Ann. Limnol. - Int. J. Lim. 53 (2017) 401–410
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DOI: 10.1051/limn/2017024

Available online at:
www.limnology-journal.org

RESEARCH ARTICLE

Molecular phylogeny and population genetic differentiation patterns in *Brachionus calyciflorus* Pallas (Rotifera) complex from two lakes in China

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Received: 9 February 2017; Received in final form: 15 September 2017; Accepted: 22 September 2017

Abstract – The spatio-temporal patterns of population genetic structures have been attracting increasing attention of late. Few of these efforts, however, have focused on the underlying mechanisms of the seasonal alterations of in rotifer clone groups. To gain insights into the seasonal variation patterns of genetic differentiation in rotifer populations, 158 clones of the *Brachionus calyciflorus* Pallas species complex, with seasonal sampling from Guangzhou and Wuhu in China, were sequenced and analyzed based on the nuclear internal transcribed spacer (ITS) region and the mitochondrial cytochrome c oxidase subunit I (COI) gene. DNA taxonomy provided estimates of evolving entities ranging from 3 to 21 by combining the mitochondrial and nuclear markers with the different ABGD, PTP, and GMYC models. The most conservative number of the three evolving entities was used for further phylogenetic and population structure analyses. The *B. calyciflorus* species complex was found to have extreme nucleotide diversity, revealing twice the diversity from the COI dataset than from ITS. The similar patterns of seasonal succession in *B. calyciflorus* were investigated in the two habitats of Guangzhou and Wuhu, and the clonal groups involved in seasonal succession were actually sibling or cryptic species within the *B. calyciflorus* complex. We assume that these cryptic species are specialized for their ecological niches, especially in terms of temperature preference. The spatio-temporal patterns of population genetic structure varied with cryptic species, *e.g.* cryptic species II (-COI/-ITS) had much greater genetic differentiation among sampling sites than among seasonal populations and cryptic species III (-COI/-ITS) displayed a reverse pattern.

Keywords: rotifer / *Brachionus calyciflorus* / cryptic species / seasonal succession / DNA taxonomy

1 Introduction

Understanding the spatio-temporal patterns and processes of population genetic differentiation has been a major focus of population ecology. Until now, most of the empirical research in population genetic patterns has attempted to tear apart the dynamic consequences of spatial variation. Little attention has been paid, however, to furthering the understanding of how the genetic structure of the population changes over time, since most spatial studies implicitly assume temporal genetic stability (King, 1972; Lessios *et al.*, 1994; Heath *et al.*, 2002; Arnaud and Laval, 2004; Bousset *et al.*, 2004; Lee and Boulding, 2009; Chan and Hadly, 2011). In water habitats, the progressive physical, chemical, and biological factors change with seasonal succession. Thus, many zooplankton species exposed to these changes often thrive during only a restricted period of the annual cycle (Gómez *et al.*, 1995; Ortells *et al.*, 2003; Vanderploeg *et al.*, 2012). During this process, selection and migration can also influence allele frequencies due to genetic drift and gene flow between populations (Hartl and Clark, 1989). Accordingly, habitat conditions have been proven to be important for shaping population genetic structure and demography (Charbonnel *et al.*, 2002; Bousset *et al.*, 2004).

Rotifers, a group of globally widespread microscopic Metazoa, play an important role in nutrient recycling and the transfer of energy in continental aquatic ecosystems (Bonecker and Aoyagui, 2005). Since the pioneering studies of the genetic structure of rotifer populations in the 1970s, several investigations have suggested extensive temporal variation in these populations (King, 1972, 1977; Snell, 1979; King and Serra, 1998). The two genetic models proposed by King (1972, 1977) showed that different genotypes are maximally adapted to different parts of the environmental spectrum over time, and that the temporal variation in the genetic structure of the

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Table 1. Summary information of sampling and characteristics of habitats for each seasonal population.

Population	Code	Sampling date	Sample size	Coordinate	*T*	pH	*N*	GenBank Acc. Nos. (ITS)	GenBank Acc. Nos. (COI)
Spring (Guangzhou)	GC	04-23-08	15	113°13'E 23°07'N	28	8.29	1.97	KX495887-KX495901	KX495818-KX495832
Summer (Guangzhou)	GX	07-27-07	15	113°13'E 23°07'N	33	8.02	1.50	GU012695-GU012709	FJ826905-FJ826909, FJ826925-FJ826933, FJ826984
Autumn (Guangzhou)	GQ	11-02-07	18	113°13'E 23°07'N	24	7.85	0.75	KX495869-KX495886	KX495800-KX495817
Winter (Guangzhou)	GD	01-27-08	19	113°13'E 23°07'N	13	8.04	4.70	FJ937493-FJ937511	GU232586-GU232604
Spring (Wuhu)	WC	04-22-08	13	118°22'E 31°21'N	27	8.24	1.95	KX495856-KX495868	KX495787-KX495799
Summer (Wuhu)	WX	07-25-07	24	118°22'E 31°21'N	34	7.57	1.99	GU012770-GU012793	FJ826934, FJ826952, FJ826956, FJ826964, FJ826982, FJ826986, FJ826987, FJ826989-FJ826992, FJ826994-FJ827001, FJ827003, FJ827005, FJ827006, FJ827008, FJ827009
Autumn (Wuhu)	WQ	10-28-07	23	118°22'E 31°21'N	20	7.88	0.57	KX495833-KX495855	KX495764-KX495786
Winter (Wuhu)	WD	01-25-08	31	118°22'E 31°21'N	8	8.18	2.60	FJ937590-FJ937620	GU232683-GU232713

T = water temperature (°C), *N* = NH_4-N (mg/l).

population occurs as the lake environment undergoes its seasonal changes. The existence of seasonally isolated populations has been shown in *Euchlanis dilatata* from a man-made lake in Illinois (King, 1972), *Asplanchna girodi* from a golf course pond in Florida, and *A. brightwelli* from Lake Thonotosassa in Florida (King, 1977; King and Snell, 1980), all of which lend support to the Nonoverlap Model (complete genetic discontinuity model). In addition, *Brachionus plicatilis* was found to support the Overlap Model (incomplete genetic discontinuity model) in Soda Lake in Nevada (King and Zhao, 1987; Zhao and King, 1989).

Cryptic speciation has become widely recognised in biodiversity analyses of rotifers (Ruttner-Kolisko, 1989; Knowlton, 1993), leading to the conclusion that the currently acknowledged level of rotifer biodiversity may even be an underestimate considering the occurrence of cryptic species (Segers, 2007, 2008), although many efforts have been made by taxonomists to obtain a comprehensive understanding (Pejler, 1977; Dumont, 1983; De Ridder and Segers, 1997). Previous studies have shown that the presence of cryptic species has been documented in many monogonont (well known in *Brachionus*, *Keratella*, and *Lecane*) (Ruttner-Kolisko, 1989; Gómez *et al.*, 2002; Segers, 2008; Segers and De Smet, 2008; Xiang *et al.*, 2011a, b; Mills *et al.*, 2017) and bdelloid (Fontaneto *et al.*, 2007, 2009, 2011) species complexes which have hitherto been difficult and unreliable to classify with traditional taxonomical methods, due to the intraspecific plasticity and interspecific similarity of morphological traits (Colbourne *et al.*, 1997; Serra *et al.*, 1997; Hebert, 1998). Nevertheless, the seasonal succession of sympatric cryptic rotifer species can be evaluated with temporal sampling. Gómez *et al.* (1995) provided evidence of seasonal succession among three groups of the *B. plicatilis* complex in a marsh along the Mediterranean coast of Spain. In contrast to the Overlap Model of *B. plicatilis* from Soda Lake in Nevada, this case provides strong support for the Nonoverlap Model with distinguished temporal heterogeneity. We now know that the clonal groups or genotypes involved in a seasonal succession are in fact sibling or cryptic species that are both genetically and ecologically isolated.

B. calyciflorus Pallas is a well-known complex of cryptic species (Gilbert and Walsh, 2005; Cheng *et al.*, 2008; Li *et al.*, 2008; Xiang *et al.*, 2011a, b; Papakostas *et al.*, 2016), although it has been considered to be a traditional cosmopolitan species for a long time (Segers, 2008). In previous studies, our research group demonstrated that this complex is composed of several cryptic species in eastern China, with different composition patterns at different sites (Xiang *et al.*, 2011a), so we wanted to investigate whether the molecular phylogeny and patterns of population genetic differentiation in *B. calyciflorus* complexes were similar in two different climate zones when seasonally surveyed. To determine the constitution of cryptic species and identify the patterns of seasonal genetic variation in the *B. calyciflorus* complex, all 158 clones of this complex were sequenced and analyzed based on the nuclear internal transcribed spacer (ITS) region and mitochondrial cytochrome c oxidase subunit I (COI) gene, as obtained by seasonal sampling from Guangzhou and Wuhu (1300 km apart from each other). We then investigated the seasonal change mechanisms of the genetic structure of the *B. calyciflorus* complex and evaluated the effects of environmental water fluctuation on the seasonal succession in rotifers.

2 Materials and methods

2.1 Sample collection and culture

The rotifer *B. calyciflorus* complex was collected from Lake Liuhua in Guangzhou and Lake Jinghu in Wuhu at four different time points (representative of all four seasons) over the course of one year (Tab. 1). Guangzhou is located in the southern subtropical zone of China with an oceanic monsoon climate that is fairly warm in winter and rather cool in summer, with a higher annual average temperature (23 °C) and a small annual temperature range. Wuhu, on the other hand, is situated in the northern subtropical zone of China and has a humid continental climate, characterized by cold and snowy winters but humid and hot summers with a lower annual average temperature (17.3 °C) and a large annual temperature range. In this study, water temperature, pH, and NH_4-N concentration were measured simultaneously with the sampling of rotifers (Tab. 1).

Stock rotifers were cultured clonally under natural light (approximately 130 lx) in a homothermal incubator with EPA medium (pH 7.4–7.8) (Peltier and Weber, 1985). The culture temperatures were set close to the sampling temperatures. The rotifers were fed daily with the algae *Scenedesmus obliquus* at 1.0–2.0 × 10^6 cells/ml, which was grown using HB-4 medium (Li *et al.*, 1959) under a semi-continuous culture condition and daily replenishment at 20%. Rotifer clones were harvested after they achieved high densities (200–300 ind/ml) and were starved for 24 h to avoid any potential intracorporal food contamination. Finally, a total of 158 *B. calyciflorus* clones were successfully established, and the sample sizes for each season were listed in Table 1.

2.2 DNA extraction, PCR amplification, and sequencing

The Wizard® Genomic DNA Purification Kit (Promega, USA) was used to isolate and purify the total genomic DNA as follows. Each rotifer clone (about 500 individuals) was transferred to a 1.5 ml microcentrifuge tube containing 35 μl of chilled Nuclei Lysis Solution, and incubated at 56 °C for 2 h. The sample was allowed to cool to room temperature for 5 min, then 15 μl of chilled Protein Precipitation Solution was added and the mixture was vortexed vigorously at high speed for 10 s. The sample was chilled on ice for 5 min, before being centrifuged for 10 min at 18 °C and 15 000 rpm. Next, the supernatant containing the DNA was removed and transferred to a sterile 1.5 ml microcentrifuge tube containing 50 μl of isopropanol and 0.5 μl of glycogen (20 mg/ml). The solution was then mixed *via* inversion, the samples were incubated at room temperature for 30 min, and the solution was centrifuged for 10 min at 15 000 rpm. The supernatant was subsequently decanted and 30 μl of 70% ethanol was added and centrifuged for 5 min at 15 000 rpm. Next, the ethanol was aspirated using a pipette tip, the tube was inverted on clean absorbent paper, and the pellet was air-dried for 2 h. Finally, 30 μl of deionized H_2O was added to rehydrate the DNA by incubating at 65 °C for 1 h. The DNA was then stored below −20 °C until ready to use.

Following the procedures detailed by Xiang *et al.* (2011a, b), the nuclear ITS region and mitochondrial COI gene were amplified and sequenced. All of the ITS and COI sequences derived from the samples in this study were deposited in GenBank (the accession nos. are listed in Tab. 1).

2.3 Sequence alignment and phylogenetic analyses

After mtCOI sequence alignment was performed with ClustalX 1.81 (Thompson *et al.*, 1997) using default parameters and nuITS alignment was performed with MAFFT v6.814b using the Q-INS-I algorithm (Katoh *et al.*, 2009), nucleotide diversity (π) and haplotype diversity (h) were calculated using DnaSP 5.10.1 (Librado and Rozas, 2009). The DNASTAR computer package (DNASTAR Inc., Madison, WI, USA) was used to test the percentage of sequence divergence among and within the main phylogenetic clades.

The phylogenetic relationships were reconstructed using four optimality criteria, Neighbor-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML), and Bayesian Inference (BI). The most optimal sequence evolution parameters and models (TVM+G and GTR+G), as selected by Modeltest 3.7 (Posada and Crandall, 1998), were used as settings in PAUP and Bayesian phylogenetic analyses based on the ITS and COI partial sequences. NJ, MP, and ML trees were constructed with the program PAUP* 4.0b10 (Swofford, 2002). Support for each node was tested with standard bootstrap analysis with 1000 replications for the NJ and MP trees and 100 replications for the ML trees. Two independent Bayesian analyses with the Markov Chain Monte Carlo (MCMC) method were conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), with four chains per analysis and randomly chosen starting trees. The Markov chains were run for 10 000 000 generations with trees being sampled every 100 generations. The first 25 0000 generations were discarded as burn-in, and the remaining trees were used to estimate Bayesian posterior probabilities. The sequences of *B. forficula* and *B. plicatilis* (GenBank Acc. Nos. DQ834362 and EF524553) were used as outgroups in the phylogenetic reconstruction based on the ITS and COI partial sequences.

Three DNA taxonomy methods were used to independently identify putative species with the COI and ITS alignments, including the Automatic Barcode Gap Discovery (ABGD), Poisson Tree Process (PTP), and Generalised Mixed Yule Coalescent (GMYC) models (Fontaneto *et al.*, 2015; Mills *et al.*, 2016). The ABGD model (available from http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) attempts to automatically discover the barcode gap instead of using one or several predefined distance thresholds for species delimitation. The identified groups were considered to be equivalent to species (Puillandre *et al.*, 2012). The PTP model (Zhang *et al.*, 2013) was applied to the input ML trees using coalescence theory to distinguish between the population- and species-levels. The PTP method was used through the online tool (http://species.h-its.org/) with default settings, and the output of the ML and BI optimisation algorithms was reported. For the identification of potential cryptic species representing independently evolving entities, an ultrametric tree was constructed based on Bayesian analysis using the penalized likelihood (PL) method and the truncated Newton (TN) algorithm on r8s software (Sanderson, 2002). A generalized mixed Yule coalescent (GMYC) model with multiple thresholds (Pons *et al.*, 2006; Monaghan *et al.*, 2009; Fontaneto *et al.*, 2009, 2011) was run on the ultrametric gene tree with R software. In the case of discordance in the amount of splitting, we chose to keep the smallest number of entities, in order to avoid over-splitting the species complex; thus, if a mistake was made in the identification of taxa, it was

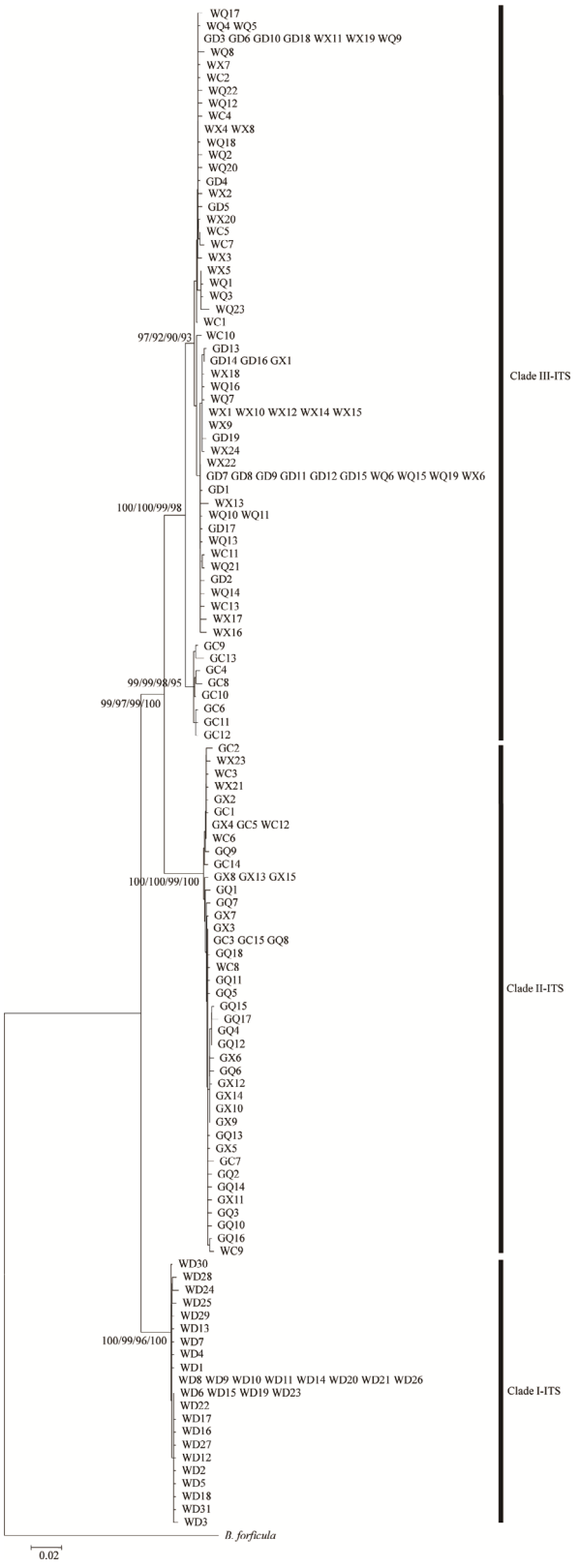

Fig. 1. The ML phylogenetic tree based on ITS sequences. Values isolated by slashes represent Bayesian, ML, MP, and NJ bootstrap support.

made in the direction of being more conservative in the amount of cryptic diversity (Mills *et al.*, 2016).

2.4 Analyses of population genetic differentiation

The population genetic differentiation was analyzed for the *B. calyciflorus* cryptic species, consisting of two or more populations. Based on the haplotype frequencies with gaps coded as missing data, the percentages of variation within and among populations were evaluated using Analyses of molecular variance (AMOVA) in Arlequin 3.1 (Excoffier and Schneider, 2005) by calculating pairwise F_{st} values. In order to assess the relative contribution of spatial partitioning *vs.* seasonal differentiation to the total genetic variation, the relative levels of fixation indices (F_{CT}, among groups of populations; F_{SC}, among populations within groups; F_{ST}, within populations) were detected for the different cryptic species based on two molecular markers using a hierarchical AMOVA. The *P*-values were determined using 1000 random permutations. Partial Pearson's correlations with two-tailed tests of significance were performed between water environment parameters (water temperature, pH, and NH_4-N concentration) and genetic diversity (F_{st} values, haplotype diversity, and nucleotide diversity), all while controlling for the effects of seasons and coordinates using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

3 Results

In this study, the ITS sequences from the *B. calyciflorus* complex ranged from 759 to 765 bp. A total of 158 clones were defined as 118 unique ITS haplotypes, of which 12 were shared, showed a wide geographical distribution. The assessment of genetic diversity showed that the Wuhu nucleotide diversity (0.0373 ± 0.0183) was higher than that of Guangzhou (0.0299 ± 0.0148), and the patterns of variation in nucleotide diversity among the four seasons were identical for both sampling sites, as follows: spring > summer > autumn > winter.

The length of the sequenced partial COI gene in the eight seasonal populations of Guangzhou and Wuhu was 712 bp. A total of 113 haplotypes were defined by the 158 sequences, including 9 shared haplotypes. The genetic diversity analyses indicated that the nucleotide diversity (π) of Wuhu (0.0966 ± 0.0465) was also higher than that of Guangzhou (0.0680 ± 0.0330), based on the COI sequences, confirming the results discovered with the ITS sequences. The changes in nucleotide diversity (π) at the two sites were similar to each other, and the change in the order of nucleotide diversity in Wuhu was spring > summer > autumn > winter, with spring and winter also corresponding to the highest and lowest levels in Guangzhou.

3.1 DNA taxonomy and phylogenetic relationships in the *B. calyciflorus* species complex

DNA taxonomy tools based on the nuITS and mtCOI sequences provided estimates of evolving entities, ranging from 3 to 21. Based on nuITS dataset, the minimum estimate of 3 groups was provided by the ABGD model with 1.2% prior maximal distance, yet the estimated number of putative species was between 3 and 16 (mean 4.96) using the PTP method with an acceptance rate of 0.47, where the most supported partition

found with a simple heuristic search was 3 species. The GMYC model on ITS sequence gave optimal solutions of 3 evolving entities, which was in agreement with the above two detections. In the case of the mtCOI gene, different species delimitation methods yielded different results. Four groups were found with the prior maximal distance of 0.06 using the ABGD method, while species number estimates ranged from 6 to 21 using the PTP model, and the most conservative estimate of 3 evolving entities was shown in the GMYC analysis. According to a report by Papakostas *et al.* (2016), species delimitations in the *B. calyciflorus* complex based on nuclear DNA markers proved to be a more reliable predictor of morphological variation than delimitations using the mitochondrial COI gene. Also, in cases where there was discordance in the amount of splitting, we chose to keep the conservative number of entities for the further phylogenetic and population structure analyses. Therefore, the most consistent number of lineages was the estimate of 3 species obtained from ITS (Fig. 1), and the three potential species were also the main well-supported lineages seen on the phylogenetic trees with the mtCOI dataset (Fig. 2).

The topological structure of phylogenetic trees, as reconstructed by the NJ, MP, ML, and Bayesian methods, was accordant in the clades close to the root node (Fig. 1). With the ML phylogenetic tree, the eight seasonal populations of the *B. calyciflorus* species complex from Guangzhou and Wuhu were grouped into three clades with strong support. Clade I-ITS, containing a unique geographical and seasonal population, was made up of 21 haplotypes of *B. calyciflorus* from Wuhu in winter (WD); the clade II-ITS consisted of 40 haplotypes from Guangzhou in spring, summer, and autumn, and from Wuhu in spring and summer. The remaining 57 haplotypes from Guangzhou (spring, summer, and winter) and Wuhu (spring, summer, and autumn) were all incorporated into clade III-ITS. The percentages of sequence divergence within and among the three clades of the ML phylogenetic tree based on the ITS sequences are available in the Supplementary material (Tab. S1).

The phylogenetic trees based on the COI partial sequences were reconstructed with the ML, MP, NJ, and Bayesian methods. The topologies of all of the phylogenetic trees were similar with well-supported main nodes differing only in minor rearrangements of the terminal branches (Fig. 2; the latter three trees are not shown). Based on the ML phylogenetic tree, 113 haplotypes of the COI partial sequences were split into three clades with strong support. The I-COI clade was made up of 22 haplotypes of *B. calyciflorus* from only Wuhu in winter (WD); the II-COI clade consisted of 34 haplotypes from Guangzhou in spring, summer, and autumn, and from Wuhu in spring and summer; and the III-COI clade was composed of the remaining 57 haplotypes from Guangzhou (spring, summer, and winter) and Wuhu (spring, summer, and autumn). The percentages of sequence divergence within and among the three clades of the ML phylogenetic tree based on the COI partial sequences are shown in Supplementary Table S2. Except for the GC4, WX21, WC3, WC9, and WC12 haplotypes, the phylogenetic relationships of all haplotypes based on the COI partial sequences were matched with those from the ITS sequences.

Three independently evolving entities were confirmed in this species complex by the ABGD, PTP, and GMYC models, coherent with the criterion of the molecular experiments and

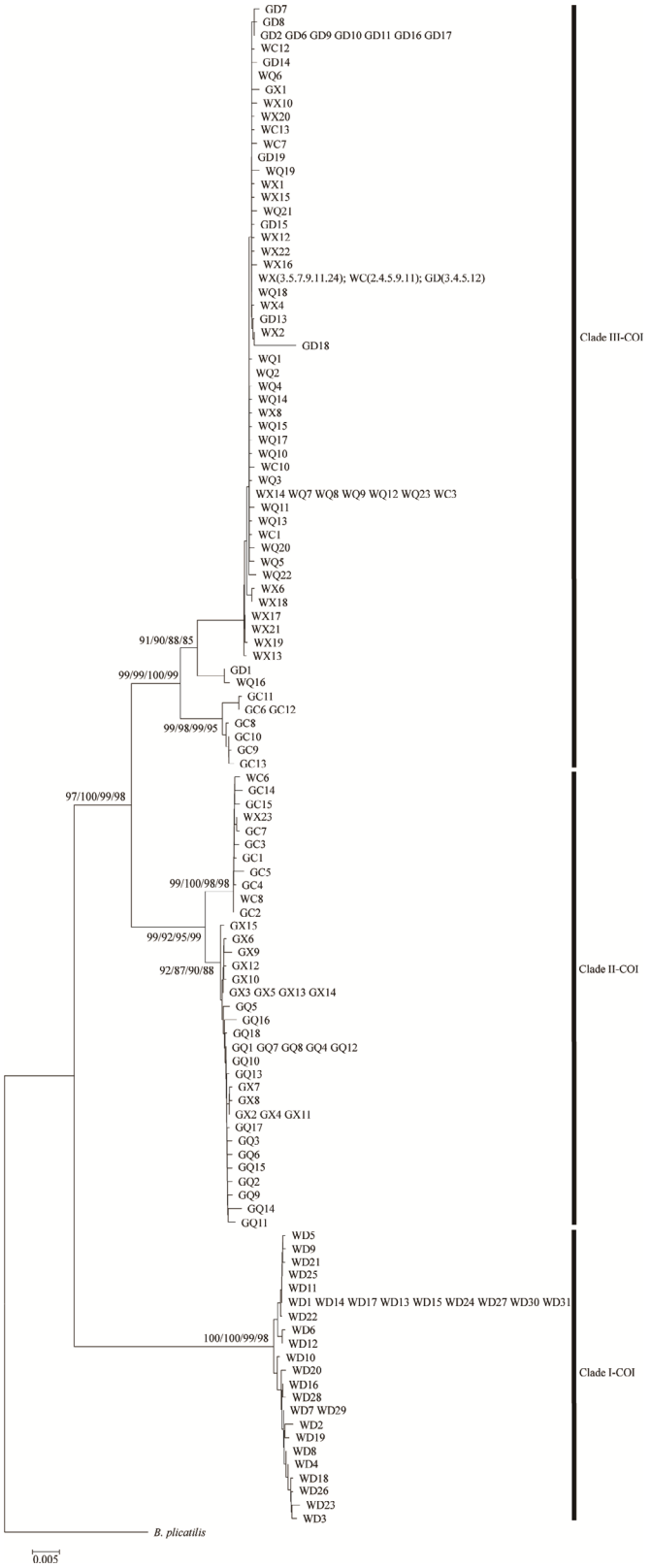

Fig. 2. The ML phylogenetic tree based on COI sequences. Values isolated by slashes represent Bayesian, ML, MP, and NJ bootstrap support.

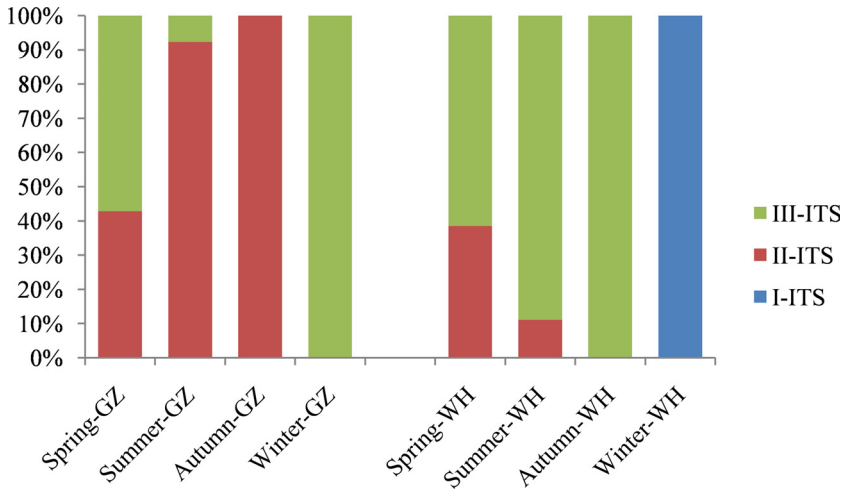

Fig. 3. Distribution of cryptic species in Guangzhou and Wuhu over four seasons based on the ITS sequences.

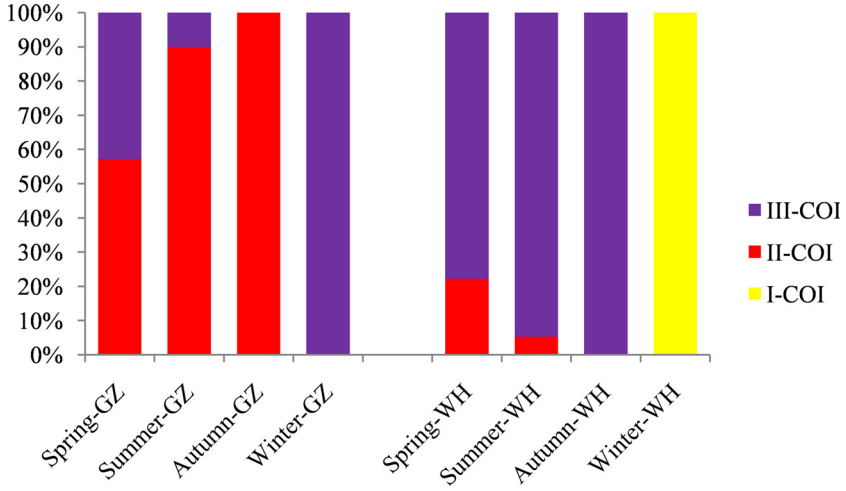

Fig. 4. Distribution of cryptic species in Guangzhou and Wuhu over four seasons based on the COI gene partial sequences.

mating tests developed by Xiang *et al.* (2011a) and Gilbert and Walsh (2005), which designated the lineages as separate cryptic species accompanied by sequence divergences >9.6% (COI) and >3.8% (ITS). Accordingly, we here refer to the three evolving entities as distinct cryptic species that were labeled I-ITS, II-ITS, and III-ITS or I-COI, II-COI, and III-COI, because no named species were described from China. The distributions of all cryptic species in each season are shown in Figures 3 (ITS) and 4 (COI).

3.2 Population genetic differentiation of *B. calyciflorus* cryptic species

Considering the composition of just one population in cryptic species I-ITS or I-COI, we did not analyze the population-level genetic differentiation of this cryptic species.

Five populations in the cryptic species II-ITS, including spring (5) and summer (2) in Wuhu, as well as spring (6), summer (12), and autumn (18) in Guangzhou (the figure in parentheses represents number of haplotypes; the same below) were included in the analysis of molecular variance. According to the sampling site, the GC, GX, and GQ populations were included in one group, and the WC and WX populations were placed into the other group. The results of the hierarchical AMOVA showed that the percentage of variation among groups (F_{CT}) was 5.73%, that among populations within the groups (F_{SC}) was 3.95%, and that within populations (F_{ST}) was 90.33%, suggesting a much greater genetic differentiation among different sampling sites than that among seasonal populations within a geographical site. The F_{st} values among and within five populations of cryptic species II-ITS are showed in Supplementary Table S3.

The analyses of molecular variance in cryptic species II-COI included five populations: WC (2), WX (1), GC (8), GX (9), and GQ (14). Based on the sampling sites, the percentages of variation among groups (F_{CT}), among populations within the groups (F_{SC}), and within populations (F_{ST}) were 43.96%, 20.18%, and 35.86%, respectively, suggesting a higher genetic differentiation between sampling sites than between seasons at the same sampling site, which is in accordance with the results of the ITS sequences. The F_{st} values among and within five

Table 2. The water environment parameters and genetic diversity indices in all populations of two *B. calyciflorus* cryptic species based on the ITS sequences.

Population	T	pH	NH_4-N[a]	Season	Latitude	Cryptic species II-ITS			Cryptic species III-ITS		
						h	π[a]	F_{st}	h	π	F_{st}
GC	28	8.29	1.97	1	23.04	0.952	0.0041	0.0860	1.000	0.0072	0.3298
GC	28	8.29	1.97	1	23.07	0.952	0.0041	0.0860	1.000	0.0072	0.3298
GX	33	8.02	1.50	2	23.07	0.967	0.0045	0.3834	1.000	0.0000	0.3834
GQ	24	7.85	0.75	3	23.07	1.000	0.0058	0.0358	–	–	–
GD	13	8.04	4.70	4	23.07	–	–	–	0.871	0.0052	0.3416
WC	27	8.24	1.95	1	31.21	1.000	0.0047	0.0798	1.000	0.0090	0.3163
WX	34	7.57	1.99	2	31.21	1.000	0.0039	0.1259	0.948	0.0066	0.3298
WQ	20	7.88	0.57	3	31.21	–	–	–	0.980	0.0075	0.3223

The superscripts "a" indicates there is significantly negative correlativity between environment parameter (NH_4-N) and genetic diversity indices (π in cryptic species II-ITS) with the same superscript alphabet ($P < 0.05$).

Table 3. The water environment parameters and genetic diversity indices in all populations of two *B. calyciflorus* cryptic species based on the COI sequences.

Population	T	pH[a]	NH_4-N	Season	Latitude	Cryptic species II-COI			Cryptic species III-COI		
						h	π	F_{st}	h[a]	π	F_{st}
GC	28	8.29	1.97	1	23.07	0.893	0.0050	0.4858	0.714	0.0257	0.1660
GX	33	8.02	1.50	2	23.07	1.000	0.0178	0.3788	1.000	0.0000	0.2621
GQ	24	7.85	0.75	3	23.07	0.967	0.0060	0.4746	–	–	–
GD	13	8.04	4.70	4	23.07	–	–	–	0.836	0.0016	0.2554
WC	27	8.24	1.95	1	31.21	1.000	0.0098	0.4807	0.727	0.0043	0.2451
WX	34	7.57	1.99	2	31.21	1.000	0.0000	0.5240	0.996	0.0363	0.1106
WQ	20	7.88	0.57	3	31.21	–	–	–	0.917	0.0070	0.2322

The superscript "a" indicates there is significantly negative correlativity between two parameters with the same superscript alphabet ($P < 0.05$).

populations of cryptic species II-COI are shown in Supplementary Table S4.

The cryptic species III-ITS consisted of six populations: WC (8), WX (16), WQ (19), GC (8), GX (1), and GD (10). The hierarchical AMOVA results indicated that the fixation indices of F_{CT}, F_{SC}, and F_{ST} were −5.00%, 36.59%, and 68.41%, respectively, based on the sampling site grouping. Using the COI data set, six populations in the cryptic species III-COI were analyzed with AMOVA, including WC (7), WX (18), WQ (19), GC (6), GX (1), and GD (9). The F_{CT}, F_{SC}, and F_{ST} were −6.03%, 23.99%, and 82.04% respectively, indicating that the genetic differentiation between geographical populations was smaller than that among seasonal populations in the cryptic species III-ITS or III-COI for the two molecular markers. The F_{st} values among and within six populations of cryptic species III-ITS and III-COI are shown in Supplementary Tables S5 and S6, respectively.

3.3 The relationship between water environment parameters and genetic diversity

The partial correlation analyses controlling for the potentially confounding factors (season and latitude) were conducted between water environment parameters, including water temperature, pH value, and NH_4-N concentration, and genetic diversity indices, such as F_{st} value, haplotype diversity, and nucleotide diversity in two cryptic species (II-ITS and III-ITS) using SPSS (Tab. 2). The results show that the nucleotide diversity of cryptic species II-ITS was significantly negative in correlation with NH_4-N concentration ($r = -0.998$, $P < 0.05$). Based on the COI sequences, the correlation analyses in the two cryptic species (II-COI and III-COI) suggested that the haplotype diversity of cryptic species III-COI was negatively related to the pH value ($r = -0.976$, $P < 0.05$) (Tab. 3).

4 Discussion

4.1 Cryptic species division and genetic diversity

Understanding the patterns and processes influencing biological diversity is a critical task given the current rapid environmental change (Mills *et al.*, 2017). The biodiversity of zooplankters would be underestimated and misunderstood without taking cryptic speciation into consideration. With such increasing evidence as mating behavior, allozyme patterns, microsatellite markers, and DNA sequencing, the conception of a species complex has been proposed for both rotifers (Ruttner-Kolisko, 1989; Gómez and Snell, 1996; Segers, 2007) and cladocerans (Frey, 1982, 1987). In zooplankton, mate recogni-

tion systems based on contact chemoreception have evolved, in order to distinguish conspecifics from other species and to discriminate males from females (Snell and Morris, 1993; Snell *et al.*, 1995), suggesting that morphological features are not the species boundaries in rotifers, and that speciation can proceed independently of morphological differentiation. In addition, the characteristics of cyclical parthenogenesis, island-like habitats, and seasonal preferences in rotifers all promote their rapid genetic differentiation and cryptic speciation (Serra *et al.*, 1997). In one of the most recent publications, using *B. calyciflorus* as a representative case, the potential of integrative taxonomy for guiding species delimitation by combining molecular, morphological, and ecological approaches was demonstrated in the presence of discordance between mitochondrial and nuclear markers. From this study, species delimitations based on the nuclear DNA markers were proven to be a more reliable predictor of morphological variation and competitive ability than delimitations using the mitochondrial COI gene (Papakostas *et al.*, 2016). In another case, the existence of 15 *B. plicatilis* cryptic species was confirmed by applying three different approaches in DNA taxonomy (ABGD, PTP, and GMYC) (Mills *et al.*, 2017).

In this study, various methods with different criteria were used to delineate species and, as a result, we were met with some degree of incongruence in the estimates of species numbers. DNA taxonomy based on the nuITS and mtCOI sequences provided estimates of evolving entities ranging from 3 to 21. According to the conclusions drawn by Papakostas *et al.* (2016), which proved that nuclear DNA markers were more reliable tools than the mitochondrial COI gene, a conservative number (3) of entities was chosen for future phylogenetic and population structure analyses. Certainly, the three potential species are also the main well-supported lineages on the phylogenetic trees in the mtCOI dataset. In addition, extreme nucleotide diversity was revealed in the *B. calyciflorus* species complex by two different molecular markers. In opposition, the nucleotide diversity was not high in any of the cryptic species. With the ITS fragment, the nucleotide diversity of the species complex was 0.0399 ± 0.0194, but was 0.0983 ± 0.0471 for the COI gene sequences, thus the mixture of multiple cryptic species might be responsible for the high diversity. Even so, there was still relatively little nucleotide diversity occurring in the winter populations of Guangzhou and Wuhu, and the autumn populations had the second lowest, which may be due to the composition of single-species. On the whole, the nucleotide diversity of Wuhu was larger than that of Guangzhou, which is closely related to the distribution of more cryptic species in Wuhu.

4.2 Seasonal succession and preference of cryptic species

For zooplankton, most of the fluctuations in the physical, chemical, and biotic environments may have been relatively minor, so that, to some extent, individuals in the population could adapt by modifying their underwater position and regulating their physiological actions. When exposed to more unfavorable environments, populations could be selected by nature, motivating the changes in population genetic structure, including the numbers of genotypes and their relative frequencies throughout both space and time. As far as the temporal pattern is concerned, seasonal variation in population genetic structure is the most common type of temporal differentiation (King and Serra, 1998). Different genotypes of *E. dilatata*, *A. girodi*, *B. plicatilis,* and *B. calyciflorus* have been reported to dwell in sympatric shallow lakes exhibiting distinct ecological preferences (King, 1972, 1980; Snell, 1980; King and Zhao, 1987). *Daphnia* is also a globally distributed complex of cryptic species, and compared to the species complex, each of component species is distributed in and tolerates a narrower ecological niche (Cerný and Hebert, 1999). These cases suggest that ecological preference plays an important role in the seasonal variation of genetic structure and the maintenance of higher rotifer species diversity.

In this study, the compositions of cryptic species was substantially identical based on ITS fragments and COI partial sequences, and each cryptic species was specialized in particular ecological niches, with strong temperature preferences. In Guangzhou, the temperature in summer and autumn is highest among the four seasons, and the dominant species was cryptic species II (-COI/-ITS), which prefers a higher temperature, while cryptic species III (-COI/-ITS) occurred rarely in summer, and disappeared completely in autumn. The water temperature was relatively lower in winter, and the composition consisted entirely of cryptic species III (-COI/-ITS), so this species might prefer a moderate temperature. With the onset of spring, the diapausing eggs of cryptic species II (-COI/-ITS) began to germinate and gradually occupy the majority of the ecological niches in the water. Simultaneously, the component ratio of cryptic species III (-COI/-ITS) decreased, until only one clone remained in the summer. In Wuhu, located at the northern subtropical zone, the water temperature was lower than that in Guangzhou over time, so it was hard for cryptic species II (-COI/-ITS), which prefers higher temperatures, to hold a dominant position, and its genotype frequencies were low. As a result, cryptic species III (-COI/-ITS) dominated the water column in spring, summer, and autumn. In winter, however, the monopolizer was cryptic species I (-COI/-ITS), which favors lower temperatures and was not found at all in Guangzhou, where the temperatures were higher. The clonal groups or genotypes involved in each seasonal succession were in fact the sibling or cryptic species in *B. calyciflorus*.

Due to the high degree of specialization in ecological features of each cryptic species, although environments tend to promote the elimination of cryptic species with low fitness through selection or competition in any given period of time, environments change before the death of all individuals, and, in turn, the new situation benefits this cryptic species. Finally, the result could be that none of these cryptic species will be eliminated, which is consistent with the "incomplete genetic discontinuity" model (King, 1972, 1977). In contrast, if the environment changes slowly, natural selection could exert distinguishing effects on different cryptic species for a long time, thus resulting in the complete elimination of certain genotypes that might regenerate until the occurrence of new suitable environments. The second case matches the "complete genetic discontinuity" model (King, 1972, 1977). Therefore, the seasonal succession patterns in zooplankton depend upon the stability of the environment and the degree of environment change. The two genetic models are not contradictory, but are merely two scenarios under the influence of different

intensities of environment change. The life history regime of seasonal succession in zooplankton depends upon the environmental conditions in which they live, rather than their species type. In this study, similar patterns of seasonal succession in *B. calyciflorus* were revealed in two distant habitats, as assessed with nuclear and mitochondrial markers.

4.3 Genetic differentiation of rotifer populations

Rotifers are characterized by island-like habitats, so their geographical distribution is necessarily patchy with a lack of hybridization or clear geographical isolation barriers between populations (Boileau *et al.*, 1992; De Meester *et al.*, 2002; Gómez *et al.*, 2007). On the other hand, rotifer populations in capricious environments are often seasonal or ephemeral, and sympatric species can occur in seasonal succession (Gómez *et al.*, 1995). In this study, the patterns of genetic differentiation in the two cryptic species were diverse from each other based on either the ITS fragment or the COI gene. In cryptic species II (-COI/-ITS), the genetic differentiation between geographical populations was obviously greater than that among seasonal populations. In opposition, seasonal changes seemed to have a stronger impact on the population genetic differentiation in cryptic species III (-COI/-ITS), suggesting that genetic differentiation was largely dependent upon the evolutionary force shaping seasonal differentiation, rather than spatial subdivision. Accordingly, the spatio-temporal patterns of population genetic differentiation in zooplankton varied with species or cryptic species.

Supplementary Material

Supplementary tables.
The Supplementary Material is available at http://www.limnology-journal.org/10.1051/limn/2017024/olm.

Acknowledgments. The authors thank the Shenzhen Nobel Science and Technology Service Co. Ltd for the language editing service. We appreciate Dr. Tylor Johnson and Prof. Ruanbao Zhou at South Dakota State University for their comments, which greatly improved the manuscript. We are grateful to the anonymous reviewers for their valuable comments and constructive suggestions. This research was funded by the Natural Science Foundation of China (31200324, 31470015), the Natural Science Foundation of Anhui Province (1708085MC79), and the Foundation of Provincial Key Laboratories of Conservation and Utilization for Important Biological Resources in Anhui.

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Cite this article as: Xiang X-L, Xi Y-L, Wen X-L, Ge Y-L. 2017. Molecular phylogeny and population genetic differentiation patterns in *Brachionus calyciflorus* Pallas (Rotifera) complex from two lakes in China. *Ann. Limnol. - Int. J. Lim.* 53: 401–410