Confirmation of species independence and affinity of *Musa huangbaioa* (Musaceae) – rare endemic banana of China – according to the molecular phylogenetic data

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**Summary.** In this article, we research the phylogenetic position of the rare endemic banana, *Musa huangbaioa*, which was described only in Chinese journal in 1987. This banana was found at the foot of the Mount Emei in Sichuan Province and has remarkable morphological features, e. g., undulated petiole margins, ribbed fruits and irregular form of the seeds, which are rather unusual in the genus and distinguish it from all other species. In addition, due to its uncertain affinity, we researched the position of *M. huangbaioa* in the Musaceae family with the aid of molecular phylogenetic analysis of two marker sequences, nrITS and *trnL*–*trnF*. We found that this species belongs to the large and rather complicated group of Chinese bananas, *M. basjoo*–*M. itinerans* clade. According to the ITS data, *M. huangbaioa* is monophyletic with one *M. basjoo* specimen that was cultivated in Central America. Probably, this fact represents that this species can be modern hybrid with one of the genomes inherited from *M. basjoo* s. l. The whole group *M. basjoo*–*M. itinerans*, which *M. huangbaioa* belongs to, is well separated within the sect. *Musa* and could be prone to frequent hybridizations in the natural environment; it requires an additional research for more precise differentiation of the group.

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Подтверждение видовой самостоятельности и родства *Musa huangbaioa* (Musaceae) – редкого эндемичного банана из Китая – по молекулярно-филогенетическим данным

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Ключевые слова: гибридизация, Китай, новые виды, nrITS, trnL–trnF.

Аннотация. В этой статье мы исследуем филогенетическое положение редкого эндемичного банана, Musa huangbaioa, который был описан только в китайском журнале в 1987 г. Этот банан был найден у подножия горы Эмей в провинции Сычуань и имеет примечательные morphологические черты, такие как волнистый край черешка листа, ребристые плоды и неправильную форму семян, которые достаточно необычны для рода и хорошо отделяют этот вид от остальных. Кроме того, из-за его неопределенного рода, мы исследовали положение M. huangbaioa в семействе Musaceae с помощью молекулярно-филогенетического анализа двух маркерных последовательностей, nrITS и trnL–trnF. Мы обнаружили, что этот вид принадлежит к большой и достаточно сложной группе китайских бананов, кладе M. basjoo–M. itinerans. По данным ITS, M. huangbaioa монофилетичен с одним образцом M. basjoo, культивировавшимся в Центральной Америке. Возможно, этот факт показывает то, что M. huangbaioa может быть современным гибридом с одним из геномов, унаследованным от M. basjoo s. l. Вся группа M. basjoo–M. itinerans, к которой принадлежит M. huangbaioa, хорошо отграничена внутри секции Musa и может быть склонна к частым гибридизациям в естественной среде; она требует дополнительного изучения для более точной дифференциации этой группы.

Introduction

Musaceae is a small paleotropical family which is one of the most important food crops in the world. Its morphological taxonomy is rather well established, nevertheless, there are some difficulties in establishing more precise phylogenetic system basing on molecular genetic data.

As a genus, Musa was established by Linnaeus (1753), but the first infrageneric classification was performed only more than a century later by Sagot (1887). He divided bananas into three groups: giant bananas, fleshy (edible) bananas and ornamental bananas with upright inflorescences and brightly colored bracts. Subsequently, Baker following Sagot’s classification formally divided bananas into 3 subgenera: Physocaulis Baker, Eumusa Baker and Rhodochlamys Baker (Baker, 1893). Then Cheesman elevated the first group, giant bananas, to the generic level as the genus Ensete Bruce ex Horan. (Cheesman, 1947). The genus Musa, he divided into four sections basing on morphology and chromosome numbers: Eumusa (x = 11), Rhodochlamys (x = 11), Australimusa Cheesman (x = 10) and Callimusa Cheesman (x = 9, 10). This classification was widely accepted and used in many treatments of the genus. Later Argent (1976) proposed a new section, Ingentimus Argent, with the lowest chromosome number, x = 7. The section is monotypic and distributed only in New Guinea.

New methods of analysis become actual due to the rather morphological unity of the genus (Li et al., 2010). After the development of molecular phylogenetic methods, many phylogenetic schemes basing on molecular data were created. RFLP and AFLP revealed some inconsistencies with the traditional system of the genus Musa, e. g., sect. Rhodochlamys grouped in the sect. Musa and sect. Australimusa occurred in the sect. Callimusa (Li et al., 2010). In addition, some phylogenetic analyses were performed according to the nuclear and chloroplast gene data. Two main clades were found in the whole genus. The first clade comprised sections Musa and Rhodochlamys and the second species of sections Callimusa, Australimusa and Ingentimus (Li et al., 2010). Within the whole family genera Musa, Musella (Franch.) H. W. Li and Ensete were monophyletic, but none of the sections previously defined by morphological features. For example, authors suggested the sections Musa and Rhodochlamys combining into the sect. Musa because of the common basic chromosome number, x = 11, and also some morphological characters (Li et al., 2010; Liu et al., 2010). The species from the second clade also have common basic chromosome number x = 7, 9, 10. Previous studies revealed that infrageneric classification of bananas is closely correlated with basic chromosome numbers, which lead to the reproductive isolation (Cheeseman, 1947). Of course, new methods of molecular phylogeny will help us clarifying the affinity of the rare and new Musa species.

The genus Musa comprises about 70 species (Simmonds, Weatherup, 1990; De Langhe, 2000; Wu, Kress, 2000; Wong et al., 2002; Häkkinen, Väre, 2008; Häkkinen et al., 2008; Häkkinen, 2009, 2013; Lý et al., 2012). Despite rather knowledge of the genus, some new species have been recently discovered (Lý et al., 2012, Singh, 2014). In 1987 banana sample with clear morphological distinction from the other species was found in China (Zhu, 1987). Its relationships are not very clear. In addition, this species was described only in Chinese local journal and was very little known for the scientists.
A series of morphological peculiarities of this newly discovered banana and its probable species status raise a question of molecular phylogenetic analysis of this sample and some other bananas from its possible affinity. Although quite a few bananas were studied by different molecular phylogenetic approaches, such analysis can be rather useful in clarifying the relationships of the newly discovered species and especially in possible hybridization cases. Because of the unusual morphological characters of *M. huangbaioa* we took into analysis species from different banana sections and clades. For our analysis we used marker sequences from two genome regions: nuclear (ITS) and chloroplast (*trnL–trnF*). These sequences are widely applied for phylogenetic reconstruction and species identifying (DNA-barcoding) (Hollingsworth et al., 2011). In bananas, as in other families, the usage of different marker sequences is necessary, because *Musa* is prone to hybridization in some cases (Swangpol et al., 2007).

### Materials and methods

Plant samples of the studied species were grown from the seeds collected in December 2016 in Sichuan Province in the territory of the Sichuan Academy of Natural Resource Science Emei Mountain Biology Resource Experiment Station and kept in Botanical Garden of Peter the Great, St.-Petersburg, Russia. Other *Musa* samples were obtained from greenhouses of Komarov Botanical Institute of the RAS (BIN RAS) and collected during the expedition of the Department Saint-Petersburg Botanical Garden of Peter the Great to Japan in 2019.

We added other sequences from the international GenBank database [https://www.ncbi.nlm.nih.gov/nuccore/?term=](https://www.ncbi.nlm.nih.gov/nuccore/?term=). All the sequences used for our analysis are presented in the Table. Totally we analyzed 28 ITS and 26 *trnL–trnF* sequences using *Musella lasiocarpa*, a species of closely related genus, as outgroup.

### Table

| Name of the species | Genbank number | Origin of the sample |
|---------------------|----------------|----------------------|
| *Musa acuminata* Colla | MW054208, MW066471 | Botanical garden of Peter the Great, St.-Petersburg, Russia |
| *Musa acuminata* Colla | –, FJ621283.1 | China; Liu et al., 2010 |
| *Musa acuminata* Colla | FJ428089.1 – | China; Li et al., 2010 |
| *Musa acuminata* Colla | KT696474.1 – | Hapsari et al., 2018 |
| *Musa acuminata* Colla | KT696473.1 – | Hapsari et al., 2018 |
| *Musa acuminata* Colla | KT696472.1 – | Hapsari et al., 2018 |
| *Musa acuminata* Colla | KT696471.1 – | Hapsari et al., 2018 |
| *Musa acuminata* subsp. *truncata* (Ridl.) Kiew | –, KU215218.1 | Janssens et al., 2016 |
| *Musa balbisiana* Colla | KT696445 – | Indonesia: Eastern Java; Hapsari et al., 2018 |
| *Musa balbisiana* Colla | –, KU215202.1 | Janssens et al., 2016 |
| *Musa banksii* F. Muell. | FJ428097.1 – | Li et al., 2010 |
| *Musa banksii* F. Muell. | –, KU215211.1 | Janssens et al., 2016 |
| *Musa banksii* F. Muell. | –, KU215208.1 | Janssens et al., 2016 |
| *Musa basjoo* Siebold et Zuccarini ex Inuma | MW054204, MW066468 | Botanical Garden of Peter the Great, St.-Petersburg, Russia |
| *Musa basjoo* Siebold et Zuccarini ex Inuma | MW054205, MW066469 | Botanical Garden of Peter the Great, St.-Petersburg, Russia |
| *Musa basjoo* Siebold et Zuccarini ex Inuma | MW054206 – | Garden of Kyoto, Japan |
| *Musa basjoo* Siebold et Zuccarini ex Inuma | FJ626374.1, FJ621270 | China; Liu et al., 2010 |
| *Musa basjoo* Siebold et Zuccarini ex Inuma | –, GG374828 | Gayral et al., 2010 |
| *Musa basjoo* Siebold et Zuccarini ex Inuma | KU215073.1, KU215195.1 | Janssens et al., 2016 |
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Genomic DNA from the leaf material was extracted using Qiagen DNeasy Plant Mini Kit according to the product manual. The ITS1-5.8S rRNA gene-ITS2 was amplified with primers ITS 1P (Ridgway et al., 2003) and ITS 4 (White et al., 1990). Amplification parameters for this region were: one

| Name of the species                  | Genbank number | Origin of the sample                                      |
|--------------------------------------|----------------|----------------------------------------------------------|
| *Musa coccinea* Andrews              | MW054210       | Botanical Garden of Peter the Great, St.-Petersburg, Russia |
| *Musa coccinea* Andrews              | FJ428062.1     | China; Li et al., 2010                                   |
| *Musa coccinea* Andrews              | –              | Janssens et al., 2016                                    |
| *Musa coccinea* Andrews              | KY214932.1     | Iles et al., 2016                                       |
| *Musa exotica* R. V. Valmayor        | FJ428063.1     | Li et al., 2010                                          |
| *Musa fragrans* Andrews              | –              |                                                         |
| *Musa fragrans* Andrews              | –              |                                                         |
| *Musa huangbaidaoa* Z. Y. Zhu        | MW054207       | Botanical Garden of Peter the Great, St.-Petersburg, Russia |
| *Musa itinerans* Andrews             | FJ428098.1     | Li et al., 2010                                          |
| *Musa jaceyi* W. Hill                | HQ331362       | Hřibová et al., 2010                                    |
| *Musa jaceyi* W. Hill                | –              |                                                         |
| *Musa lolodensis* Cheesman           | –              |                                                         |
| *Musa maclayi* F. Muell.             | FJ428068       | Li et al., 2010                                          |
| *Musa maclayi* F. Muell.             | –              |                                                         |
| *Musa maclayi* var. namatani Argent  | –              | Janssens et al., 2016                                    |
| *Musa maclayi* F. Muell.             | –              | Janssens et al., 2016                                    |
| *Musa mannii* H. Wendel. ex Baker    | –              | Liu et al., 2010                                         |
| *Musa ornata* Roxb.                  | FJ428096.1     | Li et al., 2010                                          |
| *Musa ornata* Roxb.                  | –              |                                                         |
| *Musa peekelli* subsp. angustigemma  | –              | Janssens et al., 2016                                    |
| *Musa rubinea* Hākkinen et C. H. Teo | FJ428093.1     | Li et al., 2010                                          |
| *Musa schizocarpa* N. W. Simmonds   | FJ428088.1     | Li et al., 2010                                          |
| *Musa schizocarpa* N. W. Simmonds   | HQ331332.1     | Hřibová et al., 2010                                    |
| *Musa schizocarpa* N. W. Simmonds   | –              | Janssens et al., 2016                                    |
| *Musa schizocarpa* N. W. Simmonds   | –              | Janssens et al., 2016                                    |
| *Musa textilis* Née                   | MW054211       | Garden of Kyoto, Japan                                   |
| *Musa textilis* Née                   | FR727896.1     | Hřibová et al., 2011                                    |
| *Musa textilis* Née                   | –              |                                                         |
| *Musa tonkinensis* R. V. Valmayor, L. D. Danh et Hākkinen | FJ428099.1 | China; Li et al., 2010                                   |
| *Musa velutina* H. Wendel. et Drude  | MW054209       | Botanical Garden of Peter the Great, St.-Petersburg, Russia |
| *Musa velutina* H. Wendel. et Drude  | –              |                                                         |
| *Musa violascens* Ridl.              | FJ428071       | Li et al., 2010                                          |
| *Musa violascens* Ridl.              | –              | Ning et al., 2014                                        |
| *Musa yunnanensis* Hākkinen et H. Wang | FJ428095.1 | Li et al., 2010                                          |
| *Musa yunnanensis* Hākkinen et H. Wang | –        | Thailand; Somana et al., 2015                            |
| *Musella lasiocarpa* (Franch.) H. W. Li | –             | Prince, Kress, 2004                                     |
| *Musella lasiocarpa* (Franch.) H. W. Li | –             | Thailand; Somana et al., 2015                            |

Table (end)
cycle of 95 °C for 5 min., 35 cycles: 95 °C for 40 s., 52–56 °C for 40 s., 72 °C for 40 s., final elongation 72 °C for 10 min. Marker region of chloroplast genome, including trnL gene, trnL intron and trnL–trnF intergenic spacer was amplified with primers tabC and tabF (Taberlet et al., 1991), according to the following program: one cycle of 95 °C for 5 min., 35 cycles: 95 °C for 1 min., 52–56 °C for 1 min. 10 s., 72 °C for 1 min. 10 s., final elongation 72 °C for 10 min. TrnL–trnF region was sequenced using the primer set (forward–reverse): tabC–tabD and tabE–tabF. All sequencing was performed on the sequencer ABI PRIZM 3100 sequencer at the Center of Collective Usage (CUS) BIN RAS with a BigDyeTM Terminator Kit ver. 3.1 set of reagents. Chromatograms were analyzed with the aid of Chromas Lite ver. 2.01. Alignment of the sequences was done by Muscle algorithm (Edgar, 2004) included in MEGA ver. 7.0 (Kumar et al., 2016) and adjusted manually.

Molecular phylogenetic analysis was performed by the Bayesian method and Maximum Likelihood. Models for phylogenetic tree estimation were selected using Akaike Information Criterium (AIC) with MEGA 7.0 (Kumar et al., 2016) and jModelTest 2.1.6 (Darriba et al., 2012). Unambiguous indels were coded with SeqState 1.4.1 (Müller, 2005) and then used in Bayesian analysis as binary characters (“restriction” option). We used Musella lastiocarpa (Franch.) H.W. Li as outgroup, because according to the previous research it occupies rather distant position in the family Musaceae (Simmonds, 1962; Li et al., 2010). Bayesian analysis was carried out with 1–1.5 million generations until the standard deviation of split frequencies was lower than 0.01. The first 25 % of trees were discarded, option “burn-in”. Maximum Likelihood analysis was conducted by MEGA 7.0; 1000 bootstrap replications. In the resulting trees, posterior probability is shown at the nodes as the first value, bootstrap value as the second. Clades with 100–90 % of posterior probability and bootstrap index we consider as strongly supported, 89–70 % – as moderately supported and 50–69 % – as weakly supported. Indexes below 50 % we regard as no support for the clade.

**Taxonomic treatment**

*M. huangbaioa* is an endemic plant growing in subtropical conditions of China. We studied a specimen growing in culture at Sichuan Academy of Natural Resource Science Emei Mountain Biology Resource Experiment Station. This species was described by Z. Y. Zhu in Chinese local journal, and thus was forgotten for the majority of researchers. Here we need to remind of its existence and to provide a correct link to the description of this species.

**Musa huangbaioa** Z. Y. Zhu, 1987, in Chuan Yao Xiao Kan 1(9): 41.

**Holotype:** [China] “25 IX 1983. Z. Y. Zhu 1278 Typus Sichnahn [Sichuan] School of Chinese Materia Medica); Emei Shan, alt. 470 m. Z. Y. Zhu 2341 (species sativa)”. Holotype in EMA.

**Etymology.** Probably from the Chinese name of Amur corktree, Huang Bai, or from the Chinese word that means “cork”.

**Distribution and habitat.** Grows at the foot of the Emei Mount, Sichuan Province, ca. 470 m alt., cultivated. The original specimen is cultivated in the Botanical Garden of Peter the Great, St.-Petersburg, Russia, and now this banana successfully flowers and propagates.

The plant appearance and some morphological features are presented in Fig. 1.

**Results**

*Musa huangbaioa*, a rare endemic banana, forms a clade with various *M. basjoo* Sieb. et Zucc. ex Inumia samples according to the nuclear and chloroplast datasets (PP = 99 %, BS = 98 % – ITS data, PP = 87, BS unsupported – trnL–trnF) (Fig. 2, 9). It falls into the Clade I that was defined by previous analyses (see Li et al., 2010; Liu et al., 2010; Feng et al., 2016). This species with *M. basjoo* forms a separate subclade in the Clade I representing the section *Musa*. It is notable that *M. huangbaioa* has the same ITS sequences as one sample of *M. basjoo* cultivated in Mexico (Fig. 2).

According to the ITS data (PP = 99 %, BS = 98 %), the sister subclade in the Clade I corresponds to the sections *Musa* and *Rhodochlamys* (Fig. 2). *M. banksii* F. Muell occupies an uncertain position in this subclade. The sect. *Rhodochlamys* forms a highly supported group (PP = 100 %, BS = 88 %) within this subclade; *M. rubinea* Häkkinen et C. H. Teo is sister to the samples of *M. velutina* H. Wendl. et Drude by ITS data (Fig. 2). ITS sequences of *M. yunnanensis* Häkkinen et H. Wang (sect. *Musa*) are related to *M. ornata* Roxb. (sect. *Rhodochlamys*) and *M. balbisiana* Colla (sect. *Musa*) has a sister
position to all other species in the Clade I according to the ITS analysis. Chloroplast trnL–trnF sequences show slightly other picture (Fig. 3). The second subclade of the sections Musa and Rhodochlamys is present as well (PP = 98 %, BS unsupported) but M. acuminata forms a polytomy rather than any of the monophyletic groups (Fig. 3).

As in previous studies (Li et al., 2010; Liu et al., 2010; Feng et al., 2016), Clade II comprises species of the sections Australimusa and Callimusa (x = 9, 10). It is well supported according to the ITS dataset (PP = 100 %, BS = 98 %, Fig. 2) but has weak support basing on trnL–trnF analysis (PP = 60 %, BS = 62 %, Fig. 3). ITS sequences of the M. coccinea Andrews sample cultivated in greenhouses of BIN RAS (St.-Petersburg) were identical with the sequences from GenBank database. This species is monophyletic with M. exotica R.V.Valmayor (PP = 94 %, BS = 91 %) by ITS data but takes an isolated position according to the trnL–trnF sequences whereas M. exotica forms a strongly supported clade with M. violascens Ridl. (PP = 99 %). M. violascens (sect. Callimusa) is a sister to the subclade that contains sect. Australimusa (PP = 100 %, BS = 98 %) in ITS dataset.

Fig. 1. Musa huangbaioa in the greenhouses of the Botanical Garden of Peter the Great, St.-Petersburg and its characteristic features: A – Leaves and inflorescence of Musa huangbaioa, St.-Petersburg; B – Leaf sheaths and part of the pseudostem; C – Inflorescence of Musa huangbaioa; D – Male flowers of Musa huangbaioa; E – Musa huangbaioa, ripe fruit; F – Musa huangbaioa, seeds.
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Fig. 2. Phylogenetic placement of *Musa huangbaioa* in the genus *Musa* according to the analysis of ITS1–5.8S rRNA gene–ITS2 sequence data; BI posterior probability is shown the first, ML bootstrap index – the second.
Fig. 3. Phylogenetic placement of *Musa huangbaioa* in the genus *Musa* according to the analysis of *trnL–trnF* sequence data; BI posterior probability is shown the first, ML bootstrap index – the second.

**Discussion**

Species of the most part of banana family (Musaceae) mainly have wide natural range, but *M. huangbaioa* is described from the foot of Emei mountain and thus is narrow endemic. This is rather unusual feature for the bananas. The morphological characters of *Musa huangbaioa* show that it belongs to the sect. *Musa*: its pseudostems are tall, bracts are yellow and sometimes brownish, leaves are with glaucous tinge. This species also differs from all other banana species by unusual ribbed shape of the fruits and undulated petiole margins. The floral characters of *M. huangbaioa*, as well as the plant height, mostly resemble *M. itinerans*. Seeds also resemble that of the sect. *Musa*; they are angular,
more or less tetrahedral. According to the ITS and trnL-trnF data, this species is closely related to _M. basjoo_. It is taller than _M. basjoo_, its height is almost like that of _M. acuminata_. The floral characters resemble _M. itinerans_ Cheesem, but the stipule is longer than in _M. itinerans_. Moreover, according to the ITS data, _M. huangbaioa_ is identical to the sample of _M. basjoo_ cultivated in Central America and has the same trnL-trnF sequences as _M. basjoo_.

Speaking of this group as a whole, Chinese group of _M. basjoo_ and _M. itinerans_ is rather uniform and well separated within the sect. _Musa_ (see Simmonds, 1962; Simmonds, Weatherup, 1990; Li et al., 2010). It could originate in Oligocene (Christelová et al., 2011) and, according to the morphological data, form after the separation of _M. balbisiana_, _M. acuminata_ and _M. schizocarpa_ lineages (Simmonds, 1962). As shown in our previous article, _M. basjoo_ affinity contains complex hybrid species, probably, with the different maternal genomes. We can assume that this banana, _M. huangbaioa_, can be modern hybrid with the maternal genome inherited from _M. basjoo_. Unusual fruit shape can be adaptation to the mountain conditions.

According to our data, _Musa_ sect. _Rhodochlamys_ is nested with the sect. _Musa_ in the clade that also comprises _M. acuminata_ affinity group. This well corresponds with the hypothesis by N. Simmonds (1962), that _M. acuminata_ “stock” could be ancestral for the whole section _Rhodochlamys_. Nevertheless, we do not combine this section with the section _Musa_ due to morphological distinction between these sections. Bananas, as well as many other flowering plants, are subject of reticulate evolution and, thus, their natural system can be the network rather than dichotomous picture. This may really complicate their taxonomy.

The second clade that contains sections _Callimusa_ and _Australimusa_ also presents the reticulation evidence. _Musa exotica_ from the sect. _Callimusa_, which is rather morphologically distinct from the other species of the sections _Callimusa_ and _Australimusa_ in seed characters (Li et al., 2010), falls into the clade with its relatives with similar seeds, _M. coccinea_. This fits well with the previous studies (Li et al., 2010; Liu et al., 2010; Christelová et al., 2011; Feng et al., 2016). But maternal genome of _M. exotica_, as it appears, is related to another species of the sect. _Callimusa_, _M. violascens_. _M. exotica_ can be the introgressant that originated from the hybridization between members of two clades/lines from the sect. _Callimusa_ retaining the seed traits of _M. coccinea_ group. As in previous cases, this possible hybrid species retains the initial chromosome number of the section, 2n = 20. We can assume that bananas in natural conditions form homoploid hybrids without chromosome number duplication (Fe-liner et al., 2017).

**Conclusions**

Molecular phylogenetic methods allowed us to establish the clear relationship of the rare endemic banana species, _Musa huangbaioa_, which has peculiar and unusual morphological features. _M. huangbaioa_ appeared to be related to the separate Chinese banana group, _M. basjoo-M. itinerans_ affinity; this placement was supported by the seed shape of _M. huangbaioa_ along with the color and shape of male bracts and male flowers. At the same time, our studies showed interesting events of the possible reticulation within this group. This new species, _M. huangbaioa_, can be hybridogenous and, possibly, its morphological characters formed because of the gene combination. We also confirmed convenience and suitability of the marker sequences from the different genomes, nrITS and trnL-trnF for the identification of affinity in plants, “molecular barcoding” (see Hollingsworth et al., 2011).

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