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ORIGINAL PAPER

Environment health and intraspecific biodiversity in *T. tubifex*: a preliminary analysis of a population from Apennines springs

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Abstract The cosmopolitan freshwater oligochaete *Tubifex tubifex* is an important health indicator of the aquatic environment. Water pollutants can affect the intraspecific biodiversity grade of local *Tubifex* colonies. A genetic profile of specimens collected in an environment with reduced anthropic presence is particularly important to define genetic variability in unperturbed *Tubifex* populations, and it is still missing in the international literature. Therefore, it has been performed the analysis of lineage composition in a *T. tubifex* colony from high mountain spring ponds in Central Italy, characterized by a very low concentration of heavy metals. The sampling was performed during spring 2010 and 2011, in the Sett'acque valley (1,900 m above mean sea level), within the township of Lucoli. Data presented in this work depicted a peculiar composition of the population, characterized by a reduced complexity compared to other urban environments, and with no specimens belonging to the lineage I, largely described in many European populations. Interestingly, lineage 2e, previously discovered only in a natural reserve, results to be the most common lineage in this population. Considerations on environmental health and genetic evolution are discussed.

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Introduction

The analysis of aquatic pollution is a major issue of modern society. The need to preserve water basin from dangerous chemical compounds or physical agents is strictly correlated with the improvement of ecological analysis tools. Biological indicators are largely used to assess health condition of aquatic environment and are based on the identification of several species of invertebrates present in the water site. Oligochaete are included in the list of species to be identified in many index, e.g., Extended Biotic Index (Woodiwiss 1978).

A specific oligochaete, *Tubifex tubifex*, have been the focus of systematic (Beauchamp et al. 2001; Sjölin et al. 2005; Crottini et al. 2008; Doumen 2010), ecological (Coto and Szostak 1995; Sturmbauer et al. 1999; Schaller et al. 2011), toxicological (Bouché et al. 2000; Rathore and Khangarot 2002; Maestre et al. 2009; O'Connor et al. 2012) and parasitological (Baxa et al. 2008) studies. This worm is one of the most abundant oligochaete present in the European springs, where it plays a pivotal role in the detritus food chain. However, due to its elevated tolerance to pollutants and hypoxia, it can be found also in sloughs of region with intense anthropic activity.

The taxonomical identification of *T. tubifex* is mainly based on the morphology of the *setae* and of the reproductive organs. These structure show an important intraspecific variation, been probably a key feature for the adaptation to similar but distinct microhabitats. According to Paoletti et al. (1999), this oligochaete live in complex communities made of sympatric cryptic species,

parthenogenetic clones, and polyploidy strains. Distinct water basins can be characterized by *Tubifex* communities with different complexity and internal variability. A population study based on morphological observation can be cumbersome. Allozyme tests shifted the population analysis to a molecular level giving the opportunity to define different genetic lineages with distinct ecological preferences, but the procedure remain far from been high throughput.

In the last two decades, PCR-based methods and DNA sequencing have became the gold masters in molecular taxonomy. Hallet et al. (2005), after a large analysis of the nuclear ribosomal gene from many species of oligochaete, identified a primer set annealing in the internal transcribed spacer region 1 (ITS1) to be used in a PCR reaction for the discrimination of *T. tubifex* from other related species. A phylogeny of *T. tubifex*, based on a segment of the mitochondrial 16S rDNA, has been provided by Sturmbauer et al. (1999); moreover, they developed a PCR screening analysis by using a combination of several specific oligonucleotides. Recently, Crottini et al. (2008) highlighted the complexity of the *T. tubifex* system by studying populations around the world, and in particular the Lambro River. The authors have in fact found additional lineages and further sub-divided the ones from Sturmbauer et al. (1999) and assessed the co-existence of several cryptic species of *T. tubifex*. Thus, genetic characterization of morphologically indistinguishable *T. tubifex* has revealed significant genetic diversity (Analuf 1994; Anlauf and Neumann 1997; Sturmbauer et al. 1999; Crottini et al. 2008).

Interestingly, the genetic diversity observed in *Tubifex* communities around the world has been associated with different degrees of susceptibility to infection of a freshwater myxozoan parasite: *Myxobolus cerebralis* (DuBey and Caldwell 2004; Kerans et al. 2004: Arsan et al. 2007; Hallett et al. 2009; Lodh et al. 2011; Zielinski et al. 2011). The latter infects numerous salmonid species to varying degrees (MacConnell and Vincent 2002; Sollid et al. 2002), and the infection may culminate in whirling disease. Numerous studies on *T. tubifex* susceptibility provided evidence that different genotypes may be used as indicator to assess the risk of fish contamination (DuBey and Caldwell 2004; Arsan et al. 2007).

To understand the intraspecific biodiversity grade that exists in wild population of *T. tubifex* from region with reduced anthropic activity, we performed a genotype screening in Abruzzo region (Central Italy). The area selected is a valley at 1,900 m above mean sea level (amsl) in the township of Lucoli, close to Abruzzo National Park and in the nearby of the Calderone glacier (Apennines, Italy), the southernmost glacier in Europe. The sampling was performed during spring 2010 and 2011. In our experience, this is the first *T. tubifex* lineage study conducted with sampling in uncontaminated sites from the Italian peninsula. The collected data can be associated with relevant ecological parameters.

Materials and methods

Sampling

Samples were obtained from sediments of Sett'acque spring, located in a faunistic and reserve area in a valley at 1,900 m amsl, within the township of Lucoli (L'Aquila, Abruzzo region, Italy). Sampling was undertaken during spring season, and samples were collected from mud by qualified scientists. Several individuals were isolated from each sampling, washed, and singularly stored in cryovials at −20 °C.

The spring water and a pool of *T. tubifex* specimens were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). Water samples were directly analyzed after a simple acidification by nitric acid, while organic samples were previously treated by a mixture of nitric acid and oxygen peroxide for mineralization, according to the standard procedures (Boutakhrit et al. 2011).

Genotyping and phylogenetic analysis

Whole genomic DNA ($n = 56$ individuals) was extracted using GenElute kit (Sigma NA2010) following tissue extraction protocol; subsequently, samples were PCR amplified using Sturmabuer et al. (1999) PCR-based genetic 16S rDNA screening method. Briefly, a PCR cocktail containing four lineage-specific primers and the 16sbr universal primer was used for the amplification of lineage-specific region of the 16S gene. The annealing temperature was defined both using specific softwares, both performing empirical investigation on a limited number of specimens. It has been selected an annealing at 42 °C, a temperature that allowed clean and stable results. The PCR products were analyzed on 2.2 % agarose gel; each lineage was identified by a specific electrophoresis pattern.

The DNA of the same individuals was PCR amplified by using the universal primers 16sar and 16sbr (Kessing et al. 1989), with an annealing at 52 °C. The obtained PCR product was purified and sequenced using the 16sar primer (ABI 3730XLs). A common region of 373 bp was selected for subsequent analysis and GenBank submission.

Sequences were aligned by using MEGA software, version 5.0 (Tamura et al. 2011). The alignment also included two groups of published sequences: (1) *T. tubifex* by Sturmbauer et al. (1999), accession numbers:

Table 1 Metals concentration in water and *T. tubifex*

	Li	Be	Al	Ti	V	Cr	Mn	Fe
Water	0.10	0.01	5.70	0.33	1.11	0.11	0.46	0.44
Tubifex	35.46	2.25	12,375.65	192.08	51.06	1,374.82	2,718.79	150,573.76

	Co	Ni	Cu	Zn	Ga	As	Se	Rb
Water	0.10	0.10	1.00	15.06	2.56	0.50	0.21	0.05
Tubifex	871.28	74.23	3,789.72	22,848.46	462.41	2.96	3.55	396.34

	Sr	Ag	Cd	Sn	Sb	Ba	Pb
Water	36.14	0.92	0.28	0.52	0.10	5.96	0.39
Tubifex	632.39	0.95	374.23	8.27	44.80	11,512.06	240.66

Total metals levels in water and *Tubifex* samples from Sett'acque expressed in nanogram/gram per dry weight. Concentrations in water of Al, V, Cr, Mn, Fe, Ni, Cu, As, Cd, Sb, Pb are ranging from 10 to 1,000 times under the threshold level of the Italian low for potability (Dlgs 31/2001)

AJ225907, AJ225903, AJ225904, AJ225905, AJ225906, AJ225911, AJ225908, AJ225909, AJ225910, (2) *T. tubifex* by Crottini et al. (2008), accession numbers: EU117485, EU117486, EU117500, EU117487, EU117492, EU117504, EU117489, EU117498, EU117491, EU117493, EU117499, EU117501, EU117527, EU117526, EU117541.

Phylogenetic trees were constructed by using MEGA software, version 5.0 (Tamura et al. 2011) including all the above-mentioned sequences. The phylogenetic analysis was carried out by applying a neighbor-joining bootstrapping method (1,000 replicates).

Results and discussion

Metals quantification

The level of heavy metals in *T. tubifex* tissues and in the water of Sett'acque spring was analyzed by ICP-MS. Up to 23 elements where included in the analysis. As shown in Table 1, it has been confirmed that *T. tubifex* can accumulate metals, in line with previous studies (Singh et al. 2007). Iron shows the highest level of concentration, and this is strictly correlated with its biological activity inside the giant globins of oligochaetes (Bailly et al. 2007; Stern et al. 1990). Other metals, as well transition metals and metalloids, are present in the animal tissues with specific range of accumulation, that in the case of Cr reach a ratio of 10^4 with respect to the water where worms live.

Table 2 Comparison of heavy metals levels in *T. tubifex* between Sett'acque (Italy) and Blantyre City (Malawi) samples (Kaonga and Kumwenda 2010)

Heavy metals (mg/kg)	Sett'acque (Italy)	Blantyre City (Malawi)*
Zn	22.85	45.0–82.2
Mn	2.72	1.21–3.69
Cu	3.79	1.6–4.7
Pb	0.24	ND
Cd	0.37	1.08–2.18

ND not detected

* Kaonga and Kumwenda 2010

Levels of heavy metals (Zn, Mn, Cu, Pb, Cd) found in Sett'acque specimens have been compared with data from Kaonga and Kumwenda (2010) referred to Blantyre City (Malawi). As reported in Table 2, Sett'acque water samples show very low levels of heavy metals, reflected by also a reduced *Tubifex* bioaccumulation in comparison with that one observed for Blantyre City. Low heavy metals levels of Abruzzo region were in part previously assessed by Oddis et al. (2010) .

The absence of heavy metals harmful effect on *Tubifex* population was speculated also by a preliminary observation of specimens, where no abnormal autotomy events were noticed (data not shown). In fact, it has been reported that toxic effect of several compounds can lead to the cutting of the posterior part of the worm, probably as a way of pollutants excretion from the body tissues (Back et al. 1990; Lucan-Bouché et al. 1997; Paris-Palacios et al. 2010). Cd and Zn concentrations in *Tubifex* tissues are clearly lower than those observed in urban river, while Cu and Mn seem to be less variable. The data collected confirm bioaccumulation capacity for this oligochaete; the differences between the analyzed elements can be explained in a biochemical manner, considering that many metals are important cofactors in enzymatic activity.

Genotyping of *T. tubifex* population in Sett'acque

The purified genomes of fifty-six *T. tubifex* individual from Sett'acque valley were used in PCR-based genetic

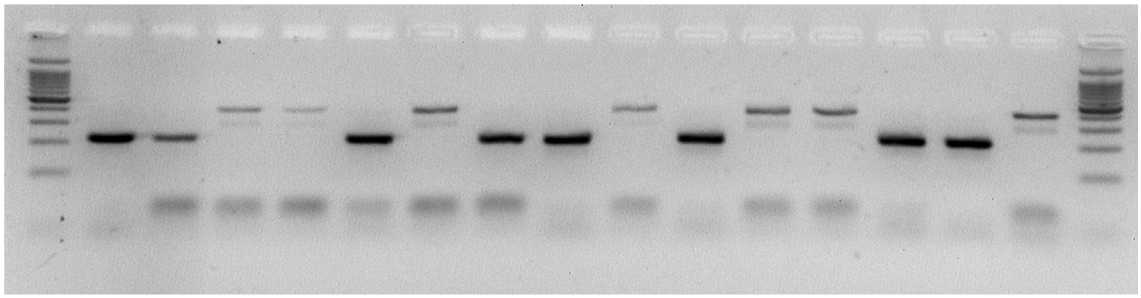

Fig. 1 PCR-based genetic 16S rDNA profiling: gel electrophoresis on 2.2 % agarose gel of several specimens from Sett'acque population. The population is mainly composed of two lineages that generate specific electrophoretic patterns: a first one with a single band around 200 bp, a second one with an upper band around 400 bp, and a faint lower band around 300 bp. *Lanes* 1 and 17 contain a DNA size standard ladder from 100 to 1,500 bp

16S rDNA profiling according to Sturmbauer et al. (1999) method. All the samples gave amplification products of the expected size range between 215 and 400 bp: In very few situations, the amplification bands were faint probably due to a reduced amount of starting material. The technique showed highly reproducible results and was useful for an immediate foresight of the intraspecific biodiversity of the population. More than 30 % of amplified samples showed a single band around 200 bp, corresponding to the expected profile for lineage III. A few samples showed a double band around 200 and 300 bp, corresponding to the expected profile for lineage IV. But the large majority of specimens were characterized by an electrophoresis pattern not described by Sturmbauer et al. (1999), been composed of an upper band around 400 bp and a faint lower band around 300 bp (Fig. 1).

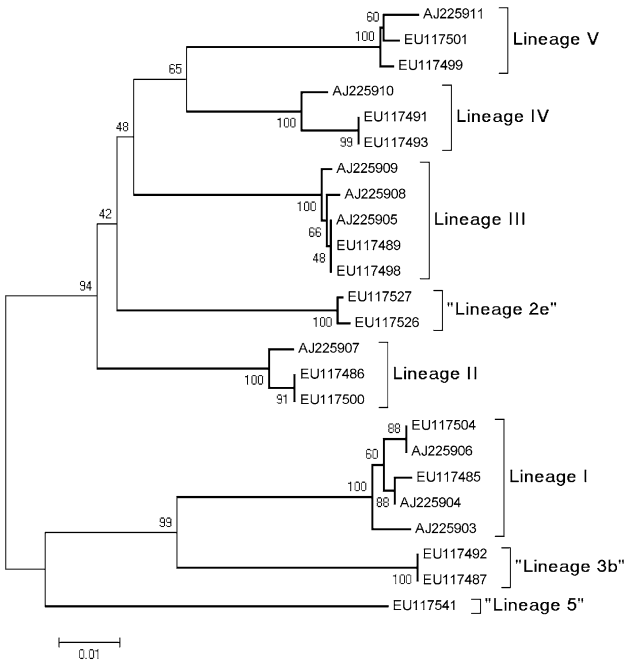

Fig. 2 NJ phylogenetic tree of 16S rDNA from European samples of *Tubifex tubifex* previously published (Sturmbauer et al. 1999; Crottini et al. 2008). Brackets define the lineages

To better investigate the complexity of the population and to clearly identify the lineage grouping in the majority of individuals, we performed a sequencing of a fragment of the 16S rDNA of all the fifty-six worms. Moreover, before using the sequence for the construction of a phylogenetic tree for Sett'acque valley, a phylogenetic analysis was performed with only published sequences from only European and Italian locations (Sturmbauer et al. 1999, Crottini et al. 2008), with the aim to highlight the complexity of *T. tubifex* system in this continent and set up stringency condition for the local analysis (Fig. 2).

Second, Sett'acque sequences were added to the above-mentioned ones in the phylogenetic analysis (Fig. 3). The 56 specimens are grouping in three different clades: lineage 2e, lineage III, and lineage IV, characterized by a frequency of 53.57, 41.07, and 5.36 %, respectively (Fig. 4).

This lineage composition of the *T. tubifex* population in Sett'acque valley is clearly different from others previously described in the literature, as depicted in Fig. 4, with the larger number of individuals belonging to the lineage 2e, and no specimens belonging to the lineage I, described on the contrary in many European populations. Moreover, the population complexity is low compared to that one described in Lambro River in North Italy. This situation can be probably explained considering the Sett'acque community as a population close to the equilibrium. The geographical isolation and the absence of chemical constrains probably acted toward a genetic uniformation. Interestingly lineage III, described by

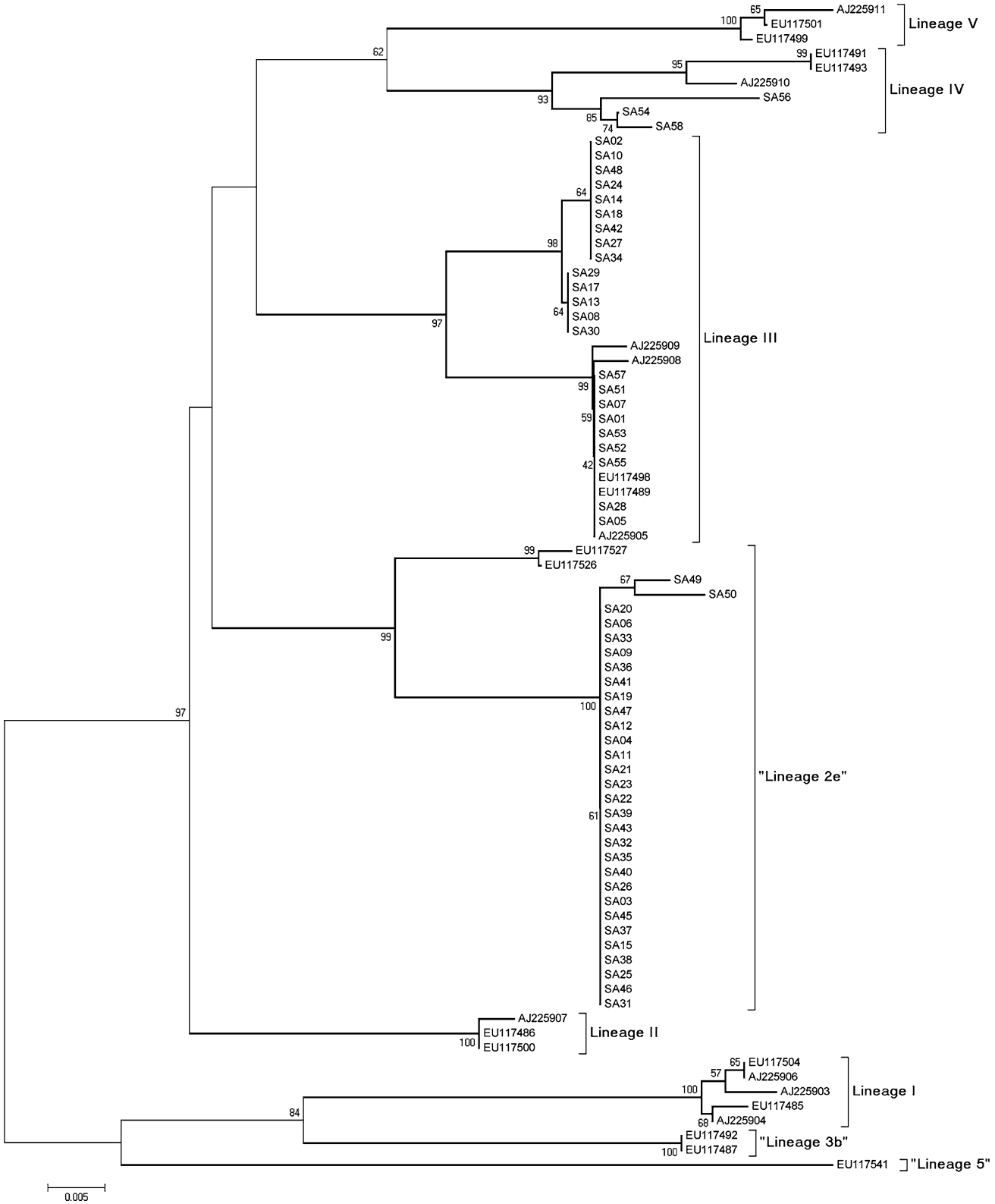

Fig. 3 NJ phylogenetic tree based on a fragment of 373 bp of the 16S rDNA for a total of 80 *T. tubifex* sequences of which 56 represents individuals from Sett'acque valley (SA)

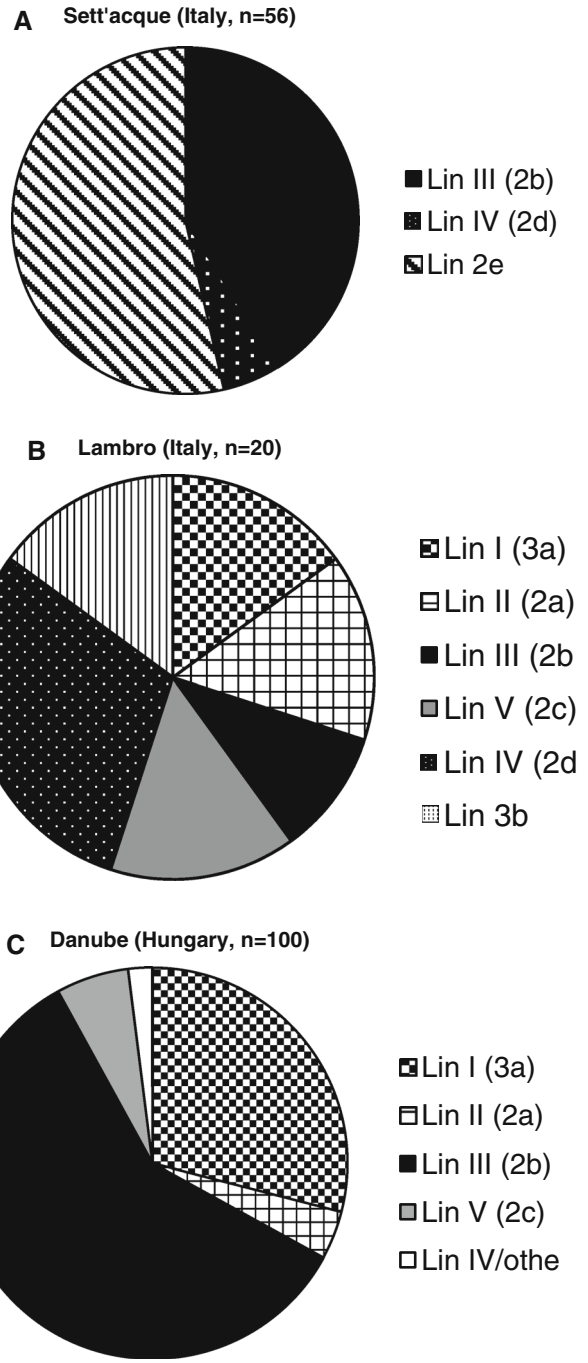

Fig. 4 *Tubifex. tubifex* lineages distribution in three water locations: **a** Sett'acque valley (data from the current study), **b** Lambro river (Crottini et al. 2008), **c** Danube (Sturmbauer et al. 1999). Countries and sample size are in *brackets*

Sturmbauer et al. (1999) as particularly cadmium-resistant, is largely represented in Sett'acque population. One hypothesis to explain this situation may be that lineage III, present also in other spring waters, is particularly suitable for the physical stress that characterizes a high mountain valley, snow covered for more than 6 months per year. Moreover, evolutionary explanations may well be found, considering that the distribution of many animal species in Italian Apennines has been strongly affected by the last glacial era: Probably, *Tubifex* colonization of this region started in subsequent warm era. It may also be speculated that lineage III may be one of the ancestral lineages, thus more resistant, from which the others have evolved accordingly to different environmental conditions; the latter would explain its high frequency in Sett'acque community.

Interestingly, lineage 2e, the most common lineage in this study, groups with specimens from locations close to a Spanish National Park (Gorbea Vizcaya, Spain) was reported in Crottini et al. (2008), which is in line with the Abruzzo's environment. Additionally, none of the other specimens were analyzed by the same authors grouped in this lineage.

Thus, to better clarify the latter points, it may be particularly interesting to investigate the oligochaete population present in other valleys of Abruzzo. Moreover, it may be particularly interesting to investigate the presence of *M. cerebralis* in Sett'acque specimens: In fact, the expectation is that this *Tubifex* population may be myxozoan parasites free, as there are no salmonids present in this valley. This is a relevant issue in zooprophylaxis studies.

Finally, we can confirm that Sturmbauer molecular method for the lineage investigation is a quick and strong tool based on a wise primer selection on hot region of the 16S DNA. The method gives a rapid idea of the intraspecific biodiversity, but is also able to discriminate the lineage 2e (according to Crottini et al. 2008) with a specific electrophoretic pattern, not yet previously described.

Conclusion

Tubifex tubifex is a freshwater oligochaete that plays a major role in decomposition of organic matter. Intraspecific biodiversity of *Tubifex* populations can be an interesting parameter for the evaluation of different levels of environmental stress. Moreover, it could be of particular interest a better characterization of lineage 2e both at molecular and toxicological levels. To strengthen the possible utilization of lineage analysis as a tool for environmental health investigations, it will be crucial to perform additional samplings from other protected areas. Statistical analysis from a larger set of data is also needed to support the speculative association of lineage 2e with low pollution levels.

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References

Analuf A (1994) Some characteristics of genetic variants of *Tubifex tubifex* (Muller, 1774) (Oligochaeta: Tubuficidae) in laboratory cultures. Hydrobiologia 278(1–3):1–6

Anlauf A, Neumann N (1997) The genetic variability of *Tubifex tubifex* (Muller) in 20 populations and its relation to habitat type. Arch Hydrobiol 139(2):145–162

Arsan EL, Hallett SL, Bartholomew JL (2007) *Tubifex tubifex* from Alaska and their susceptibility to *Myxobolus cerebralis*. J Parasitol 93(6):1332–1342

Back H et al (1990) Epidermal uptake of Pb, Cd, and Zn in tubificid worms. Oecologia 85:226–232

Bailly X et al (2007) Globin gene family evolution and functional diversification in annelids. FEBS J 274(10):2641–2652

Baxa DV et al (2008) Arrested development of the myxozoan parasite, *Myxobolus cereb*ralis, in certain populations of mitochondrial 16S lineage III *Tubifex tubifex*. Parasitol Res 102(2):219–228

Beauchamp KA et al (2001) Molecular phylogeny of Tubificid Oligochaetes with special emphasis on *Tubifex tubifex* (Tubificidae). Mol Phylogenet Evol 19(2):216–224

Bouché ML et al (2000) Toxic effects and bioaccumulation of cadmium in the aquatic oligochaete *Tubifex tubifex*. Ecotoxicol Environ Saf 46(3):246–251

Boutakhrit K et al (2011) Comparison of four analytical techniques based on atomic spectrometry for the determination of total tin in canned foodstuffs. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28(2):173–179

Coto JM, Szostak A (1995) Estudio preliminary sobre aspectos quimicos y biologicos de las aguas de Rio Segundo, Costa Rica, usadas para riego. Agronomía Costarricense 19(1):69–77

Crottini A et al (2008) The world in a river? A preliminary analysis of the 16S rDNA variabilità of *Tubifex* species (Clitellata: Tubificidae) from Lambro River. Mol Phylogenet Evol 48(3):1189–1203

Doumen C (2010) cDNA identification, comparison and phylogenetic aspects of lombricine kinase from two oligochaete species. Comp Biochem Physiol B Biochem Mol Biol 156(2):137–143

DuBey R, Caldwell CA (2004) Distribution of *Tubifex tubifex* lineages and *Myxobolus cerebralis* infection in the tailwater of the San Juan River, New Mexico. J Aquat Anim Health 16(4):179–185

Hallet SL, Atkinson SD, Bartholomew JL (2005) Countering morphological ambiguities: development of a PCR assay to assist the identification of *Tubifex tubifex* oligochaetes. Hydrobiologia 543(1):305–309

Hallett SL et al (2009) Propagation of the myxozoan parasite *Myxobolus cerebralis* by different geographic and genetic populations of *Tubifex tubifex*: an Oregon perspective. J Invertebr Pathol 102(1):57–68

Kaonga CC, Kumwenda J (2010) Accumulation of lead, cadmium, manganese, copper and zink by sludge worms; *Tubifex tubifex* in sewage sludge. Int J Environ Sci Tech 7(1):119–126

Kerans BL et al (2004) Differential propagation of the metazoan parasite *Myxobolus cerebralis* by *Limnodrilus hoffmeisteri*, *Ilyodrilus templetoni*, and genetically distinct strains of *Tubifex tubifex*. J Parasitol 90(6):1366–1373

Kessing B et al (1989) The simple fools guide to PCR. University of Hawaii, Honolulu

Lodh N, Stevens L, Kerans B (2011) Prevalence of *Myxobolus cerebralis* infections among genetic lineages of *Tubifex tubifex* at three locations in the Madison River, Montana. J Parasitol 97(3):531–534

Lucan-Bouché ML et al (1997) Étude expérimentale des effets létaux induits par le cuivre et/ou le plomb chez l'Oligochète Tubifex tubifex. Bull Soc zool Fr 122:389–392

MacConnell E, Vincent ER (2002) The effects of *Myxobolus cerebralis* on the Salmonid Host. In: Bartholomew JL, Wilson JC (eds) Whirling disease: reviews and current topics. American Fisheries Society symposium series 29. American Fisheries Society, Bethesda

Maestre Z, Martinez-Madrid M, Rodriguez P (2009) Monitoring the sensitivity of the oligochaete *Tubifex tubifex* in laboratory cultures using three toxicants. Ecotoxicol Environ Saf 72(8):2083–2089

O'Connor TP et al (2012) Trace metal accumulation in sediments and benthic macro invertebrates before and after maintenance of a constructed wetland. Water Environ Res 84(4):370–381

Oddis M et al (2010) Heavy metals bioaccumulation in truffle and quality assessment of the product from Abruzzi. Micol Veget Medit 25(1):33–46

Paoletti A, Pagani M, Leoni B (1999) Enzymatic variability in sympatric species of *Tubifex* (Clitellata: Tubificidae). Hydrobiologia 406:83–86

Paris-Palacios S et al (2010) Toxic effects and bioaccumulation of the herbicide isoproturon in *Tubifex tubifex* (Oligocheate, Tubificidae): a study of significance of autotomy and its utility as a biomarker. Aquat Toxicol 98(1):8–14

Rathore RS, Khangarot BS (2002) Effects of temperature on the sensitivity of sludge worm *Tubifex tubifex* Müller to selected heavy metals. Ecotoxicol Environ Saf 53(1):27–36

Schaller J et al (2011) Metal/metalloid accumulation/remobilization during aquatic litter decomposition in freshwater: a review. Sci Total Environ 409(23):4891–4898

Singh RK, Chavan SL, Sapkale PH (2007) Heavy metal concentrations in water, sediments and body tissues of red worm (*Tubifex* spp.) collected from natural habitats in Mumbai, India. Environ Monit Assess 129(1–3):471–481

Sollid SA et al. (2002) Relative susceptibility of selected Deschutes and Columbia River *Salmonid* species to experimentally induced infection by *Myxobolus cerebralis*. In: Bartholomew JL, Wilson JC (eds) Whirling disease: reviews and current topics. American Fisheries Society Symposium Serie 29. American Fisheries Society, Bethesda

Sjölin E, Erséus C, Källersjö M (2005) Phylogeny of Tubificidae (Anellida, Clitellata) based on mitochondrial and nuclear sequence data. Mol Phylogenet Evol 35(2):431–441

Stern MS et al (1990) Amino acid sequence of the monomer subunit of the giant extracellular hemoglobin of the aquatic oligochaete *Tubifex tubifex*. Eur J Biochem 194(1):67–73

Sturmbauer C et al (1999) Mitochondrial DNA reveals cryptic oligochaete species differing in cadmium resistance. Mol Biol Evol 16(7):967–974

Tamura K, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum Parsimony methods. Mol Biol Evol [Epub ahead of print]

Woodiwiss FS (1978) Comparative study of biological-ecological water quality assessment methods. Second practical demonstration. Summary Report. Commission of the European Communities

Zielinski CM et al (2011) Comparative susceptibility of Deschutes River, Oregon, *Tubifex tubifex* populations to *Myxobolus cerebralis*. J Aquat Anim Health 23(1):1–8