The complete mitochondrial genome of the Chinese *Daphnia pulex* (Cladocera, Daphniidae)

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Academic editor: Saskia Brix | Received 23 March 2016 | Accepted 9 August 2016 | Published 7 September 2016

http://zoobank.org/73EDAF86-13FF-401A-955D-37CC9A56E047

Citation: Geng X, Cheng R, Xiang T, Deng B, Wanga Y, Deng D, Zhang H (2016) The complete mitochondrial genome of the Chinese *Daphnia pulex* (Cladocera, Daphniidae). ZooKeys 615: 47–60. doi: 10.3897/zookeys.615.8581

Abstract

*Daphnia pulex* has played an important role in fresh-water ecosystems. In this study, the complete mitochondrial genome of *Daphnia pulex* from Chaohu, China was sequenced for the first time. It was accomplished using long-PCR methods and a primer-walking sequencing strategy with genus-specific primers. The mitogenome was found to be 15,306 bp in length. It contained 13 protein-coding genes, two tRNA genes, 22 tRNA genes and a typical control region. This research revealed an overall A+T content of 64.50%. All of the 22 typical animal tRNA genes had a classical clover-leaf structure except for *trnS1*, in which its DHU arm simply formed a loop. The lengths of small and large rRNA were 744 bp and 1,313 bp, respectively. The A+T-rich region was 723 bp in length, which is longer than that from the North American species (689 bp). In terms of structure and composition, many similarities were found between the Chinese and North American *Daphnia pulex*.

Keywords

*Daphnia pulex*, gene order, mitochondrial genome, secondary structure

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Introduction

Cladocerans (“water fleas”) are an important component of the microcrustacean zooplankton. Their habitats are mostly continental fresh and saline waters (Forró et al. 2008). *Daphnia pulex* has become a well-known model species for studying evolutionary biology, environmental biology and ecology (Miner et al. 2013, Geng et al. 2016). Although other related research has been done (Roland et al. 2011, Geng et al. 2014), there are still some difficulties with species identification. In this study, meaningful data to assist in the taxonomy of different species of *Daphnia* is provided, and variations in similar morphological groups using molecular tools are analysed (Petrusek et al. 2012).

The sequence and structure of mitochondrial genomes has been frequently used to study phylogenetic relationships of animal taxa. More specifically, the unusual characters of mitochondrial genome DNA, for instance its small size, fast evolutionary rate, simple structure, maternal inheritance and high informational content, have been widely regarded as a molecular marker for phylogenetic analysis (Wilson et al. 2000, Chao et al. 2014, Ma et al. 2015).

All metazoan animals contain their own circular mitochondrial genome with two strands (a J-strand and an N-strand) (Simon et al. 2014), which range from 14 kb to 42 kb in length (Wolstenholme 1992). These typically encoded 37 genes, namely: 2 rRNA genes (*16S rRNA* and *12S rRNA*), 22 tRNA genes, and 13 protein-coding genes (*COI, COII, COIII, Cytb, ATP6, ATP8, ND1, ND2, ND3, ND4, ND4L, ND5, ND6*) (Boore 1999). Moreover, the non-coding region (also called the control region or D-loop), which with significant functions in the regulation and initiation of mitochondrial DNA transcription and replication (Brown et al. 1979, Shadel and Clayton 1993, Zhang and Hewitt 1997). Complete mitochondrial genome sequences are more informative than shorter sequences of individual genes but also provide a set of genomic characters. This led to the recognition of relative positions of different genes, RNA secondary structures and modes of control of replication and transcription (Masta and Boore 2008). However, the complete mitochondrial genome sequences data on *Daphnia* released in Genbank is far from enough.

The main purpose of this study was to disclose the complete mitochondrial genome sequence of the Chinese *Daphnia pulex* for the first time, and to compare its features with other available cladoceran mitochondrial genomes.

This study also served as a useful source of information for both nuclear and mitochondrial markers in comparative analyses of the evolution of mitochondrial genomes in Cladocerans.
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Materials and methods

Samples and DNA extraction

Total DNA was extracted from individual specimens using a TIANamp Micro DNA Kit (TIANGEN BIOTECH (BEIJING) CO., LTD) following manufacturer protocols. DNA samples were stored at -20 °C until further use.

PCR amplifications and sequencing

The Daphnia pulex mitochondrial genome was amplified using five pairs of primers (Table 1). To obtain the complete sequences of Chinese Daphnia pulex, short-PCR and long-PCR methods were used. The primers employed in this study were designed based on the mitochondrial genomes of the North American Daphnia pulex (GenBank accession number AF117817) (Crease 1999) by using an NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

The PCRs were performed by using an Eppendorf Thermal Cycler (5331AH760577, Eppendorf, Germany) with a 25 µL volume reaction mixture containing 2.5 µL 10×LA-Taq Buffer II (Mg²⁺ plus), 4 µL dNTP Mixture (2.5 mM), 2 µL DMSO, 1 µL genomic DNA, 1 µL 10 µM of each primer, 0.5 µL MgCl₂ (25 mM) and 0.25 µL 2.5 units of LA Taq polymerase (TaKaRa Biomedical, Japan), and 12.25 µL distilled water.

The reaction conditions were one cycle of denaturation at 95 °C 5 min, 35 cycles of denaturation at 95 °C 30 s, annealing at 50 °C 30 s, extension at 72 °C for 2 to 8 min and a final extension at 72 °C for 10 min. Each amplicon (5 µL) was examined with agarose gel electrophoresis to validate amplification efficiency. PCR products were sequenced directly by primer walking from both directions after purification.

Table 1. Details of the primers used to amplify the mitogenome of Chinese Daphnia pulex.

| Primer pair | Size (bp) | Primer sequence(5’-3’)                        |
|-------------|-----------|-----------------------------------------------|
| F1          |           | AGAAGGGAATTTGAGCTCTTTTWGT                     |
| R1          | 5450      | TTAGCCCTAGGGATAACACGCTAA                      |
| F2          | 2221      | TCGTCTCGTATCCATCGAGAC                       |
| R2          |           | GTGCCAGCAGYYGCGGGTTANAC                      |
| F3          | 3122      | AATAGGGGATTATCCATCTTTACGC                    |
| R3          |           | ACTTCGWTGATGCTCCYAAYTC                      |
| F4          | 4000      | ACTACGCCGCAAACCGTCTTTG                        |
| R4          |           | TGGGATGCTTTTGGGGCTAA                       |
| F5          | 750       | AGGGTTATTTTTTATTTCC                         |
| R5          |           | TGGGCTTCGGCAGCAGGATAG                      |
Analysis and annotation

The raw sequences of mitochondrial genome were edited and assembled by using the program Seqman (DNAStar, Inc.) and then adjusting them manually. Protein-coding genes and rRNA genes were identified by the MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py) and the similarity between *Daphnia pulex* and that published in NCBI database were distinguished by BLAST search function (http://www.ncbi.nlm.nih.gov/BLAST/). Nucleotide sequences of PCGs were translated using the invertebrate mitochondrial genetic code. The tRNA genes were initially identified by the MITOS WebServer (http://mitos.bioinf.uni-leipzig.de/index.py) and their secondary structures were predicted and modified based on other metazoan's secondary structure of tRNA genes.

The exact initiation and termination codons were identified by using Clustal X version 2.0 (Larkin et al. 2007) and relied on reference sequences from other invertebrates. Nucleotide composition and codon usage were calculated with MEGA 6.0 software (Tamura et al. 2013). The sequence data has been deposited into GenBank database under the accession number KT003819.

Results and discussion

Genome organization and base composition

The mitochondrial genomes of the Chinese *Daphnia pulex* used in this study were similar to that of the *Daphnia pulex* in North America (Crease 1999). The complete mitochondrial genome of Chinese *Daphnia pulex* was a circular molecule 15,306 bp in size, containing 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes for both the small and large subunits (*rrnS* and *rrnL*) and a putative control region (Fig. 1). Among all the 37 genes, 23 genes were encoded on the J-strand. The remaining genes were encoded on the N-strand. 8 overlaps were found between adjacent genes (29 bp in total), among which the longest was 10 bp located at *trnS2* and *ND1*. This included 15 intergenic spacers that ranged from 1 to 31 bp (84 bp in total), of which only one spacer was longer than 10 bp. That occurred between *ND4L* and *trnT*.

The mitochondrial genome of the Chinese *Daphnia pulex* has an A+T content of 64.50%, which is a little higher than that of the North American species (62.26%). Furthermore, it was determined that the AT skew was 0.006, and the GC skew was -0.107. AT skew and GC skew for a given strand were calculated as (G-C)/(G+C) and (A-T)/(A+T), respectively, with negative values in skewness meaning the coding strand is enriched for T or C. In contrast, positive values infer more As and Gs. On the whole, AT skew was slightly negative, or positive in the third codon position of vestimetiferans, and GC skew was more negative than AT skew (Table 2). Nucleotide bias can also be reflected by codon usage. We found that the RSCU (Relative Synonymous Codon Usage) value of NNA and NNU codons were greater than 1,
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**Figure 1.** Structure of Chinese *Daphnia pulex* mitochondrial genome. **COI**, **COII**, **COIII** refer to the cytochrome oxidase subunits, **Cytb** refers to cytochrome b, **ND1** - **ND6** refer to NADH dehydrogenase components, and **rrL** and **rrnS** refer to rRNAs. tRNA genes are denoted by one letter symbol according to the IUPUC-IUB single-letter amino acid codes. L1, L2, S1 and S2 denote tRNALeu(CUN), tRNALeu(UUR), tRNASer(AGN) and tRNA Ser(UCN), respectively. D-loop indicates A+T-rich region. Gene names outside the ring are coded on the majority strand while those inside are on the minority strand.

which indicates that codons were biased in favor of codons with A or T in the third position (Table 3).

Amino acids are denoted as one-letter symbol according to the IUPAC-IUB single letter amino acid codes.

**Protein-coding genes**

The complete mitochondrial DNA of Chinese *Daphnia pulex* from Chaohu had 13 protein-coding genes. Nine of these genes were located on the J-strand while the others
Table 3. Codon usage of the Chinese *Daphnia pulex* mitogenome.

| Codon | Count | RSCU | Codon | Count | RSCU | Codon | Count | RSCU | Codon | Count | RSCU |
|-------|-------|------|-------|-------|------|-------|-------|------|-------|-------|------|
| UUU(F) | 20.1 | 1.39 | UCU(S) | 8 | 2.04 | UAU(Y) | 7.9 | 1.27 | UGU(C) | 3.5 | 1.3 |
| UUC(F) | 8.8 | 0.61 | UCC(S) | 3 | 0.76 | UAC(Y) | 4.5 | 0.73 | UGC(C) | 1.9 | 0.7 |
| UUA(L) | 12.3 | 1.76 | UCA(S) | 3.7 | 0.94 | UAA(*) | 5.9 | 1.01 | UGA(*) | 4.5 | 0.77 |
| UUG(L) | 6.3 | 0.9 | UCG(S) | 1.8 | 0.45 | UAG(*) | 7.2 | 1.22 | UGG(W) | 3.8 | 1 |
| CUA(L) | 5.2 | 1.32 | CGC(P) | 5 | 1.71 | CAU(H) | 3.3 | 1.23 | CGU(R) | 1.2 | 0.63 |
| CUC(L) | 5.2 | 0.74 | CCC(P) | 3.2 | 1.08 | CAC(H) | 2.1 | 0.77 | CGC(C) | 1.1 | 0.59 |
| CUA(L) | 5.3 | 0.76 | CCA(P) | 1.5 | 0.5 | CAA(Q) | 3.5 | 1.08 | CGA(R) | 1.9 | 1.05 |
| CUG(L) | 3.6 | 0.52 | CCG(P) | 2.1 | 0.71 | CAG(Q) | 2.9 | 0.92 | CGG(R) | 1 | 0.55 |
| AAU(I) | 12.2 | 1.57 | ACU(T) | 6 | 1.88 | AUA(N) | 5.6 | 1.4 | AGU(S) | 4.6 | 1.18 |
| AUC(I) | 5 | 0.64 | ACC(T) | 2.4 | 0.75 | AAC(N) | 2.4 | 0.6 | AGC(S) | 2.5 | 0.63 |
| AUA(I) | 6.2 | 0.79 | ACA(T) | 2.9 | 0.92 | AAA(K) | 4.4 | 1.1 | AGA(R) | 3.8 | 2.06 |
| AUG(M) | 5 | 1 | ACG(T) | 1.5 | 0.46 | AAG(K) | 3.6 | 0.9 | AGG(R) | 2.1 | 1.13 |
| GUU(V) | 5.2 | 1.41 | GCU(A) | 4 | 1.81 | GAU(D) | 4.3 | 1.23 | GGU(G) | 2.4 | 0.62 |
| GUC(V) | 2.3 | 0.62 | GCC(A) | 1.8 | 0.83 | GAD(C) | 2.7 | 0.77 | GGC(G) | 2.1 | 0.54 |
| GUA(V) | 4.6 | 1.24 | GCA(A) | 2.2 | 0.97 | GAA(E) | 1.8 | 0.69 | GGA(G) | 4.4 | 1.13 |
| GUG(V) | 2.7 | 0.73 | GCG(A) | 0.8 | 0.38 | GAG(E) | 3.4 | 1.31 | GGG(G) | 6.6 | 1.71 |

RSCU: Relative Synonymous Codon Usage.
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Ten out of these 13 protein-coding genes initiated with typical ATN codons. ND2, COII, ATP6, COIII, ND4, Cytb and ND1 started with ATG, COI initiated with ATA, and moreover ND3 and ND6 used ATC as the initiating codon. The ATP8 and ND5 genes used GTG. The ND4L gene used none of these as initiating codon, but GCT.

As is the case with some other arthropod species, the initiation functions of the COI gene has not been fully investigated. Atypical initiating codons for the COI gene in mitochondrial genomes have been reported in many studies, examples of these genes are: CGA (Gong et al. 2012), GTG (He et al. 2011), TTG (Hu et al. 2010, Li et al. 2012), ACG (Wilson et al. 2000), CCG (Fenn et al. 2007), ACC (Yamauchi et al. 2004), and TTA (Yamauchi et al. 2002). In Drosophila, Locusta and Daphnia, there are occasionally some uncommon quadruplets like, ATAA or ATTA, that may serve as an initiation codon (Wilson et al. 2000). One example of this is the COI gene in the North American Daphnia pulex initiating with ATTA (Crease 1999). However, the COI gene of Chinese Daphnia pulex started with classical ATA.

Nine of the 13 protein-coding genes used the typical termination codon TAN. ND2 and ATP8 terminated with TAG. COIII, ND3, Cytb, ATP6, ND4L, ND6 and ND1 all terminated with TAA. COI, COII, ND4 and ND5 used the incomplete termination codon T. Both of the complete termination codons TAG and TAA and two additional abbreviated termination codons T and TA were found in the North American Daphnia pulex (Table 4).

The use of incomplete termination codons on these genes might serve the purpose of avoiding overlapping nucleotides between adjacent genes (He et al. 2012). The incomplete termination codons would become functional termination codons after polycistronic transcript cleavage and polyadenylation processes have occured (Ojala et
Table 4. Organization of the mitochondrial genomes of *Daphnia pulex* from Chinese Chaohu (Ch) and that from North America (Na).

| Gene/strand | position | length | Start/stop codon |
|-------------|----------|--------|------------------|
|             | Ch       | Na     | Ch               | Na   | (Ch/Na) | Taa/Taa |
| trnI/J      | 1–64     | 1–64   | 64               | 64   | ATG/ATG | TAG/T__ |
| trnQ/N      | 66–133   | 66–133 | 68               | 68   | ATG/ATG | Taa/Taa |
| trnM/J      | 134–197  | 134–197| 64               | 64   | ATG/ATG | Taa/Taa |
| ND2/J       | 198–1139 | 198–1185| 942              | 988  | ATG/ATG | TAG/T__ |
| trnW/J      | 1138–1202| 1186–1251| 65               | 66   | ATG/ATG | Taa/Taa |
| trnC/N      | 1206–1268| 1253–1316| 63               | 64   | ATG/ATG | Taa/Taa |
| trnY/N      | 1278–1340| 1328–1391| 63               | 64   | ATG/ATG | Taa/Taa |
| COI/J       | 1350–2886| 1397–2934| 1537             | 1538 | ATG/ATG | Taa/Taa |
| trnL2/J     | 2887–2954| 2935–3002| 68               | 68   | ATG/ATG | Taa/Taa |
| COII/J      | 2956–3634| 3004–3682| 679              | 679  | ATG/ATG | TAG/TAG |
| trnK/J      | 3635–3704| 3683–3752| 70               | 70   | ATG/ATG | Taa/Taa |
| trnD/J      | 3709–3773| 3757–3821| 65               | 65   | ATG/ATG | Taa/Taa |
| ATP8/J      | 3774–3935| 3821–3982| 162              | 162  | GTG/ATG | Taa/Taa |
| ATP6/J      | 3929–4603| 3976–4649| 675              | 674  | ATG/ATG | Taa/Taa |
| COIII/J     | 4603–5391| 4650–5438| 786              | 789  | ATG/ATG | Taa/Taa |
| trnG/J      | 5393–5456| 5439–5499| 64               | 61   | ATC/ATT | Taa/Taa |
| ND3/J       | 5457–5810| 5500–5852| 354              | 353  | ATC/ATT | Taa/Taa |
| trnA/J      | 5811–5874| 5853–5918| 64               | 66   | ATG/ATG | Taa/Taa |
| trnR/J      | 5876–5940| 5920–5984| 65               | 65   | ATG/ATG | Taa/Taa |
| trnN/J      | 5943–6010| 5985–6051| 68               | 67   | ATG/ATG | Taa/Taa |
| trnS1/J     | 6011–6075| 6052–6116| 65               | 65   | ATG/ATG | Taa/Taa |
| trnE/J      | 6076–6141| 6117–6184| 66               | 68   | ATG/ATG | Taa/Taa |
| trnF/N      | 6141–6205| 6184–6249| 65               | 66   | ATG/ATG | Taa/Taa |
| ND5/N       | 6207–7913| 6250–7957| 1707             | 1708 | GTG/ATG | Taa/Taa |
| trnH/N      | 7908–7971| 7952–8015| 64               | 64   | ATG/ATG | Taa/Taa |
| ND4/N       | 7972–9292| 8016–9336| 1321             | 1321 | ATG/ATG | Taa/Taa |
| ND4L/N      | 9295–9570| 9339–9614| 276              | 276  | GCT/ATT | Taa/Taa |
| trnT/J      | 9602–9664| 9646–9710| 63               | 65   | ATG/ATG | Taa/Taa |
| trnP/N      | 9665–9730| 9711–9775| 66               | 65   | ATG/ATG | Taa/Taa |
| ND6/J       | 9733–10245| 9778–10290| 513              | 513  | ATC/ATT | Taa/Taa |
| Cytb/J      | 10245–11378| 10298–11431| 1134             | 1134 | ATG/ATG | Taa/Taa |
| trnS2/J     | 11379–11447| 11432–11500| 69               | 69   | ATG/ATG | Taa/Taa |
| ND1/N       | 11438–12373| 11494–12426| 936              | 936  | ATG/ATG | Taa/Taa |
| trnL1/N     | 12377–12443| 12430–12496| 67               | 67   | ATG/ATG | Taa/Taa |
| rnl/J       | 12454–13766| 12506–13819| 1313             | 1314 | ATG/ATG | Taa/Taa |
| trnV/N      | 13769–13840| 13821–13892| 72               | 72   | ATG/ATG | Taa/Taa |
| trnS/N      | 13840–14583| 13892–14644| 744              | 753  | ATG/ATG | Taa/Taa |
| D-loop/J    | 14584–15306| 14645–15333| 723              | 689  | ATG/ATG | Taa/Taa |

Note: J and N refer to the majority and minority strand, respectively. Position numbers refer to positions on the majority strand.
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Figure 3. Inferred secondary structure of 22 tRNA genes in Chinese Daphnia pulex mtDNA genome.
These incomplete codons and this mechanism has been commonly found in metazoan mitochondrial genomes (Wei et al. 2009, Liao et al. 2010). The total length of the 13 protein-coding genes was found to be 11,026 bp for the Chinese *Daphnia pulex*, which accounts for 63.37% of the total mitogenome length.

Many composition similarities were noted between the two different species compared in this study (Fig. 2).

**tRNA genes**

All of the 22 typical arthropod tRNAs were found in the Chinese *Daphnia pulex* mitochondrial genome. They ranged from 63 to 72 bp in size. A schematic drawing of their respective secondary structures is shown in Figure 3. All tRNA genes had a clover-leaf structure except for *trnS*, in which its DHU arm simply formed a loop. This loop in *trnS* is not uncommon in metazoan mitochondrial genomes (Crease and Little 1997). Whether or not the aberrant tRNAs lose their respective functions is still unknown. However, it’s possible this anomaly may be rectified by subsequent RNA-editing mechanisms (Lavrov et al. 2000, Masta and Boore 2004, Li et al. 2012).

Non-canonical pairs, which possessed non Watson-Crick matches, commonly manifest in mitochondrial tRNA gene secondary structures. There are 30 base pair mismatches present in the tRNA secondary structures of Chinese *Daphnia pulex* mtDNA, including 15 wobble G-U pairs, 13 U-G pairs, two U-U pairs, one A-A pair and one U-C pair mismatch (Fig. 3). Nevertheless, the post-transcriptional RNA-editing mechanism can rectify these mismatches to maintain tRNA functions (Tomita et al. 2001, Wang et al. 2014).

**rRNA genes**

Both the *rrnL* and *rrnS* genes were present in Chinese *Daphnia pulex* mitochondrial genome. They were located between *trnL* and the non-coding putative control region and separated by *trnV*, as similarly found in vertebrate mitochondrial genomes (Delisle and Strobeck 2002, Hwang et al. 2008, Chao et al. 2014).

Large and small ribosomal RNA genes (*rrnL* and *rrnS*) in Chinese *Daphnia pulex* were 1,313 bp and 744 bp long, respectively. The lengths of the two rRNAs were almost similar to that of the *Daphnia pulex* in North America (1,314 bp and 753 bp, respectively).

**Non-coding sequence**

There are 15 non-coding regions ranging from 1 to 31 bp except for the A+T-rich region in the Chinese *Daphnia pulex* mitochondrial genome.

A 31 bp intergenic sequence was present between *ND4L* and *trnT*, which is also found in the North American *Daphnia pulex* mitochondrial DNA. The longest inter-
The complete mitochondrial genome of the Chinese Daphnia pulex was the A+T-rich region. It was between *rrnS* and *trnI* with the length of 723 bp. It has an A+T content of 65.42%. It was a little longer than that of the North American *Daphnia pulex* mitochondrial DNA (689 bp), but lower in A+T content. This region usually contains replication and transcription areas in both vertebrates and invertebrates (Zhang and Hewitt 1997, Boore 1999). The stem-loop structure and the quantity of multiple repeats of AT sequences are notable features of the control region, ranging from 200 bp to 1,300 bp, and determine the difference in arthropod mitochondrial DNA size (Boore 1999).

**Phylogenetic analyses**

The phylogenetic relationships among the *Daphnia pulex* from different areas were reconstructed based on nucleotide sequences of the *COI* gene by using the maximum likelihood (ML) method (Fig. 4). The phylogenetic analyses show that the Chinese and North American *Daphnia pulex* are recovered as two monophyletic clades with strong bootstrap support values (bs=100). They maybe evolved into two different species.

**Conclusion**

The mapping of the mitochondrial genome of the Chinese *Daphnia pulex* was completed in this study. It was found to be 15,306 bp in length and had a similar composition in size and structure to the *Daphnia pulex* mitochondrial DNA in North America published in GenBank AF117817 (Crease 1999). However, the phylogenetic analysis showed that the Chinese and North American *Daphnia pulex* maybe evolved into two different species (Fig. 4). The complete mitogenome of the Chinese *Daphnia pulex* reported here is expected to supply more molecular information for further studies of the *Daphnia* phylogeny and for analyses on the taxonomic status of the Cladocera.
Acknowledgments

The authors are grateful to Jun Li for his help with experiments. This work was supported by the National Natural Science Foundation of China (81272377, 31370470), the Natural Science Foundation of Anhui Province of China (1208085MC45) and the open-ended fund of Anhui Key Laboratory of Plant Resources and Biology (ZY-ZWSW2014014).

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