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PHYLOGENETIC RELATIONSHIPS OF TWO REPRESENTATIVE *LEPIDIUM* SPECIES (BRASSICACEAE) IN QINGHAI PROVINCE

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Abstract

Lepidium belongs to Tribe Lepidieae of the family Brassicaceae. There are about 15 species and one variety in China, which are distributed throughout the country. So far, the systematic studies of this genus at the species level still depends on morphological characters, and there is little data on their molecular phylogeny. A total of 162 specimens of *Lepidium latifolium* and *Lepidium obtusum* were collected from Qinghai Province. Combined with sequences from GenBank, the phylogenetic relationships of *Lepidium* were analyzed based on nrITS gene. The results showed that *Lepidium* clustered into a large clade (Clade A), in which *L. hirtum* together with *L. campestre* as a sister group of *L. perfoliatum* formed a sister clade (98/1) and was located at the bottom. In the middle part, *L. densiflorum* clustered with *L. virginicum* (100/1). At the top *L. sativum*, *L. obtusum* and *L. latifolium L. apetalum L. ruderale* formed parallel clades (61/0.83). These results were inconsistent with morphological data. Meanwhile, the haplotype network based on the sequenced nrITS sequences indicated that 6 haplotypes (Hap4-Hap9) of *L. obtusum* were obtained through a 22-step mutation from haplotype Hap1 from *L. latifolium*, and the initial haplotype (Hap9) was collected in Xining City, followed by one-step mutation into Hap4 which was widely spread in Qinghai Province. Hap4 transformed into other haplotypes by undergoing one-step mutation (Hap6, Hap7 and Hap8) or two-step mutation (Hap5), respectively. These results will provide the basis for future experiments and further accumulate data for molecular systematic studies of the Chinese *Lepidium* at species level.

Key words: *Lepidium latifolium*, *Lepidium obtusum*, nrITS gene, Phylogenetic relationship.

Introduction

Lepidium, belonging to Tribe *Lepidieae* of the family *Brassicaceae*, is an annual to perennial herb or under-shrub. *Lepidium* is a cosmopolitan genus and there are about 150 species all over the world. Fifteen species and one variety are distributed in China. *L. latifolium* is the type species of the genus *Lepidium*, commonly known as Dalala, Lalagen and Alfalfa, usually grow in salty beaches, fields and roadsides. The morphological characters of *Lepidium obtusum* are similar to *L. latifolium*, but the tip of the leaves are blunt, the base of the short-horned fruit is heart-shaped, and the raceme is densely integrated in the fruit. *L. obtusum* often grows in grassland, filed edge, Gobi desert, wasteland and also on the flat or the slopes from flat to 1,800 meters above sea level. Only six species of *Lepidium* are reported from in Qinghai Province *L. latifolium* attracted the attention of many scholars because of its medicinal value (Chen *et al*., 2005; Ge *et al*., 2016; Sun *et al*., 2016). However, to date, the molecular phylogenetic studies of *Lepidium* are mainly carried out at the generic level and only foreign scholars did researches at species level of *Lepidium* (Mummenhoff *et al*., 2001; Lee *et al*., 2002; Smith *et al*., 2009). At present, indigenous domestic *Lepidium* species is classified are mainly on morphological characters, such as leaf characters, flower and fruit structure, and inflorescence characters. Morphologically, *L. campestre* is close to *L. sativum*, and three species *L. perfoliatum, L. latifolium* and the *L. obtusum* are also close, as well as the relationships among *L. virginicum*, *L. ruderale*, *L. densiflorum* and *L. apetalum* is also obvious (Flora of China, 1987).

The nuclear internal transcribed spacer region (nrITS) includes the ITS1, ITS2, and 5.8S regions, which require a large number of ribosomes during rapid growth, which is quite high in the higher plant nuclear genome (Eickbush & Eickbush, 2007). Repetitive, usually in the form of unequal crossover, gene amplification and gene conversion (Hillis *et al*., 1991; Gonzalez & Sylvester, 2001; Kovari *et al*., 2004), concerted evolution within or between loci occur, whereby different nrITS sequences will tend to be identical or absolutely identical. Meanwhile, nrITS is widely used in phylogenetic research because it has a fast evolution rate that can provide suitable mutations and information sites. In addition, nrITS is also proposed as an important plant barcode by several workers (Kress *et al*., 2005; Chen *et al*., 2010; Li *et al*., 2011). In the present study, nrITS was used as a molecular marker to analyze the systematic relationship between the two species of *Lepidium* in Qinghai Province and further to accumulate data on the molecular systematic studies of the Chinese *Lepidium* species.

Materials and Methods

Sampling: Sampling was carried out at nine sampling sites in Qinghai Province (Fig. 1). A total of 162 samples were collected and the sampling information was shown in Supplementary material A1. The fresh leaves were collected, then dried in silica gel for DNA extraction. According to the forms of the two plants, a total of 60 specimens of *L. obtusum* (D group) and 102 specimens of *L. latifolium* (K group) were grouped. Additionally, nrITS of 22 species close to *L. latifolium* was downloaded from GenBank for reconstruction of phylogenetic trees (Supplementary material A2).

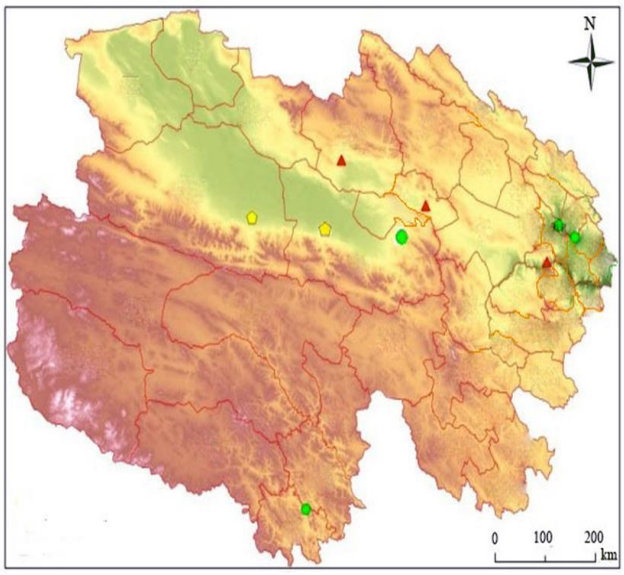

Fig. 1. Sampling sites in Qinghai Province
Yellow regular pentagon indicates sampling sites of *L. obtusum*, green round indicates sites of *L. latifolium* and red triangle indicates shared sampling sites.

DNA extraction, PCR amplification and sequencing: The modified CTAB method was used (Doyle & Doyle, 1990; Schmiderea *et al*., 2013). After precipitating the DNA with isopropyl alcohol, the DNA was washed twice with 70% ethanol, and the DNA was again precipitated with absolute ethanol, and the DNA was collected by centrifugation and air-dried in a clean bench. Then the DNA was dissolved with a suitable amount of TE solution, and stored at 4℃ for PCR.

The primers for the nrITS were ITS-F 5' GGA AGG AGA AGT CGT AAC AAG G 3' and ITS-R 5' TCC TCC GCT TAT TGA TAT GC 3' (White *et al*., 1990). All PCR systems were 50 μL in volume. Amplification conditions included an initial denaturation step at 94℃ for 4 min followed by 35 cycles at 94℃ for 40 s, 50.3℃ for 40 s, 72℃ for 40 s; with a final extension of 8 min at 72℃. Configured 1.2% agarose gel, spot 2 μL, electrophoresis at 120 V for 15 - 20 min, and saved the image by electrophoresis imager. The PCR products with a bright-purpose band were selected and the remaining 48 μL PCRs were sent to Beijing Bomed (Beijing) for purification and sequencing.

Data analysis

Sequences were first spliced using Contigexpress 9.1 (Khaokhajorm *et al*., 2015). All sequences were aligned in Clustal X 1.83 (Thompson *et al*., 1997). Then sequences were corrected and analyzed in MEGA 6.0 (Tamura *et al*., 2013). Preliminary analysis and alignment for species confirmation were accomplished in the GenBank database. The nrITS sequence haplotypes and genetic diversity of the two populations were analyzed in DnaSP v 5.1 (Librado & Rozas, 2009). In the phylogenetic relationship analysis, *Cleome spinosa* was used as the outgroup (Beilstein *et al*., 2006) and combined with the results of haplotype analysis, one or two samples were selected for each species at each sampling site. Phylogenetic analysis was performed using ML and BI in RAxML 7.2.8 (Stamatakis *et al*., 2008) and Mr. Bayes 3.2.4 (Huelsenbeck & Ronquist, 2005). Finally, haplotype networks of two sampled *Lepidium* species were drawn in Network 5.0 (Bandelt & Rohl, 2008) for further unravel the evolutionary relationship between the two species of *Lepidium* in Qinghai Province.

Results

nrITS sequence analysis: 152 nrITS sequences with 598 bp length were obtained from162 individuals (94 individuals in group K, 58 individuals in group D), and 10 sequences were deleted from the data for their fragment length less than 598 bp). Thirty polymorphic sites (5.0%) and 26 parsimony informative sites (4.3%) were found in nrITS sequences. There were three insertion or deletion sites located at 101, 492, and 493 bp, respectively. The average base composition was 22.16% A, 21.01% T, 27.68% G, and 29.15% C, and the (G + C) content was 56.83%, which was higher than (A + T) (43.17%).

Nine haplotypes were obtained (GenBank accession numbers were: MH507026 - MH507034), in which K group and D group contained 6 haplotypes and 3 haplotypes, respectively. And the haplotype diversity was 0.1229 (K) and 0.2835 (D), respectively. There is no shared haplotype between the two species of *Lepidium*. The results of genetic diversity analysis showed that the nucleotide diversity values of K and D groups were 0.00023 and 0.00050, respectively, and that of group D was slightly higher than that of group K. The nucleotide diversity of the two species in a whole was lower. The genetic distance analysis based on K2P model showed that the average genetic distance within group was 0.00% in group K and 0.06% in group D. The genetic distance between the two groups was 4.01%. The genetic distance between species was much larger than the genetic distance within the species.

Phylogenetic relationship: The phylogenetic relationship between the two species of *Lepidium* in Qinghai Province was reconstructed by ML and BI methods, in which *Cleome spinosa* was used as the outgroup. The results showed that the phylogenetic tree constructed by the two methods had the identical topologic structure (Fig. 2). As can be seen from Fig. 2, the sequences of these species were mainly clustered into three major clades (A, B and C).

Clade A was composed of *Lepidium*, clade B consisted of *Cardamine* and *Rorippa*, and clade C mainly included *Capsella* and *Arabidopsis*. In clade A, the sequences of the two species of *Lepidium* in Qinghai Province were separately clustered into independent clades, which formed a sister clade with other species of *Lepidium*. At the bottom of clade A, *Lepidium hirtum* together with *L. campestre* as a sister cluster of *L. perfoliatum* formed a sister clade (98/1); then this cluster formed a sister cluster with the other clades composed of *Lepidium* with strong support (100/1). In the middle of clade A, the *L. densiflorum* and the *L. virginicum* formed a sister clade (100/1). At the top of clade A, Parallel clades were formed with clades composed of *L. sativum, L. obtusum,* and *L. latifolium / L. apetalum / L. ruderale*, though the nodal support was weak (61/0.83).

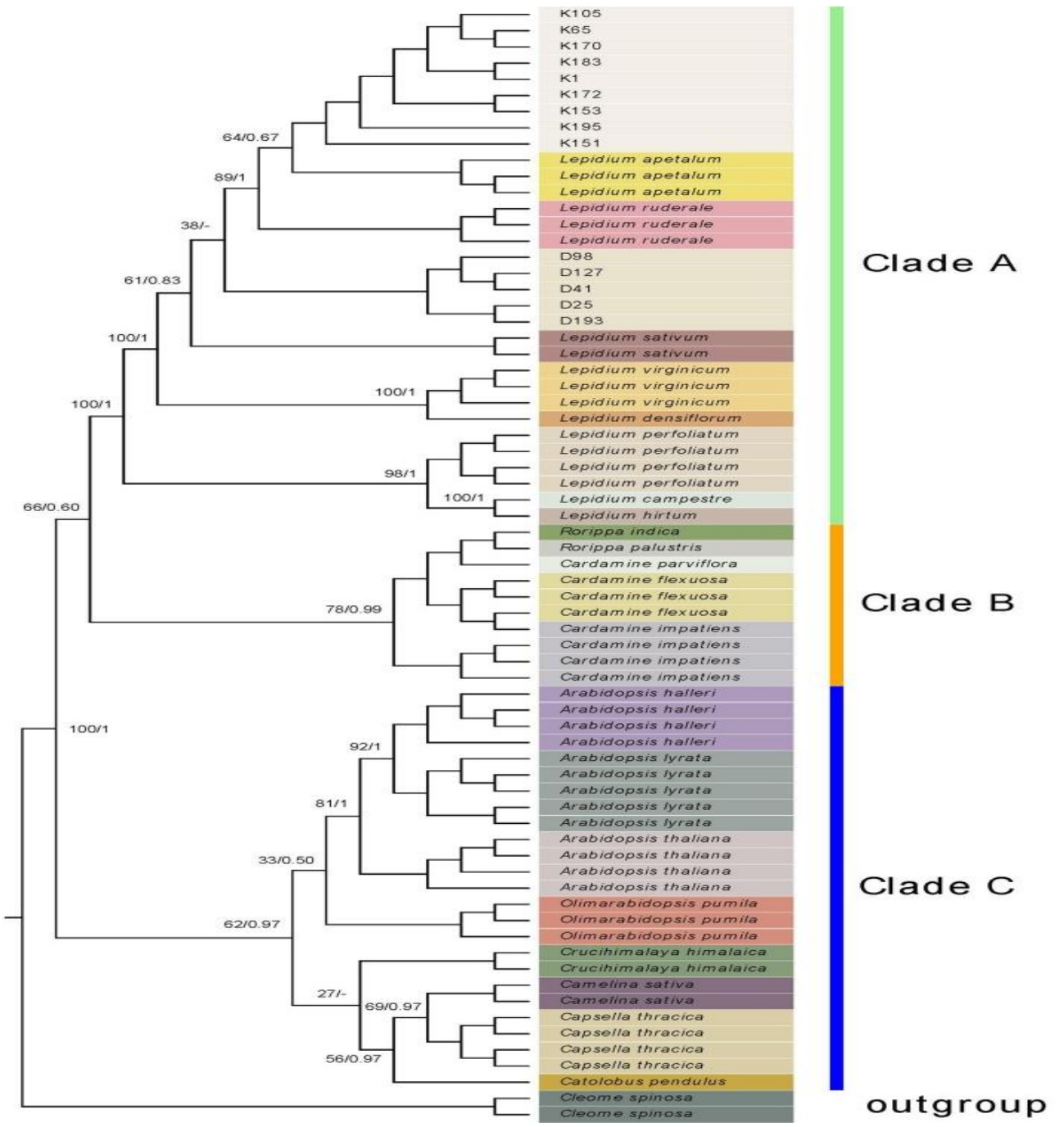

Fig. 2. Phylogenetic tree of *Lepidium* based on nrITS sequences. The values on the nodes represented the nodal support obtained through both ML and BI. The nodal support of ML tree was assessed using the nonparametric bootstrap method with 1000 replicates. 10,000,000 generations of 4 simultaneous MCMC chains were run in MrBayes, and 50% burn-in trees were discarded, the posterior probabilities (BBP) of nodes were estimated based on the 50% majority rule consensus of the trees. "-" indicated nodal support value was less than 0.50.

Median-joining network analysis: It is from the phylogenetic tree indicated that *L. latifolium* might be originated from their recent common ancestors earlier than *L. obtusum*. In order to uncover the genetic relationship between the two species of *Lepidium* in Qinghai Province, we constructed the haplotype network only using the nrITS sequences (Fig. 3). The results showed that the two haplotypes (Hap1 and Hap9) linked K group to the D group were the most mutated, and there was no haplotype missing between them. Among all haplotypes, Hap4 was the most widely distributed, followed by the distribution of Hap1. The Hap9 from K group was formed after the Hap1 from D group undergoing 22-step mutation, and then transformed into Hap4 after undergoing 1-step mutation. The distribution of haplotype Hap4 covered all sampling sites of group K. Then the Hap4 transformed into other haplotypes by undergoing one-step mutation (Hap6, Hap7 and Hap8) or two-step mutation (Hap5), respectively. Similarly, in group D, the distribution of Hap1 covered all sampling sites in group D, and both Hap2 and Hap3 were formed via 1-step mutation from Hap1.

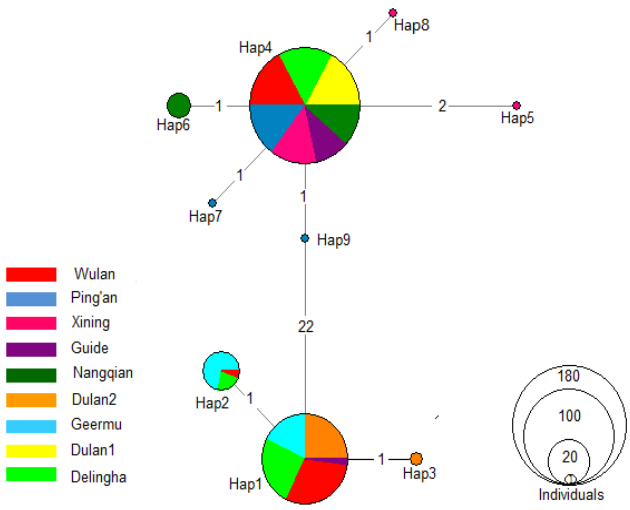

Fig. 3. Median-joining network of the two species of *Lepidium* in Qinghai Province.

Discussion

In this study, the phylogenetic relationships of the ten species of *Lepidium* were carried out based on the nrITS sequences. The results showed that the phylogenetic tree topology obtained by the two methods was almost the same and can be divided into three clades A, B and C (Fig. 2). The single system of clustering in phylogenetic tree can indicate consistent levels of morphology and nrITS sequence grouping (Funk & K.Omland, 2003; Rubinoff, 2006). In clade A, the two species of *Lepidium* were clustered into single independent clades and formed a sister clade with other *Lepidium*, which was reasonable in topological structure and high in resolution. At the bottom of the phylogenetic tree, *L. hirtum* and *L. campestre* clustered together with *L. perfoliatum* to form a sister clade (100/1); indicating that *L. campestre* had a close relationship with *L. perfoliatum*. In addition, in the middle of the tree, *L. densiflorum* and *L. virginicum* formed a sister clade (100/1). At the top of the tree, parallel clades were formed with *L. sativum, L. obtusum, and L. latifolium /L. apetalum /L. ruderale* (61/0.83). The results showed the relationship between *L. ruderale*, *L. apetalum* and *L. latifolium /L. obtusum* tended to be closer than that between *L. perfoliatum* and *L. latifolium /L. obtusum*, which was inconsistent with the morphological data. Yang *et al*., (Yang *et al*., 1998) found that *Cardamine* and *Rorippa* were close in system relationship based on 5S rRNA sequence analysis. In this study, the clade B consisted of *Cardamine* and *Rorippa* (78/0.99), indicated that the species of these two genera is closely related to phylogeny, which was further confirmed by the conclusion from Yang *et al*. (Yang *et al*., 1998). The clade C is mainly composed of *Capsella* and *Arabidopsis*. The *Arbideae* included *Arabidopsis* which is a paraphyletic group. In addition, *Capsella* from *Lepidieae* inserted to *Arabidopsis* (Fig. 2), indicating that the *Arbideae and Lepidieae* are not monophyletic group, which was consistent with the conclusions of most workers (Mitchell & Heenan, 2000; Koch *et al*., 2001; Heenan *et al*., 2002). The above results indicated that using nrITS gene to construct the phylogenetic relationship of partial *Lepidium* species in china, in some extend, had certain value for reference.

When the sequence nucleotide diversity was low, the networks between haplotypes could depict the relationship between sequences more effectively (Crandall & Templetion, 1993). In the present study, the nucleotide diversity of *L. latifolium* and *L. obtusum* was 0.00050 and 0.00023, respectively, which was generally low. We used the nrITS sequences from two sampled specimens to construct haplotype network (Fig. 3), and further explored the genetic relationship between the two species of *Lepidium* in Qinghai Province. The results showed that the haplotype (Hap9) of *L. latifolium* was formed through 22-step mutation from the haplotype Hap1 of *L. obtusum*. Hap9 was collected from Xining and transformed into Hap4 after 1-step mutation. Hap4 was distributed in 7 sampling sites (Fig. 1) of 9 sampling sites, which was more widely distributed. In China, the *L. obtusum* is widely distributed in Gansu, Qinghai and Xinjiang, the original variant of *L. latifolium* is native to Mongolia and Tibet and the cosmopolitan species of *L. latifolium* is widely distributed in the northeast, north and northwest of China. Meanwhile, Xining is a major transportation hub in Qinghai Province. The flow of foreigners and traffic is large, and it is the first stop for exotic species to enter Qinghai Province. Based on the results, therefore, it is inferred that the samples of the *L. latifolium* in this study may be variant. The pioneer species may be that the original varieties/variants of other regions are brought to Xining by human or other external forces, and then radiated to other areas of Qinghai Province. Obviously, to verify this inference, it is also necessary to collect samples from more populations of other species of *Lepidium* outside Qinghai Province. The results will provide the groundwork for future experiments and further accumulate data about molecular systematic studies of the Chinese Lepidium at species level. Besides, further study of the origin of these two species and their adaptive evolution on the Tibetan Plateau also requires other genes, such as organelle genes (Kress *et al*., 2005; Smith *et al*., 2009), to avoid the conflict caused by maternal inheritance when reconstructing phylogenetic relationships.

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References

Bandelt, H. and A. Rohl. 2009. Quasi-median hulls in hamming space are steiner hulls. *Discrete Appl. Math.,* 157(2): 227-233.

Beilstein, M., I. Al-Shehbaz and E. Kellogg. 2006. Brassicaceae phylogeny and trichome evolution. *Am. J. Bot*., 93(4): 607-619.

Chen, H., R. Qualls and R. Blank. 2005. Effect of soil flooding on photosynthesis, carbohydrate partitioning and nutrient uptake in the invasive exotic *Lepidium latifolium*. *Aquat. Bot*., 82(4): 250-268.

Chen, S., H. Yao, J.P. Han, C. Liu, J.Y. Song, L.C. Shi, Y.J. Zhu, X.Y. Ma, T. Gao, X.H. Pang, K. Luo, Y. Li, X.W. Li, X.C. Jia, Y.L. Lin and C. Leon. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *Plos One*, 5(1): e8613.

Chinese Flora Editorial Board of the Chinese Academy of Sciences. 1987. Flora of China: Volume 33.1. Science Press, Beijing: 47-58.

Crandall, K. and A. Templetion. 1993. Empirical test of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genet*., 134(3): 959-969.

Doyle, J. and J. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus (San Francisco, Calif.), 12: 13-15.

Eickbush, T.H. and D.G. Eickbush. 2007. Finely orchestrated movements: evolution of the ribosomal RNA genes. *Genet*., 175(2): 477-485.

Funk, D.J. and K.E. Omland. 2003. Species-level paraphyly and polyphyly: frequency causes, and consequences, with insight from animal mitochondrial DNA. *Ann. Rev. Ecol. Evol. Syst*., 34(0): 397-423.

Ge, S., T. Chen and C. Xu. 2016. Optimization of extraction for chlorogenic acid from *Lepidium latifolium* by response surface methodology. *Cereals & Oils,* 29(5): 82-85.

Gonzalez, I.L. and J.E. Sylvester. 2001. Human rDNA: Evolution patterns within the genes and tandem arrays derived from multiple chromosomes. *Genomics*, 73(3): 255-263.

Heenan, P.B., A.D. Michell and M. Koch. 2002. Molecular systematic of the New Zealand Pachycladon (Brassicaceae) complex: generic circumscription and relation to Abrabidopsis sens. lat. and Arabis sens. *Lat. N.Z J. Bot*., 40: 543-562.

Hillis, D.M., C. Moritz, C.A. Porter and R.J. Baker. 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. *Sci*., 251(4991): 308-310.

Huelsenbeck, J. and F. Ronquist. 2005. Bayesian analysis of molecular evolution using MrBayes, ed, M Gail, K Dietz, K Kricheberg, A Tsiatis, J Samet, 183-226 vols. *Stat. Methods Mol. Evol*. (USA): 183-226.

Khaokhajorm, P., S. Samipak, S. Nithithanasilp, M. Tanticharoen and A. Amnuaykanjanasin. 2015. Production and secretion of naphthoquinones is mediated by the MFS transporter MFS1 in the entomopathogenic fungus *Ophiocordyceps* sp. BBC1869. *World J. Microbiol. Biotechnol.,* 31: 1543-1554.

Koch, M., B. Haubold and T. Michell-Olds. 2001. Molecular systematics of the Brassicaceae: evidence from coding plastic matK and nuclear Chs sequences. *Am. J. Bot.,* 88(3): 534-544.

Kovarik, A., R. Matyasek, K.Y. Lim, K. Skalicka, B. Koukalova, S. Knapp, M. Chase and A.R. Leitch. 2004. Concerted evolution of 18-5.8-26S rDNA repeats in Nicotiana allotetraploids. *Biol. J. Lin. Soc*., 82: 615-625.

Kress, W., K.J. Wurdack, E.A. Zimmer, L.A. Weigt and D.H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci. U.S.A.*, 102(23): 8369-8374.

Lee, J.Y., K. Mummenhoff and J.L. Bowman. 2002. Allopolyploidization and evolution of species with reduced floral structures in *Lepidium* L. (Brassicaceae). *Proc. Natl. Acad. Sci. U.S.A.*, 99(26): 16835-16840.

Li, D.Z., L.M. Gao, H.T. Li, H. Wang, X.J. Ge, J.Q. Liu, D.Z. Chen, S.L. Zou, S.L.Chen, J.B. Yang, C.X. Fu, C.X. Zeng, H.F. Yan, Y.J. Zhu, Y.S. Sun, S.Y. Chen, L. Zhao, K. Wang, T. Yang and G.W. Duan. 2011. Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proc. Natl. Acad. Sci. U.S.A.*, 108(49): 19641-19646.

Librado, P. and J. Rozas. 2009. DnaSP v5: Asoftware for comprehensive analysis of DNA polymorphism data. *Bioinf*., 25(11): 1451-1452.

Mitchell, A.D. and P.B. Heenan. 2000. Systematic relationships of New Zealand endemic Brassicaceae inferred from nrDNA ITS sequence data. *Syst. Bot*., 25(1): 98-105.

Mummenhoff, K., H. Bruggemann and J.L. Bowman. 2001. Chloroplast DNA phylogeny and biogeography of *Lepidium* (Brassicaceae). *Am. J. Bot*., 88(11): 2051-2063.

Rubinoff, D. 2006. Utility of mitochondrial DNA barcodes in species conservation. *Conserv. Biol*., 20(4): 1026-1033.

Schmiderea, C., B. Lukas and J. Novak. 2013. Effect of different DNA extraction methods and DNA dilution on the amplification success in the PCR of different medicinal and aromatic plants. *Zeitschrift Fur Arznei Und Gewurzpflanzen*, 18(2): 65-72.

Smith, J., A. Stillman, S. Larson, M. Culumber, I. Robertson and S. Novak. 2009. Phylogenetic relationships among *Lepidium papilliferum* (*L. henderson*) A. Nels. & J. F. Macbr., L. montanum Nutt., and L. davisii Rollins (Brassicaceae). *J. Torr. Bot. Soc*., 136(2): 149-163.

Stamatakis, A., P. Hoover and J. Rougemont. 2017. A rapid bootstrap algorithm for the RAxML Web servers. *Syst. Biol*., 57(5): 758-771.

Sun, S.H., Y.R. Du and D.S. Qi, L.P. Cheng and J B. Ma. 2016. Effect of different polar fractions of *Lepidium latifolium* L. on mice gastrointestinal motility, MTL and GAS. *Hubei Agri. Sci*., 55(18): 4790-4792.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. . *Mol. Biol. Evol*., 30(12): 2725-2729.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res*., 25(24): 4876-4882.

White, T., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: (Eds.): Inns, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White. *PCR protocols: A guide to methods and applications*. Academic Press, San Diego, California, USA: 315-322.

Yang, Y., P. Tseng, P. Tai and C. Chang. 1998. Phylogenetic position of *Raphanus* in relation to *Brassica* species based on 5S rRNA spacer sequence data. *Bot. Bull. Acad. Sin*., 39: 153-160.

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