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rDNA ITS sequences among morphotypes of *Keratell cochlearis*, *Keratell quadrata* and *Brachionus forficula* (Rotifera)

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ABSTRACT. Morphological variation in rotifers is affected by environmental conditions, making it hard to identify some rotifer taxa. We examined the rDNA ITS sequences of 10 unspined (KCU1-KCU10) and 17 spined (KCS1-KCS17) *Keratell cochlearis* clones, 26 two-spined (KQT1-KQT26), 18 single-spined (KQS1-KQS18) and 9 unspined (KQU1-KQU9) *K. quadrata* clones, and 17 long-spined (BL1-BL17) and 11 short-spined (BS1-BS11) *Brachionus forficula* clones collected from Lake Tingtang in Wuhu city, China. Molecular phylogenetic trees were constructed by neighbor-joining, maximum-likelihood, maximum parsimony, and Bayesian inference methods using *B. calyciflorus* as an outgroup. The *K. cochlearis* clones included 20 haplotypes, the *K. quadrata* clones included 37 haplotypes, and the *B. forficula* clones included 25 haplotypes. Different morphotypes of each rotifer species had shared haplotypes. Sequence divergences were 0.1-8.9% among different *K. cochlearis* haplotypes, and 8.1-8.9% between KCHAP1 (KCU1 and KCU10), KCU3, KCU4 and KCU6, and the other haplotypes. Sequence divergences were 0.1-14.5% among different *K. quadrata* haplotypes, and 11.9-14.5% between KQS17 and the other haplotypes. Sequence divergences were 0.1-11.7% among different *B. forficula* haplotypes, 11.0-11.7% between

BL15 and the other haplotypes, 9.3-10.1% between BS3 and the other haplotypes, and 11.7% between BL15 and BS3. The four phylogenetic trees all supported that KCHAP1, KCU3, KCU4, KCU6 and the other 16 haplotypes among the 20 *K. cochlearis* haplotypes, KQS17 and the other 36 haplotypes among the 37 *K. quadrata* haplotypes, and BL15, BS3 and the other 23 haplotypes among the 25 *B. forficula* haplotypes all belonged to their own isolated clades. The morphological variation of the three rotifer species was attributed mainly to phenotypic plasticity.

Key words: Morphotype; Molecular difference; rDNA ITS sequence; Rotifer

INTRODUCTION

With the application of molecular tools in studies on rotifer taxonomy, *Brachionus plicatilis* (Ciros-Pérez et al., 2001; Gómez et al., 2002; Gómez, 2005; Papakostas et al., 2005), *Keratella cochlearis* (Derry et al., 2003), *B. calyciflorus* (Gilbert and Walsh, 2005; Li et al., 2008; Zhang et al., 2010), *Epiphanes senta* (Schröder and Walsh, 2007), and *Philodina flaviceps* (Fontaneto et al., 2008) have been recognized as a cryptic species complex consisting of several sibling species. Seasonal succession of sibling species caused by variations in water environment is one of the possible proximate causes of cyclomorphosis in rotifers (Gómez, 2005). However, studies on genetic differentiation among various rotifer morphotypes are still relatively scant. Derry et al. (2003) have found that *K. cochlearis* morphotypes with posterior spines (*faluta* and *robusta*) and without (*tecta*) have 4.4% mean cytochrome oxidase I (COI) nucleotide sequence divergence and 0.9% mean amino acid sequence divergence and that they belong to 2 isolated sibling species. However, one- and two-spined morphotypes of *K. hiemalis* had only 0.21% mean COI nucleotide sequence divergence and no amino acid sequence divergence, and they showed no differentiation of sibling species. Li et al. (2008) and Zhang et al. (2010) have found that various *B. calyciflorus* morphotypes did not belong to isolated sibling species.

K. cochlearis is one of the most polymorphic planktonic rotifer species. In particular, the length and width of the lorica and the presence and size of the posterior spines are the most variable external features of this species. Similarly, the presence and number of posterior spines of *K. quadrata* and the posterior spine length and body size of *B. forficula* are also variable. However, studies on the taxonomic status of various *K. quadrata* and *B. forficula* morphotypes have not been reported. Therefore, investigating the genetic differences among various *K. cochlearis* and *K. quadrata* as well as *B. forficula* morphotypes by applying molecular techniques will help to determine their taxonomic status and the possible proximate causes of rotifer cyclomorphosis.

The eukaryotic internal transcribed spacer (ITS) sequence is an intergenic spacer region without transcription functions in tandem duplication of ribosomal RNA genes (ribosomal DNA [rDNA]) (Felleisen, 1997). It has been applied extensively as a molecular marker to investigate phylogenetic relationships among species, geographic populations, and strains of rotifers (Gómez et al., 2002; Tang et al., 2002; Xi et al., 2003; Gilbert and Walsh, 2005; Xiang et al., 2006; Zhang et al., 2010). In this study, we examined the rDNA ITS sequences of various *K. cochlearis*, *K. quadrata*, and *B. forficula* morphotypes and analyzed their genetic differentiation and taxonomic status.

MATERIAL AND METHODS

Sample collection and clonal culture

Individuals displaying various morphotypes of *K. cochlearis* (spined and unspined), *K. quadrata* (single and 2 spined and unspined), and *B. forficula* (short and long spined) were collected from Lake Tingtang in Wuhu City in April, December, and August 2009, respectively. After collection, females with amictic eggs from these species were randomly collected and clonally cultured at 15° ± 1°C, 10° ± 1°C, and 25° ± 1°C (close to the water temperatures of the sampling locations), respectively, under an illumination of 1300 lX and a 16:18-light:dark cycle in incubators. They were fed daily with the algae *Scenedesmus obliquus*. The algae were grown in Holley & Baker-4 medium (Li et al., 1959) under a semi-continuous culture condition by replenishing daily at 20%, and those in exponential growth were harvested by centrifugation and then resuspended in the rotifer medium prepared as described by Gilbert (1963). In total, 17 spined (KCS1-KCS17) and 10 unspined (KCU1-KCU10) *K. cochlearis* clones, 26 two-spined (KQT1-KQT26), 18 single-spined (KQS1-KQS18), and 9 unspined (KQU1-KQU9) *K. quadrata* clones, and 17 long-spined (BL1-BL17) and 11 short-spined (BS1-BS17) *B. forficula* clones were successfully cultured. When they achieved higher densities (200-300 individuals/mL), the animals were starved for 24 h to avoid potential contamination by the genomes of food organisms, harvested, and immediately stored below -20°C until use.

DNA extraction

The glass milk method (Hao et al., 2003) was used to isolate and purify total genomic DNA as follows: the rotifers were put into 1.5-mL Eppendorf tubes containing 500 µL DNA isolation buffer (0.5% sodium dodecyl sulfate, 25 mM ethylenediaminetetraacetic acid, 25 mM NaCl, 100 mM Tris-HCl, pH 8.0) and 20 µL proteinase K (20 µg/mL) and bathed in water (60°C) for 2 h. Then, 500 µL 8 M guanidinium thiocyanate and 40 µL 50% clean glass milk liquor were added, and the tubes were bathed in water (37°C) for 1 h and centrifuged at 4000 rpm for 1 min. The supernatant was discarded, and the sediment was cleaned twice with 70% alcohol and once with acetone and then dried completely in a vacuum drier at 45°C for 30 min and added to 40 µL TE (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0), bathed in water at 56°C for 30 min, and centrifuged at 4000 rpm for 1 min. The supernatant was placed into a centrifuge tube and refrigerated at -20°C for use.

Polymerase chain reaction (PCR) amplification and sequencing

The rDNA ITS sequences were amplified using the primers LH2 (5'-GTCGAATTCGT AGGTGAACCTGCGGAAGGATCA-3') and Dlam (5'-CCTGCAGTCGACA[TG]ATGCTT AA[AG]TTCAGC[AG]GG-3') (Xi et al., 2003). The reaction volume of 25 µL for PCR was composed of 0.2 µM of each primer, 1X reaction buffer, 200 µM deoxyribonucleotide triphosphate, 3.3 mM $MgCl_2$, 2 U *Taq* polymerase (Takara), and 2.0 µL template DNA. The PCR procedure consisted of predenaturation at 94°C for 5 min and 30 cycles of denaturation at 94°C for 40 s, annealing at 68°C for 45 s, elongation at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were resolved using electrophoresis on a 1.5% agarose gel and

purified with an AxyPrep™ PCR Cleanup kit (AXYGEN), then ligated into pMD 19-T Vector (Takara) and transferred to competent *Escherichia coli* DH5α cells using standard protocols. The plasmid containing the desired fragment was extracted using a Plasmid Extraction Kit (Qiagen) and sequenced on an ABI-PRISM 3730 automated sequencer using the universal sequencing primers M13-47 and RV-M.

Data analysis

All sequences were aligned using ClustalX version 1.8 (Thompson et al., 1997) with default parameters and manually checked. DNAStar was used to test the percentage of sequence divergence. Variable sites, polymorphic sites, parsimony informative sites, nucleotide diversity (π), and haplotype diversity (h) were calculated using DnaSP 5.0 (Librado and Rozas, 2009). All nucleotide positions were weighted equally, and gaps were treated as missing data. Phylogenetic relationships were reconstructed using 4 optimality criteria: maximum parsimony (MP), maximum likelihood (ML), neighbor joining, and Bayesian inference. To determine which model of evolution was the most appropriate for a dataset, log-likelihood scores were obtained with Phylogenetic Analysis Using Parsimony (PAUP*) 4.0b10 (Swofford, 2002) and then used to test evolution models with the Akaike information criterion in Modeltest 3.7 (Posada and Crandall, 1998). ML and MP trees were constructed based on the rDNA ITS sequences using PAUP* 4.0b10 with *B. calyciflorus* as the outgroup (GenBank accession No. GUO12723.1).

The most appropriate model (TVM+G) selected by Modeltest was used as a setting in PAUP* to generate an ML tree. The tree with the best fit was found using a heuristic search with tree bisection reconnection branch swapping. Support for each node was tested with standard bootstrap analysis through 1000 replications for the MP trees and 100 replications for the ML trees. Two independent Bayesian analyses using the Markov chain Monte Carlo method were conducted in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), with 4 chains per analysis and randomly chosen starting trees. The optimal parameters of sequence evolution obtained by Modeltest were used to explore the Bayesian analyses. The Markov chains were run for 10,000,000 generations with trees being sampled every 100 generations. The first 25,000 generations were discarded as burn-in, and the remaining trees were used to estimate Bayesian posterior probabilities. Megalign (DNAStar Inc., Madison, WI, USA) was used to estimate the percentage of sequence divergence among the main phylogenetic clades. This parameter was then used to delineate putative cryptic species in the rotifer complex.

RESULTS

Sequence variation and genetic diversity

Eighty-two unique haplotypes from the 108 individual sequences of 3 rotifer species were defined. The GenBank accession numbers in the National Center for Biotechnology Information database for the rDNA ITS sequences of all samples are JN574757-JN574838.

Twenty-seven samples (clones) of *K. cochlearis* included 20 haplotypes in which haplotype KCHAP1 included the KCU1 and KCU10 clones, and haplotype KCHAP2 included the KCU5, KCU7, KCU8, KCU9, KCS2, KCS7, and KCS11 clones. Thereafter, the 2 *K. cochlearis* morphotypes had shared haplotypes. The sequence alignment gave us a data matrix of 793-802

bp: KCHAP1, KCU3, KCU4, and KCU6 were 793 bp, and the other haplotypes were 800-802 bp. Of the aligned characters, 80 bp were polymorphic sites, 84 bp were variable sites, and 58 bp were parsimony informative sites. Mean base frequencies were 31.0% (A), 33.8% (T), 18.2% (G), and 17.0% (C). The content of A base plus T base (64.8%) was larger than that of G base plus C base (35.2%). The sequence divergences were 0.1-8.9% among all the haplotypes, with an average of 2.4%. The sequence divergences were 8.1-8.9% between KCHAP1, KCU3, KCU4, and KCU6 and the other haplotypes, with an average of 8.5%. However, the sequence divergences were only 0.6% among all the other haplotypes. KCHAP1, KCU3, KCU4, and KCU6 belonged to a sibling species, and the other 16 haplotypes belonged to a different sibling species.

Fifty-three samples of *K. quadrata* included 37 haplotypes in which haplotype KQHAP1 included the KQU1, KQU3, KQU7, KQU8, KQU9, KQS3, KQS6, KQS9, KQS12, KQT2, KQT6, KQT8, KQT9, KQT10, KQT12, KQT13, and KQT16 clones. The 3 *K. quadrata* morphotypes had shared haplotypes. The sequence alignment gave us a data matrix of 786-800 bp: KQS17 was 786 bp, KQS4, KQS7, and KQT23 were 800 bp, and the other haplotypes were 799 bp. Of the aligned characters, 146 bp were polymorphic sites, 148 bp were variable sites, and 5 bp were parsimony informative sites. Mean base frequencies were 31.6% (A), 36.2% (T), 16.7% (G), and 15.5% (C). The content of A base plus T base (67.8%) was larger than that of G base plus C base (32.2%). The sequence divergences were 0.1-14.5% among all the haplotypes, with an average of 1.1%. The sequence divergences were 11.9-14.5% between KQS17 and the other haplotypes, with an average of 12.3%. However, the sequence divergences were only 0.5% among all the other haplotypes. KQS17 and the other 36 haplotypes belonged to isolated sibling species.

Twenty-eight samples of *B. forficula* included 25 haplotypes in which haplotype BHAP1 included the BL8 and BL11 clones, and haplotype BHAP2 included the BL9, BL17, and BS10 clones. The 2 *B. forficula* morphotypes had shared haplotypes. The sequence alignment gave us a data matrix of 813-815 bp: BL3 and BL4 had 812 bp, BS6 had 815 bp, and the other haplotypes had 813 bp. Of the aligned characters, 146 bp were polymorphic sites, 162 bp were variable sites, and 29 bp were parsimony informative sites. Mean base frequencies were 30.2% (A), 35.6% (T), 17.6% (G), and 16.6% (C). The content of A base plus T base (65.8%) was larger than that of G base plus C base (34.2%). The sequence divergences were 0.1-11.7% among all haplotypes, with an average of 2.1%. The sequence divergences were 11.0-11.7% between BL15 and the other haplotypes, with an average of 11.4%. The sequence divergences were 9.3-10.1% between BS3 and the other haplotypes, with an average of 9.7%. However, average sequence divergence was only 0.6% among all other haplotypes except BL15 and BS3. The sequence divergence was 11.7% between BL15 and BS3. BL15, BS3, and the other 23 haplotypes belonged to 3 isolated sibling species.

Phylogenetic relationships

The topologies of the Bayesian interference, neighbor joining, MP, and ML trees for all *K. cochlearis*, *K. quadrata*, and *B. forficula* haplotypes were largely congruent, differing only in minor rearrangements of the leaves. The rDNA ITS sequences based on the complete data set revealed a well-resolved phylogeny that divided the complex of *K. cochlearis* into 2 clades (KCHAP1, KCU3, KCU4, and KCU6 belonged to a unique clade, and the other 16 haplotypes belonged to a different unique clade), the complex of *K. quadrata* into 2 clades (KQS17 and the other 36 haplotypes belonged to 2 different clades), and the complex of *B. forficula* into 3 clades (BL15, BS3, and the other 16 haplotypes belonged to 3 different clades) (Figure 1A, B and C).

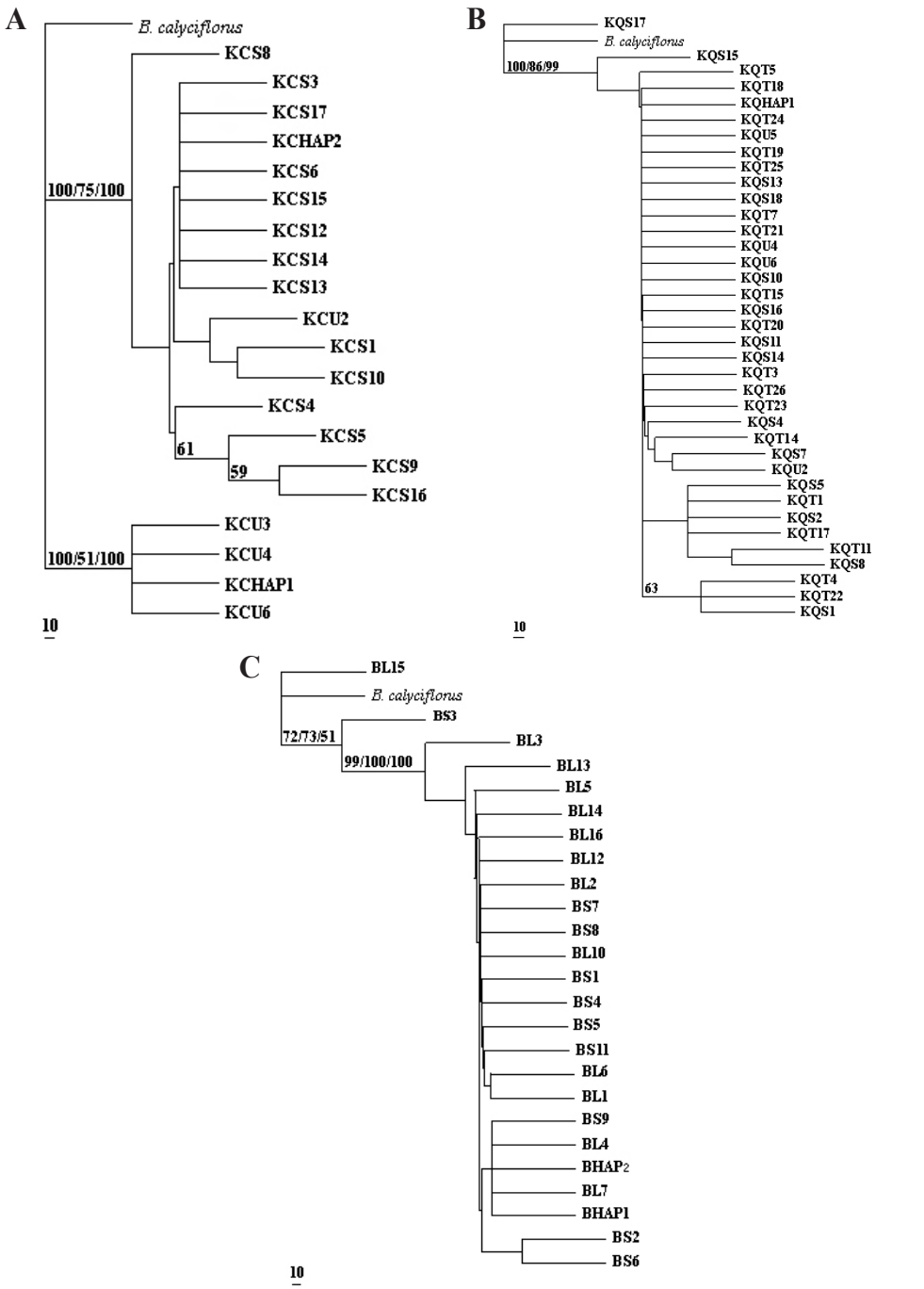

Figure 1. Maximum parsimony phylogenetic tree of *Keratell cochlearis* (**A**), *Keratell quadrata* (**B**) and *Brachionus forficula* (**C**) morphotypes based on rDNA ITS sequences. Values isolated by slashes represent maximum parsimony, maximum likelihood and neighbor joining bootstrap support, respectively.

Combined with the above striking percentages of rDNA ITS sequence divergence among the clades of the 3 rotifer species, the conclusion might be drawn that the 2 isolated clades of *K. cochlearis* as well as those of *K. quadrata* and the 3 isolated clades of *B. forficula* belong to different sibling species. Considering that a sibling species of *K. cochlearis*, *K. quadrata*, and *B. forficula* included 16 haplotypes consisting of 2 morphotypes, 36 haplotypes consisting of 3 morphotypes, and 23 haplotypes consisting of 2 morphotypes, respectively, however, the various morphotypes of all 3 rotifer species did not belong to different sibling species.

DISCUSSION

Sibling species differentiation of rotifers

In recent years, studies on the population genetics differentiation of rotifers through alignment of rDNA ITS sequences have been occasionally reported (Gómez et al., 2002; Gilbert and Walsh, 2005; Li et al., 2008; Cheng et al., 2008; Zhang et al., 2010) and the results have shown that *B. calyciflorus* strains from Australia and from Florida, Georgia, and Texas in the United States make up different sibling species because of their 3.8% divergence of rDNA ITS sequences and reproductive isolation (Gilbert and Walsh, 2005). *B. calyciflorus* populations from Lake Liantang consisted of 2 sibling species because of their >5.1% divergence of rDNA ITS sequences and reproductive isolation, but their different morphotypes do not represent distinct sibling species (Li et al., 2008; Cheng et al., 2008; Zhang et al., 2010). Spined and unspined *K. cochlearis* morphotypes from lakes near Fort McMurray, Alberta, Canada, are different sibling species because of their 4.4% COI nucleotide sequence divergence, but 1- and 2-spined morphotypes of *K. hiemalis* are not different sibling species because of their only 0.21% mean COI nucleotide sequence divergence and no amino acid sequence divergence (Derry et al., 2003).

In the present study, the rDNA ITS sequence divergences among *K. cochlearis*, *K. quadrata*, and *B. forficula* clones were 8.5, 12.3, and 9.7-11.4%, respectively, indicating that they are species complexes. Contradictory to the results obtained by Derry et al. (2003), however, the results of the present study indicated that the various morphotypes of *K. cochlearis* were not sibling species because every sibling species consisted of spined and unspined morphotypes. Further research is necessary to determine whether the discrepancy between our result and that of Derry et al. (2003) is related to the smaller samples used by the latter. Similar to the morphotypes of the *K. cochlearis* species complex, the 3 *K. quadrata* morphotypes and the 2 *B. forficula* morphotypes were not different sibling species.

Factors influencing morphological variation of rotifers

In general, the noticeable morphological variation in rotifer has been associated with various environmental and genetic conditions. L, M, and S types of the marine rotifer *B. plicatilis* (3 sibling species of *B. plicatilis* in reality) are mainly induced by genetic factors (Fu et al., 1991; Segers, 1995; Gómez and Serra, 1995, Gómez et al., 2002; Suatoni, 2003; Derry et al., 2003; Papakostas et al., 2005), but the morphological variations of *K. hiemalis* and *B. calyciflorus* are mainly induced by environmental factors, a phenomenon that reflects the phe-

notypic plasticity of rotifers (Derry et al., 2003; Li et al., 2008; Zhang et al., 2010). Our results suggested that the various morphotypes of *K. cochlearis*, *K. quadrata*, and *B. forficula* were mainly induced by environmental factors because we detected no deep genetic divergence between spined and unspined *K. cochlearis* morphotypes, among single-, two- and unspined *K. quadrata* morphotypes, or between short- and long-spined *B. forficula* morphotypes.

Phenotypic plasticity and adaptation of rotifers

Phenotypic plasticity of an organism is an expression capability of various morphotypes that shows the developmental response to biotic or abiotic factors. Bradshaw (1965) thought that a single genotype could express different phenotypes in different environments and that the polymorphism of an organism could be attributed to its developmental instability. Many phenotypes of a single genotype can, in part, help an organism better adapt to an environment, but these phenotypes cannot be transmitted to future generations or promote the adaptive evolution of a species. Phenotypic plasticity is also recognized as one of the possible important causes of rotifer polymorphism. Temperature, food abundance and quality, competition of zooplankton such as cladocerans, and predation by plankton predators such as cyclopoid copepods and *Asplanchna* all may cause spine changes in rotifers (Hillbricht-Ilkowska, 1972; Karabin, 1982; Stemberger and Gilbert, 1984; Sarma and Rao, 1987; Green, 2005). Rotifers with large phenotypic plasticity might have selective advantages and obtain genetic variations more quickly, increasing their fitness (Schlichting, 1986; Sultan, 2000; Weiner, 2004; Miner et al., 2005). Therefore, polymorphism is favorable for evolution in rotifers. Some studies have shown, however, that many plasticity variations are closely related to the plasticity expression of developmental regulation genes in various surroundings and can be inherited by future generations through epigenetics. Some plasticity variations are also fixed through genetic assimilation and significantly affect individual development patterns, the genetic composition and structure of a population, and the evolutionary potentiality of species, among other characteristics (Sultan, 2000; Schlichting and Smith, 2002; Pigliucci et al., 2006; Richards, 2006).

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