NOTE / NOTE

ITS sequence variation supports the hybrid origin of *Malus toringoides* Hughes

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Abstract: *Malus toringoides* (Rehd.) Hughes was suggested to have originated from hybridization between *Malus transitoria* Schneid. and *Malus kansuensis* Rehd., followed by repeated backcrossing to one of the putative parents. In the present study, the sequence information of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) was used to re-examine the origin of this species. A total of 69 accessions from three natural populations (Maerkang, Xiaba and Kehe, Aba Autonomous Region, Sichuan, China) of *M. toringoides* and 10 accessions of its putative parents were analyzed. Using *Malus angustifolia* (Ait.) Michx., *Malus ioensis* (Wood) Britt. and *Malus doumeri* Chev. as outgroups, our phylogenetic analysis of the ITS sequences of *M. toringoides* and its putative parents showed that *M. toringoides* was not monophyletic, and two different types of ITS sequences which were obtained from each of the six accessions of *M. toringoides* were found to have clustered separately with those of the two putative parent species on the gene tree. A comparison of the sequence variation between *M. toringoides* and its putative parents revealed an additive variation pattern of ITS sequences in the putative hybrid species. These results are consistent with the previous morphological and amplified fragment length polymorphism (AFLP) data which suggested that *M. toringoides* was of hybrid origin. Our ITS data provide new molecular evidence for the hybrid origin hypothesis of *M. toringoides* and these results are of great importance for future study on hybridization, polyploid speciation and evolution of the genus *Malus* Miller.

Key words: Malus toringoides, hybrid origin, ITS.

Résumé : On a suggéré que le *Malus toringoides* (Rehd.) Hughes proviendrait d'une hybridation entre le *Malus transitoria* Schneid. et le *Malus kansuensis* Rehd., suivie de nombreux rétrocroisements avec un des parents présumés. Afin de revoir l'origine de cette espèce, les auteurs ont utilisé l'information séquentielle de l'espaceur interne transcrit (ITS) de l'ADN nucléique ribosomal. Ils ont analysé 69 accessions provenant de trois populations naturelles (Marerkang, Xiaba et Kehe et région autonome de Aba, du Sichuan, en Chine) du *M. toringoises,* et 10 accessions de ses présumés parents. Utilisant comme groupes externes les *Malus angustifolia* Michx., *Malus ioensis* Britt. et *Malus doumeri* Chev., l'analyse phylogénétique des séquences ITS du *M. toringoides,* et de ses parents présumés, montre que le *M. toringoides* n'est pas d'origine monophylétique. On constate plutôt que deux types distincts de séquences ITS, provenant de chacune de six accessions du *M. toringoides,* se regroupent séparément avec ceux des deux espèces parentales présumées, sur l'arbre phylogénétique. Une comparaison de la variation séquentielle entre le *M. toringoides* et ses parents présumés révèle un patron de variation additif des séquences ITS, chez les espèces hybrides présumées. Ces résultats concordent avec les données morphologiques et celles du polymorphisme de la longueur des fragments amplifiés (AFLP), suggérant que le *M. toringoides* provienne d'hybridation. Les données ITS fournissent une nouvelle preuve moléculaire supportant l'hypothèse de l'origine hybride du *M. toringoides,* et ces résultats sont d'une grande importance pour les études futures sur l'hybridation, la spéciation polyploïde et l'évolution du genre *Malus* Miller.

Mots-clés : Malus toringoides, origine hybride, ITS.

[Traduit par la Rédaction]

Received 11 April 2007. Published on the NRC Research Press Web site at canjbot.nrc.ca on 16 August 2007.

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 doi:10.1139/B07-050

Introduction

The origin of plant species via hybridization is a common phenomenon and has been well documented in higher plants (Sang et al. 1995; Rieseberg 1997; Ellstrand and Schierenbeck 2000). Hybridization and introgression (the gradual infiltration of the genetic material of one species into another as a result of hybridization and repeated backcross) may have different evolutionary consequences (Arnold 1992), such as increased intraspecific genetic diversity, the origin and transfer of genetic adaptations, the origin of new ecotypes or species, and the reinforcement or breakdown of reproductive barriers (Rieseberg 1997; Ellstrand and Schierenbeck 2000). Thus, the formation of new hybrid species is one of the major evolutionary contributions of hybridization in plants (Rieseberg and Carney 1998).

Morphological characters (Chen 1999; Hardig et al. 2000; Lau et al. 2005), species-specific isozyme markers (Marchelli and Gallo 2001), and diagnostic allozyme markers (Toumi and Lumaret 1998; Santalla et al. 2002) were the traditional markers used to detect hybridization and (or) introgression events in plant species. However, results based solely on morphology can be potentially misleading (Rieseberg 1995; Rauscher et al. 2002), and allozyme markers may not offer sufficient variability to differentiate closely related putative parental taxa (Wolfe et al. 1998). In recent years, DNA molecular data have been demonstrated to be useful to test hypotheses of hybridization (Hegarty and Hiscock 2005). The internal transcribed spacer (ITS) region is a part of nuclear ribosomal DNA (nrDNA), which in higher plants is grouped into arrays consisting of hundreds to thousands of tandem repeats. This region includes two spacers (ITS1 and ITS2) that separate the 18S, 5.8S, and 26S genes of nuclear ribosomes (Baldwin et al. 1995). Since 18S-26S rDNA arrays reside in the nuclear genome, ITS sequences are biparentally inherited in most angiosperms, and are thus distinguished from maternally inherited chloroplast DNA (cpDNA) in widespread use (Baldwin 1992; Alvarez and Wendel 2003). ITS sequence data have been extensively documented to provide insights into phylogenetic history, polyploid ancestry, genome relationships, and historical introgression (Alvarez and Wendel 2003; Bailey et al. 2003). Arising from mechanisms such as unequal crossing-over and high-frequency gene conversion, the ITS paralogues are generally homogenized by concerted evolution (Wendel et al. 1995; Alvarez and Wendel 2003). As the mode and rate of rDNA concerted evolution are different among various plant groups (Buckler et al. 1997; Fuertes Aguilar et al. 1999), coexistence of multiple divergent ITS repeats in one genome is quite common as a consequence of well-known organismal processes such as hybridization and polyploidization, and by genomic processes like gene and chromosome segment duplication and various forms of homologous and nonhomologous recombination (Bailey et al. 2003). Because of its nuclear inheritance, ITS sequence data may detect hybridization events and provide information on both the maternal and paternal progenitor lineages (Alvarez and Wendel 2003). Moreover, additivity of parental rDNA sequences is often observed in the origin of allopolyploid species (Mcfadden and Hutchinson 2004). ITS sequences have been successfully used to document the hybrid origin of several species in the past decade, for example, *Paeonia* (Sang et al. 1995), *Arabidopsis* (O'Kane et al. 1996), *Amelanchier* (Campbell et al. 1997), *Oxalis* (Emshwiller and Doyle 1998), *Cardamine* (Franzke and Mummenhoff 1999), *Draba* (Widmer and Baltisberger 1999), *Miscanthus* (Hodkinson et al. 2002), *Eupatorium* (Siripun and Schilling 2006).

The genus *Malus* Miller (Rosaceae) is composed of 30 to 35 species that are widely distributed in China, North America, and Europe (Zhou 1999). Because of its economic importance in temperate fruit production, *Malus* has long been the focus of studies on genetic resources (Zhou 1999), interspecific relationships (Zhou and Li 2000), and taxonomy (Robinson et al. 2001). Robinson et al. (2001) suggested that hybridization and introgression might have played an important role in the evolution of *Malus*. However, the role of hybridization, polyploidization, and apomixes in the complex interspecific relationships of the genus *Malus*, remains poorly understood. *Malus toringoides* (Rehd.) Hughes belongs to the section *Sorbomalus* Schneid. based on Phipps' classification of *Malus* (Phipps et al. 1990). In horticulture, *M. toringoides* is used as a rootstock in apple breeding and has excellent agronomic attributes including resistance (or tolerance) to drought (Xiang et al. 1995), cold (Cheng et al. 1987), salt (Li et al. 1996), waterlogging (Cheng et al. 1996), and diseases, such as *Valsa mali* Miyabe et Yamada (Liu et al. 1990). In natural populations, *M. toringoides* exhibits continuous (sometimes discrete) morphological variation which ranges from *M. kansuensis* to *M. transitoria*. The patterns of morphological variation shows the characteristics of hybridization and introgression (Cheng et al. 1992). Geographically, in the Aba Autonomous Region of the western Sichuan Province of China, *M. toringoides* is still in sympatry with *M. transitoria* and *M. kansuensis* (Zhou 1999). Therefore, *M. toringoides* is thought to be of hybrid origin based on morphological characteristics (Cheng et al. 1999). With all of the above taken together it is, therefore, of importance to have a better understanding of the phylogenetic origin of *M. toringoides*. The objective of this study was to use the variation in the ITS sequence of nrDNA to test the hypothesis that *M. toringoides* is a hybrid species derived from *M. transitoria* and *M. kansuensis*.

Materials and methods

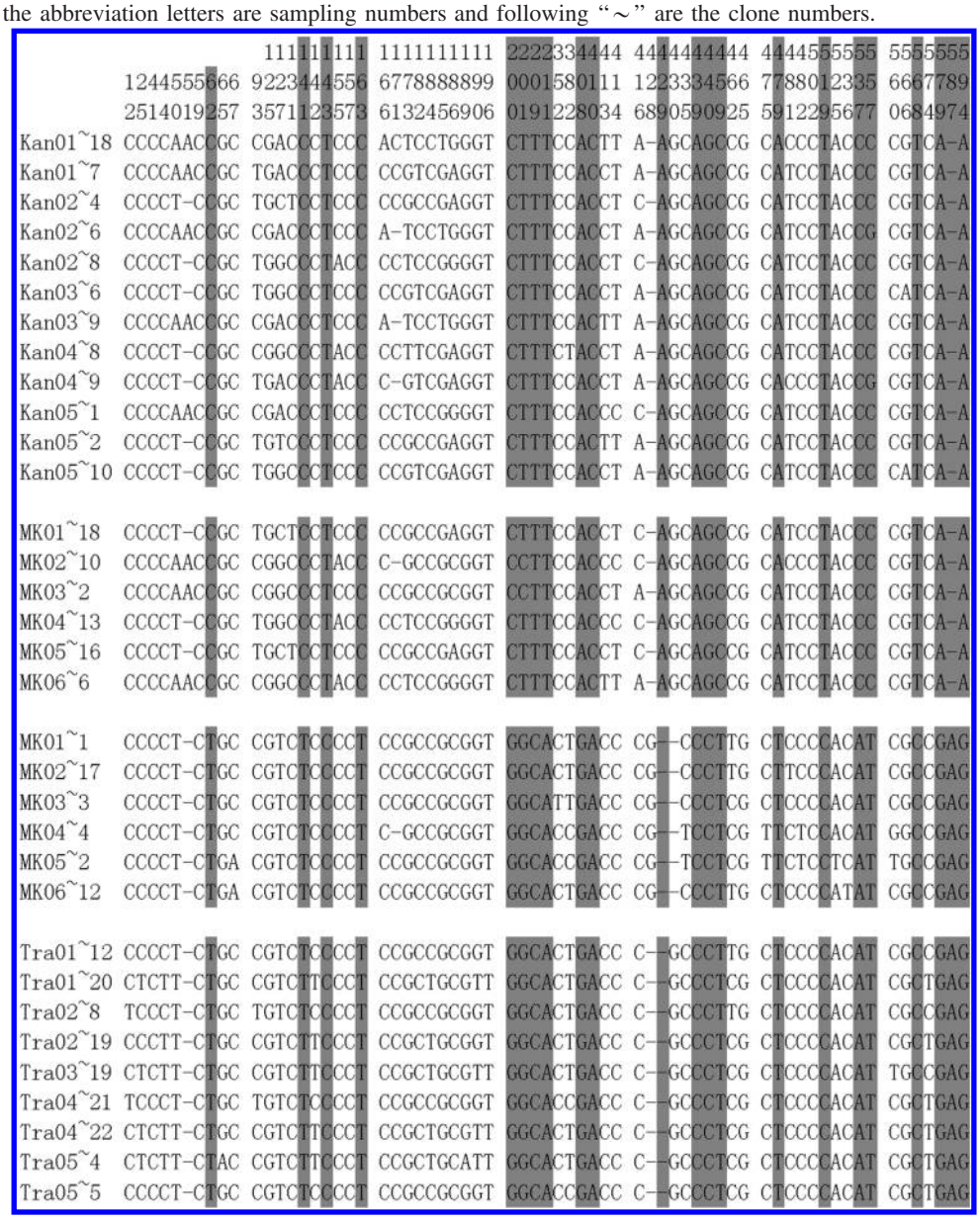
Sampling of plant materials

Three natural populations of *M. toringoides* at Maerkang (MK), Kehe (KH), and Xiaba (XB), all in the Aba Autonomous Region of western Sichuan Province of China, were chosen as sampling sites. From each population, 22, 21, and 26 accessions, respectively, were collected. For the two putative parents of *M. toringoides*, five accessions of *M. transitoria* were collected from Xiaba and five accessions of *M. kansuensis* were collected from Maerkang. Voucher specimens of all samples were deposited in the herbarium of Southwest University, Chongqing, China.

DNA isolation, ITS amplification, cloning and sequencing

Total genomic DNA was isolated from silica-gel dried leaves using the CTAB method as described by Doyle and Doyle (1987). The ITS fragments including ITS1, ITS2, and 5.8S were amplified with primers AB101 (5′-ACGAATTCATGGTCCGGTGAAGTGTTCG-3′) and AB102 (5′-TA-

Fig. 1. The additive variation pattern of the ITS sequences of *Malus toringoides.* Twenty-two nucleotide sites (shaded in gray) were found to be consistently different between *Malus transitoria* and *Malus kansuensis*. Two different ITS types were observed in each of the six accessions (MK01–MK06) of *M. toringoides*. Kan, *M. kansuensis*; Tra, *M. transitoria*; MK, *M. toringoides* of Maerkang population. The numbers following the abbreviation letters are sampling numbers and following "~" are the clone numbers.



GAAT TCCCCGGTTCGCTCGCCGTTAC-3′) (Robinson et al. 2001). The PCR reaction was carried out in a volume of 25 μL containing 5–50 ng of template DNA, 0.4 μmol/L of each primer, 0.2 μmol/L of each dNTP, 0.75U of *Taq* DNA polymerase and 10× *Taq* DNA polymerase buffer (TakaRa Biotech Co., Dalian, China). Amplification was performed in a Peltier Thermal Cycler (PTC-200, PE) using the following protocol: 2 min at 96 °C, followed by 30 cycles of 1 min at 96 °C, 1 min at 65 °C and 2 min at 72 °C, and finally 10 min at 72 °C. PCR products were electrophoresed on 1.5% agarose gels and bands of expected size were excised from gels and purified using Gel Band Purification kit (Amersham Pharmacia Biotech, Piscataway, N.J.) or DNA Fragment Quick Purification/ Recover kit (Dingguo, Beijing, China). The purified PCR products were cloned using pGEM-T Easy Vector (Promega) following the manufacturer's instructions. For each accession, at least 16 positive clones with correct inserts (determined by *Eco*R I enzyme digestion analysis) were screened for different ITS types using restriction enzymes *Sty* I (C↓CWWGG, W = A or T) and *Sac* I (GAGCT↓C), which may detect the species-specific ITS types among three species. The selected clones were sequenced in both directions using T7 and SP6 plasmid primers. Sequencing reactions were conducted using the DYEnamic ET Terminator Kit (Amersham Pharmacia Biotech) and the sequencing reaction product was purified by precipitation with 95% ethanol and 3 mol/L NaAc (pH 5.2). Sequencing was done on a Megabase 1000 Automatic DNA sequencer (Amersham Biosciences, Buckinghamshire, UK).

Fig. 2. Strict consensus tree obtained from parsimony analysis of three *Malus* species ITS sequence data (CI = 0.9303; RI = 0.9141). Bootstrap values (1000 replicates) are above branches. Tra and Kan indicate the species of *M. transitoria* and *M. kansuensis*. MK, KH, and XB indicate the populations of *M. toringoides*. The numbers in front of "∼" are the sampling numbers of each population, the numbers following "∼" are the clone numbers of each accession. The clones with gray shading indicate the accessions of *M. toringoides* with two sets of putative parental ITS paralogous loci.

Malus kansuensis-specific ITS amplification

Species-specific PCR can recover rare ITS copies that are difficult to obtain by direct sequencing or cloning (Rauscher et al. 2002). To further test the intra-genomic ITS diversity of *M. toringoides*, a *M. kansuensis*-specific primer named ITS-kan (5′-CTTCCGCCCCTCCGACGCTCCCTAAGGAG-3′) was designed based on the ITS sequences of *M. kansuensis* newly obtained in the present study and downloaded from GenBank (accession number AF186512). Using the primers AB101 and ITS-kan, PCR amplifications were performed in 25 μL reactions containing 10–20 ng genomic DNA, 10× PCR buffer (Promega), 1.3 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, 0.75 unit of *Taq* polymerase, 0.4 μmol/L of each primer and 50% Dimethylsulphoxide (DMSO). PCRs were performed using the following protocol: 2 min at 96 °C, followed by 30 cycles of 1 min at 96 °C, 1 min at 68 °C, and 2 min at 72 °C, and finally 10 min at 72 °C, and hold at 4 °C. The product of specific-PCR was cloned and sequenced using the procedure described above to test the identity of the ITS repeats amplified.

Data analysis

The forward and reverse sequences of each clone were checked and assembled manually into a single consensus sequence using ContigExpress computer program (InforMax, Inc., Bethesda, Md.) with 100% overlap. The boundary of the two ITS regions and the coding sequences of 5.8S rDNA were determined by comparison of the newly obtained sequences with those downloaded from GenBank (Robinson et al. 2001). Sequences were aligned using Clustal × 1.8.1 computer program (Thompson et al. 1997) and refined manually. MEGA program version 2.1 (Kumar et al. 2001) was employed to estimate the GC content and nucleotide substitution of the ITS sequences. Phylogenetic relationships among the ITS sequences were analyzed using the neighbor joining (NJ) and the maximum parsimony (MP) approaches. Sites with missing or unreliable data (such as polyA/T and single sequence repeats) were excluded in the phylogenetic analyses. The ITS sequences of *M. angustifolia*, *M. ioensis*, and *M. doumeri* (downloaded from GenBank, accession numbers AF186523, AF186526, and AF186529) were used as outgroups in the phylogenetic analysis based on the work of Robinson et al. (2001). The NJ (uncorrected "p" distance) and MP trees were constructed using PAUP* version 4.0b10 program (Swofford 2002). For parsimony analysis, a heuristic search with 1000 random addition replicates and tree–bisection–reconnection (TBR) branch swapping were used, and the gaps were treated as missing data. Bootstrap analysis (Felsenstein 1985) was also performed with 1000 replicates to test the robustness of tree branches.

Results

ITS sequence characteristics of *Malus toringoides* and its putative parents

Ninety-six ITS sequences were obtained from 69 *M. toringoides* accessions and 10 accessions of its putative parents in the present study (GenBank accession numbers EF113154-EF113249). The total length of ITS sequences of the three species varied from 590 to 595 bp. The ITS1 region ranged from 223 bp to 225 bp, and the ITS2 region ranged from 201 bp to 204 bp. As expected, the 5.8S subunit region was conserved in length (166bp). The G+C content of the sequence ranged from 63.8% to 65.1%.

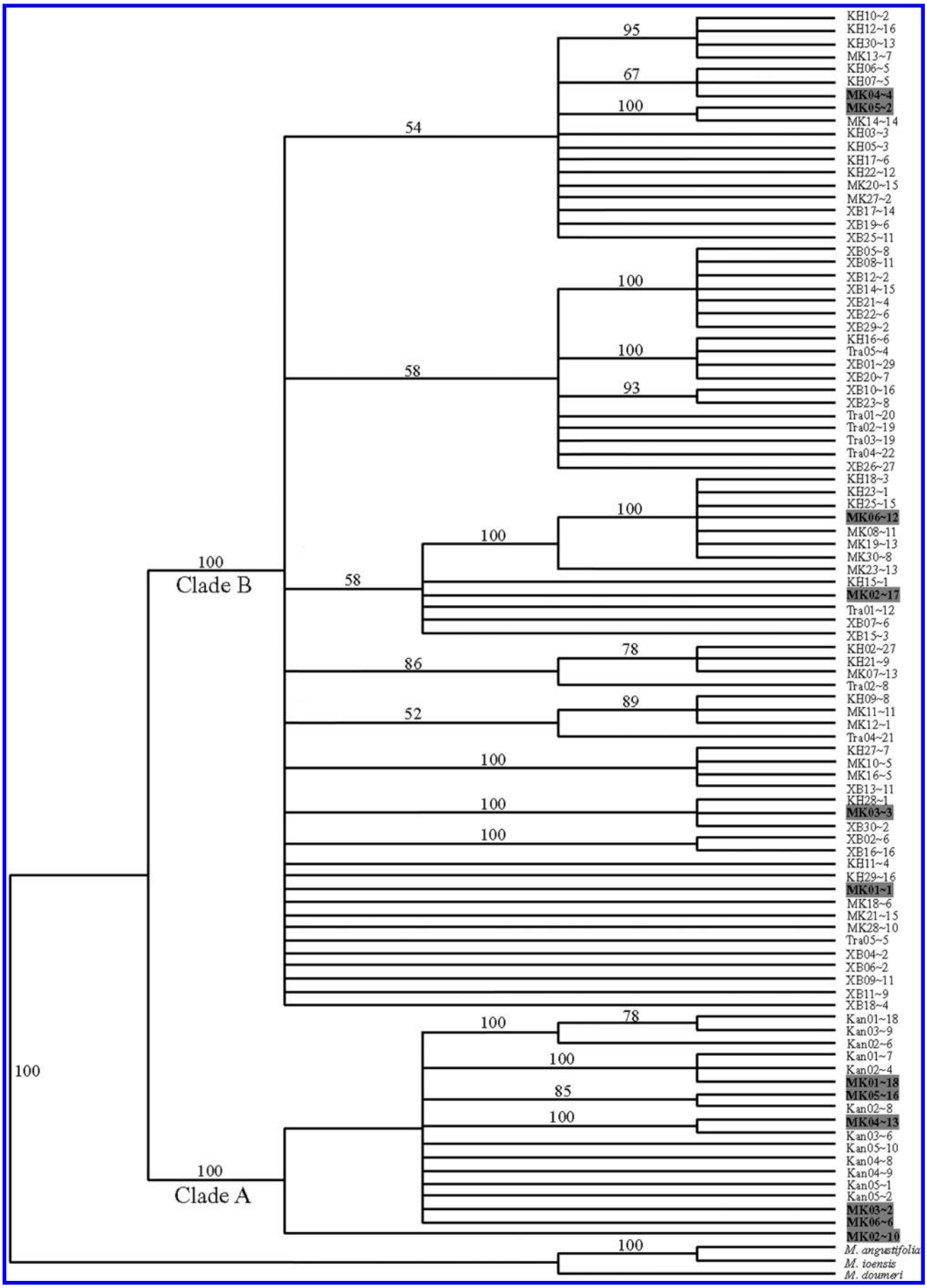
The sequence variations of *M. toringoides* and its putative parents are summarized in Fig. 1. Twenty-two variation sites including 20 substitutions and two indels between *M. transitoria* and *M. kansuensis* in ITS sequences were found. Most accessions of *M. toringoides* had ITS sequences similar to those of *M. transitoria* (GenBank accession numbers EF113154–EF113162). However, six accessions (MK01–06) of *M. toringoides* had two different ITS sequence types which were either similar to those of *M. transitoria* or to those of *M. kansuensis* (Fig. 1). These results suggest that an additive variation pattern exists in the ITS sequences of *M. toringoides*.

Phylogenetic analysis of the ITS sequences

To test the phylogenetic origin of the ITS sequences of *M. toringoides*, we constructed both the NJ and MP trees of all ITS sequences obtained in the present study using *M. doumeri*, *M. angustifolia,* and *M. ioensis* as outgroups. Within the data set, there were 91 variable sites, of which 75 were parsimony informative. The two phylogenetic analyses resulted in the same tree topology regarding the general relationships among ITS sequences of the three species. The maximum parsimony analysis resulted in 296 equally most parsimonious trees of 155 steps. The consistency index (CI) was 0.9303 and the retention index (RI) was 0.9141. The strict consensus tree of the MP trees (Fig. 2) showed that the ITS sequences of the three species grouped into two strongly supported clades, Clade A and Clade B. The Clade A consisted of all 12 sequences from *M. kansuensis* and 6 sequences from *M. toringoides* (MK01–MK06) with a bootstrap support of 100%. Clade B comprised all 9 sequences from *M. transitoria* and 69 sequences from *M. toringoides* with also a bootstrap value of 100%. It was evident that of the three species analyzed only *M. toringoides* was not monophyletic, that is, individual cloned sequences of this species were grouped with either *M. transitoria* or *M. kansuensis*. Beyond our expectation, two different types of ITS sequences that were obtained from each of only six individuals of the MK population were found scattered in the clades of the two putative parent species. The remaining ITS sequences from *M. toringoides* were grouped very closely to *M. transitoria* or formed distinctive subclades within Clade B.

The ITS repeat types of *M. toringoides*

The results show that 6 (MK population) out of the 69 *M. toringoides* accessions analyzed in the present study



have either *M. kansuensis* type or *M. transitoria* type of ITS sequences. To ascertain the absence of *M. kansuensis*-specific ITS type in the remaining two populations (KH and XB populations) of the *M. toringoides*, two accessions were randomly chosen from each of the KH and XB populations and their ITS repeat types were exhaustively examined by sequencing 16–28 clones using one sequencing primer SP6. The result (data not shown) indicated that none of the sequences obtained belonged to the ITS repeat types of *M. kansuensis* absolutely, which confirmed the above results. Moreover, to recover low-copy ITS repeats that otherwise could not be obtained by the direct cloning method, we carried out species-specific PCR using AB101 and ITS-kan as PCR primers and all the *M. toringoides* accessions as templates, with their putative parents as positive and negative controls. The results (data not shown) showed that the ITS repeat types of *M. kansuensis* genome were not found in the putative hybrid accessions of *M. toringoides*, except for the six accessions collected from MK population.

Discussion

Because two different genomes of putative parents are recombined into a new genetic background after hybridization (Hegarty and Hiscock 2005), additive inheritance of nuclear sequences that are otherwise unique in the putative parental species is a convincing evidence of hybridity (Rieseberg 1997; Rieseberg and Carney 1998). In the present study, sequence data indicate that the ITS sequences from *M. transitoria* and *M. kansuensis* are obviously different in 22 nucleotide sites (Fig. 1), and both types of ITS sequences from the two putative parents are found in some samples of *M. toringoides* (Fig. 2). This suggests that *M. toringoides* contains two ITS paralogous loci of its putative parents, and additivity of ITS sequences from its putative parents can be observed in *M. toringoides*. Therefore, the molecular evidence supports the hypothesis that *Malus toringoides* is of hybrid origin with *M. transitoria* and *M. kansuensis* as putative parental species. In addition, our results provide another example to back up the phenomenon that the additivity of rDNA sequences in the putative hybrid species is intuitively expected.

The putative hybrid species is intermediate between the two putative parental species in morphological features, as has been noted for many hybrids and hybrid derivatives (Rieseberg 1995). Previous studies using pictorial scatter diagrams and hybrid index to investigate the morphological variations and divergence (relationships) among species *M. toringoides*, *M. transitoria* and *M. kansuensis* suggested that *M. toringoides* has rich intraspecific morphological diversity which is represented by the existence of a series of transitions between *M. transitoria* and *M. kansuensis* (Deng et al. 2002). AFLP analysis also showed close genetic relationships among *M. toringoides*, *M. transitoria* and *M. kansuensis* (Shi et al. 2005). In the study of Shi et al. (2005), the banding patterns and genetic similarity of *M. toringoides* are intermediate between *M. transitoria* and *M. kansuensis*, and they argued that the variation types of *M. toringoides* are the results of introgressive hybridization between *M. toringoides* and *M. transitoria* or between *M. toringoides* and *M. kansuensis*. Cytological data indicated that *M. toringoides* is cytologically complex, in that diploid, triploid, and tetraploid individuals were observed within the species (Liang and Li 1993; Schuster and Büttner 1995). It has also been suggested that in the genus *Malus*, differences in ploidy levels in the same species are likely to be due to frequent hybridization (Tatum et al. 2005). In addition, *M. toringoides* is geographically sympatric with its putative parental species (Zhou 1999). The distributions of the three species overlap in the western part of Sichuan Province and the southeastern part of Gansu Province (Cheng et al. 1999). The geographical overlapping of the three species facilitates genetic introgression through hybridization owing to incomplete reproductive isolation. Finally, the artificial hybrids of *M. transitoria* and *M. kansuensis* were reported to be very similar to *M. toringoides* morphologically (Deng 2002). Therefore, our ITS data (Fig. 2) was consistent with previous studies in that *M. toringoides* is a hybrid species derived from *M. transitoria* and *M. kansuensis*.

With respect to only 6 (out of 69) accessions analyzed containing both *M. transitoria* and *M. kansuensis* ITS sequences, our ITS data might suggest genome homogenization by concerted evolution in *M. toringoides*. For most of the *M. toringoides* accessions analyzed in the present study, backcrossing to *M. transitoria* or introgression from *M. transitoria* to *M. toringoides* might be the main reason for the absence of the ITS sequence types of one putative parental species *M. kansuensis* in *M. toringoides*. The different flowering time and the geographical isolation between *M. kansuensis* and *M. toringoides*, for example in the KH and XB populations, might explain a lack of further backcrossing between *M. kansuensis* and *M. toringoides.* As for the many distinctive ITS sequence types of *M. toringoides* found in Clade B (Fig. 2), both the incomplete concerted evolution and random point mutations in ITS loci are possibly an explanation.

Acknowledgments

The authors thank the two reviewers and all editors for their insightful comments and suggestions on the manuscript. We thank Dr. Sheng-You Shi for help in field collection and Miss Wan-Qing Jin for help in sequencing. We are also grateful to Dr. Jin Pan and Dr. Jordi López-Pujo for helpful suggestions and discussions. This study was supported by the National Natural Science Foundation of China (NSFC Grant no. 30471206).

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