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Morphological and molecular differentiation of genus *Corbicula* suggests that two species are sympatrically distributed in Datong Lake in the Central Yangtze River Basin

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Abstract

Background: White and purple color morphs of *Corbicula* have been identified all over the world. Previous studies suggested that the distinct difference of inner shell color, especially for sympatric individuals, derived mainly from their different genetic constitutions, not just environmental conditions. Two color morphs of *Corbicula* sympatric in Datong Lake were compared by both morphometric methods (shell length, shell height, and shell width) and genetic analysis (based on the mitochondrial cytochrome *c* oxidase subunit I gene *COI*-mtCOI) to explore their taxonomic relationship.

Results: Morphological analysis showed that there were no significant differences in the values of SH/SL, SW/SL, and SW/SH ($P > 0.05$) between the two color morphs. Meanwhile, there were no significant differences in measurable parameters among the four mitochondrial COI haplotypes (DT-1, DT-7, DT-19, and DT-31) ($P > 0.05$). The sequences of four haplotypes (DT-1, DT-7, DT-19, and DT-31) were identical to FW1, FW5, FW4, and FW3, respectively. Restriction fragment length polymorphism (RFLP) analysis showed that only the sequences of haplotype DT-7 could be digested by the restriction enzyme *Sac*I into two fragments with 200/500 bp. Haplotype DT-31 was found only in one individual (white morph), while each of the other three haplotypes shared with both two color morphs. Phylogenetic analysis demonstrated that the four haplotypes were subdivided into two divergent clades among freshwater clades, and haplotype DT-7 was a divergent sister taxon to the other three.

Conclusions: The results indicated that there were no distinct differences on morphological measures between the two color morphs, and each color morph shared with all four mitochondrial COI haplotypes. Two species (DT-7 referred as *Corbicula fluminea* and DT-1, DT-19, and DT-31 as *Corbicula leana*) might coexisted in the Datong Lake. Therefore, a comprehensive study combining nuclear and mitochondrial data along with biological information should be performed to confirm this assumption.

Keywords: *Corbicula*; Mitochondrial DNA COI; Phylogeny; Taxonomy

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Background

At the beginning of the 20th century, the modern native distribution of the genus *Corbicula* was confined in Asia, Africa, and Australia (Sousa et al. 2008). *Corbicula* has been dispersed worldwide rapidly by a combination of human and natural dispersion mechanisms (Araujo et al. 1993; Sousa et al. 2007). The first documented record of this genus outside its native range was in British Columbia in Canada in the 1920s (Counts 1981) and then rapidly spread throughout the continent. Afterwards, it was extended to South America in the 1970s (Lee et al. 2005; Pigneur et al. 2011) and Europe in the 1980s (Karatayev et al. 2007). Nowadays, the genus *Corbicula* has colonized almost all over the world (Renard et al. 2000; Pfenninger et al. 2002), which caused great ecological and economic impacts on introduced ecosystems in the invasive range. Although its invasion and rapid expansion have been of intensive research topic in freshwater ecology, the taxonomy and systematic status of freshwater clam *Corbicula* in its native range is still not clearly resolved (Park and Kim 2003). Several systematic uncertainties using molecular methods were clarified in the last decades, such as mitochondrial and nuclear marks (Hedtke et al. 2008; Komaru et al. 2012, 2013). However, it still remained confusing, mainly due to the polymorphism of the morphology, color of the shell, and complex reproduction modes. Therefore, more information on genus *Corbicula* in its native range will help solve this confusion.

In China, the genus *Corbicula* has been consumed as food since ancient times (Liao et al. 2013). In addition, it has also been explored for medicine with its pharmacological activity (Liu et al. 1979). However, the taxonomy and systematicness of the genus *Corbicula* were poorly understood. Seven species have been reported in China, including *Corbicula fluminea*, *Corbicula fluminalis*, *Corbicula nitens*, *Corbicula aurea*, *Corbicula largillierti*, *Corbicula tenuis*, and *Corbicula scholastica*, which were nominated mainly according to morphology (Tchange and Li 1965; Tchange et al. 1965; Huang and Cheng 2003). It is not clear if the same occurs just like in America and Europe.

The shell color of the genus *Corbicula* was highly polymorphic (Morton 1987). Generally, two different color morphs (white and purple) have been identified by significantly different dimension ratios (Hillis and Patton 1982; Tsoi et al. 1991; Park et al. 2002). These two morphs were usually nominated to be one (Morton 1986; Qiu et al. 2001) or two different species (Britton and Morton 1986; Morton 1987; Park et al. 2002). It was inferred that the differences between the shell color morphs of *Corbicula* mainly derived from their different genetic constitutions, not just environmental condition, especially for those sympatric individuals (Komura and Konishi 1999; Qiu et al. 2001; Park et al. 2002). In the present study, two shell color morphs (white and purple) of the genus *Corbicula* were discovered sympatrically in an aquaculture lake (Datong Lake) in the Central Yangtze River Basin. A combination of morphological methods (shell length (SL), shell height (SH), and shell width (SW)) and genetic analysis (based on the mitochondrial cytochrome *c* oxidase subunit I gene *COI*-mtCOI) were used to explore their systematic relationship. In addition, mtCOI sequences of individuals in the present study were compared with preexisting mtCOI sequences in China. The results will provide more valuable information to clarify the taxonomy and systematicness of the genus *Corbicula*.

Methods

Study area and sampling

Datong Lake was located at 29°05′ ~ 29°16′N and 112°26′ ~ 112°35′E in the nearly middle part of Hunan Province, China. It was separated from Dongting Lake since the reclamation of land in 1951 (Li et al. 2012). Now, it covers a water surface area of 82.7 km^2 (Figure 1). Sixty individuals of *Corbicula* clams were collected from the Datong Lake by using Peterson grab sampler in August 2012. All specimens were cleaned using a sieve with 0.5-mm mesh and then placed immediately on ice for transportation.

Morphological analysis and statistics

For conventional morphometric analysis, three morphometric variables in 60 individuals were measured after wet weight (WW) was taken: shell length (the greatest anteroposterior dimension), shell height (the maximal dorsoventral dimension of the shell taken at the umbo), and shell width (the maximal width of the articulated paired valves) (Li et al. 2013). The accuracy of weighing was 0.01 g, and the morphological variable was 0.01 mm using a digital caliper.

All values were presented as means ± SE. All data were tested by using Shapiro-Wilk's *W* test for normality, while using Levene's test for homogeneity of variances. Differences between two shell morphs were compared by *t*-test. Differences among haplotypes were analyzed using one-way ANOVA. All analyses were performed using Statistic 6.0 software (StatSoft, Inc., Tulsa, OK, USA), with $P < 0.05$ considered statistically significant.

Genetic analysis

Genomic DNA was extracted from the mantle tissue of 60 individuals using TIANamp Marine Animals DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). A 710-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) designed by Folmer et al. (1994).

Figure 1 Location map of the study area.

Polymerase chain reaction (PCR) was performed in 25 μl, including 100 ng of template DNA, 0.5 μM of each primer, 2.5 μM of each dNTP, 1.5 mM $MgCl_2$, 1× Taq reaction buffer, 2.5 U of Taq polymerase, and ddH_2O. The PCR conditions were as follows: following initial denaturation of 5 min at 94°C, 35 cycles of denaturation at 94°C for 60 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min, followed by a final extension for 5 min at 72°C were done. A negative control reaction (no template DNA) was performed alongside the experimental PCR. The PCR products were purified using a TIANgel Midi Purification Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) and sequenced with each of the same primers by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). In order to avoid erroneous sequence determination at the 5′- and 3′-terminal regions, only a 614-bp fragment was selected for further phylogenetic analysis. The PCR products of the four haplotypes were digested with a restriction enzyme *Sac*I. The digestion reaction was performed according to the procedures provided by the enzyme supplier (Fermentas Inc., Burlington, Ontario, Canada).

The haplotype sequences in the Datong Lake were identified by comparing with those from previously published studies on *Corbicula* using the BLAST program (Altschul et al. 1997): FW1 (GenBank, AF196269), FW2 (AF457989), FW3 (AF457990), FW5 (AF196268), FW11 (AF457994), FW12 (AF457995), FW13 (AF457999), and FW4 (AF269096-8). The sequences were visualized and aligned using BioEdit 7.0.5.3 software. Haplotypes were determined using the DnaSP program (Rozas et al. 2003). MEGA version 5.1 software was employed for the construction of the neighbor-joining (NJ) tree and maximum likelihood (ML) tree, estimating genetic distances according to Tamura (1992) 3′ parameters method. Two published sequences of Zebra mussels (U47648 and U47652) were designed as outgroups. The reliability of nodes on trees was obtained using bootstrap analysis with 1,000 replicates (Felsenstein 1985; Sousa et al. 2007).

Results

Shell morphology

Two distinct color forms were recognized (white or purple morph) (Figure 2). Both of the two shell color morphs shared more or less centrally located umbo, glossy periostracum, prominent and raised sculpture, and regular concentric ribs. The purple morph with purple internal and dark green external was featured whitish from the pallia line to the umbo. The white morph, described as having white internal and light green external, was also characterized by the purple flashes along its anterior and posterior lateral teeth.

WW, SL, SH, and SW were not significantly different between the two color morphs ($P > 0.05$). There were no significant differences among four haplotypes for WW, SL, SH, and SW and three ratios of SH/SL, SW/SL, and SW/SH ($P > 0.05$) (Table 1) either. The SW/SL value of DT-7 (0.68 ± 0.01, $N = 16$) was much larger than those of DT-1, DT-19 and DT-31 (0.65 ± 0.01, $N = 44$) ($P < 0.05$), whereas SH/SL and SW/SH of the two groups were similar.

Genetic variability

Among the 60 individuals, four haplotypes (DT-1, DT-7, DT-19, and DT-31) were identified. In addition, haplotype DT-31 was found only in one individual (white morph); the other three haplotypes (DT-1, DT-7, and DT-19) were discovered to have both white and purple morphs. Thirty-eight of the 60 sequences corresponded to the haplotype DT-1, and 16, 5, and 1 represented the other three haplotypes (Figure 3). Alignment of the identified sequences revealed that the four haplotypes were resolved differing by one point (0.2% divergence) to 16 sites (2.6% divergence).

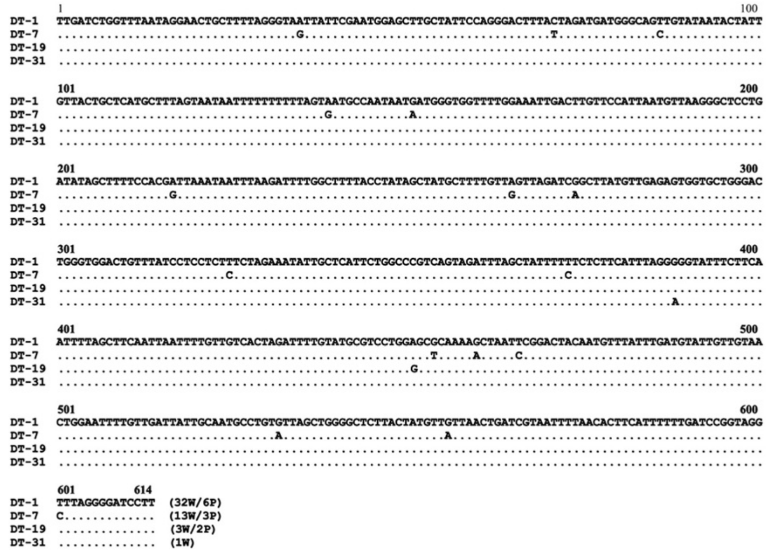
Figure 2 Shell morphology of two color morphs of *Corbicula* clams in Datong Lake.

No novel haplotype of *Corbicula* was discovered in the present study, and the sequences of DT-1, DT-7, DT-19, and DT-31 were identical to FW1, FW5, FW4, and FW3 that were found in Asia, respectively (Park and Kim 2003). Restriction fragment length polymorphism (RFLP) analysis of the four haplotypes showed that only the 710-bp fragment of haplotype DT-7 was digested by the one-site restriction enzyme *Sac*I and provided two fragments with 200/500 bp.

Phylogenic trees constructed by NJ and MP methods showed similar tree topologies (Figure 4). The results demonstrated that the analyzed haplotypes were subdivided into two divergent clades-estuarine (composed exclusively of brackish members) and freshwater (exclusively comprising freshwater members) clades. Among the freshwater *Corbicula* members, haplotype DT-7 and FW5 occupied a well-supported basal position rather than the remaining three (Figure 4).

Table 1 Morphometric data of the shells of *Corbicula* clams from Datong Lake, with mitochondrial COI haplotypes

Haplotypes	Number	WW	SL	SH	SW	SH/SL	SW/SL	SW/SH
DT-1	38	2.93 ± 0.23	19.39 ± 0.48	18.26 ± 0.43	12.61 ± 0.27	0.94 ± 0.01	0.65 ± 0.01	0.69 ± 0.01
DT-7	16	2.78 ± 0.29	18.57 ± 0.69	17.88 ± 0.65	12.53 ± 0.39	0.96 ± 0.01	0.68 ± 0.01	0.70 ± 0.01
DT-19	5	2.93 ± 0.62	18.88 ± 1.75	18.01 ± 1.56	12.28 ± 1.09	0.96 ± 0.02	0.65 ± 0.02	0.68 ± 0.01
DT-31	1	2.90	18.98	18.37	12.27	0.97	0.65	0.67



```
        1                                                                                               100
DT-1    TTGATCTGGTTTAATAGGAACTGCTTTTAGGGTAATTATTCGAATGGAGCTTGCTATTCCAGGGACTTTACTAGATGATGGGCAGTTGTATAATACTATT
DT-7    ..................................G...................................T..............C..............
DT-19   ....................................................................................................
DT-31   ....................................................................................................

        101                                                                                             200
DT-1    GTTACTGCTCATGCTTTAGTAATAATTTTTTTTTAGTAATGCCAATAATGATGGGTGGTTTTGGAAATTGACTTGTTCCATTAATGTTAAGGGCTCCTG
DT-7    ......................................G...........A.................................................
DT-19   ....................................................................................................
DT-31   ....................................................................................................

        201                                                                                             300
DT-1    ATATAGCTTTTCCACGATTAAATAATTTAAGATTTTGGCTTTTACCTATAGCTATGCTTTTGTTAGTTAGATCGGCTTATGTTGAGAGTGGTGCTGGGAC
DT-7    ................G...............................................G........A..........................
DT-19   ....................................................................................................
DT-31   ....................................................................................................

        301                                                                                             400
DT-1    TGGGTGGACTGTTTATCCTCCTCTTTCTAGAAATATTGCTCATTCTGGCCCGTCAGTAGATTTAGCTATTTTTTCTCTTCATTTAGGGGGTATTTCTTCA
DT-7    ........................C...............................................C...........................
DT-19   ....................................................................................................
DT-31   .......................................................................................A............

        401                                                                                             500
DT-1    ATTTTAGCTTCAATTAATTTTGTTGTCACTAGATTTTGTATGCGTCCTGGAGCGCAAAAGCTAATTCGGACTACAATGTTTATTTGATGTATTGTTGTAA
DT-7    .....................................................T.....A.....C..................................
DT-19   ..................................................G.................................................
DT-31   ....................................................................................................

        501                                                                                             600
DT-1    CTGGAATTTTGTTGATTATTGCAATGCCTGTGTTAGCTGGGGCTCTTACTATGTTGTTAACTGATCGTAATTTTAACACTTCATTTTTTGATCCGGTAGG
DT-7    ...............................A.......................A............................................
DT-19   ....................................................................................................
DT-31   ....................................................................................................

        601        614
DT-1    TTTAGGGGATCCTT  (32W/6P)
DT-7    C.............  (13W/3P)
DT-19   ..............  (3W/2P)
DT-31   ..............  (1W)
```

Figure 3 Alignment of four haplotypes and variable sites in partial mtDNA COI sequence of *Corbicula*. Of the 60 analyzed sequences, 32 white morphs and 6 purple morphs correspond to DT-1, 13 white and 3 purple to DT-7, 3 white and 2 purple to DT-19, and 1 white to DT-31.

Discussion

Two shell color morphs of *Corbicula* clam have already been described from a number of regional populations, such as North America (Hillis and Patton 1982; Siripattrawan et al. 2000), Japan (Houki et al. 2011), Thailand (Kijviriya et al. 1991), Hong Kong (Tsoi et al. 1991), Taiwan (Komura and Konishi 1999), and China (Qiu et al. 2001). It was inferred that not only environmental condition but also the different genetic constitutions resulted in the differences between the two color morphs (Qiu et al. 2001), especially for those sympatric individuals. Most researchers have reached an agreement that the two morphotypes should be relegated to two separate species, for *C. fluminea* (white morph) and *C. leana* (purple morph) (Choe et al. 1999; Kwon et al. 2001; Park et al. 2002). In Datong Lake, two sympatric shell color morphs were generally to be identified as *C. fluminea* on account of their similar external shell morphology (Li et al. 2011, 2013). Similar results of *C. fluminea* have also been reported in Sichuan Province, China (Qiu et al. 2001). Morphological analysis in the present study further supported that there were no significant differences between the two color morphs except for their different internal color in Datong Lake. This was inconsistent with that in Sichuan Province (Qiu et al. 2001), in which the three ratios of SW/SL, SW/SH, and SW/(SL + SH + SW) were significantly larger in white morph than in purple morph. Therefore, these results supported the assumption that the origin of differences between two color morphs should have been derived from genetic constitutions.

DNA sequencing analysis revealed four distinct haplotypes in 60 individuals in Datong Lake. No novel haplotype was discovered, comparing with those available sequences in Asia including the two largest freshwater lakes of China (Poyang Lake and Dongting Lake) (Park and Kim 2003). Because Datong Lake was once part of Dongting Lake 60 years ago, the results may suggest that at least four haplotypes coexisted in the Dongting Lake not just three haplotypes reported previously (Park and Kim 2003). Nowadays, eight haplotypes have been identified, including FW1/DT-1, FW2, FW3/DT-31, FW4/DT-19, FW5/DT-7, FW11, FW12, and FW13. Therefore, more extensive samplings especially for species in Qingshan Lake which has novel haplotypes (FW11, FW12, and FW13) should be carried out to clarify the taxonomy and phylogeny of *Corbicula* in China. In addition, three major haplotypes (DT-1, DT-7, and DT-19) each was shared with both two color morphs. Thus, more individuals in Datong Lake should be analyzed to make sure whether it was true for DT-31. The genetic analysis based on phylogenic trees strongly supported the sister status of the estuarine clade to all its freshwater congeners, which was consistent with previous results (Park and Kim 2003; Pigneur et al. 2011). Among the freshwater clades, haplotype DT-7 was a divergent sister taxon to the other three, obviously supported by maximum bootstrap values (100%). In the present study, both of the haplotype DT-7 and FW5 (referred as *C. leana* in Japan) shared identical sequence, similar RFLP result, and robust support (a bootstrapping value of 100%). Hence, the individuals with haplotype DT-7 could be assigned as *C. leana*, which was one of the most

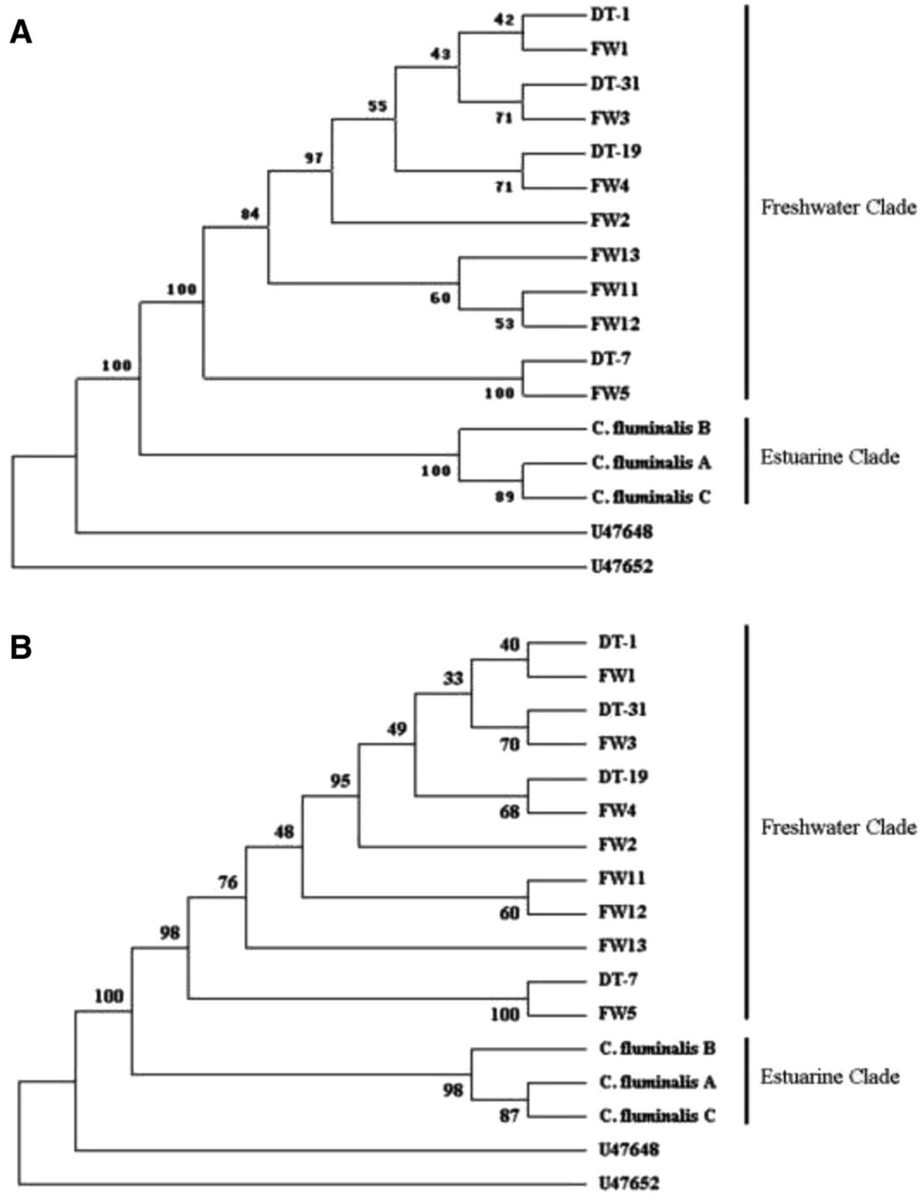


Figure 4 Phylogenic trees constructed by NJ (A) and ML (B) methods. The construction of the trees was based on a 614-bp fragment of the mitochondrial gene COI dataset. Sequences of U47648 and U47652 were used as outgroups. Bootstrap proportions and posterior probabilities are shown close to the nodes.

common recorded freshwater system members in Asia. Accordingly, the individuals with haplotypes DT-1, DT-19, and DT-31 could be described as *C. fluminea* (FW1, FW3, and FW4 were referred to as *C. fluminea*). This assumption would also be supported by the minor significant differences in shell morphological characters between DT-7 and the group of haplotypes DT-1, DT-19, and DT-31.

Therefore, all results in the present study showed that two species (*C. leana* and *C. fluminea*) of genus *Corbicula* may coexisted in Datong Lake, and each species shared both two color morphs. This was significantly inconsistent with the previous results that the two morphotypes should be relegated to two separate species, for white morph *C. fluminea* and purple morph *C. leana* (Choe et al. 1999; Kwon et al. 2001; Park et al. 2002), or only to be identified as *C. fluminea* (Qiu et al. 2001; Li et al. 2011). However, this assumption should be confirmed further. As it was reported, most phylogenetic studies on *Corbicula* based on mitochondrial data could lead to inaccurate species delimitation because of mitochondrial/morphotype-nuclear mismatch (Pigneur et al. 2011). In addition, the special androgenesis and hermaphrodite characteristics that were recorded in most *Corbicula* species made the delineation of *Corbicula* even more complicated. Hence, a comprehensive study combining nuclear and mitochondrial data, along with morphology and biological information will do help to distinguish the taxonomy and systematic status of the two color morphs in further studies.

Conclusions

Two color morphs (white and purple) of *Corbicula* sympatric in Datong Lake were compared by morphometric methods and genetic analysis to explore their taxonomic relationship. The results showed that there were no distinct differences on morphological measures in the aspects of the two color morphs and the four mitochondrial COI haplotypes (DT-1, DT-7, DT-19, and DT-31). Each color morph was shared by all four mitochondrial COI haplotypes. Phylogenetic analysis demonstrated that the four haplotypes were subdivided into two divergent clades among freshwater clades, and haplotype DT-7 was a divergent sister taxon to the other three. Thus, two species (DT-7 as *C. leana* and DT-1, DT-19, and DT-31 as *C. fluminea*) may coexisted in Datong Lake. However, this assumption should be confirmed further because of the potential inaccurate species delimitation resulting from the mitochondrial/morphotype-nuclear mismatch in *Corbicula* genus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

This study was performed in collaboration among all authors. GPW carried out the experiment, participated in the statistical analysis, and drafted the manuscript. TZ provided expertise in drafting, reviewing, and revising the manuscript for intellectual content. JZ participated in collecting the samples of *Corbicula*. DLL conceived of the study, designed the experiment, and drafted the manuscript. TYX provided purely technical help. All authors read and approved the final manuscript.

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