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Population structuring in the monogonont rotifer *Synchaeta pectinata*: high genetic divergence on a small geographical scale

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SUMMARY

1. Like many other zooplankton species, individual species of planktonic freshwater rotifers often possess a cosmopolitan distribution despite inhabiting isolated habitats (e.g. ponds or lakes) that present little opportunity for direct gene flow. This 'everything is everywhere' distribution is typically ascribed to aspects of the life history of these animals (heterogonic reproductive strategy) in combination with the high dispersal capabilities presented by a dormant stage (resting eggs).
2. Recent molecular analyses indicate the presence of strong population structuring in many rotifer species, including both phylogeographic structuring and the potential for cryptic speciation. Building on these studies, we investigated the intraspecific genetic structuring in the mitochondrial barcoding marker cytochrome *c* oxidase subunit I (COI) among mid-European populations of the cosmopolitan rotifer *Synchaeta pectinata* (Rotifera, Monogononta). These data were analysed using a combination of phylogenetic analysis and haplotype networks as well as population-genetic methods to assess the degree of population and geographic structuring.
3. Gene flow among four neighbouring populations in north-west Germany (126 individuals; regional scale) and between them and a set of populations from northern Italy (an additional 48 individuals based on literature data; mid-European scale) was generally low. Paradoxically, however, higher genetic similarity occurred across the broader mid-European scale than within the regional scale. Nevertheless, no significant correlation with spatial distance was detected at the former scale, rejecting an isolation-by-distance model for population differentiation.
4. Most populations comprised several distinct haplotype clusters, each corresponding to ancient mitochondrial lineages of *S. pectinata*. Although it is common to infer cryptic speciation from results such as these, the pattern we observed can also arise through historical colonisation events and/or persistent founder effects (Monopolization hypothesis) and we present potential arguments against the default assumption that *S. pectinata* comprises a complex of cryptic species.

Keywords: cryptic species, cytochrome *c* oxidase subunit I, phylogeography, population differentiation, *Synchaeta pectinata*

Introduction

Standing waters are species-rich, complex ecosystems as a result of their highly structured nature and diversity of microhabitats. Despite being isolated 'islands', standing waters regularly provide refuge to the same microscopic zooplankton species (Shurin *et al.*, 2000). Indeed, many zooplankton organisms tend to have broad, ubiquitous distributions beyond individual lakes, often to the point of individual species being

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distributed on a worldwide scale (Mayr, 1963; De Meester *et al*., 2002).

In attempting to explain this perhaps counterintuitive observation, Baas-Becking postulated the well-known Everything is Everywhere hypothesis in 1934 to explain the apparent global distribution of many microscopic species such as bacteria or zooplankton, contingent on the proper (micro)habitat being present ('but the environment selects', the equally important, but often forgotten second part of the hypothesis) (Jenkins & Buikema, 1998). Today, it is understood that the global distributions of diverse microscopic species under this hypothesis require the presence of gene flow to maintain the low overall intraspecific genetic diversities observed (Bilton, Freeland & Okamura, 2001; Fenchel, 2005). Gene flow, in turn, probably occurs mainly by migration, which is assumed to be facilitated by certain properties that most microscopic species share, including their small body size and high dispersal ability, the latter often via dormant stages in the life cycle (Fenchel & Finlay, 2004; Kellogg & Griffin, 2006). Indeed, dormant stages serve to not only ensure survival during adverse living conditions, but can also function as an important dispersal stage (Gilbert, 1974; Pourriot & Snell, 1983), where, analogous to seed dispersal in plants, they can be distributed by wind, zoochoric events (e.g. via waterfowl along major bird migration routes) or potentially even via the hydrological cycle. Long-range gene flow can also occur via sequential, short-range migration events, thereby resulting in a distribution pattern consistent with an 'isolation-by-distance' model (Wright, 1943), where genetic differences between populations increase with geographic distance. This pattern has already been found for some zooplankton species (Gómez *et al*., 2002).

Contrasting with the general pattern underlying the Everything is Everywhere hypothesis are numerous phylogeographic studies of otherwise ubiquitous species of microorganisms that reveal strong genetic differentiation among populations in close proximity, where the populations have been inferred to exist as pools of stable independent strains that have been subject to rapid expansions and bottlenecks (Carvalho, 1994; Achtman, 1997; Spratt & Maiden, 1999; De Gelas & De Meester, 2005). De Meester *et al.* (2002) combined the various stochastic and selection-driven processes that had been proposed to explain the paradox between apparently high dispersal capabilities of the focal organisms, but the limited amount of gene flow observed (e.g. Boileau, Hebert & Schwartz, 1992; Hairston, 1996; Okamura & Freeland, 2002) as the Monopolization hypothesis. Here, initial colonisation of new waters by zooplankton is generally rapid, but occurs mainly through a limited number of founder individuals and, in the extreme, only a single such individual. Populations, therefore, are often established with low variability among (the few) alleles (Boileau *et al*., 1992). Reinforcing these founder effects is the initial adaptation to the local environment and the continual, successive recruitment of older genotypes (including founder genotypes) from the dormant stages (Hairston, 1996), leading potentially to a pattern of high genetic diversity between populations over small geographical ranges or even neighbouring ponds as well as populations that are resistant to invasion by less adapted, immigrant genotypes (Boileau *et al*., 1992). It has been argued that the effects of this selection-driven process might already be present after a few growing seasons and could play a significant role in developing and maintaining population divergence (Okamura & Freeland, 2002), possibly leading to (cryptic) speciation in the long term.

In this context, monogonont rotifers represent an interesting study system to investigate evolutionary aspects of demographic population structuring, differentiation and speciation in aquatic microorganisms, with a view to the extremes presented by the Everything is Everywhere and the Monopolization hypotheses. This clade of aquatic animals successfully populates fresh, brackish and saline waters and even some terrestrial habitats (Wallace & Snell, 1991). Occurrences have been reported from all continents, and many species show an apparent global distribution in accordance with the Everything is Everywhere hypothesis (Fontaneto *et al*., 2006; Segers, 2008), whereas cryptic speciation has recently been implicated for other species, including *Brachionus plicatilis* (Gomez, 2005), *Brachionus manjavacas* (Montero-Pau *et al*., 2011) and *Synchaeta pectinata* (Obertegger, Fontaneto & Flaim 2012). Many habitats in which these organisms are present show high temporal variability in terms of their environmental characteristics (e.g. temperature or oxygen levels) whether or not they are prone to periodic desiccation events. Finally, monogonont rotifers are also characterised by a heterogonic life cycle, with a predominantly parthenogenetic life cycle alternating with the production of sexual, resting eggs under specific, usually deteriorating, environmental conditions (Birky & Gilbert, 1971), that can accumulate as resting egg banks in the sediment to enable the population to overwinter adverse living conditions (Brendonck & De Meester, 2003).

We focus in this study on the monogonont rotifer species *S. pectinata* (Ehrenberg, 1832), which represents one of the commonest truly planktonic rotifers inhabiting

freshwater systems. Due to its comparatively large size for a rotifer (240–550 μm), the species is quite well catalogued (Nogrady & Segers, 2002), with occurrences documented worldwide, including all of Europe (Arnemo *et al*., 1968; Koste & Voigt, 1978; Smirnov, 1986; Marcé *et al*., 2005; Wolska & Piasecki, 2007; Pociecha, Higgins & Mccarthy, 2010) as well as parts of Asia, Australia, North and South America and the Arctic (Stemberger, 1979; Hawkins, 1988; Chengalath & Koste, 1989; Sanoamuang, 1998; Frutos, Poi De Neiff & Neiff, 2006). Nevertheless, a recent molecular phylogeographic study of populations in the South Tyrol region of Italy argued in favour of cryptic speciation in this species (Obertegger *et al*., 2012). Using the mtDNA barcoding marker cytochrome *c* oxidase subunit I (COI), we expand on the latter study to investigate the genetic structuring of *S. pectinata* at three different geographical scales: (i) within each of four populations of (more or less) neighbouring bodies of water around Oldenburg-Eastern Friesland (northern Germany), (ii) between these populations and (iii) between these populations and those in South Tyrol examined by Obertegger *et al.* (2012) as part of a mid-European continental metapopulation. Given the apparent worldwide distribution of *S. pectinata* and the assumption that this species is following the Everything is Everywhere model, our null hypothesis is one of the genetic homogeneity throughout our study range. In testing this hypothesis, we make use of a diverse set of phylogenetic and population-genetic analyses given that historical events such as range expansion, bottlenecks, priority effects and founder events can complicate the interpretation of the effects of contemporary natural selection across heterogeneous landscapes and obscure current patterns of gene flow and drift (Hutchison & Templeton, 1999) as well as potentially mislead assessments of new (cryptic) speciation. In so doing, we also hope that our study will serve as a framework for phylogeographic studies of zooplankton species in general.

Methods

The sampling of natural populations of *S. pectinata* took place during March and April 2012 in the region of Oldenburg-Eastern Friesland (Fig. 1). Oldenburg (11 m above sea level on average; 53°10′N, 08°10′E) is located in the Atlantic to sub-Atlantic climate zone in a lower moraine landscape that is typical of north-west Germany. Average annual rainfall is 823 mm and average annual temperature is 9.5 °C (long-term data from the German Weather Service; www.dwd.de).

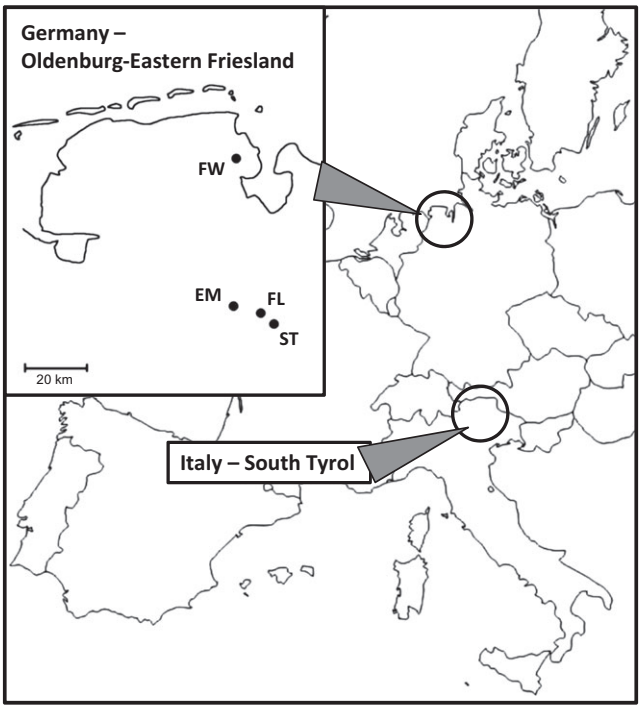

Fig. 1 Sampling sites in two regions of Europe, including four separate sites in Oldenburg-Eastern Friesland (Germany; n = 129 individuals in total) and eight pooled sites in South Tyrol (Italy; n = 48; from Obertegger *et al*., 2012). The larger European map was adapted from d-maps.com.

Four freshwater lakes and ponds were sampled: Großes Engelsmeer (EM), Schlossgartenteich (ST), Feuerlöschteich (FL) and Fedderwarden (FW) (Table 1). Geographical distances between these waterbodies range from 4 to 50 km (see also Appendix S1). All water samples were collected using a plankton net (mesh size of 55 μm) that was cast nine times from the shore. Concurrently, water temperature, pH, conductivity and oxygen concentrations for each waterbody were measured (WTW Multi 340; WTW, Weilheim, Germany) (Table 1). Samples were stored at 4 °C with sufficient oxygen exchange until processing. Specimens were typically isolated and all were identified as *S. pectinata* (*sensu* Ehrenberg, 1832) using stereomicroscopy (Leica M80; Wetzlar, Germany), which usually suffices for the correct taxonomic determination of this species. Additionally, individuals from a subset of these specimens were confirmed as *S. pectinata* using differential interference light microscopy (Leica DMLB) and were also documented photographically.

For each body of water, total DNA from each of 40 *S. pectinata* individuals was extracted. Extraction and sequencing failures, however, meant that the actual sample size per population could be lower than this value.

Table 1 Summary information for the sampled populations of *Synchaeta pectinata* and their corresponding waterbodies in the Oldenburg-Eastern Friesland region. Population densities of *S. pectinata* in a sample were estimated as A = one individual, B = some individuals, C = many individuals and D = plentiful

Sampling site	Code	Collection date	Geographical coordinates	Size of water (ha)	Water temp. (°C)	pH	Conductivity ($\mu S\ cm^{-1}$)	Oxygen ($mg\ L^{-1}$)	Sample size (*n*)	Population density
Oldenburg, Schlossgartenteich	ST	23-03-12	53°08′N 08°12′E	0.65	10.2	7.0	496.0	10.3	28	C
Oldenburg, Feuerlöschteich	FL	29-03-12	53°09′N 08°10′E	0.073	10.3	7.1	488.0	11.7	37	D
Bad Zwischenahn, Großes Engelsmeer	EM	20-03-12	53°09′N 08°03′E	1.7	7.7	4.0	52.7	11.4	32	D
Wilhelmshaven, near Fedderwarden	FW	12-04-12	53°34′N 8°01′E	3.778	8.3	8.6	670.0	12.1	32	C

To avoid possible contamination from algae in the digestive tract, all individuals were starved in filtered pond water prior to DNA extraction before being transferred to separate 0.2 μL Eppendorf tubes using an extracted glass pipette. Thereafter, 50 μL of InstaGene Matrix was added (Bio-Rad, Hercules, CA, U.S.A.), with DNA extraction following the standard protocol of Montero-Pau, Gomez & Munoz (2008). Amplification of the COI marker gene used the standard Folmer *et al.* (1994) primers LCO (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCOI (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′). The PCR volume of 20 μL was composed of 0.5 μM of each primer; 10× reaction buffer including 2 mM $MgCl_2$, 0.2 μM of each dNTP and 5 units of Taq polymerase (Jena Bioscience, Jena, Germany); and 3 μL of template DNA. PCR procedures consisted of initial denaturation for 2 min at 94 °C; 40 cycles of denaturation for 60 s at 94 °C, annealing for 60 s at 50 °C and elongation for 75 s at 75 °C; and final extension for 7 min at 72 °C. The presence and quality of the PCR products were checked by electrophoresis on a 0.8% agarose gel. Purification and forward sequencing were outsourced to Macrogen Europe (Amsterdam, the Netherlands) using the LCO primer. Thereafter, the quality of the sequences was checked by eye using Chromas lite (Version 2.01; Technelysium Pty Ltd, Tewantin, Qld, Australia). In so doing, we searched for signs of contamination, particularly the co-amplification of NUMTs (nuclear insertions of mitochondrial DNA) as indicted by the presence of stop codons, indels and signs of multiple mitochondrial copies (heteroplasmy) as suggested in Jordal & Kambestad (2014). Noisy base calls were recoded as missing data, whereas apparent, ambiguous sequencing mistakes in five sequences were corrected using IUPAC ambiguity codes (Tipton, 1994). All COI sequences from this study have been uploaded to GenBank (Accession Numbers KP875588–KP875716).

Analogously, virtually complete 18S rDNA sequence data were obtained for a subset of 18 individuals comprising all Oldenburg-Eastern Friesland populations (EM, $n = 2$; FL, $n = 1$; FW, $n = 3$; and ST, $n = 12$). PCR primers for the amplification were 18A1 mod and 1800 mod (Raupach *et al.*, 2009); forward primers for one-way sequencing by Macrogen Europe were 18A1 mod as well as two self-designed primers 18SV4f (5′-ATT GGA GGG CAA GTC TGG TGC-3′) and 18SV7f (5′-TAT GGT TGC AAA GCT GAA AC-3′). PCR volumes and composition were the same as for COI. The PCR temperature profile consisted of an initial denaturation at 94 °C (5 min); 40 cycles at 94 °C (30 s, denaturation), 52 °C (40 s, annealing) and 72 °C (90 s, annealing); and a final extension at 72 °C (10 min). Alignments were constructed as for the COI sequences, and the number of pairwise nucleotide differences as well as corrected p-distances was calculated. In addition, analogous p-distances for COI for only these 18 individuals were calculated as a reference. All 18S rDNA sequences from this study have similarly been uploaded to GenBank (Accession Numbers KP875570–KP875587).

To investigate potential metapopulation structuring over a broad geographical distance (Table S1), the COI data set was expanded through 48 homologous COI sequences collected from several South Tyrolean (Italy) populations by Obertegger *et al.* (2012; for accession numbers, see Table S2) who examined for the occurrence of potential cryptic species within the genus *Synchaeta* in this area. These latter samples were obtained from nine bodies of water (LA, LE, WO, SC, VA, TE, LS, FI and SE; see Table S1 for full names of waterbodies), with 3–13 individuals per population. Thus, in total, the data set for our analyses comprised 177 *S. pectinata* sequences, including 129 novel sequences on the regional geographical scale. The data set was completed through the inclusion of the homologous GenBank

sequences from *Synchaeta grandis* and *Synchaeta lakowitziana* (Accession Numbers JN936517 and JN936539, respectively), which served as outgroups.

The sequences were aligned in BioEdit 7.0.9.0 (Hall, 1999) using the default settings of ClustalW (Thompson, Higgins & Gibson, 1994) and subsequently corrected manually. The number of pairwise nucleotide differences as well as p-distances at the different hierarchical levels (i.e. within and among populations and at the metapopulational level) was calculated using MEGA 4.1 (Tamura *et al.*, 2007). Reconstruction of a neighbour-joining (NJ) tree was conducted using PAUP* v4.0b10 (Swofford, 2003) under the Kimura-2-parameter (Kimura, 1980) model of evolution typically used for barcoding sequences. All nucleotide positions were weighted equally. Statistical support for the nodes in the NJ tree was estimated by a nonparametric bootstrap analysis (Felsenstein, 1985) of 1000 replicates. Using this phylogeny as well as pairwise genetic distances, we also examined for the presence of cryptic species among the different clades using the 'four-times' rule (K/theta) test of Birky *et al.* (2010, 2011) as implemented in the Perl script fourX.pl v1.0 (available from the last author on request). Briefly, based on population-genetic considerations, the test holds sister clades as representing distinct species if the mean pairwise genetic distance between the clades is more than four times that within either clade (for more information, see Birky *et al.* 2010, 2011).

To reconstruct the haplotype network and derive the F_{ST} statistics, the data set was pruned to 174 sequences (aligned length of 573 bp) to prevent errors or ambiguities arising from the presence of missing data and IUPAC ambiguity codes (Gourraud, Génin & Cambon-Thomsen, 2004; Joly, Stevens & Van Vuuren, 2007). Haplotype networks were reconstructed for the populations of the Oldenburg-Eastern Friesland region and the mid-European continental level using Network v4.6.1.1 (Fluxus Technology Ltd., Clare, Suffolk, England Bandelt, Forster & Röhl, 1999).

Both the degree of genetic differentiation and population structuring were estimated using Arlequin v3.5.1.2 (Excoffier & Lischer, 2010) on three different hierarchical levels: populational, regional and continental. All individuals from a given body of water were assumed *a priori* to represent a single population. The regional level describes all individuals of the waterbodies in a given region. In this case, only the Oldenburg-Eastern Friesland region was analysed because of insufficient sample sizes from the populations in the South Tyrol region. Finally, the continental level describes all individuals on the mid-European scale. The percentage of variation for the different population levels was calculated using the fixation index F_{ST}. The number of haplotypes in total and of shared haplotypes at the relevant hierarchical levels were determined using both Arlequin and Network; slight differences in how the haplotypes are determined between the programmes means that although the results are generally similar, some small differences do exist (see Results). Because of the greater information provided by Arlequin in this context (e.g. calculations of haplotype diversity), we focus our discussion on its values for the haplotype distributions, leaving Network to determine primarily the haplotype network.

Furthermore, to assess population differentiation, both an analysis of molecular variance (AMOVA) and a hierarchical AMOVA were performed using Arlequin. Whereas the former compares the variation among and within populations, the latter analyses three different, nested levels: among populations within a region (F_{SC}), among all populations (F_{ST}) and between the two regions (F_{CT}; continental level). For these calculations, we pooled the South Tyrolean populations because of their small individual sample sizes. P-values testing the significance of the observed values were obtained through 10 100 permutations of the data set. To test for recent demographic expansion events (as expected under the Monopolization hypothesis) versus demographic equilibrium (null hypothesis; as expected under the Everything is Everywhere hypothesis), a mismatch analysis of the distribution of the observed number of differences between all pairs of haplotypes was performed for each of the Oldenburg-Eastern Friesland populations (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) using the sum of square deviations between the observed and the expected mismatches and their associated P-values as the test statistic. Finally, the correlation between genetic population structure (pairwise F_{ST} values) and geographical distance was analysed using a Mantel test (Mantel, 1967) to test for possible isolation-by-distance effects.

Results

The final alignment of the mitochondrial COI fragment for the 177 *S. pectinata* individuals plus the two outgroup sequences from *S. grandis* and *S. lakowitziana* had a length of 628 bp and did not contain any gaps. Within *S. pectinata*, 20.1% of base pairs (126 bp) were variable and 18.6% (117 bp) were parsimony informative. Within populations, the number of polymorphic sites varied from three (population FL) to 116 (population ST). At the more inclusive levels, 123 polymorphic sites were

presented among sequences from the Oldenburg-Eastern Friesland region compared to 101 for South Tyrol; these two regions shared 88 polymorphic sites. Base frequencies were distributed unequally, with an overabundance of Ts compared to Cs and Gs (T = 46.8%, C = 14.0%, A = 25.1% and G = 14.1%; $\chi^2 = 12.412$, d.f. = 3, $P = 0.0061$), although there was no significant heterogeneity in state frequencies across all *S. pectinata* sequences (all positions: $\chi^2 = 59.913$, d.f. = 528, $P = 1.00$; informative positions: $\chi^2 = 354.922$, d.f. = 528, $P = 1.00$). Our data set revealed no non-functional COI sequences or other indications of NUMTs or false amplifications. Five ambiguous base calls were present, but were scattered throughout the phylogeny and were not associated with any specific clade.

The NJ phylogeny (Fig. 2) shows *S. pectinata* to be clearly distinct from the outgroups. Within *S. pectinata*, applying the 'four-times' method of species delineation revealed the existence of eight clades with significant *K*/theta ratios (Fig. 2). The basal nodes of these clades are supported by bootstrap values from 71.0 to 100.0. Except for clade 6, which comprises exclusively individuals from South Tyrol, all clades contain individuals from ST and thus from the Oldenburg region. Individuals of EM and FL are restricted to clade 1, whereas those from FW are present in clades 3 and 8 only. Individuals from South Tyrol are found among all clades except clade 1 and 7. Phylogenetic analyses under a maximum-likelihood framework using RAxML v7.2.8 (Stamatakis, 2006) in combination with the four-times method also support this pattern (results not shown). Average pairwise p-distances within each of the 'four-times' clades ranged from 0.0 to 0.006 and from 0.004 to 0.121 between all pairs of them (see also Appendix S3).

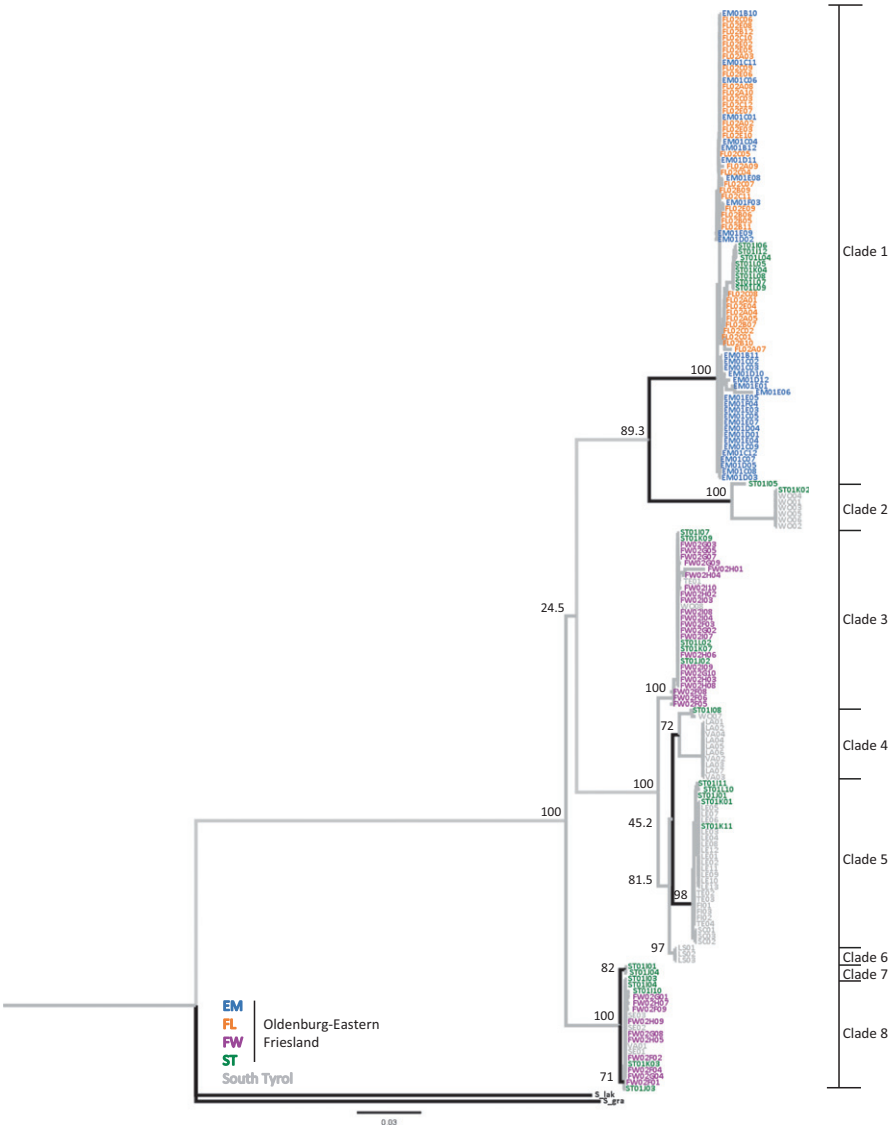

Fig. 2 Neighbour-joining tree based on Kimura-2-parameter distances for COI sequences for *Synchaeta pectinata* individuals at the mid-European continental level using *S. grandis* and *S. lakowitziana* as outgroups. Populations are colour-coded and the eight clades identified by the four-times method are marked with thick, black branches.

	Intrapopulational level	Regional level		
		EM	ST	FL
EM	0.002 (±0.001)			
ST	0.074 (±0.008)	0.071 (±0.009)		
FL	0.001 (±0.001)	0.003 (±0.002)	0.071 (±0.013)	
FW	0.036 (±0.006)	0.100 (±0.013)	0.066 (±0.013)	0.100 (±0.007)

Table 2 Average pairwise p-distances (±SE) for the COI marker within and among the four populations of *Synchaeta pectinata* sampled in the Oldenburg-Eastern Friesland region as determined using MEGA under a Kimura-2-parameter model. See Table 1 for sampling site codes

Similarly, average intrapopulational pairwise p-distances were generally low (Table 2), with a maximum of 0.074 ± 0.008 for population ST, which was also the most widely distributed across the tree. This value is on the same order of magnitude as most of the average interpopulational p-distances, with the exception of the comparison between populations EM and FL, which was noticeably lower at 0.003 ± 0.002 (Table 2). Average p-distances within each of the two regions were similar with values of 0.057 ± 0.007 and 0.046 ± 0.006 for Oldenburg-Eastern Friesland and South Tyrol, respectively. Between these two regions, the average p-distance was slightly higher at 0.085 ± 0.010.

The 18S rDNA alignment of 18 sequences comprised all 'four-times' COI clades except for clade 6 (which comprises individuals from the South Tyrolean region only). The aligned length was 1674 bp, although some sequences were shorter (1198–1554 bp). The analysis of 18S rDNA gene revealed an average pairwise p-distance of 0.00032 and the presence of only a single-point mutation (an A/C transversion) at position 1587 (within the V9 hypervariable region) for the four individuals of clade 1 (FL2C8, ST18, EM1D12, EM1E8). The average p-distance based on mitochondrial COI of the same 18 individuals was 0.069 ± 0.006, with values ranging between 0.000 and 0.113. Application of the 'four-times' method showed that no clades were distinguished by significant *K*/theta ratios.

The pruned COI alignment of 174 sequences used for reconstructing the haplotype network and the calculations for the F_{ST} statistics had a length of 573 bp. We found a total of between 27 (Network; see also Appendix S4) and 30 (Arlequin; Tables 3 and 4) COI haplotypes among all *S. pectinata* sequences. As calculated by Arlequin, the number of haplotypes within any given population from the Oldenburg-Eastern Friesland region ranged from four to nine, with the corresponding haplotype diversities ranging between 0.458 ± 0.078 (population FL) and 0.858 ± 0.036 (population ST) (Table 3). At a more inclusive level, each of the Oldenburg-Eastern Friesland and South Tyrolean regions presented high haplotype diversities of around 0.85 despite the different numbers of haplotypes present within each region (25 and 10, respectively; Table 3). Arlequin indicated five major haplotypes (HT) for the entire mid-European continental metapopulation (HT1, HT2, HT8, HT10 and HT13), each of which was sampled between 15 and 35 individuals (Table 4). Seventeen private haplotypes sampled from only a single individual were indicated and then almost exclusively from the Oldenburg-Eastern Friesland region. Population FL was characterised by the major haplotype HT1 together with several less frequent haplotypes. Populations EM and FW were each dominated by two major haplotypes (HT1/HT2 and HT8/HT10, respectively), whereas population ST consisted of eight haplotypes of roughly equal frequency, including the major haplotypes HT8, HT9 and HT10.

Analyses of mismatch distributions show two distinctly different patterns among the Oldenburg-Eastern Friesland populations. Populations EM and FL (Table 3;

Table 3 Haplotype diversity indices and demographic expansion for the COI marker among the four populations of *Synchaeta pectinata* in the Oldenburg-Eastern Friesland region as well as for the pooled population in the South Tyrol region as determined using Arlequin. Demographic expansion was calculated using the sum of square deviation (SSD) between observed and expected mismatches and the corresponding *P*-value as the test statistic. See Table 1 for sampling site codes

	Tyrol region	Oldenburg-Eastern Friesland region	EM	ST	FL	FW
n	48	126	31	26	37	32
No. of haplotypes	10	25	9	8	4	7
No. of polymorphic sites	91	119	12	102	4	50
Haplotype diversity (SD)	0.854 (±0.026)	0.857 (±0.018)	0.697 (±0.067)	0.858 (±0.036)	0.458 (±0.078)	0.575 (±0.087)
Demographic expansion SSD (*P*-value)	–	–	0.089 (0.140)	0.061 (0.000)	0.009 (0.180)	0.437 (0.000)

Table 4 Haplotype distribution of the COI marker among populations of *Synchaeta pectinata* in the Oldenburg-Eastern Friesland and South Tyrol regions as determined using Arlequin. See Table 1 for sampling site codes

Haplotype	Total number	EM	ST	FL	FW	South Tyrol	Individuals
HT1	35	9		26			EM01B10, EM01B12, EM01C01, EM01C06, EM01C11, EM01D02, EM01E08, EM01E09, EM01F03, FL02A02, FL02A03, FL02A08, FL02A10, FL02B05, FL02B06, FL02B09, FL02B11, FL02B12, FL02C03, FL02C04, FL02C05, FL02C06, FL02C07, FL02C09, FL02C10, FL02C11, FL02C12, FL02E02, FL02E03, FL02E05, FL02E06, FL02E07, FL02E08, FL02E09, FL02E10
HT2	15	15					EM01B11, EM01C02, EM01C03, EM01C05, EM01C07, EM01C09, EM01C12, EM01D01, EM01D03, EM01D04, EM01D05, EM01E03, EM01E04, EM01E07, EM01F04
HT3	1	1					EM01E01
HT4	1	1					EM01E06
HT5	1	1					EM01D10
HT6	1	1					EM01D12
HT7	2		2				ST01I01, ST01J04
HT8	16		5		7	4	ST01I03, ST01I04, ST01I10, ST01J03, ST01K03, FW02F01, FW02F02, FW02F04, FW02G04, FW02G08, FW02H05, FW02H09, VA01, SE01, SE02, SE03
HT9	7		7				ST01I06, ST01I12, ST01K04, ST01L05, ST01L07, ST01L08, ST01L09
HT10	26		5		20	1	ST01I07, ST01J02, ST01K07, ST01K09, ST01L02, FW02F03, FW02F05, FW02F06, FW02F08, FW02G02, FW02G03, FW02G05, FW02G07, FW02G09, FW02G10, FW02H02, FW02H03, FW02H06, FW02H08, FW02I03, FW02I04, FW02I07, FW02I08, FW02I09, FW02I10, WO08
HT11	2		1			1	ST01I08, WO07
HT12	3		3				ST01I11, ST01J01, ST01L10
HT13	15		2			13	ST01K01, ST01K11, LE01, LE02, LE03, LE04, LE05, LE06, LE07, LE08, LE09, LE10, LE11, LE12, LE13
HT14	7		1			6	ST01K02, WO01, WO02, WO03, WO04, WO05, WO06
HT15	9			9			FL02A01, FL02A04, FL02A05, FL02B07, FL02B10, FL02C01, FL02C02, FL02C08, FL02E04
HT16	1			1			FL02A07
HT17	1			1			FL02A09
HT18	1				1		FW02F09
HT19	1				1		FW02G01
HT20	1				1		FW02H01
HT21	1				1		FW02H04
HT22	1				1		FW02H07
HT23	10					10	LA01, LA02, LA03, LA04, LA05, LA06, LA07, VA02, VA03, VA04
HT24	3					3	SC01, SC02, SC03
HT25	1					1	TE01
HT26	6					6	TE02, TE03, TE04, FI01, FI02, FI03
HT27	3					3	LS01, LS02, LS03
HT28	1	1					EM01C04
HT29	1	1					EM01C08
HT30	1	1					EM01E05

Fig. 3) present unimodal distributions typical of a single major haplotype associated with many others at low frequencies (see also Fig. 4). By contrast, the large range of highly different haplotypes present in each of populations ST and FW (Fig. 4) results in the null distribution to be rejected significantly in each case in favour of one indicating demographic equilibrium.

Shared haplotypes were more frequent between the Oldenburg-Eastern Friesland and South Tyrolean regions (5; including the major haplotypes HT8, HT10, HT13 and HT14) than between populations of the former region (3; including the major haplotypes HT8 and HT10). The median-joining network obtained with Network (Fig. 4) reveals four major haplotype clusters that are separated by a large number of mutations (40, 43 and 48), with each cluster containing individuals from each of the two regions on the mid-European continental scale.

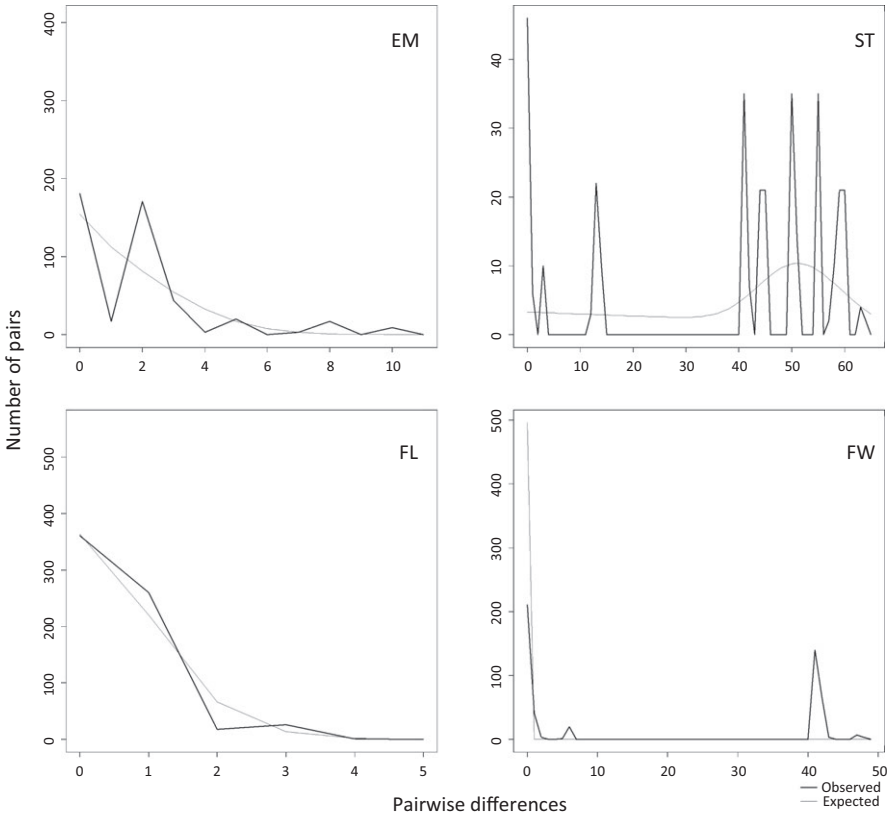

Fig. 3 Mismatch distributions showing the demographic expansion of the four *Synchaeta pectinata* populations in the Oldenburg-Eastern Friesland region based on mtCOI sequences. Associated SSD and *P*-values are given in Table 3.

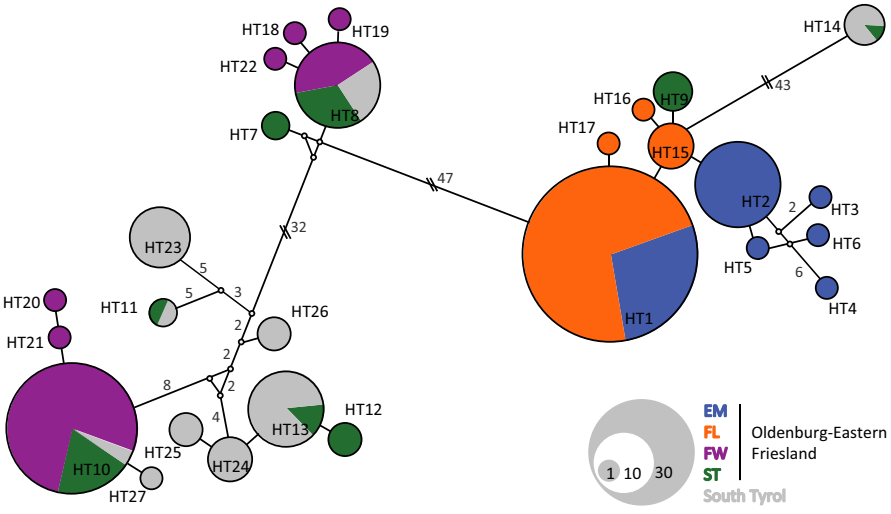

Fig. 4 Median-joining network of individuals of *Synchaeta pectinata* at the mid-European continental level. Haplotypes are represented as circles, the size of which represents the number of individuals possessing the haplotype and the distance between which represents the number of mutational steps. Populations are colour-coded as in Fig. 2.

Gene flow was generally low at all hierarchical levels for *S. pectinata*. For instance, pairwise F_{ST} values among all four populations on the regional level of Oldenburg-Eastern Friesland were often very high (see also Appendix S5), with an AMOVA indicating strong genetic differentiation among the four populations (F_{ST} = 0.63204; P = 0.00; Table 5). A similar tendency was found on the mid-European continental level when comparing the two regions (F_{ST} = 0.37144; P = 0.00; Table 5), but might derive in part through the high differentiation among the populations within the regions insofar as the F_{CT} value of 0.277 is substantially lower than the analogous values for F_{SC} (0.652) and F_{ST} (0.758) and is non-significant (P = 0.09347; Table 6). Finally,

Table 5 AMOVA of the four *Synchaeta pectinata* populations sampled in the Oldenburg-Eastern Friesland region as well as at the mid-European continental level. All calculations were made using Arlequin, with significance values determined on the basis of 10 100 permutations

Level	Source of variation	d.f.	Sum of squares	Variance component	% of Variation
Oldenburg-Eastern Friesland	Among populations	3	1069.07	11.16	63.2
	Within populations	122	792.91	6.5	36.8
	Total	125	1861.98	17.66	
				F_{ST} = 0.63204 (P = 0.000)	
Middle Europe	Among populations	1	597.37	8.39	37.14
	Within populations	172	2441.64	14.2	62.86
	Total	173	3039.01	22.58	
				F_{ST} = 0.37144 (P = 0.000)	

Table 6 Hierarchical analysis of variance and *F*-statistics from an AMOVA at the metapopulation level of *Synchaeta pectinata*, including both the Oldenburg-Eastern Friesland and South Tyrol regions. F_{SC} = among populations in the two regions, F_{ST} = among all populations, and F_{CT} = among regions. All calculations were made using Arlequin

Source of variation	d.f.	Sum of squares	Variance component	% of Variation
Among regions	1	597.37	6.33	27.68
Among populations within regions	11	1514.36	10.77	47.12
Among individuals within populations	161	927.28	5.76	25.20
Total	173	3039.28	22.85	
Fixation indices (*P*-value)			F_{CT} = 0.27676 (0.09347 ± 0.003)	
			F_{SC} = 0.65157 (0.000)	
			F_{ST} = 0.75800 (0.000)	

there was no significant correlation between the logarithm of the genetic pairwise F_{ST} and the geographical distance separating the populations (Mantel test $r = -0.175$; $P = 0.92$; see also Appendix S6).

Discussion

At the species level, many monogonont rotifers show wide distributional areas in accordance with the Everything is Everywhere hypothesis (Segers, 2008). This also appears to be true for *S. pectinata*, a species that has been documented as occurring across Europe (if not globally; see Introduction) based on both morphological and molecular zooplankton recordings (Koste & Voigt, 1978; Smirnov, 1986; Dokulil & Herzig, 2009; Apaydın Yağcı, 2013). However, our genetic results mirror those of Obertegger *et al.* (2012) to reveal a large amount of variation in the COI marker gene and thus high potential biodiversity of European *S. pectinata*, even across the relatively restricted geographic area examined. In particular, the geographical genetic pattern is one of the isolated populations, each characterised by low genetic divergence and largely dominated by a few major haplotypes (exception: population ST). Furthermore, the haplotypes cluster in distinct, well-defined groups separated by large numbers of mutations, with low sharing of haplotypes between populations. Together with the absence of significant gene flow among the Oldenburg-Eastern Friesland populations (geographical distances of only 3.6–50.4 km), this fact leads to an extremely patchy genetic structuring on the smaller geographical scale. Higher genetic similarity, but still insignificant gene flow, points to slightly more genetic homogeneity on the broader, mid-European continental scale. The presence of shared haplotypes of COI in both metapopulations separated by a geographical distance of ca. 800 km is evidence for long-range dispersal.

This genetic pattern conflicts with the expectation of low overall intraspecific diversity under an Everything is Everywhere scenario, even taking an isolation-by-distance model into account. Thus, despite having the appearance of a single species morphologically, this significant genetic structuring and divergence could be taken as evidence for past, cryptic speciation events in *S. pectinata*, as was inferred for *B. plicatilis* (Gomez, 2005) and *B. manjavacas* (Montero-Pau *et al.*, 2011). Indeed, such a scenario was postulated for *S. pectinata* by Obertegger *et al.* (2012). The identification of eight major clades from the 'four-times' method of Birky *et al.* (2010, 2011) also serves to reinforce the hypothesis that cryptic speciation could explain the widely separated mtDNA haplotype clusters within *S. pectinata*. Nevertheless, despite the fact that similar levels of COI sequence divergence have previously been interpreted in favour of the existence of cryptic species (Stoeckle, 2003; Hebert *et al.*, 2004), we argue that our results might equally well be explained by historic colonisation events (De Meester *et al.*, 2002) and that conclusions about cryptic speciation

are potentially premature in our case for three main reasons.

First, it is important to critically examine the marker gene COI. Because of its high intraspecific variability, COI often represents the population-genetic marker of choice, as in the present case. By now, it also represents the default marker for questions of DNA taxonomy and identification of cryptic speciation events (e.g. Hebert *et al*., 2004). However, mitochondrial DNA in rotifers is, as for most animals, maternally inherited and thus does not undergo recombination (Birky *et al*., 2011), such that we are merely reconstructing mitochondrial lineages that cannot mix. Thus, although nuclear, and therefore recombining, markers are arguably more appropriate for molecular species identification, suitable markers are generally rare (e.g. microsatellite loci are virtually unknown in rotifers outside of the genus *Brachionus*; Gómez & Carvalho, 2000). One option is the use of ribosomal DNA because its hypervariable regions often differ between closely related metazoan species (Lee & Foighil, 2004; Hasegawa *et al*., 2009). However, in contrast to the results from the COI data, our analyses of a smaller data set of 18S rDNA sequences (18 sequences) revealed virtually no variation along the gene (1674 bp) between the four Oldenburg-Eastern Friesland populations of *S. pectinata*, representing seven of the eight 'four-times' clusters. At most, one-point mutation in four individuals of the same clade was found in the hypervariable V9 region of the gene. Moreover, the 18S rDNA revealed an average pairwise p-distance of 0.00032 within *S. pectinata* compared to 0.005 between *S. pectinata* and its presumably close relative and undisputed species *S. grandis*. The very high levels of COI divergence combined with essentially no differentiation at the 18S rDNA sequences require further consideration. Our results mirror those of other species groups such as mites (Ros & Breeuwer, 2007; Rosenberger *et al*., 2013), where genetic diversity differed between mitochondrial COI and ribosomal (and also other nuclear) marker genes such as 18S. The incongruence between COI and 18S haplotypes might be explained by a very rare occurrence of sexual reproduction, probably caused by the rare presence of males. In this case, genetic variability in both genes is only driven by a mutation rate that could be probably higher for COI than 18S (Lee & Foighil, 2004; Hasegawa *et al*., 2009; Rosenberger *et al*., 2013). Indeed, males in *S. pectinata* are barely mentioned in zooplankton reports in the field, and only occasionally in the laboratory (Gilbert & Schreiber, 1995). In addition, a retention of ancestral polymorphism may cause molecular divergence to be substantially older than species divergence (Hebert *et al*., 2004). A different explanation involves selection, which might have driven homogenisation of 18S rDNA sequence (presumably after lineages came into contact after millions of years of divergence), while at the same time selection was maintaining the coexistence of highly divergent mtDNA lineages. Further studies are necessary to elucidate this variability pattern. Thus, there is no evidence for cryptic speciation at the nuclear genetic level and our results point to the importance of not relying on a single marker for species delineation.

Second, and reinforcing the previous argument that the use of COI reconstructs clonal mitochondrial lineages in the first instance, are the ages of these lineages as inferred through the application of a strict molecular clock based on observed substitution rates for mtDNA that range between 0.5 and 6.6% per million years (Crandall *et al*., 2012). Our reconstructed ages are on the scale of hundreds of thousands, if not millions, of years (see also Appendix S7) and are therefore much older than the bodies of water *S. pectinata* inhabit, all of which must have arisen sometime after the last Pleistocene glaciation event that ended *c.* 12 500 years ago. The existence of virtually absolute morphological stasis over such an extended timescale in organisms with such short generation times also seems unlikely. Likewise, most of the observed mutations within COI are silent with respect to amino acid changes and therefore presumably neutral. In total, only five non-synonymous mutations were present across the entire alignment and all but one were essentially restricted to single individuals. The fifth mutation induced a serine to proline change and characterised all individuals of clades 3 and 5. Interestingly, however, these two clades, although closely related, are not sister groups, indicating that the mutation was derived in parallel rather than being evidence of cryptic species status. Admittedly, the use of a strict molecular clock, and especially one based on average substitution rates derived from other organismal groups, is often highly problematic (Ho & Larson, 2006); however, it does serve to provide a very rough first estimate in the absence of any robust external calibration information (as is the case here).

How then can we explain our observed pattern of local divergence despite greater overall 'global' similarity? Looking at other species with similar reproductive and dispersal characteristics (*Daphnia*, Thielsch *et al*., 2009), we can see that these phylogeographic patterns are not unusual in zooplankton. Instead, the population demographic history of such animals is greatly influenced by their specific ecology and life history. Key here for monogonont rotifers are the resting eggs, which,

together with the small size of these organisms, enable a high dispersal ability that potentially facilitates mixing over broad geographic scales (Fenchel & Finlay, 2004; Kellogg & Griffin, 2006). Indeed, as demonstrated in water beetles, widespread geographical ranges in lentic freshwater taxa might result from strong selection for dispersal in these relatively short-lived habitats (Arribas *et al.*, 2012). However, high dispersal ability facilitated by resting eggs might simultaneously yield strict local population structuring (Brendonck & De Meester, 2003) given that the potential for the eggs to accumulate in very large numbers in the sediment can, as under the Monopolization hypothesis, reinforce both uniform intrapopulational structures and potentially high degrees of genetic differentiation within a given region through any or all of priority effects, persistent founder effects or haplotype selection (exclusion) (Hairston, 1996; De Meester *et al.*, 2002; Okamura & Freeland, 2002). In particular, priority effects could come into force during initial colonisation when populations reestablish themselves following overwintering under suboptimal conditions. Here, the resting egg bank functions as a sleeping gene pool of a diversity of (ancient) haplotypes. In the absence of strong haplotype selection, haplotypes from eggs that hatch earlier under the more favourable conditions can use this temporal competitive advantage to establish themselves through the rapid population growth facilitated by asexual reproduction and impose a relatively uniform local haplotype structure (Boileau *et al.*, 1992). In this context, the mismatch analyses show that the EM and FL populations in northern Germany indeed do show strong signatures of a relatively recent population expansion, pointing to a likely reestablishment of the population from a few individuals (or resting eggs) with the associated priority effects.

Furthermore, during a long-lasting founder effect, a resident, steadily occurring population could persist over long timescales with considerable fluctuations in population size (De Meester *et al.*, 2002), which, in turn, can maintain high levels of population divergence between isolated bodies of water (Okamura & Freeland, 2002), as was observed here. Importantly, through chance, priority effects can also favour rare haplotypes that are under-represented in the resting egg banks and across the species as a whole, thereby inflating apparent population structuring. Finally, over and above these factors, is the potential for selective advantages among haplotypes leading to competitive exclusions. Environmental change (e.g. in food levels) and predator–prey communities as well as seasonal patterns can differentially influence population dynamics (Fussmann, 2011) and/or put some haplotypes at a competitive disadvantage (Snell, 1979). Indeed, De Meester *et al.* (2002) suggested that local adaptation has the potential to reduce gene flow among populations, such that the patterns of strict genetic differentiation would then often reflect historical colonisation events.

Given the high potential impact on genetic structuring of repeated colonisation events together with strong fluctuations in population size, long-term temporal sampling schemes are the inevitable next step in analysing the demographic history of rotifer populations (Papakostas *et al.*, 2013) to assess the degree to which isolated results represent biased snapshots of the entire gene pool. Furthermore, data from sequential population samples can be correlated with environmental variables to potentially provide evidence of clonal selection as well as ecological specialisation and, in the present context, to provide a more comprehensive assessment of the species status of *S. pectinata*.

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Supporting Information

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

Appendix S1. Matrix of geographical distances (in km) between the sample sites.

Appendix S2. Accession numbers and quantity of the South Tyrolean (Italy) populations from Obertegger *et al.* (2012) used in this study.

Appendix S3. Average pairwise p-distances (±SE) for the COI marker within and among the five clades identified on the neighbour joining tree of *S. pectinata* as determined using MEGA under a Kimura-2-parameter model.

Appendix S4. Haplotype distribution of the COI marker among populations of *S. pectinata* in the Oldenburg-Eastern Friesland and South Tyrol regions as determined using Network.

Appendix S5. Pairwise F_{ST}-values (below diagonal) and corresponding *P*-values (above diagonal) for the COI marker among the four populations of *S. pectinata* sampled in the Oldenburg-Eastern Friesland region as determined using Arlequin.

Appendix S6. The relationship between genetic distance (pairwise F_{ST}) and geographical distance at the mid-European continental level for *S. pectinata*.

Appendix S7. Timetree showing the estimated divergence times (in millions of years, Ma) within *S. pectinata* assuming a strict molecular clock.

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