Genetic Differentiation and Phylogeography of Rotifer *Polyarthra Dolichoptera* and *P. Vulgaris* Complexes Between Southern China and Eastern North America: High Intercontinental Differences

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Research Article

Keywords: rotifer, phylogeography, cryptic species, genetic distance, spatio-temporal pattern, long-distance dispersal

Posted Date: February 25th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-242024/v1>

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Abstract

Genetic differentiations and phylogeographical patterns of small organisms may be shaped by spatial isolation, environmental gradients and gene flow. However, knowledge about genetic differentiation of rotifers on intercontinental gradient is still limited. *Polyarthra dolichoptera* and *P. vulgaris* are cosmopolitan rotifers and tolerant to environmental changes, offering an excellent model to address the research gap. Here, we investigated the populations in Southern China and eastern North America, and evaluated the phylogeographical patterns from their geographical range sizes, geographic-genetic distance relationships and their response to spatial-environmental factors. Using mitochondrial cytochrome c oxidase subunit I gene as the DNA marker, we analyzed a total of 170 individuals. At least 24 putative cryptic species, including 20 of *P. dolichoptera* and 4 of *P. vulgaris* were detected based on three delimitation methods. Our results showed that some cryptic species were widely distributed but most of them were limited to single areas. The divergence of *P. dolichoptera* and *P. vulgaris* complexes indicated that gene flow between continents was limited while that within each continent was stronger. Furthermore, on the intercontinental scale spatial distance had a stronger influence than physicochemical variables on the genetic differentiations of *P. dolichoptera* and *P. vulgaris* complexes. However, the relationship between genetic distance and geographic distance was not continuously linear and the *P. dolichoptera* data best fitted the power-law model. This might be due to the effects of habitat heterogeneity, long-distance colonization and oceanographic barriers. Outliers above the correlation line between geographic distance and genetic distance suggest a significant dispersal barrier on large geographic scales studies.

1. Introduction

One of the most important questions in ecology and biogeography is how and why species composition differs between geographic locations (Dambros et al. 2020). Environmental factors, geographic distance, and dispersal barriers are potential drivers. Some scholars believe that genetic diversity should be related to geographical distances by a classical distance–decay relationship (Gómez-Rodríguez et al., 2020 ; Gómez et al., 2002). On the contrary, others hold that environmental conditions, not dispersal, control the patterns of genetic differentiation (Sbabou et al., 2016 ; Fenchel and Finlay, 2006).

Isolation by distance (IBD), proposed by Wright (1943), predicts that the degree of genetic differentiation increases with geographic distance due to dispersal limits (Tisthammer et al., 2020), analogously to a distance–decay relationship at the community level (Gómez-Rodríguez et al., 2020). The IBD pattern, respecting the association between genetic and geographic distances, is common in many plants (e.g. *Rhododendron* spp, *Mikania micrantha*) and even the single-celled phytoplankton cyanobacteria (Banerjee et al., 2020 ; Ribeiro et al., 2020 ; Zhang et al., 2020). For heterotrophic protists (unicellular eukaryotes) such as *Noctiluca scintillans*, an intercontinental spatial barrier (the Pacific Ocean) seems to impose a limitation on gene flow and induces an increase in genetic distance (Pan et al., 2016). However, weak or no genetic/geographic correlation has been found in most invertebrates including *Tetranychus*, *Thrips tabaci* and *Periplaneta americana* (Jin et al., 2020 ; Li et al., 2020 ; Ma et al., 2019). Rotifers, cosmopolitan microscopic organisms, have been proposed as a model of distance–decay biogeographic patterns (Fontaneto et al., 2008a). But to date, findings are inconsistent. A study of *Brachionus calyciflorus* phylogeography in eastern China showed no significant association between geographical and genetic distances (Xiang et al., 2011). Conversely, a significant albeit weak correlation was found in *Euchlanis dilatata* phylogeography in North America (Kordbacheh et al., 2017). The relationship between geographical distance and genetic distance is thus far from clear in rotifers. Because the IBD pattern is non-linear at very short and long average geographic distances (Bradbury and Bentzen, 2007), studies at intercontinental geographic scale are needed.

In contrast to the IBD hypothesis, the Baas-Becking's hypothesis, known as "everything is everywhere, but the environment selects" (EisE) posits that for small species, differences from different places occur because of environmental variation, and not because of restricted dispersal (Fenchel and Finlay, 2006). In addition, it is widely recognized that cryptic diversity is currently underestimated, which results in underappreciation of cosmopolitan organisms (Leal et al., 2019). According to EisE, two identical individuals can be found in the same kind of environments in two far separated locations. Most studies of environmental-spatial selection to date focused on small geographic or continental scales, and genetic differentiation across intercontinental scales has been underexplored.

Morphological and genetic variations commonly occur between populations and are mainly influenced by two factors. On one hand, genetic drift, mutations and natural selection will lead to the genetic differentiation of local populations. On the other hand,

gene flow produces genetic homogeneity by the movement of gametes, individuals and even entire populations, and blockage of gene flow creates genetic differentiation between populations. The latter can lead to speciation (Slatkin, 1987).

With the development of molecular tools, increasing numbers of cryptic species have been discovered in various morphological species of rotifers, such as *Brachionus calyciflorus* (Papakostas et al., 2016), *B. plicatilis* (Mills et al., 2017), *Keratella cochlearis* (Derry and Prepas, 2003), *Polyarthra dolichoptera* (Obertegger et al., 2015), *Lecane spp.* (García-Morales and Elías-Gutiérrez, 2013), *Testudinella clypeata* (Leasi et al., 2013), and some bdelloid rotifers (Fontaneto et al., 2008a). Moreover, coexistence of cryptic species is common in a single water body and can be mediated by ecological forces including seasonal changes, food resources and competition (Wen et al., 2016).

Rotifers undergo periodic parthenogenesis, with little genetic recombination and intraspecific hybridization. Thus, compared with cladocerans and copepods, rotifers are advantaged for the study of phylogeography. Studying the genetic differentiation of rotifers is of great significance for understanding the dispersal pattern of gene flow and adaptive evolution mechanisms in microscopic organisms (Zhang et al., 2018b). *Polyarthra dolichoptera* and *P. vulgaris*, cosmopolitan species of rotifera, are more tolerant to seasonal changes than other rotifers and exist in almost all kinds of water bodies (Liang et al., 2020). It has been suggested that *Polyarthra* cryptic species distribution might reflect genotypic adaptations to temperature differences and food resources (Obertegger et al. 2015). Temperature influences all metabolic processes, and it plays an important role in rotifer habitat selection (Obertegger et al., 2015). Also, phytoplankton biomass has marked effects on rotifer communities and it has strong seasonalities in most water bodies (Liang et al., 2019). A strongly positive correlation has been found between *Polyarthra* abundance and chlorophyll-*a* (Liang et al., 2020).

In this study, we attempted to address the IBD versus EisE hypothesis by investigating the phylogeographic patterns of *P. dolichoptera* and *P. vulgaris* across the America and the Eurasia continents. The specific objectives were: (1) to estimate the relationship between geographic distance and genetic distance on the intercontinental scale (2) to understand whether physicochemical or spatial variables are the key factors affecting genetic differentiation.

2. Methods

2.1 Sampling

Samples were collected from 28 sites including rivers, ponds and lakes in both eastern North America and Southeastern China, during June 2018 to September 2019 (Fig. 1, Table 1). For determining detailed cryptic species structure on a small geographic scale, five sites in Guangzhou city, China and five ponds in New London Country, U.S.A., were sampled. In consideration of temperature effects on cryptic species structure, four seasons were sampled in Lake Liuye, China (6_liuye, 9_liuye, 12_liuye and 3_liuye; Table 1).

Table 1
Details of sampling localities and the environmental parameters

Population	Abbreviation	Collection date	Longitude	Latitude	Altitude (m)	Temperature (°C)	Chlorophyll-a (ug/L)	Salinity (‰)
March_Lake Liuye	3_liuye	2019.3	111.72917	29.109722	30	14	10	0.11
June_Lake Liuye	6_liuye	2019.6	111.72722	29.048333	30	28	18	0.13
September_Lake Liuye	9_liuye	2018.9	111.70911	29.12	30	25	5	0.12
December_Lake Liuye	12_liuye	2018.12	111.76028	29.069167	30	8	1	0.13
September_the Chuanzi River	9_chuanzi2	2018.9	111.69194	29.053889	29	30	17	0.15
December_the Chuanzi River	12_chuanzi	2018.12	111.69194	29.053889	29	8	1	0.15
The pond of Haizhu Park	haizhu	2018.12	113.22833	23.122778	2	11	8	0.12
The pond of Minghu	minghu	2019.1	113.34323	23.134201	11	17	72	0.11
The pond of Nanhu	nanhu	2019.1	113.34434	23.131315	15	18	18	0.14
Guangzhou Segment of the Pearl River	pearlriver2	2019.3	113.34718	23.042379	1	17	1	0.15
The pond of Zhujaing Park	zhujiangpark	2019.4	113.33385	23.122431	3	25	17	0.13
The reservoir of Jiukeng	jiukeng	2019.4	112.5482	23.231587	39	25	1	0.12
The pond of Xiamen University	XM	2018.8	118.30997	24.620833	47	27	78	0.16
The brook of Xiamen University	XX	2018.8	118.3093	24.613373	21	27	8	0.1
The pond near Lake Donghu	WHS7	2018.7	114.165	30.528611	18	27	107	0.14
Thames river	Thames	2019.5	-72.07313	41.47805	0	16	1	6
Housatonic river	Housatonic	2019.9	-73.12371	41.340767	8	25	78	0.14
Quinnipiac river	Quinnipiac	2019.9	-72.86769	41.398428	-2	24	10	0.13
Lake Success	SUC	2019.6	-73.70729	40.763248	59	22	65	0.12
Niagara waterfall	Niagara	2019.8	-79.06275	43.081979	166	25	1	0.1
Pattagansett Lake	P2	2019.6	-72.22837	41.376893	20	23	8	0.11
Norwich Pond	P4	2019.6	-72.30391	41.384799	25	24	2	0.15
Powers Lake	P5	2019.6	-72.2559	41.393302	48	24	3	0.12
Amos Lake	P7	2019.7	-71.97741	41.516628	39	25	6	0.14

Population	Abbreviation	Collection date	Longitude	Latitude	Altitude (m)	Temperature (°C)	Chlorophyll-a (ug/L)	Salinity (‰)
Mirror Lake	P9	2019.9	-72.24724	41.806832	179	24	3	0.14
Swan Lake	P10	2019.9	-72.25277	41.81083	184	24	5	0.16
Moodus Reservoir	P14	2019.9	-72.40739	41.509835	109	24	10	0.12

All rotifer samples were collected by towing a plankton net (mesh size 30 μm) horizontally at surface and subsurface depths and preserved in a 50 mL centrifuge tube. To prevent changes in DNA, samples were fixed on site immediately with neutral Lugol's solution at 2% final concentration and transported in a cooler before storing at -20 °C. In vivo semi-quantitative measurements of chlorophyll-a (Chl-a) were obtained using a FluoroSense™ handheld fluorometer (Turner Designs, USA). Water temperature (Temp) and salinity, were measured on site. Also, GPS coordinates and altitude values were recorded using a GPS application.

2.2 Species identification and isolation

Species identification was based on Dumont (2002), the latest and most authoritative rotifer taxonomy system. Identification relies on morphological differences of body forms, sizes, fins, lateral antennae and vitellarium. The species of *Polyarthra dolichoptera* and *Polyarthra vulgaris* were isolated with micropipette under the stereo microscope. Single individuals were rinsed several times and transferred into PCR tubes for DNA analysis.

2.3 Selection of mt COI as the DNA marker

DNA markers including 18S ribosomal RNA, nuclear internal transcribed spacer (ITS) and mitochondrial cytochrome c oxidase subunit I gene (COI) are widely used as DNA barcodes for identification (Papakostas et al., 2016). Unlike nuclear DNA, mitochondrial DNA such as COI generally does not undergo genetic recombination as it is transmitted directly from the mother to the offspring, and can be an effective single haplotype marker (Freeland et al., 2011). Moreover, COI evolves more rapidly than ITS in animals, and is thus the better marker for phylogeography and cryptic species delimitation (Mills et al., 2017).

2.4 DNA extraction and amplification

DNA from each single animal was extracted following the HotSHOT protocol (Montero-Pau et al., 2008). Then, the partial cytochrome c oxidase subunit I (COI) mtDNA gene was amplified and sequenced using primers LCOI (5'-GGT CAA CAA ATC ATA AAG ATA TTGG-3') and HCOI (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al., 1994). PCR was processed according to the TaKaRa exTaq protocol with 5 μL of extracted DNA. Cycle conditions were initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 45 s. The amplification ended with a final extension of 72 °C for 8 min. Successful amplification products were then purified using the TaKaRa Minibest agarose Gel DNA extraction Kit before being sent to TsingKe company for sequencing.

2.5 Sequences alignment and phylogenetic analyses

Sequences were aligned by Mega X using Clustal-W and then visually checked. Each sequence was verified by BLAST search in NCBI GenBank (Sayers et al., 2018). Within-species genetic distances should be less than 14% for mt COI (Obertegger et al., 2015; Mills et al., 2017). The closest sequences with the highest similarity scores were obtained from GenBank for comparison (Accession #: KJ460388, LC215566, LC215573, KC618934, KC619030, JN936500, KJ460383, KC619195 and LC215562). Population genetic statistics (average number of nucleotide differences between haplotypes, number of haplotypes, haplotype diversity [H_d] and nucleotide diversity [π], average number of nucleotide differences [K], average number of segregating sites [S]) were calculated using DNASP 5.1 (Librado and Rozas, 2009).

Bayesian phylogenetic trees were run in BEAST v1.8.4 using a HKY + I + G model, separately for the two data sets (108 *P. dolichoptera* and 64 *P. vulgaris* sequences). For this analysis, an uncorrelated lognormal relaxed clock, the Yule process speciation prior (rate of linear birth in the Yule model of speciation set as lognormal); the default settings of prior, and the MCMC of 10^7 generations with sampling every 1000 generations were used. Tracer v1.6 was used for evaluating effective sample size (ESS > 200). Trees were summarised using TreeAnnotator v1.8.4 with a 20% burn-in. For *P. dolichoptera* phylogenetic reconstructions,

congener *P. vulgaris* (KJ460388) was included as outgroup, and *P. dolichoptera* (KC618934) was included as outgroup for *P. vulgaris*.

2.6 Cryptic species delimitation

Generalised Mixed Yule Coalescent (GMYC), Automatic Barcode Gap Discovery (ABGD) and Poisson tree processes (PTP) models are widely used approaches for cryptic species (entities) delimitation (Kordbacheh et al., 2017). An ultrametric tree generated by BEAST was required for both GMYC and PTP delimitations. The GMYC delimitation analysis was processed on software R 3.6.1 using the 'rnc1' and 'splits' packages, (R Core Team 2019). The GMYC model is a likelihood method for delimiting species by fitting within and between species branching models to reconstructed gene trees (Fujisawa and Barraclough, 2013 ; Pons et al., 2006).

The ABGD model was performed for primary species delimitation and was processed on the website <https://bioinfo.mnhn.fr/abi/public/abgd/> (Accessed January 10, 2020). ABGD classifies sequences into putative cryptic species based on pairwise genetic distances without any prior assumptions (Puillandre et al., 2012).

PTP is a tree-based method that uses the number of substitutions to distinguish intraspecies processes from interspecies processes. This method considers two classes of Poisson processes, speciation (higher substitution rate associated to interspecies events) and coalescence (within species events) (Zhang et al., 2013). PTP model for cryptic species delimitation was run using the online tool at <http://species.h-its.org/ptp/> (Accessed January 8, 2020).

2.7 Geographical and genetic distance analysis

The pairwise geographic distance matrices were calculated in R 3.6.1 (R Core Team, 2019) using the 'geosphere' package (Ding et al., 2019). The pairwise genetic distance matrixes were calculated with Kimura 2-parameters model, pairwise deletion, transitions + transversions using the Mega X program. Linear regression analysis and Generalized additive models (GAM) of the relationships between geographic distance and genetic distance were processed in R 3.6.1 using the package 'ggplot2'. To determine the significance of differences ($p < 0.05$) in geographic distances and genetic distances among different groups, analysis of variance (ANOVA) with TukeyHSD test was conducted, using the software R 3.6.1, 'agricolae', 'car' and 'multcomp' packages. To characterize the shape of the relationships within the two species, three different GLM models including linear, exponential and power-law were applied using the package 'betapart' and 'pscl'.

2.8 Relationships between cryptic species and environmental factors

Redundancy analysis (RDA) or Canonical Correlation Analysis (CCA) was performed to explore the relationships between cryptic species and environmental factors using the 'vegan' and 'ggplot2' packages in R. CCA or RDA model is determined based on the community composition by Detrended Correspondence Analysis (DCA). If the longest gradient is > 4 , the unimodal method (CCA) will be applied. On the other hand, if that value is < 3 , the linear method (RDA) is a better choice. In the range between 3 and 4, both methods can be applied (ter Braak and Smilauer, 2002). Varying inflation factors less than 10 ($VIF < 10$) were included in the analysis and the envfit (permu = 999) function was used to determine the significant ($p > 0.05$) variables (Oksanen et al., 2010).

3. Results

3.1 Genetic diversity

We obtained 107 COI sequences of *P. dolichoptera* and 63 sequences of *P. vulgaris*, with aligned lengths of 562 bp and 589 bp, respectively (Accession numbers: Table S1). A total of 64 *P. dolichoptera* haplotypes were detected with haplotype diversity (h) of 0.98 and nucleotide diversity (π) of 0.175. For *P. vulgaris* a total of 36 haplotypes were found with a haplotype diversity of 0.96 and nucleotide diversity of 0.106 (Table 2).

Table 2
Genetic diversity summary statistics, as calculated by DNASP 5

Population	Individuals	Haplotypes	Haplotype diversity (H_d)	Nucleotide diversity (π)	Average number of nucleotide differences (K)	Average number of segregating sites (S)
Total <i>P. dolichoptera</i>	103	64	0.979	0.175	94.87	330
<i>P. dolichoptera</i> in China	70	41	0.96	0.149	80.97	230
<i>P. dolichoptera</i> in the USA	33	23	0.978	0.156	84.51	281
Total <i>P. vulgaris</i>	63	36	0.957	0.106	60.73	227
<i>P. vulgaris</i> in China	42	24	0.93	0.048	27.56	156
<i>P. vulgaris</i> in the USA	21	12	0.895	0.026	15.11	91

For *P. dolichoptera* complex, a larger genetic variation was observed in eastern North America than in southeastern China, with higher haplotype diversity (H_d ; 0.978), nucleotide diversity (π ; 0.156), average number of nucleotide differences (K; 84.51) and average number of segregating sites (S; 281). For *P. vulgaris* complex, higher levels of H_d (0.93), π (0.048), K (27.56) and S (156) were detected in the southeastern China samples (Table 2).

3.2 Phylogenetic analysis and cryptic species delimitation

Using Bayesian phylogenetic analysis, *P. dolichoptera* complex were reconstructed (Fig. 2). All the individuals analyzed were binned into three groups. Group 1 was composed of 54 individuals, which were all from Southeastern China. This group was composed of similar clusters from different sites in Southeastern China. Group 2 consisted of 20 individuals from eastern North America and 17 individuals from Southeastern China, which formed independent clades by continents, showing high genetic divergence between the two geographic communities. Group 3 consisted of 13 individuals from the eastern North America and three individuals from Southeastern China, which formed many divergent clusters within eastern North America and an independent clade for the individuals from Southeastern China. The Bayesian tree analysis clustered the *P. vulgaris* samples into two groups with strong support values (Fig. 3). All of the 42 individuals from Southeastern China were in Group 1, while the 18 individuals from eastern North America were in Group 2 with strong support values.

A large number of cryptic species were detected using three independent methods (Figs. 2, 3). The ABGD method produced 20 cryptic species for *P. dolichoptera* complex and four for *P. vulgaris*. GMYC analysis revealed 21 cryptic species in *P. dolichoptera* complex and five in *P. vulgaris* complex. Using the PTP method, *P. dolichoptera* complex was delimited into 24 cryptic species and *P. vulgaris* complex into 13. The most conservative estimate of cryptic species was obtained using ABGD, while PTP method gave the greatest number of cryptic species. All three methods shared common species boundaries for the smallest number of cryptic species, however. As the results from ABGD and GMYC were similar, unless specified otherwise, cryptic species determination will be discussed based on ABGD results.

3.3 Geographical distribution

The range size of geographic distances declined as the resolution of classification increased from morphological species to cryptic species to haplotype (Fig. 4). Both *P. dolichoptera* and *P. vulgaris* species were widely distributed, with the geographic range sizes up to 12903 km and 12836 km, respectively. Their range sizes at cryptic species and haplotype levels decreased to 2726 km and 411 km, respectively. These correspond to the mean range sizes at species level (*P. dolichoptera*: 5374 $\pm$ 5665 km; *P. vulgaris*: 5517 $\pm$ 5720 km) significantly larger than at cryptic species (463 $\pm$ 815 km) and haplotype level (12 $\pm$ 61 km). However, there was no significant difference in range size between cryptic species and haplotype ranges.

Most of the cryptic species were limited to single areas, but some were widely distributed. For example, cryptic species one of *P. vulgaris* complex (V1) comprised individuals from as distant areas as Changde (liuye), Wuhan (WHS7), Xiamen (XM) and Guangzhou (zhujiangpark), in China (Fig. 3). In addition, cryptic species 10 of *P. dolichoptera* complex (D10) were as widely distributed as from Connecticut (P4), Long Island (SUC) and Niagara (upstate New York), in USA. In contrast, some cryptic species only occurred in one sampling site, such as D7 and D16 (Fig. 2). These results indicated that the cryptic species and haplotypes tended to be regionally restricted. Although some of them can be widely spread into different habitats (sampling sites), no cryptic species or haplotype was found to occur on both continents.

3.4 Phylogeographical patterns and genetic structure

The relationships between dependent variables for genetic distance and independent variables for geographic distance were examined by linear regression (Fig. 5). The genetic distance of *P. dolichoptera* complex showed significant positive correlation with geographic distance ($R^2 = 0.18, p < 0.01$). The genetic distance of *P. vulgaris* complex was also positively correlative with geographic distance ($R^2 = 0.53, p < 0.01$) (Fig. 5). Fitting the data to the smooth function of the generalized additive model (GAM) indicated that the relationship between geographic distance and genetic distance was not simply linear. Genetic distances increased with geographic distances initially but then decreased rapidly when geographic distances increase within 1300 km, before it rose again at greater distances of around 10000 km.

To characterize the shape of these relationships within these two species complexes, GLM analysis was carried out based on AIC values. Our results showed that the power-law model fitted the *P. dolichoptera* data better than either the exponential or linear models, while the linear model fitted the *P. vulgaris* data better than other models (Table 3).

Table 3
Comparison of three GLM models (linear, exponential and power-law) assessing the shape of the relationship between geographic distance and genetic distance for *P. dolichoptera* and *P. vulgaris* complexes. Models are evaluated according to their AIC and the lowest values of AIC are shown in bold.

	<i>P. dolichoptera</i>	<i>P. vulgaris</i>
linear	-24567	-9323
exponential	-24551	-9310
power-law	-25185	-9131
<i>p</i> < 0.01 in all cases		

Our results indicated that in both *P. dolichoptera* and *P. vulgaris* complexes, the mean genetic distances between the two continents were significantly higher than those within either eastern North America or Southern China ($p < 0.05$) (Fig. 6). The mean genetic distance in the *P. dolichoptera* complex decreased in the order: Southern China VS eastern North America (0.248 ± 0.052) > within eastern North America (0.188 ± 0.111) > within Southern China (0.173 ± 0.089) (Fig. 6A). The mean genetic distances in the *P. vulgaris* complex decreased in the order: Southern China VS eastern North America (0.223 ± 0.02) > within Southern China (0.055 ± 0.088) > within eastern North America (0.049 ± 0.067) (Fig. 6B). However, there was no significant difference in the mean genetic distance values for the Southern China and the eastern North America groups in the *P. vulgaris* complex. These results indicated that the genetic divergences between these two continents were significantly higher than those within a single continent ($p < 0.05$). This suggests that there is a higher level of gene flow and high frequency of recombination within continents than between continents.

3.5 Relationships between environmental factors and cryptic species distributions

As the longest gradient performed by Detrended Correspondence Analysis (DCA) was 7.5 (larger than 4), a Canonical Correlation Analysis (CCA) model was chosen for estimating the relationship between cryptic species and environmental factors. The first two ordinate axes explained 61% of the cryptic species-environment variability in the CCA ordination (Table S2). The CCA ordination

showed that four variables including longitude, latitude, altitude and temperature were significantly related to the cryptic species distributions ($p < 0.05$) (Table S3). However, the environmental factor chlorophyll-*a* was not significant variable affecting the cryptic species structure (Fig. 7). Figure 7 clearly showed that the Southern China populations (red) mostly stayed on the left of the figure, while the eastern North America populations (blue) were mostly on the right of the figure. In addition, the spatial variables of longitude and latitude showed positive correlation with axis 1, which indicated that longitude and latitude were the key factors for the variation of the cryptic species structure. Furthermore, the cryptic species-variables relationship was similar to that of sampling sites-variables, which indicated that most cryptic species tended to be restricted to specific regions.

Discussion

4.1 The hidden diversity in species complexes

Cryptic species have been found in almost all groups of animals (Tang et al., 2012 ; Fossen et al., 2016), and rotifers seem to be one of the invertebrates hosting the highest potential cryptic diversity in the world (Fontaneto et al., 2009 ; Fontaneto, 2014). For instance, eight potential cryptic species of *Brachionus calyciflorus* were found in eastern China (Xiang et al., 2011). Also, more than seven cryptic species of *Euchlanis dilatata* were defined in North America (Kordbacheh et al., 2017). By the end of 2017, there were 15 cryptic species of *B. plicatilis* were recorded in the world, as a conservative estimate (Mills et al., 2017). Our results indicated that both *P. dolichoptera* and *P. vulgaris* are complexes of cryptic species, with at least 17 taxa of *P. dolichoptera* and 3 taxa of *P. vulgaris* in our study areas. Some cryptic species of *P. dolichoptera*, such as D11 and D12, were only found in one site, which could be due to small sample sizes. Obertegger et al. (2015) reported that at least 12 cryptic species of *P. dolichoptera* had been found in 35 lakes along an altitudinal gradient in Italy. Given that the small sample sizes in this study impeded a thorough detection of cryptic species, the degree of cryptic diversity in Southeastern China and eastern North America is likely to be higher than what we reported here.

Leaving out the data from NCBI, which gave two cryptic species based on our analysis, our most conservative delimitation based on ABGD gave 20 cryptic species in the *P. dolichoptera* complex and 4 in the *P. vulgaris* complex from our current data. The results from the GMYC method were similar (21 in the *P. dolichoptera* complex and 5 in the *P. vulgaris* complex). PTP-based estimates, 24 in the *P. dolichoptera* complex and 13 in the *P. vulgaris* complex might be a result of overestimation by the method, as has been suspected in previous studies on *E. dilatata* (Kordbacheh et al., 2017) and *B. plicatilis* (Mills et al., 2017). Since our cryptic species delimitation was based on molecular methods with less morphological evidence, the conservative estimate is a better choice.

4.2 Genetic divergence and geography distribution

Long-distance dispersal of cryptic species has been reported not only in Monogononta including *Brachionus*, *Polyarthra*, *Euchlanis* and *Lecane*, but also in Bdelloidea including *Philodina* and *Rotaria* (Kordbacheh et al., 2017 ; Fontaneto et al., 2008b). The cryptic species D1 was found in Guangdong and Hunan provinces, separated by > 500 km (e.g. liuye, chuanzi2, minghu, pearlriver2). Also, D10 was found across a range of > 500 km in the US states of New York and Connecticut (e.g. SUC, Niagara, P2, P4). In addition, V1 was widely distributed in southeastern of China, while V3 was widespread in Connecticut.

The cosmopolitan distribution of rotifers could be attributed to long-distance dispersal. Colonization and long-distance dispersal to different waters across whole or even multiple continents, which may be mediated by waterfowl, have been observed in a number of zooplankton species (Gómez et al., 2002). Secondly, some areas that are widely separated share haplotypes and therefore appear genetically connected (Xiang et al., 2011). Sasaki and Dam (2019) found that the widely distributed genetic clades of a marine copepod shared haplotypes between geographically distant populations. This implies that gene flow can be strong enough to overcome long distances at least within a continent.

Most small organisms do have very widespread distributions, but some are limited to distinct geographical areas (Savary et al., 2018). Our results showed that the cryptic species of *P. dolichoptera* D5 and D7 only occurred in Xiamen and Wuhan, respectively. This is consistent with the study of *Brachionus calyciflorus* cryptic diversity in eastern China. Though most cryptic species of *Brachionus calyciflorus* were widely distributed, one clade was only found in Danzhou, China (Xiang et al., 2011). Although high genetic distances of *Adineta* can be found at different geographical distances, closely related individuals were only found at geographical scales < 2000 km (Fontaneto et al., 2008a). In the current study, we found that the range sizes of geographic distance in both genera declined as resolution increased from species to cryptic species to haplotype. This suggests that the restricted cryptic

species in our study are not simply an artifact of sampling fewer individuals at lower levels. Interestingly, even though *Polyarthra* was widely distributed as a genus, none of the cryptic species or haplotypes was found on both continents.

Our results indicated that all of the cryptic species from eastern North America formed independent strains that were separate from the Chinese ones, indicating high divergence. These results are consistent with the study in *Noctiluca*, a heterotrophic dinoflagellate (Pan et al., 2016). The haplotypes of *Noctiluca* within China were geographically quite homogeneous, but were generally different, compared to the American population suggesting basin or continental-scale endemism. In addition, a study of the cryptic species of *B. plicatilis* revealed existence of four clades associated to four geographic regions (one in North America, two in Europe and one in Australia) (Mills et al., 2017).

Levels of gene flow can be estimated by producing the visible patterns using allele frequencies and DNA sequence differences (Slatkin, 1987). Lack of differentiation in mitochondrial COI sequences of geographically distant populations usually indicates strong effects of gene flow (Sasaki and Dam, 2019). Our study showed that the genetic distances of Southern China VS eastern North America were significantly higher than those within each continent. The divergence of populations between Southern China and eastern North America indicates limited gene flow between the two continents. The relatively low genetic divergence in the populations within continents of both *P. dolichoptera* and *P. vulgaris* complexes suggests strong gene flow within Southeastern China and within eastern North America.

4.3 Relationship between geographic and genetic distance

In the present study, a significantly positive correlation between genetic and geographic distance was found in both *Polyarthra* species complexes. Similar results have been obtained for *E. dilatata* in the North America (Kordbacheh et al., 2017). Moreover, it was reported that there was a strong positive correlation between genetic distance and geographic range when comparing samples on small geographical scales (Kordbacheh et al., 2017). Habitat heterogeneity and temporal variation can generate high genetic diversity on small geographic scale study (Fontaneto et al., 2009).

However, as the geographical scope of the study becomes broader, this correlation may weaken or disappear. A study of *Geomalacus* revealed that the genetic distance increased rapidly along with the geographic distance, but as the geographic distance continues to expand, the genetic distance reached a plateau (Gómez-Rodríguez et al., 2020). In another example, no significant associations between geographical and genetic distances were found for *B. calyciflorus* across eastern China. The nonsignificant correlation may result from the effects of long-distance colonization and secondary contact, combined with monopolization effects which reduce gene flow among established populations (Xiang et al., 2011; Kordbacheh et al., 2017). In our results, Guangdong and Hunan provinces shared *P. dolichoptera* cryptic species D1 and *P. vulgaris* cryptic species V1, while New York and Connecticut states shared the D10 and V3. Thus, long-distance intra-continental dispersal and colonization are responsible for depressing the geographic-genetic correlation in the present study.

Interestingly, GAM analysis indicated that genetic distance suddenly increased when the geographic distance was extremely high. Since the between continent genetic distances were significantly higher than those of within continent, a stronger positive correlation between genetic and geographic distance was observed, in consideration of datasets from the two continents. Furthermore, the significant genetic difference between the trans-Pacific regions suggests gene flow limitation (Pan et al., 2016). Therefore, effects of the Pacific barrier leads to the restriction of gene flow and results in an increase in genetic distance.

The relationship between geographic distance and genetic distance may not be simply linear. Generally, declines in the IBD slope are associated with increases in the geographic scales of observation, and the IBD pattern is non-linear at small scale and large scale geographic distances (Bradbury and Bentzen, 2007). The GLM analysis of the *P. dolichoptera* complex showed that genetic distance was significantly related to geographic distance, and the power-law model fitted the data better than the exponential and linear models. GAM models showed that genetic distance increases rapidly with geographical distance on small geographic scales because of the coexistence and habitat heterogeneity among lakes, streams, and rivers. However, as the geographic distance extends to an entire continent, long-distance colonization leads to the decrease of the genetic distance. Extreme barriers to dispersal, such as separate oceanographic basins, lead to an increase in genetic distance at larger geographic scales. As a power-law model is expected when there is no dispersal limitation (Gómez-Rodríguez et al., 2020), outliers above the correlation line between geographic distance and genetic distance might suggest a significant dispersal barrier on large geographic scales.

4.4 Key factors for phylogeographical patterns of rotifers

CCA analysis indicated that spatial variables including longitude, latitude and altitude were key factors in controlling the cryptic species structure rather than environmental factors such as temperature and Chlorophyll-*a*. For most microeukaryotic communities, both abundant and rare communities exhibited a stronger response to environmental factors than spatial factors (Zhang et al., 2018a). But for populations, genetic differentiation depend largely upon the evolutionary force regulating spatial patterns rather than seasonal differentiation (Xiang et al., 2011 ; Obertegger et al., 2015). It was reported that although cryptic diversity of *P. dolichoptera* changed along an altitudinal gradient in the Trentino–South Tyrol region, environmental parameters such as temperature and trophic status might also affect the distribution of cryptic species (Obertegger et al., 2015). However, in the absence of geographical barriers, genetic divergence might be more explained by environmental gradients (Tisthammer et al., 2020).

The present study compared populations with similar food resource levels (Chl-*a*) at different sites and we found that the haplotypes belonged to different clades. Therefore, food sources level might not be an influencing factor for their genetic divergence. Dispersal, genetic diversity and gene flow can be strongly affected by temperature changes (Sasaki and Dam, 2019). Although the ambient temperature of our samples ranged from 8 to 30°C (Table 1), *Polyarthra* rotifers in eastern North America experience greater interannual changes in temperature. As cytochrome c oxidase subunit I is the terminal enzyme of the mitochondrial respiratory chain (Afkhami et al., 2020), extreme low temperature in winter may lead to differences in mitochondrial functions. Thus mutations may also exist in the coding sequence for COI.

Rotifers possess the ability for passive long-distance dispersal through their diapausing stages including resting eggs and xerosomes (Walsh et al., 2017). In this way, hydrology influencing community composition and wind influencing dispersal could also play an important role in rotifers dispersal (Rivas et al., 2018 ; Liang et al., 2019). In addition, human-mediated transport has likely facilitated species' persistence since its initial colonization, through the ongoing introduction and inter-continental spread of genetic variation (Baird et al., 2020). As boating is one of the popular recreational activities for Americans in summer, rotifers and resting eggs can spread over North American lakes by launching boats. However, wind and migratory bird-mediated transport, which could operate on larger scales than this are impeded by the oceanographic barriers.

Conclusion

1. Cryptic diversities of *Polyarthra dolichoptera* and *P. vulgaris* are definitely underestimated in the world.
2. The divergence of *P. dolichoptera* and *P. vulgaris* complexes indicates that gene flow between eastern North America and Southeastern China is limited while that within eastern North America or Southeastern China was higher.
3. Genetic distance and geographic distance do not show a simple linear relationship and the power-law model fitted the data of *P. dolichoptera* better than the exponential and linear models. This may result from the effects of habitat heterogeneity, long-distance colonization and oceanographic barriers to dispersal.
4. Spatial variables are key factors in affecting the genetic differentiation of rotifers when compared with physicochemical variables on the intercontinental scale.
5. Outliers above the correlation line between geographic distance and genetic distance might suggest a significant dispersal barrier on large geographic scales studies.

Declarations

Acknowledgement

We gratefully acknowledge Dr. Susan Smith from the University of Connecticut for assistance with DNA extractions and PCR. Many thanks to Lingjie Zhou and Xiaotong Ye for samples assistance and sequencing.

Declarations

We thank National Natural Science Foundation of China (41673080) and Jinan University for financial support. The study complies with current ethical guidelines. The data and materials are available. The COI sequences lacked internal stop codons of this study have been submitted to the NCBI database. We also assert that all of the listed authors have participated in the work and have approved the submission. Yang Y. conceived and designed the research. Lin S. guided the writing direction and revision of the manuscript. Liang D. carried out the experiment, analysis and wrote the manuscript. McManus G. revised the manuscript. Wang Q.

and Sun X. provided scientific comments to the manuscript. Liu Z. participated in the R code writing. All authors have no conflict of interest associated with this work.

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Figures

Figure 1

Location of the sampling sites in Southern China and eastern North America. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

The Bayesian phylogenetic tree of the *P. dolichoptera* complex based on 108 COI gene sequences. Posterior probabilities (> 0.5) from Bayesian reconstruction are shown at each node. Putative cryptic species detected using Automatic Barcoding Gap Discovery (ABGD), Generalized Mixed Yule Coalescent models (GMYC) and Poisson Tree Process (PTP) are shown. Red branch, individuals from Southeastern China; Blue branch, individuals from eastern North America; Abbreviation: D, *P. dolichoptera* cryptic species; V, *P. vulgaris* cryptic species, which was included as the outgroup.

Figure 3

The Bayesian phylogenetic tree of the *P. vulgaris* complex based on 64 COI gene sequences. Posterior probabilities (> 0.5) from Bayesian reconstruction are shown at each node. Putative cryptic species detected using Automatic Barcoding Gap Discovery (ABGD), Generalized Mixed Yule Coalescent models (GMYC) and Poisson Tree Process (PTP) are shown. Red branch, individuals from Southeastern China; Blue branch, individuals from eastern North America; Abbreviation: V, *P. vulgaris* cryptic species; D, *P. dolichoptera* cryptic species, which was included as the outgroup.

Figure 4

Boxplot of the distribution of range size of distances in kilometers for three levels of analysis: haplotype, cryptic species and the morphological species (*P. dolichoptera* and *P. vulgaris*). Differences were detected with the TukeyHSD method. Letters indicate sample means that are similar (same letter) or significantly different (different letter).

Figure 5

Relationships between pairwise genetic distance and geographic distance of the *P. dolichoptera* complex (A) and *P. vulgaris* complex (B); Black line, Linear regression; Blue line, smooth function of generalized additive model (GAM);

Figure 6

The ranges of pairwise genetic distances among the three groups of *P. dolichoptera* (A) and *P. vulgaris* complexes (B). Differences were detected with TurkeyHSD method. Letters indicate sample means that are similar (same letter) or significantly different (different letter)

Figure 7

Canonical Correlation Analysis (CCA) of the *P. dolichoptera* and *P. vulgaris* cryptic species with environmental variables. Relationship between sampling sites and environmental variables (A). Relationship between cryptic species and environmental variables (B). Red, samples and cryptic species from Southern China; Blue, samples and cryptic species from eastern North America. Abbreviations used in the figures: V, *P. vulgaris* cryptic species; D, *P. dolichoptera* cryptic species; 3_liuye, in Lake Liuye in March; 6_liuye, in Lake Liuye in June; 9_liuye, in Lake Liuye in September; 12_liuye, in Lake Liuye in December; 9_chuanzi2, in the Chuanzi River in September; 12_chuanzi2, in the Chuanzi River in December;

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