thermocycler (Stratagene, Milan, Italy), with an initial denaturation step (95 °C for 2 min) to activate the polymerase followed by 45 cycles of denaturation at 95 °C for 30 s, and annealing and extension at 60 °C for 15 s. During annealing/extension, the increase in fluorescence at 495 nm was monitored, and the threshold value was set at 30 units. To verify that the primer pairs gave specific PCR products without non-specific amplification, the DNA samples were subjected to melting curve analysis by ramping the thermocycler temperature from 50 to 95 °C at 0.05 °C/sec.

The relative expression of the Hsp70 genes was calculated by the method of Pfaffl:

$$\text{Relative expression ratio} = \frac{(E_{\text{target}})^{\Delta Ct}}{(E_{\text{ref}})^{\Delta Ct}}$$

where Ct is the PCR cycle number at which the fluorescent signal is above the set threshold, ΔCt is the Ct difference (control minus sample) of the target or reference gene, and E is the real-time PCR efficiency of the target or reference gene ($E = 10^{-1/slope}$, calculated from plots of Ct vs. cDNA input). The relative expression ratios of transcripts under investigation were tested for statistical significance by a pairwise, fixed reallocation randomization test implemented in REST MCS version 2 software.

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**References**

1. Pucciarelli, S. et al. Molecular cold-adaptation of protein function and gene regulation: the case for comparative genomic analyses in marine ciliated protozoa. *Mar Genomics* 2, 57–66. https://doi.org/10.1016/j.margen.2009.03.008 (2009).
2. Pucciarelli, S., Marziale, F., Di Giuseppe, G., Barchetta, S. & Miceli, C. Ribosomal cold-adaptation: characterization of the genes encoding the acidic ribosomal P0 and P2 proteins from the Antarctic ciliate *Euplotes* focardii. *Genome* 560, 103–110. https://doi.org/10.1016/j.jgenet.2005.06.007 (2005).
3. Pucciarelli, S. & Miceli, C. Characterization of the cold-adapted alpha-tubulin from the psychrophilic ciliate *Euplotes* focardii. *Extremophiles* 6, 385–389. https://doi.org/10.1007/s00792-002-0268-5 (2002).
4. Yang, G. et al. Characterization and comparative analysis of psychrophilic and mesophilic alpha-amyloses from *Euplotes* species: a contribution to the understanding of enzyme thermal adaptation. *Biochem Biophys Res Commun* 438, 715–720. https://doi.org/10.1016/j.bbrc.2013.07.113 (2013).
5. Prescott, D. M. The DNA of ciliated protozoa. *Microbiol Rev* 58, 233–267 (1994).
6. Mollenbeck, M. & Klobutcher, L. A. De novo telomere addition to spacer sequences prior to their developmental degradation in *Euplotes* crassus. *Nucleic Acids Res* 30, 523–531 (2002).
7. Swart, E. C. et al. The *Oxytricha trifallax* macronuclear genome: a complex eukaryotic genome with 16,000 tiny chromosomes. *PLoS Biol* 11, e1001473. https://doi.org/10.1371/journal.pbio.1001473 (2013).
8. Heyse, G., Jonsson, F., Chang, W. J. & Lips, H. J. RNA-dependent control of gene amplification. *Proc Natl Acad Sci U S A* 107, 22134–22139. https://doi.org/10.1073/pnas.1009284107 (2010).
9. Nowacki, M., Haye, J. E., Fang, W., Vijayan, V. & Landweber, L. F. RNA-mediated epigenetic regulation of DNA copy number. *Proc Natl Acad Sci U S A* 107, 22140–22144. https://doi.org/10.1073/pnas.1012236107 (2010).
10. Dayeh, V. R. et al. Comparing a ciliate and a fish cell line for their sensitivity to several classes of toxicants by the novel application of multwell filter plates to *Tetrahymena*. *Res Microbiol* 156, 93–103. https://doi.org/10.1016/j.resmic.2004.08.005 (2005).
11. Detrich, H. W., 3rd, Parker, S. K., Williams, R. C., Jr., Nogales, E. & Downing, K. H. Cold adaptation of microtubule assembly and dynamics. Structural interpretation of primary sequence changes present in the alpha- and beta-tubulins of Antarctic fishes. *J Biol Chem* 275, 37038–37047. https://doi.org/10.1074/jbc.M095899200 (2000).
12. Manska, S. W. & Moores, C. A. Microtubule structure by cryo-EM: snapshots of dynamic instability. *Essays Biochem* 62, 737–751. https://doi.org/10.1042/EBC20180031 (2018).
13. Inclan, Y. F. & Nogales, E. Structural models for the self-assembly and microtubule interactions of gamma-, delta- and epsilon-tubulin. *J Cell Biol* 114, 413–422 (2001).
14. Chiappori, F. et al. Structural thermal adaptation of beta-tubulins from the Antarctic psychrophilic protozoan *Euplotes* focardii: remodelling of interaction surfaces may enhance microtubule nucleation at low temperature. *FEBS J* 275, 5367–5382. https://doi.org/10.1111/1742-4658.12666x (2008).
15. Pucciarelli, S., Miceli, C. & Melki, R. Heterologous expression and folding analysis of a beta-tubulin isotype from the Antarctic ciliate *Euplotes* focardii. *Eur J Biochem* 269, 6271–6277 (2002).
55. Pucciarelli, S. et al. Distinct functional roles of beta-tubulin isoforms in microtubule arrays of Tetrahymena thermophila, a model single-celled organism. *PLoS ONE* 7, e39694. https://doi.org/10.1371/journal.pone.0039694 (2012).

56. Pucciarelli, S. et al. Tubulin folding: the special case of a beta-tubulin isoform from the Antarctic psychrophilic ciliate Euplotes fociardii. *Polar Biol* 36, 1833–1838. https://doi.org/10.1007/s00300-013-1390-9 (2013).

57. Pucci, F. & Rooman, M. Physical and molecular bases of protein thermal stability and cold adaptation. *Curr Opin Struct Biol* 42, 117–128. https://doi.org/10.1016/j.sbi.2016.12.007 (2017).

58. Aygüt, I., Isaksen, G. V. & Brandsdal, B. O. Computation of enzyme cold adaptation. *Nat Rev Chem* 1, 0051. https://doi.org/10.1038/s41570-017-0051 (2017).

59. Lesser, M. P. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annu Rev Physiol* 68, 253–278. https://doi.org/10.1146/annurev.physiol.68.041014.110001 (2006).

60. McMord, J. M. & Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein).

61. Pucci, F. & Rooman, M. Physical and molecular bases of protein thermal stability and cold adaptation. *Curr Opin Struct Biol* 42, 117–128. https://doi.org/10.1016/j.sbi.2016.12.007 (2017).

62. Miller, A. F. Superoxide dismutases: ancient enzymes and new insights.

63. Benov, L. T. & Fridovich, I. Escherichia coli expresses a copper- and zinc-containing superoxide dismutase.

64. Chen, X.

65. Yang, G.

66. Marklund, S. L. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem J* 222, 649–655. https://doi.org/10.1042/bj220649 (1984).

67. Bannister, J. V., Bannister, W. H. & Rotilio, G. Aspects of the structure, function, and applications of superoxide dismutase. *CRC Crit Rev Biochem* 22, 111–180 (1987).

68. James, E. R. Superoxide dismutase.

69. Ferro, D.

70. James, E. R. Superoxide dismutase.

71. Marklund, S. L. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem J* 222, 649–655. https://doi.org/10.1042/bj220649 (1984).

72. Lee, Y. M., Friedman, D. J. & Ayala, F. J. Superoxide dismutase: an evolutionary puzzle.

73. Lee, Y. M., Friedman, D. J. & Ayala, F. J. Superoxide dismutase: an evolutionary puzzle.

74. Yang, G. & Rooman, M. Physical and molecular bases of protein thermal stability and cold adaptation. *Curr Opin Struct Biol* 42, 117–128. https://doi.org/10.1016/j.sbi.2016.12.007 (2017).

75. Benov, L. T. & Fridovich, I. Escherichia coli expresses a copper- and zinc-containing superoxide dismutase.

76. Chen, X.

77. Yang, G.

78. La Terza, A., Miceli, C. & Luporini, P. The gene for the heat-shock protein 70 of Euplotes focardii, an Antarctic psychrophilic ciliate.

79. Li, Y.

80. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data.

81. Andrews, S. (2010).

82. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data.

83. Andrews, S. (2010).

84. Bankiewich, A. et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 19, 455–477. https://doi.org/10.1089/cmb.2012.0021 (2012).

85. Nênikoško, S. K., Korobeynikov, A. I. & Alekseyev, M. A. BayesHammer: Bayesian clustering for error correction in single-cell sequencing. *BMC Genomics* 14 Suppl 1, S7. https://doi.org/10.1186/1471-2164-14-S1-S7 (2013).

86. Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. QUAST: quality assessment tool for genome assemblies. *BMC Genomics* 19, 1072–1075. https://doi.org/10.1186/s12862-018-5017-x (2018).

87. Vasa, T. et al. Transcriptomic and genomic evolution under constant cold in Antarctic nototheniid fish. *Proc Natl Acad Sci U S A* 110, 12944–12949. https://doi.org/10.1073/pnas.1802432105 (2018).

88. Li, Y. et al. Comparative transcriptomic analysis reveals gene expression associated with cold adaptation in the tea plant Camellia sinensis. *BMC Genomics* 20, 624. https://doi.org/10.1186/s12864-018-5988-3 (2019).

89. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 (2014).

90. Andrews, S. (2010).

91. Conesa, A. et al. BLAST2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21, 3674–3676. https://doi.org/10.1093/bioinformatics/bti401 (2005).

92. Gotta, S. et al. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res* 36, 3420–3435. https://doi.org/10.1093/nar/gkn176 (2008).

93. Parra, G., Bradnam, K. & Korf, I. CEGMA: a pipeline to accurately annotate core genes in eukaryotic genomes. *Bioinformatics* 23, 1061–1067. https://doi.org/10.1093/bioinformatics/btm071 (2007).
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M.M., S.P., E.C.S. and C.M. wrote the main manuscript text and performed the main analyses. A.P. and C.E. performed the tRNA analysis. G.M. participated to the assembling and the cleaning of the assembly. P.B. maintained the cell cultures. All authors reviewed the manuscript.

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The authors declare no competing interests.

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