Lineage diversity, morphological and genetic divergence in *Daphnia magna* (Crustacea) among Chinese lakes at different altitudes

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Abstract

The biogeography and genetic structure of aquatic zooplankton populations remains understudied in the Eastern Palearctic, especially the Qinghai-Tibetan Plateau. Here, we explored the population-genetic diversity and structure of the cladoceran waterflea *Daphnia magna* found in eight (out of 303 investigated) waterbodies across China. The three Tibetan *D. magna* populations were detected within a small geographical area, suggesting these populations have expanded from refugia. We detected two divergent mitochondrial lineages of *D. magna* in China: one was restricted to the Qinghai-Tibetan Plateau and the

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other was present in lowland China. Several different haplotypes in the Qinghai-Tibetan Plateau were most similar to those from various parts of Siberia, suggesting that as a source region. We also found substantial genetic differentiation between *D. magna* populations from the Qinghai-Tibetan Plateau and those from lowland China. Moreover, significant morphological differences were identified: *D. magna* from the Qinghai-Tibetan Plateau had a larger head length, body length and body width than did those from lowland China. Geographical and environmental factors were correlated with the observed morphological variation and genetic divergence of *D. magna* in China. Our data offer an insight into the divergence of freshwater zooplankton due to the uplift of the Qinghai-Tibetan Plateau.

Keywords

Daphnia magna – genetic divergence – refugia – the Qinghai-Tibetan Plateau

Introduction

Freshwater invertebrates have frequently been studied to investigate biogeographical principles. Of particular interest has been the genetic diversity across their geographical range, which is often very wide (Taylor et al., 1998) as a consequence of efficient dispersal mechanisms (e.g., Havel & Shurin, 2004; Mayr, 1963). Indeed, many freshwater invertebrate species were assumed in the past to be cosmopolitan because of morphological similarities of specimens inhabiting different continents (Mayr, 1963). However, in-depth morphological analyses, especially when integrated with molecular data, have identified the diagnostic characters of specific lineages and thus led to recognition and description of new species (e.g., Juracka et al., 2010; Kotov et al., 2006; Zuykova et al., 2018b). The application of molecular tools has also demonstrated substantial genetic divergence among populations of freshwater zooplankton taxa (e.g., Rotifera and Cladocera) not only at global (e.g., Petrussek et al., 2004; Xu et al., 2009), but also at regional scale (e.g., Gomez et al., 2000; Ni et al., 2019; Penton et al., 2004).

The many species in the cladoceran genus *Daphnia* Müller, 1785 (Anomopoda: Daphniidae) are among the most important com-

ponents of freshwater ecosystems, being key grazers of phytoplankton as well as main prey for planktivorous fish (Lampert, 2011). *Daphnia magna* Straus, 1820 has been frequently subjected to ecotoxicological, ecological, evolutionary, biogeographical and physiological studies (e.g., De Gelas & De Meester, 2005; Koussoroplis et al., 2019; Lee et al., 2019; Seyoum & Pradhan, 2019). This species is widely distributed, being detected in Africa, Asia, Europe and North America (e.g., Bekker et al., 2018; Brooks, 1957; Fields et al., 2015; Xu et al., 2018). Moderate to high levels of genetic differentiation within *D. magna* are often found between different continents and within continents (e.g., De Gelas & De Meester, 2005; Fields et al., 2015). For example, moderate overall genetic divergence (based on a mitochondrial gene marker) was detected across the European range, and high genetic differentiation was even found on a local scale (De Gelas & De Meester, 2005). By using restriction-site associated DNA sequencing, a clear spatial genetic structure of *D. magna* was apparent across its Eurasian range, suggesting a geographical distance component in the genetic differentiation of *D. magna* on a continental scale (Fields et al., 2015). Applying synonymous substitutions and microsatellites as genetic markers, higher levels of divergence

were found among *D. magna* populations from northern Eurasia relative to those from southern/central Eurasia (Walser & Haag, 2012). A more recent study explored a deep split between East Asian and Western Eurasian *D. magna* lineages by using the whole mitochondrial genomes (Fields et al., 2018).

In China, *D. magna* has been found in the Eastern Plain, the Inner Mongolia-Xinjiang Plateau, the Northeastern Plain and the Qinghai-Tibetan Plateau in the 1970s (Chiang & Du, 1979). This distribution suggests that *D. magna* can occupy a wide range of habitats, from relatively high-altitude locations in the west (the Qinghai-Tibetan Plateau) to relatively low-altitude regions in the east (lowland China). The Qinghai-Tibetan Plateau, in particular, has a special environment: extremely low temperatures and strong ultraviolet radiation (Clewing et al., 2016; Niu et al., 2019). This unique environment should lead to the adaptive divergence of local species (Favre et al., 2015; Hoorn et al., 2013). A recent study using DNA barcoding evaluated the species diversity of *Daphnia* from the Qinghai-Tibetan Plateau in China and identified six species (or species complexes), including *D. tibetana* Sars, 1903, *D. longispina* species complex, *D. magna*, *D. pulex* species complex, *D. cf. himalaya* Manca, 2006 and *D. similoides* Hudec, 1991 (Xu et al., 2018). Most recently, by applying a set of high-resolution microsatellite makers, we found that *D. sinensis* Gu, 2013 populations from Eastern China and the Qinghai-Tibetan Plateau of Western China were genetically separated from each other (Ma et al., 2019a). However, there have been no specific studies on genetic diversity or population structure of *D. magna* from the Qinghai-Tibetan Plateau, or even from China overall.

In this study, we analyzed *D. magna* populations found in eight out of 303 waterbodies sampled across China. These populations differ in their types of habitat, from high-altitude populations in the west (the Qinghai-Tibetan

Plateau) to the relatively low-altitude populations in the east (here collectively termed lowland China). First, we investigated morphological variation of *D. magna* populations from the Qinghai-Tibetan Plateau relative to populations from lowland China. Then, we used the mitochondrial COI marker and a set of nuclear microsatellite loci to investigate the genetic diversity and structure of *D. magna* populations from China. We expected to detect substantial genetic divergence between *D. magna* populations from the Qinghai-Tibetan Plateau and those from lowland China, given the significant differences in geographical and environmental factors between these regions.

Materials and methods

Sample collection

Daphnia magna samples were recovered from eight of 303 waterbodies across China from 2012 to 2018 (during the growing seasons of *Daphnia*), using a 125- μ m plankton net hauled vertically at two or three sites per locality, from a boat or from the shore. Samples from the same waterbody were pooled and preserved in 95% ethanol. All *D. magna* specimens were identified morphologically (Benzie, 2005; Chiang & Du, 1979). The eight waterbodies containing this species were two natural lakes, one artificial reservoir and five ponds, representing five main geographical regions of China: Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain, Yunnan-Guizhou Plateau and Qinghai-Tibetan Plateau (table 1 and fig. 1A). For each locality, the following information was collected: geographical position (latitude and longitude), altitude, habitat origin (natural or artificial), surface area, maximum depth, predators (presence or absence of fish) and whether the waterbody freezes in winter (this information was obtained from local residents). Additionally,

the trophic status (eutrophic, mesotrophic or oligotrophic) was assigned based on Chinese literature or information from local environmental protection bureaus (table 1).

Morphometric analyses

The morphological characters of adult female *D. magna*, including head length (defined as the distance between the upper edge of the head and the lower edge of the head, HL), body width (defined as the distance between the left edge of the body and the right edge of the body, BW) and body length (defined as the distance between the lower edge of the head and the base of the tailspine, BL; supplementary fig. S1), were recorded. Five to 9 adult female *D. magna* per population were randomly selected for the morphological analyses. The ratios of head length to body length (HL/BL) and body length to body width (BL/BW) were additionally calculated to compensate for size-dependent variations in head length, body width and body length. Comparisons of these measurements between populations were done using one-way ANOVA in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). A *t*-test was performed to evaluate the differences in *D. magna* populations from the Qinghai-Tibetan Plateau versus those from lowland China (combined populations from Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain and Yunnan-Guizhou Plateau), in GraphPad Prism 5. If data did not follow a normal distribution, a Rankit transformation was applied (Conover & Iman, 1981).

DNA extraction

Between 15 and 46 adult females of *D. magna* per population were randomly selected for the genetic analyses. DNA was extracted from each specimen using 60 µL H3 buffer (Schwenk et al., 1998) with proteinase K (10 mg/ml; MERCK, Germany), containing 10 mM Tris-HCl, 0.05 M KCl, 0.005% Tween 20 and

0.005% NP-40. Individuals were incubated at 55°C for 10–16 hours in a water-bath with mild shaking. The proteinase K was then denatured via a 12 min incubation at 95°C, the tube centrifuged briefly and stored at 4°C before use.

mtDNA sequencing

Six to ten individuals per locality were selected for mitochondrial DNA analysis (i.e., 75 out of 289 individuals; table 1). The mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using standard primer pairs LCO1490 and HCO2918 (Folmer et al., 1994). The PCR reaction was performed in a total volume of 30 µL, consisting of 3 µL of genomic DNA, 0.5 µM of each primer, 2.5 mM of each dNTP, 10 mM of buffer, and 2 units of Taq HS (TaKaRa, Japan). The PCR protocol was as follows: incubation at 94°C for 1 min, followed by 40 cycles of 1 min at 94°C, 1.5 min at 40°C and 1.5 min at 72°C. This was followed by a final incubation at 72°C for 6 min. The PCR products were then purified and sequenced using the forward primer on an ABI PRISM 3730 DNA capillary sequencer at BGI Tech. (China). Only chromatograms with high quality sequences (Phred quality scores > 40) were used for the genetic analysis. All newly obtained COI sequences from the present study have been submitted to GenBank under accession numbers MN310895–MN310900.

Phylogeny and divergence time estimation

All COI sequences were aligned together with all 655 published COI sequences of *D. magna* using Clustal W (Thompson et al., 1994) in MEGA 6.0 (Tamura et al., 2013). Published sequences, retrieved from GenBank, were from *D. magna* populations in Asia, Europe, North Africa and North America (supplementary table S1). Twenty published reference sequences were excluded because of their extremely short length. A sequence from a member of the *D. similis* group was used as an outgroup.

TABLE 1 List of waterbodies in which *Daphnia magna* was detected (name, abbreviation, geographical position and environmental descriptors; see fig. 1 for their geographical position) and basic genetic characteristics of analyzed individuals.

Lake (abbreviation)	Longitude, latitude	Sampling period	Origin	Altitude (m)	Surface area (km ²)	Maximum depth (m)	Trophy	Freezes in winter	Predator	N ₁	N ₂	COI haplotype	N ₃	N ₄	MLG*	R	H _o	H _e	HWE
Eastern Plain																			
Linzhihe (LZH)	118.02E, 36.13N	2014 (Autumn)	Artificial	119	2	0.5	Mesotrophic	yes	no	10	1	HSA	38	38	29	0.76	0.47	0.4	ns
Northern Plain																			
Dongjiatunpao (DJT)	124.9E, 46.48N	2016 (Summer)	Artificial	50	3	1	Mesotrophic	yes	no	10	2	HSB, HSC	39	34	34	1	0.48	0.58	***
Daqinfujin (DQF)	124.9E, 46.3N	2016 (Summer)	Artificial	144	1	0.5	Mesotrophic	yes	no	9	3	HSA, HSB, HSC	37	36	34	0.94	0.52	0.58	***
Inner Mongolia-Xinjiang Plateau																			
Daihai (DAH)	112.7E, 40.58N	2013 (Summer)	Natural	1222	160	15	Mesotrophic	yes	yes	10	3	DAHA, HSA, HSB	37	24	20	0.83	0.39	0.54	***
Yunnan-Guizhou Plateau																			
Dianchi (DIC)	102.38E, 24.45N	2014 (Spring)	Natural	1888	48.4	8	Eutrophic	no	yes	10	1	HSB	39	29	28	0.96	0.43	0.53	***
Qinghai-Tibetan Plateau																			
Dongla z Pond (DLzP)	90.85E, 29N	2017 (Summer)	Artificial	4457	6	0.3	Oligotrophic	yes	no	10	1	HSD	15	10	10	1	0.37	0.39	*
Niulanghu (NLH)	90.45E, 28.78N	2017 (Summer)	Artificial	4437	3	1	Oligotrophic	yes	no	10	1	NLHA	46	44	42	0.95	0.46	0.38	***
Quguzhong (QGZ)	91.08E, 28.83N	2017 (Summer)	Artificial	4426	4	0.4	Oligotrophic	yes	no	6	1	HSD	38	21	21	1	0.49	0.44	ns

N₁: number of sequenced individuals for COI; N₂: number of haplotypes for COI; N₃: number of individuals genotyped at up to 11 microsatellite loci; N₄: number of individuals genotyped at all 11 microsatellite loci; MLG*: number of unique multilocus genotypes at all 11 microsatellite loci; R: relative clonal richness; H_o: observed heterozygosity; H_e: expected heterozygosity; HWE, Hardy-Weinberg equilibrium; * $P < 0.05$, *** $P < 0.001$, ns, not significant.

(A): Sampling localities for *Daphnia magna* in China. (B): Bayesian phylogenetic tree and lineage delimitation results for *D. magna*, based on the mitochondrial COI gene (413 bp). A single representative of each haplotype (including all haplotypes represented in GenBank) is included in the tree. Codes of *D. magna* individuals from this study are provided in table 1; for origin of reference sequences' IDs see supplementary table S1. Only posterior probabilities >0.70 are shown. The lineage IDs are shown in circles. Abbreviations of country names are, AM: Armenia, BE: Belgium, CA: Canada, CN: China, CS: Czech, DE: Germany, DK: Denmark, ES: Spain, FI: Finland, HU: Hungary, IL: Israel, IT: Italy, JP: Japan, LU: Luxembourg, MA: Morocco, MX: Mexico, MN: Mongolia, NO: Norway, PL: Poland, PT: Portugal, RU: Russia, SE: Sweden, TR: Turkey, UK: United Kingdom, US: United States. *Daphnia similis* was used as an outgroup. (C): Results from a Bayesian assignment analysis (STRUCTURE) of microsatellite data for all eight *D. magna* populations (the best K was 2). For lake abbreviations see table 1.

(GenBank ID: LC389171). The alignment was visually inspected and all the sequences were trimmed to the same length (413 bp). Unique haplotypes were identified in DNASP 5.10 (Librado & Rozas, 2009), and one representative of each was used in phylogenetic analysis. The COI phylogenetic tree was constructed using the Bayesian method in BEAST 2 (Bouckaert et al., 2014), run for 10^7 generations and a tree recorded every 1,000 generations. Twenty-five percent of trees were discarded as burn-in, and the final 10^4 trees summarized with Tree-Annotator. Here, HKY+G+I was estimated to be the best-fit substitution model according to the corrected Akaike information criterion (AIC), using jModelTest v. 2.1.7 (Darriba et al., 2012). The tree priors were left at their default values and a strict molecular clock was applied. To ensure that enough generations were computed, Tracer v1.7 (Rambaut et al., 2018) was used. The fossil-based calibration point for the most-recent common ancestor was set at 100 Mya and a 10% standard deviation was assigned. This calibration point was estimated according to the fossil evidence for *D. magna* and the *D. similis* group (Cornetti et al., 2019).

Detection of genetic lineages and phylogeographic analyses

The general mixed Yule coalescent model (GMYC, Pons et al., 2006) was applied to the COI phylogenetic tree to identify different lineages within *D. magna*. The GMYC model, which is a likelihood-based method, uses an ultrametric tree to delimit different species/lineages, and within- or between-species branching models were fitted to reconstruct gene trees. The GMYC modeling was performed in R 2.15 (R Development Core Team, 2009) using the “splits” package (Ezard et al., 2009). To evaluate genealogical relationships among *D. magna* clade “B”, a COI haplotype network for *D. magna* was constructed using

HAPLOVIEWER (Salzburger et al., 2011). This network comprised haplotypes of clade “B” representing all relevant world regions: China, Japan, Northern Eastern Siberia, Southern Eastern Siberia and Mongolia, USA and Canada and Western Siberia (supplementary table S2). A maximum likelihood tree constructed in MEGA 6 using the best model (HKY+G+I; by jModeltest v. 2.1.7) was used as input. A hierarchical analysis of molecular variance (AMOVA) was conducted in Arlequin 3.11 (Excoffier et al., 2005) and the genetic variance (based on COI sequences) among *D. magna* populations was partitioned into the following components: 1) among five regions (i.e., Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain, Yunnan-Guizhou Plateau and Qinghai-Tibetan Plateau), 2) among populations within regions, and 3) among individuals within populations. To estimate genetic differentiation based on the COI marker among *D. magna* populations, pairwise F_{st} values (estimated following Weir & Cockerham, 1984) were calculated for conspecific populations (in SMOGD V. 1.2.5, Crawford, 2010). By bootstrapping with 10,000 replicates, 95% confidence intervals were established, and the significance of pairwise F_{st} values was examined based on 10,000 permutations. The correlation between pairwise geographical distances among populations and pairwise F_{st} values was calculated using a Mantel test (10^4 permutations, using the Isolation by Distance Web Service, version 3.15, Jensen et al., 2005).

Microsatellite genotyping

Fifteen to 46 randomly selected adult *D. magna* females from each population were genotyped at 11 microsatellite loci (Bastiaan et al., 2011). These loci were amplified in five PCRs, two of them multiplexes (MP1: locus B107; MP2: B031, B075, B088, B135 and B155; MP3: A001; MP4: A002, B033 and B180; MP5: B081)

using the Multiplex PCR Kit (Qiagen, Germany). The PCR cycling protocol used for amplification was as follows: incubation at 95°C for 15 min, then 30 cycles of 30 s at 94°C, 1.5 min at 54°C or 56°C and 1.5 min at 72°C. This was followed by a final incubation for 6 min at 72°C. PCR products were then separated on an ABI PRISM 3730 DNA capillary sequencer (Applied Biosystems), using the LIZ 500 labeled size standard for fragment analysis. Genotypes were scored using GeneMapper version 4.0 (Applied Biosystems), and all alleles were checked manually and defined by the size of the fragments in base-pairs. Only specimens with missing data at no more than five microsatellite loci were included, resulting in a total of 289 individuals included in further analyses (table 1). As this microsatellite panel was applied to Chinese *D. magna* populations for the first time, genotyping errors (due to stuttering, large allele dropout, or presence of null alleles) were assessed in Micro-Checker 2.3.3 (Van Oosterhout et al., 2004).

Genetic diversity

Only individuals with a complete multilocus genotype (MLG) (i.e., across all 11 microsatellite loci) were used here, resulting in a final dataset of 236 individuals. To explore the level of genetic diversity, relative clonal richness R was calculated per population ($R = (G - 1) / (N - 1)$, where G is the number of MLGs and N represents sample size; Dorken & Eckert, 2001). Only populations of sample size of 10 or more were included.

Population genetic structure

The identification of MLGs, based on 11 microsatellite loci, was performed in GENALEX 6.5 (Peakall & Smouse, 2006). To distinguish genetically differentiated population groups of *D. magna*, a factorial correspondence analysis, FCA (GENETIX 4.05, Belkhir et al., 1996–2004), was performed. To identify the most likely

number of such genetic clusters, a Bayesian algorithm was applied in STRUCTURE V2.3.4 (Pritchard et al., 2000), assuming the existence of K groups. The value of K was set from 1 to 8, ten independent runs were performed for each value of K and, for each run, 10^5 iterations were carried out after a burn-in period of 10^5 iterations. The most likely K was determined by the distribution of ΔK , following the methods of Evanno et al. (2005). To display the genetic relationships among the populations, the unweighted pair-group method with arithmetic mean (UPGMA), based on pairwise Nei's genetic distances (Nei, 1978), was applied in MEGA 6.0. An AMOVA test comparable to that applied on COI sequence data (see above) was run on microsatellite data. Pairwise F_{st} values were calculated among populations based on the microsatellite data, the approach being similar to that applied to the COI sequence data. Finally, the correlation between pairwise geographical distance and pairwise F_{st} values (based on microsatellites) was calculated with and without populations from the Qinghai-Tibetan Plateau using a Mantel test.

Environmental preferences

To assess the contribution of geographical and environmental factors in explaining variation between regions (Qinghai-Tibetan Plateau and lowland China), a principal component analysis (PCA) was conducted in Past version 3.12 (Hammer et al., 2001). Next, to relate the presence or absence of *D. magna* lineages to combinations of these parameters, a PCA was calculated based on geographical position (i.e., longitude and latitude) and seven environmental factors, including altitude, maximum depth, origin (i.e., natural or artificial), predator (i.e., presence or absence of fish), surface area and trophic status (i.e., eutrophic, mesotrophic or oligotrophic) and whether or not the waterbody was frozen in

winter. Finally, to determine whether *D. magna* lineages was non-randomly distributed along each component of the PCA, a one-way analysis of variance (ANOVA) was conducted. All statistical analyses were performed using SPSS 22.0.

Results

Morphology

Significant differences in the head length, body width and body length were found between adult female *D. magna* from Qinghai-Tibetan Plateau and those from lowland China, see fig. 2. Specifically, adult female *D. magna* from the Qinghai-Tibetan Plateau were larger in all measured dimensions than those from other regions of China. However, there were no significant differences in the ratios of head length to body length and body length to body width of adult *D. magna* from the Qinghai-Tibetan Plateau and those from other regions (fig. 2). Among the populations, DJT had the shortest head length, body width and body length, whereas DL2P had the greatest head length and QGZ had the greatest body width and body length (supplementary fig. S2).

Phylogeny and divergence time estimation

Seventy-five individuals were successfully sequenced at the COI locus (413 bp in the aligned dataset); among them, six unique haplotypes were detected (table 1). The Bayesian tree together with the GMYC lineage-delimitation showed that the six Chinese haplotypes from this study fell in two different mitochondrial lineages ("B2" and "B3"), but belonged to a single group "B". Two haplotypes (i.e., HSD and NLHA), which belonged to the lineage "B2", were restricted to Qinghai-Tibetan Plateau; while the other four haplotypes (i.e., DAHA,

HSA, HSB and HSC), belonging to the lineage "B3", were present in lowland China (combined populations from Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain and Yunnan-Guizhou Plateau; fig. 1B). The most recent common ancestor of all included *D. magna* lineages based on COI was estimated to be around 17.8 Mya (95% HPD: 8.9-27.1 Mya). *Daphnia magna* from Qinghai-Tibetan Plateau and those from lowland China diverged around 6.6 Mya (95% HPD: 2.5-9.9 Mya; supplementary fig. S3).

Phylogeography

Four Chinese haplotypes (two from the present study and two from GenBank; belonging to "B2") were detected in the Qinghai-Tibetan Plateau: three were restricted to this region, and another one was present in both the Qinghai-Tibetan Plateau of China and West Siberia of Russia. Another six Chinese haplotypes (four from the present study and two from GenBank; belonging to "B3") were detected in lowland China (i.e., mix of Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain and Yunnan-Guizhou Plateau). One haplotype (HSB) was positioned in the center of the star-like haplotype network in the lineage "B3" and shared by four regions of China, including Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain, and Yunnan-Guizhou Plateau (fig. 3). No haplotype was shared between the Qinghai-Tibetan Plateau and the other regions of China.

The AMOVA test based on COI sequences revealed that the genetic variation component at the among-region level was about 10 times higher than at the within-region level (i.e., among populations), or 14 times higher than within populations (i.e., among individuals; Table 2). Pairwise F_{st} values, based on COI, ranged from 0 to 1 among *D. magna* populations. There was no significant correlation

FIGURE 2 (A) Head length, (B) Head length/Body length, (C) Body width, (D) Body length/Body width and (E) Body length of *Daphnia magna* from Qinghai-Tibetan Plateau and lowland China (combined populations from Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeast Plain and Yunnan-Guizhou Plateau). Each boxplot indicates median, interquartile range and range (minimum to maximum).

*** $P < 0.001$.

between pairwise F_{st} and geographical distance for *D. magna* based on COI ($R^2 = 0.05077$, $P = 0.249$; fig. 4A).

Genetic diversity

There was no evidence for large-allele drop-out in any of 11 microsatellite loci in this

study. Scoring error due to stuttering might have been present in 2 of the 88 analyzed population/locus cases, and null alleles may be present at loci A001, A002, B031, B075 and B107, as suggested by the general excess of homozygotes for most allele size classes. Overall, *D. magna* in China had a high clonal diversity

FIGURE 3 Haplotype network of *Daphnia magna* clade “B” mitochondrial COI sequences (413 bp). Each circle represents a unique haplotype and its size reflects the number of individuals carrying that haplotype. Color codes allow easy discrimination of geographical regions in the network. Segment sizes within circles indicate the distribution of haplotypes among different regions. Haplotypes of the same lineage are framed with dotted lines, and the lineage IDs are shown. HSB refers to the most abundant COI haplotype in China.

TABLE 2 Hierarchical analysis of molecular variance (AMOVA) for *Daphnia magna* populations, based on mtDNA and microsatellites, respectively. Among regions variation is estimated in relation to within region and within population components.

Marker type	Source of variation	DF	Explained variation (%)	P
mtDNA	Among regions	4	85.18	< 0.05
	Among populations within region	3	8.9	< 0.001
	Within population	67	5.92	< 0.001
Microsatellites	Among regions	4	23.59	< 0.001
	Among populations within region	3	17.61	< 0.001
	Within population	570	58.8	< 0.05

(average $R = 0.93$; table 1). We found the highest possible clonal richness value of 1.00 in three populations (i.e., DJT, DL2P and QGZ), indicating that each individual possessed a different MLG (table 1). Identical MLGs were detected within populations, but never between populations.

Population genetic structure

Assignment tests in STRUCTURE showed that the best estimated number of groups was $K = 2$, separating Qinghai-Tibetan Plateau and lowland China (combined populations from Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeastern Plain and Yunnan-Guizhou Plateau; fig. 1C). One has to be cautious in cases with a best $K = 2$ (Janes et al., 2017). However, specifying other values of K (i.e., $K = 3$ to $K = 8$) did not yield biologically reasonable results (i.e., to cluster the populations from lowland China; supplementary fig. S4), as evident from the FCA (as shown in fig. 5). Individuals that were genotyped at up to 11 microsatellite loci were obviously separated by the first FCA axis: one group restricted to the Qinghai-Tibetan Plateau and the other group detected in the remaining four regions (fig. 5). The FCA results were further supported by the UPGMA tree based on genetic distances, in which *D. magna* populations from the Qinghai-Tibetan Plateau were distinctly separated from those in other regions (supplementary fig. S5). The geographical separation of *D. magna* populations was further supported by AMOVA: although most of the variation component was within-populations (58.80%), values for among-region (23.59%) and within-regions (17.61%) components were also significant (table 2). Pairwise F_{st} values ranged from 0.14 to 0.54 among *D. magna* populations (averaged over all loci) based on microsatellite data. There was a significant relationship between pairwise F_{st} and geographical distance for *D. magna* based on microsatellite markers

FIGURE 4 Linear regression of pairwise geographical distances (kilometers) versus genetic distances (F_{st}): (A) based on mtDNA, (B) based on 11 microsatellites with all populations included and (C) based on 11 microsatellites after excluding populations from the Qinghai-Tibetan Plateau, among eight *Daphnia magna* populations from China.

FIGURE 5 Factorial correspondence analysis (FCA) of *Daphnia magna* populations based on 11 microsatellite loci. For abbreviations of lakes see table 1.

FIGURE 6 The PCA plot of *Daphnia magna*, showing the relationship between distribution of *D. magna* lineages and environmental parameters. Purple dots represent lakes inhabited by *D. magna* of Qinghai-Tibetan Plateau; blue dots represent lakes inhabited by *D. magna* from lowland China (combined populations from Eastern Plain, Inner Mongolia-Xinjiang Plateau, Northeast Plain and Yunnan-Guizhou Plateau).

($R^2 = 0.1822$, $P = 0.0235$; fig. 4B) when all populations were included. However, after excluding populations from the Qinghai-Tibetan Plateau, there was no significant correlation ($R^2 = 0.07527$, $P = 0.4430$; fig. 4C).

Environmental preferences

The first and second components of the PCA explained 51.23% and 32.37%, respectively, of the variability in geographical and environmental factors (fig. 6). Overall, lakes from different regions (i.e., Qinghai-Tibetan Plateau versus the remaining four regions in China) inhabited by *D. magna* were characterized by components which have been loaded on these two PCA axes. Thus, lakes from the Qinghai-Tibetan Plateau were at a lower longitude ($df = 1$, $F = 20.93$, $P = 0.004$), higher altitude ($df = 1$, $F = 57.65$, $P < 0.001$) and a lower trophic status ($df = 1$, $F = 20.25$, $P < 0.001$) than those elsewhere. In contrast, neither lake latitude ($df = 1$, $F = 3.34$, $P = 0.117$), maximum depth ($df = 1$, $F = 1.33$, $P = 0.292$), origin ($df = 1$, $F = 2.81$, $P = 0.145$), presence or absence of predators ($df = 1$, $F = 1.50$, $P = 0.267$), surface area ($df = 1$, $F = 0.89$, $P = 0.382$), nor winter freezing ($df = 1$, $F = 0.56$, $P = 0.482$; loadings on PC2) had any influence on the presence or absence of *D. magna* lineages (fig. 6).

Discussion

In the present study, two divergent mitochondrial lineages of *D. magna* were detected in China. Specifically, one was restricted to the Qinghai-Tibetan Plateau while the other was present in lowland China. Similarly, microsatellite data demonstrated substantial population-genetic differentiation between *D. magna* populations from the Qinghai-Tibetan Plateau and those from the lowlands of China. Additionally, a significant morphological difference was found in *D. magna* populations:

individuals from the Qinghai-Tibetan Plateau had a larger body size than did those from lowland China. Such morphological and genetic differences between *D. magna* populations from the Qinghai-Tibetan Plateau and those from the lowlands were attributed to differences in geographical and environmental factors, e.g., longitude, altitude and trophic status of lake occupied.

Phenotypic plasticity, such as predator-induced phenotypic changes (e.g., Rabus & Laforsch, 2011; Tollrian, 1995), have been observed in numerous *Daphnia* species. For example, *Daphnia* reproduce at smaller or larger sizes in response to threats from visually hunting (e.g., fish) or gap-limited (e.g., small invertebrates) predators, respectively (Boersma et al., 1998; Riessen, 1999). Temperature can also significantly affect the body shape of *D. magna*, *D. pulex* and *D. longispina* Muller, 1776 (Ranta et al., 1993). Therefore, the morphological variation we observed between *Daphnia* populations from the Qinghai-Tibetan Plateau and those from lowland China might be due to phenotypic plasticity in response to the different environmental conditions, such as temperature and predator pressures. Alternatively, the different morphology of high altitude *D. magna* populations, when compared with those from lowland China, might be due to the unique geographical and environmental conditions there (e.g., Chen et al., 2013; Lin et al., 2008). Individual *D. magna* with longer heads, wider and longer bodies are more likely to survive and reproduce in the harsher environments (i.e., extremely low temperature and strong ultraviolet radiation) of the Qinghai-Tibetan Plateau. Indeed, the frog *Rana kukunoris* Xie, 2000 in the Qinghai-Tibetan Plateau produces larger eggs than do those from the lowlands, which allows for increased rates of embryonic development leading to earlier hatching of tadpoles (Chen et al., 2013). The larger females of

R. kukunoris in high-altitude habitats produce more gelatinous matrix surrounding the eggs, which could protect embryos from temperature fluctuations and ultraviolet radiation (Chen et al., 2013).

In agreement with a recent study (Bekker et al., 2018), our data suggested that the included clades of *D. magna* diverged long ago. Both studies used a calibration point estimated based on fossil evidence for *Daphnia* (Kotov & Taylor, 2011). However, this divergence date differs significantly from findings by Fields et al. (2018). The latter authors applied a fully informative Gaussian prior to substitutions at the third codon position of four-fold degenerate codons. The substantial differences in the estimated coalescence time of *D. magna* clades is due to the different time calibrations used. Appropriate paleontological records will be required to resolve these differences.

Highly divergent lineages within species are often observed in Cladocera (e.g., Bekker et al., 2018; Ni et al., 2019; Woltereck, 1920). For example, using mitochondrial and nuclear genetic markers, multiple lineages were detected within *Moina* species across China (Ni et al., 2019). Another study suggested that dark-colored populations of *D. pulicaria* Forbes, 1893 from the Alps had at least two genetically divergent lineages: one genetically close to boreal haplotypes and another related to refugial populations that survived in southern Europe (Bellati et al., 2014). We found two divergent mitochondrial lineages of *D. magna* in China with a clear geographical separation: one was restricted to the Qinghai-Tibetan Plateau while the other was present in lowland China. Such geographically distinct phylogroups (based on part or all of the mitochondrial genome) within *D. magna* were also observed in Europe (De Gelas & De Meester, 2005), in Northern Eurasia (Bekker et al., 2018) and even between

East Asia and Western Eurasia (Fields et al., 2018). The observed phylogenetic structure of *D. magna* could be explained by strong founder effects that are persistent through time (De Meester et al., 2002), by genetic drift (De Gelas & De Meester, 2005), or local adaptation (Boersma et al., 1999). Indeed, a strong founder effect has been recently demonstrated in high-altitude *D. longispina* populations from the Altai and Sayan Mountains (Zuykova et al., 2019).

By applying three independent statistical approaches (i.e., FCA, UPGMA and STRUCTURE) to microsatellite data, we discovered a clear geographical separation between *D. magna* populations from the Qinghai-Tibetan Plateau and those from lowland China. The geographical distance and differences in ecology and environment might explain the genetic differences. Specifically, lowland *D. magna* mostly occur at lower altitudes in eutrophic lakes, whereas *D. magna* populations in the Qinghai-Tibetan Plateau are found at higher altitudes in oligotrophic lakes. Many studies have shown substantial genetic divergence in plant and animal populations between lowland China and the Qinghai-Tibetan Plateau (reviewed in Qiu et al., 2011; Yang et al., 2009). The rapid uplift of the Qinghai-Tibetan Plateau must have had profound effects on the evolution of resident populations and created challenging environments for immigrants, especially for freshwater zooplankton. Altitudinal gradients are characterized by steep changes in physical factors, such as temperature, oxygen concentration and UV radiation, inducing strong diversifying selection. For example, the saker falcon *Falco cherrug* Gray, 1834 shows obvious genetic divergence between populations from the Qinghai-Tibetan Plateau and those inhabiting grasslands with a lower altitude (Pan et al., 2017). Hypoxia and low temperatures were believed to be the main forces driving such genetic divergence

(Pan et al., 2017). Interestingly, we only detected eight *D. magna* populations from a large number of lakes (303) sampled across China, and the three Tibetan *D. magna* populations were detected within a small geographical area from 92 lakes we sampled from the Qinghai-Tibetan Plateau. It could be that these populations on the Qinghai-Tibetan Plateau have expanded from Pleistocene refugia and have successfully recolonized the area following the last glacial maximum. Moreover, several different haplotypes were found in the Qinghai-Tibetan Plateau, and they were most similar to those from various parts of Siberia, suggesting that as a source region. However, more intensive sampling in the Qinghai-Tibetan Plateau will be required to address this issue in the future.

Interestingly, there was a significant association between genetic distance and geographical distance for *D. magna* populations from China. A similar result was found by Fields et al. (2015), who applied RAD sequencing to a large sample of *D. magna* from Eurasia. But this was not the case in the central/southern Eurasian *D. magna* populations (Walser & Haag, 2012). In addition to isolation-by-distance, ecological differences among habitats, such as differences in trophic status, could also affect the genetic composition of the *Daphnia* assemblages (Keller et al., 2008). Therefore, a pattern of isolation-by-distance will not always be a rule, because of the selective constraints. Here, one haplotype (HSB) was shared by *D. magna* populations in all regions except the Qinghai-Tibetan Plateau. This haplotype was also present in Northeastern Siberia, Southeastern Siberia and Mongolia, being the center of the star-like network of lineage “B3”. This star-like network pattern suggests that recent dispersal and colonization events of *D. magna* have occurred in Asia, and that haplotype HSB was ancestral in the lineage “B3”. Similar evidence

of rapid expansion has also been observed in other *Daphnia* species in Asia, such as *D. mitsukuri* Ishikawa, 1896 (Ma et al., 2019b) and *D. galeata* Sars, 1864 (Ma et al., 2015). In agreement with previous observations in other *Daphnia* species, for example, *D. galeata* (Thielsch et al., 2009; Yin et al., 2018) and *D. mitsukuri* (Ma et al., 2019b), we found that *D. magna* populations, regardless of their origin, had high relative clonal richness values (average $R = 0.93$), suggesting frequent sexual reproduction. However, high genetic diversity is not always a rule in *Daphnia*, as different species have different demographic histories (e.g., Zuykova et al., 2018a).

In conclusion, we detected substantial genetic divergence (at both mitochondrial DNA and microsatellite markers) and morphological variation between *D. magna* populations from the Qinghai-Tibetan Plateau and those from lowland China. However, this result needs to be interpreted with caution because the small number of populations and microsatellite loci in this study might limit the power of analysis. Geographical and environmental factors (e.g., altitude and trophic status) were likely to have contributed to this. Our study calls for future investigations on the local adaption of zooplankton to the special habitats in the Qinghai-Tibetan Plateau, especially at the genome level.

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interpreted the data. MY and YN wrote the manuscript with the help of XM. All authors read and approved the final version. The authors declare no conflicts of interest.

Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.12248306>

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