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Full Length Article

Implications of nrDNA and cpDNA Region in *Acer* (Aceraceae): DNA Barcoding and Phylogeny

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Abstract

Acer Linn. (Aceraceae), is a genus of the most important horticultural trees. Many species in this genus are potential medicinal plants. However, *Acer* species are easily confused for their similar morphological features, and therefore the phylogenetic relationships among *Acer* species are not fully understood. This hinders the new varie breeding and genetic resource preservation. Here, six candidate DNA barcodes (*psb*A-*trn*H, *mat*K, *trn*L-*trn*F, *rbc*L, ITS2 and ITS) were evaluated for the ability to identify 69 species in *Acer*. For evaluating each barcode's ability to identify species, PCR amplification and sequencing efficiency, genetic divergence, DNA barcoding gaps and discrimination success rates were assessed. The results indicated that ITS region was suitable barcode, ITS sequence exhibited significant inter- and intra-specific variation, clear DNA barcoding gaps, and higher species identification efficiency (73.09% for "all species barcodes" analysis). In addition, Phylogenetic tree was constructed based on 171 ITS sequences from 51 *Acer* species. Species clusters observed in the trees largely agreed with morphologically-based taxa of Xu *et al.* (2013) 14 sections, and 12 sections were supported in the ML tree. However, the taxonomic status of several species should be further analyzed, such as *A. yangbiense*, *A. crassum*, *A. wardii*. Our results showed that ITS region can be used as an efficient and efficient tool for identifying the species and phylogenetic analysis of *Acer*. © 2019 Friends Science Publishers

Key words: *Acer*; DNA barcoding; ITS; Molecular identification; Phylogenetic relationship

Introduction

Acer Linn., a wide spread genus in the family Aceraceae, contains approximately 129 species distributing in temperate and tropical mountain ranges of the Northern Hemisphere (Xu *et al.*, 2013). Of which more than three quarters have been found in China, including 99 species (61 endemic), classified into 14 sections (Xu *et al.*, 2013). Many *Acer* species have important horticultural and economical values and some species, including *A. tataricum* subsp. *ginnala*, *A. tegmentosum* and *A. saccharum* are widely planted for their beautiful autumn colors, medicinal components, or raw sugar product (Tung *et al.*, 2008; Yuan *et al.*, 2011). *Acer* species have very significant morphological features that are their samaras with an elongated wing, while some other characters, such as leaf shapes and inflorescence types, are very diverse (Sipe and Linnerooth, 1995; Xu, 1996a, b; Tian *et al.*, 2002). Although the leaf shapes of most *Acer* species are simple and palmately lobed, the number of lobes is variable, even in intraspecies, such as *A. amplum*, with 3- or 5-lobed or unlobed (Tanai, 1978; Tian *et al.*, 2002; Xu *et al.*, 2013). Several types of inflorescence, such as corymbiform, umbelliform, racemose and large paniculate, occur in this genus (Tian *et al.*, 2002; Xu *et al.*, 2013). Variation in samaras shapes and blended sexuality types are also represented in this genus (Sipe and Linnerooth, 1995; Xu, 1996a; Xu *et al.*, 2013). These variances make the taxonomy of *Acer* species very difficult by analyzing gross morphology (Xu, 1998; Xu *et al.*, 2013; Gao *et al.*, 2015). In addition, the species delimitation and phylogenetic relationships in this genus are also very controversial, although some evidence, including morphological characters, chemical composition, geographical distributions, fossils and molecular data, are available (Tian *et al.*, 2002).

Molecular data has been induced in taxonomy, systematics and evolutionary biology for more than 20 years, which provided an unprecedented way to explore the width and depth of biodiversity (Fay *et al.*, 1998; Hebert *et al.*, 2003a,b; Ren *et al.*, 2009). However, there are still millions of species that await description, and plant identification remains a great challenge (Mora *et al.*, 2016). DNA barcoding, based on DNA sequencing from a standardized genome region, has proved an effective approach for species identification, biodiversity assessment,

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phylogenetic analysis, new and cryptic species investigation, and natural resources conservation (Hebert and Gregory, 2005; Lee *et al.*, 2016; Feng *et al.*, 2016). DNA barcode provides lots of advantages: (1) no request on morphological information; (2) not affected by environmental factors and individual growth habitats; (3) fast and easy to operate; and (4) available for identification of large number of samples (Wyler and Naciri, 2016). For animals, the mitochondrial *cytochrome c oxidase I* (*COI*) gene has been recognized as the official DNA barcode (Hebert and Gregory, 2005), although some other mitochondrial loci were also been proved effective for some taxa (*e.g.*, Vences *et al.*, 2005). For plants, several nrDNA and cpDNA regions, including *mat*K, *atp*F-*atp*H, *rbc*L, *rpo*B, *ycf*1, *psb*A–*trn*H, *trn*L-*trn*F, ITS and ITS2, were proposed for potential standard DNA barcoding, but no consensus has been reached until now (Kress *et al.*, 2005; Luo *et al.*, 2010; Chen *et al.*, 2010; Feng *et al.*, 2016). In 2009, the CBOL Plant Working Group suggested *rbcL* and *matK* as the core barcodes for plants, while Ren *et al.* (2009) and Yu *et al.* (2011a) demonstrated that the internal transcribed spacer (ITS) was a better choice for some plants taxa. For short length and high PCR efficiency, ITS2 was considered as a more suitable barcode marker than ITS (Yao *et al.*, 2010; Feng *et al.*, 2016). Some authors found that *trn*L-*trn*F and *psb*A-*trn*H were also two promising DNA barcoding loci (*e.g.*, Wyler and Naciri, 2016; Wang, *et al.*, 2016). These differences indicated that further testing of DNA barcodes across more species and more plant taxa should be carried out.

At present, some DNA marker systems, including RAPD, AFLP, SRAP, SSR and ISSR, have been served to genetically analyze *Acer* species (Li, *et al.*, 2010; Chen *et al.*, 2011; Lin, *et al.*, 2015). In addition, two DNA sequences, ITS and *trn*L-*trn*F regions, have been applied to reconstruct the phylogeny of Aceraceae (Tian *et al.*, 2002; Grimm *et al.*, 2006). Although the combination of *rpl*16 + *psb*A-*trn*H+ *trn*L-*trn*F was considered to be useful for taxa identification at the intraspecific level in *A. palmatum* (Gao *et al.*, 2015), no one has extensively evaluated the *Acer* genus at interspecific level. Here, we tested six candidate DNA barcoding fragments (*rbc*L, *mat*K, *trn*H–*psb*A, *trn*L-*trn*F, ITS2, and ITS) to explore a most suitable DNA barcode for different species in the genus A*cer*, and then used it to construct the phylogenetic relationships among *Acer* species.

Materials and Methods

Sample Collection

In order to select the most suitable barcoding fragment for *Acer* species, we collected totally 427 samples belonging to 69 species in this study. Fifty two specimens of 41 species were sampled in eight provinces and three municipality of China including Guangxi, Yunnan, Zhejiang, Hubei, Liaoning, Jiangxi, Jiangsu, Henan, Chongqing, Beijing, and Shanghai (Table 1). All samples were identified to species using the herbarium specimen and botanical information from Chinese Virtual Herbarium (http://www.cvh.org.cn/). Digital image information and voucher specimens of these species were stored in the herbarium of Ningbo City College of Vocational Technology. Additionally, 375 sequences were downloaded from GenBank, which comprised 61 *Acer* species (Table S1). Because the delimitation of some *Acer* species was controversial, we followed the latest classification criteria of Flora of China (Xu *et al.*, 2013) in this study.

DNA Extraction, Amplification and Sequencing

Total DNA was extracted from dried leaf tissue using the plant/Fungal DNA Miniprep Kit (Lifefeng Biotech Co., Shanghai, China). The PCR reactions were performed on an AG 22331 Hamburg Mastercycler (Eppendorf Ltd., Hamburg, Germany). Primers used for amplification and DNA sequencing were given in Table 2. The amplification reactions were carried out in 50 μL volumes containing 100 ng template DNA, 1 μL of each primer (10 μmol/L), 25 μL 2 × Taq PCR MasterMix (BioTeke Corporation, Beijing, China), and 22 μL ddH_2O. The cycling conditions were obtained from White *et al.* (1990) and Chen *et al.* (2010). All PCR products were purified using AxyPrep PCR Cleanup Kit (Axygen Biotechnology Limited, Hangzhou, China), and then sequenced using an ABI 3730xl DNA Analyzer (Applied Biosystems, CA, USA). Newly generated sequences were lodged in GenBank, and accession numbers were allocated (Table 1).

Data Analyses

The raw sequences were assembled manually using Vector NTI software (Invitrogen Inc., Carlsbad, CA, USA). Sequences containing ITS2 were trimmed and retrieved according to Chen *et al*. (2010). All *psb*A-*trn*H and *trn*L-*trn*F regions were edited and generated according to GenBank annotations. All sequences alignment and checking were produced using Clustal X 2.1. Kimura 2-Parameter (K2P) distances were calculated in MEGA version 6.0 (Tamura *et al.*, 2013). Three parameters were applied to characterize the inter-specific divergence (Meyer and Paulay, 2005; Feng *et al.*, 2016): average inter-specific distance, theta prime, and minimum inter-specific distance. Another three different parameters were used to evaluate the intra-specific variability (Chen *et al.*, 2010; Gao *et al.*, 2010a): average intra-specific distance, theta and coalescent depth. DNA barcoding gaps were evaluated by comparing the distribution of intra-versus inter-specific distances (Slabbinck *et al.*, 2008; Feng *et al.*, 2016) and Wilcoxon signed-rank tests were used as described previously (Feng *et al.*, 2016). In order to further evaluated the effectiveness of each potential DNA barcode, three criteria (Best Match: BM; Best Close Match: BCM; and All Species Barcodes:

Table 1: Samples information of *Acer* species

genera	Scetion	Samples name	Sampling location	Voucher number	GenBank Accession no.				
					ITS	*matK*	*rbcL*	*trnH-psbA*	*trnL-trnF*
Acer	*Platanoidea*	*A. miaotaiense*	Chenshan, Shanghai	AM2015SH02	KU902468	KU902505	KU902585	KU945298	KU749519
		A. miaotaiense	Taoyuanling, Hangzhou, Zhejiang	AY2015HZ05	KU902489	KU902509	KU902597	KU945299	KU749540
		A. miaotaiense	Shennongjia Forestry District, Hubei	AY2015WH07	KU902490	KU902508	KU902598	KU945300	KX494388
		A. truncatum	Yaowan, Wuhan, Hubei	AT3015WH08	KU902494	KU902538	KU902600	KU945301	KU749493
		A. Pictum subsp. *mono*	Mt. Tianmu, Hangzhou, Zhejiang	AM2015HZ10	KX494362	KU902542	KU902589	KU945342	KU749528
		A. acutum	Fragrance Hill, Beijing	AA2015BJ01	KU902474	KU902515	KU902587	KU945303	KX494394
		A. acutum	Chenshan, Shanghai	AA2015SH03	KU902473	KU902514	KU902586	KU945304	KX494393
		A. acutum	Mt. Tianmu, Hangzhou, Zhejiang	AA2015HZ02	KU902475	KU902516	KU902588	KU945302	KU749526
		A. cappadocicum subsp. *sinicum*	Kunming, Yunnan	AC2015KM06	KU902486	KU902534	KU902591	KU945305	KU749536
		A. amplum subsp. *tientaiense*	Mt. Tiantai, Taizhou, Zhejiang	AA2015HZ08		KU902531	KU902618	KU945339	KU749531
	Palmata	*A. palmatum*	Xikou, Fenghua, Zhejiang	AP2015FH07	KU902463	KX494366	KX494375	KU945341	KU749507
		A. japonicum	Chenshan, Shanghai	AJ2015SH10	KX494352	KU902537	KX494376	KU945343	KU749542
		A. pseudosieboldianum	Kuandian, Dandong, Liaoning	AP2015LN04	KX494353	KU902561	KU902576	KU945340	KU749508
		A. sinense	Hangzhou, Zhejiang	AS2015HZ06	KU902493	KU902544	KU902599	KU945312	KU749547
		A. flabellatum	Hangzhou, Zhejiang	AF2015HZ05	KU902482	KU902529	KU902590	KU945311	KU749529
		A. elegantulum	Yanshan, Guilin, Guangxi	AO2015GL01	KU902460	KU902522	KU902570	KU945345	KX494389
		A. elegantulum	Kunming, Yunnan	AO2015KM01	KU902461	KU902523	KU902571	KU945346	KU749499
		A. elegantulum	Mt. Tianmu, Hangzhou, Zhejiang	AE2015HZ04	KU902487	KU902562	KU902596	KX494382	KU749538
		A. elegantulum	Mt. Siming, Ningbo, Zhejiang	AE2015NB01	KU902488	KU902563	KX870504	KU945316	KU749546
		A. pubinerve	Chenshan, Shanghai	AP2015SH12	KX494354	KU902558	KU902584	KU945313	KU749518
		A. oliverianum	Wuhan, Hubei	AO2015WH06	KU902485	KU902532	KU902593	KU945315	KU749533
		A. wilsonii	Chenshan, Shanghai	AW2015SH04	KU902481	KX494367	KX494377	KU945347	KU749527
		A. fenzelianum	Moshan, Wuhan, Hubei	AF2015WH09		KU902521	KU902602	KU945358	KU749505
		A. fabri	Yanshan, Guilin, Guangxi	AF2015GL03	KU902465	KU902524	KU902575	KX494383	KU749506
		A. fabri	Wuhan, Hubei	AF2015WH04	KU902466	KU902525	KU902574	KU945324	KU749517
		A. pauciflorum	Mt. Zhongshan, Nanjing, Jiangsu	AC2015NJ03	K494349	KU902549	KU494372	KU945308	KU749496
	Oblonga	*A. buergerianum*	Kunming, Yunnan	AB2015KM04	KU902480	KU902512	KU902580	KU945320	KU749513
		A. buergerianum	Fragrance Hill, Beijing	AB2015BJ02	KU902477	KU902510	KU902578	KU945317	KX494390
		A. buergerianum	Mt. Lushan, Jiujiang, Jiangxi	AB2015LS02	KU902479	KU902513	KU902581	KU945318	KX494392
		A. buergerianum	Xikou, Fenghua, Zhejiang	AB2015FH02	KU902478	KU902511	KU902579	KU945319	KX494391
		A. paxii	Kunming, Yunnan	AP2015KM02	KU902464	KU902504	KU902577	KU945321	KU749511
		A. coriaceifolium	Kunming, Yunnan	AC2015KM08	KU902492	KU902543	KU902601	KU945322	KU749545
		A. oblongum	Yaowan, Wuhan, Hubei	AO2015WH02	KU902459	KU902546	KU902565	KU945348	KU749486
	Macrantha	*A. davidii* subsp. *grosser*	Anning District, Kunming,Yunnan	AG2015KM10	KX494355	KU902519	KU902609	KU945327	KU749500
		A. davidii	Kunming, Yunnan	AD2015KM03	KU902471	KX494368	KU902613	KU945351	KU749524
		A. sikkimense	Taoyuanling, Hangzhou, Zhejiang	AH2015HZ01	KU902472	KU902541	KU902614	KU945326	KU749525
		A. metcalfii	Nanshan, Chongqing	AM2015CQ02		KU902506	KU902608	KX494384	KU749520
		A. pectinatum	Kunming Botanical Garden, Yunnan	AP2015KM15	KX494356	KX494369	KX494378	KX494385	KU749491
		A. tegmentosum	Kuandian, Dandong, Liaoning	AT2015LN01	KU902470	KU902540	KU902616	KU945328	KU749523
	Lithocarpa	*A. sinopurpurascens*	Taoyuanling, Hangzhou, Zhejiang	AS2015HZ03	KU902483	KU902530	KU902603	KU945329	KU749487
		A. tsinglingense	MT. Funiu, Luanchuan, Henan	AT2015HN05	KU902469	KU902539	KX494379	KU945352	KU749488
		A. sterculiaceum subsp. franchetii	Fangxian, Shiyan, Hubei	AF2015WH01	KU902458	KU902518	KU902606	KU945330	KU749498
		A. kungshanense	Kunming Botanical Garden, Yunnan	AK2015KM11	KX494357	KU902520	KX494380	KU945354	KU749501
		A. yangbiense	Kunming Botanical Garden, Yunnan	AY2015KM07	KU902491		KU902623	KU945335	KU749541
	Ginnala	*A. tataricum* subsp. *ginnala*	Xuhui, Shanghai	AG2015SH06	KU902495	KU902555	KU902582	KU945309	KU749495
	Pentaphylla	*A. pentaphyllum*	Kunming Botanical Garden, Yunnan	AP2015KM12	KX494358	KU902533	KU902619	KU945331	KU749534
	Trifoliata	*A. griseum*	Mt. Zhongshan, Nanjing, Jiangsu	AG2015NJ02	KX494359	KU902527	KU902622	KU945332	KU749539
		A. nikoense	Mt. Lushan, Jiujiang, Jiangxi	AN2015LS01	KU902467	KU902526	KU902620	KU945355	KX494387
		A. triflorum	Kuandian, Dandong, Liaoning	AT2015LN02	KU902476	KU902507	KU902615	KU945333	KU749521
		A. mandshuricum	Kuandian, Dandong, Liaoning	AM2015LN03	KX494360	KU902528	KU902605	KU945356	KU749490
	Negundo	*A. henryi*	MT. Funiu, Luanchuan, Henan	AH2015SH08	KX494361	KU902503	KU902611	KU945334	KU749510
		A. negundo	Xuhui, Shanghai	AN2015SH01	KU902456	KX494370	KU902606	KU945357	KU749493
Dipteronia		*D. sinensis*		GenBank	AY605290				
		D. sinensis		GenBank	EU720445				
		D. sinensis		GenBank	AF401121				

ASB) were calculated for each alignment using the software Taxon DNA (Slabbinck *et al*., 2008; Lee *et al*., 2016). Phylogenetic trees were generated using MEGA version 6.0 based on the maximum likelihood (ML) method (Tamura *et al*., 2013). Bootstrap values were computed using 1000 replicates. Three *Dipteronia sinensis* samples were used as outgroups (Table 1).

Results

Character Analysis of Each Barcode

We obtained 49 ITS/ITS2, 51 *mat*K, 52 *rbc*L, 52 *trn*H–*psb*A, and 52 *trn*L–*trn*F sequences from the 52 *Acer* samples. The amplification success rate of all five regions was 100%. The percentage of successful sequencing was 100% for *rbc*L, *trn*H–*psb*A and *trn*L-*trn*F, 98.08% for *mat*K, and 94.23% for ITS/ITS2 (Table 3). The aligned lengths of the six DNA sequences ranged from 277 bp (ITS2) to 705 bp (ITS) (Table 3). Among the six DNA barcodes, ITS2 exhibited the highest percentage (49.46%) of variable sites (Table 3). Among the plastid barcodes, the non-coding region *trn*H-*psb*A had the highest percentage of variable sites, which was approximately 3.1, 1.4 and 5.4 times more variable than *mat*K, *trn*L-*trn*F and

Table 2: Primers used for PCR in this study

DNA region	Primer	Sequence	Reference
ITS	ITS4	5′-TCCTCCGCTTATTGATATG-3′	White *et al*., 1990
	ITS5	5′-GGAAGTAAAAGTCGTAACAAGG-3′	White *et al*., 1990
*mat*K	472F	5′-CCCRTYCATCTGGAAATCTTGGTTC-3′	Yu *et al*., 2011b
	1248R	5′-GCTRTRATAATGAGA AAGATTTCTGC-3′	Yu *et al*., 2011b
*rbc*L	1F	5′-ATGTCACCACAAACAGAAAC-3′	Olmstead *et al*., 1992
	724R	5′- TCGCATGTACCTGCAGTAGC-3′	Fay *et al*., 1998
*trn*H–*psb*A	trnH	5′-CGCGCATGGTGGATTCACAATCC-3′	Tate and Simpson, 2003
	psbA	5′-GTTATGCATGAACGTAATGCTC-3′	Sang *et al*., 1997
*trn*L- *trn*F	trnL	5′-CGAAATCGGTAGACGCTACG-3′	Taberlet *et al*., 1991
	trnF	5′-ATTTGAACTGGTGACACGAG-3′	Taberlet *et al*., 1991

Table 3: DNA barcoding utility of six candidate sequences for *Acer*

Potential barcode	Aligned length (bp)	No. samples species (individuals)	Conserved sites (%)	Variable sites (%)	Informative sites (%)	PCR efficiency (%)	Sequencing efficiency (%)
ITS	705	51(171)	428(60.71)	271(38.44)	237(33.62)	52/52 (100)	49/52 (94.23)
ITS2	277	51(171)	136(49.10)	137(49.46)	121(43.68)	52/52 (100)	49/52 (94.23)
*mat*K	691	46(91)	647(93.63)	44(6.37)	31(4.49)	52/52 (100)	51/52 (98.08)
*rbc*L	599	51(102)	577(96.33)	22(3.67)	13(2.17)	52/52 (100)	52/52 (100)
*trn*H-*psb*A	511	56(110)	389(76.13)	101(19.76)	77(15.07)	52/52 (100)	52/52 (100)
*trn*L-*trn*F	403	60(158)	332(82.38)	55(13.65)	42(10.42)	52/52 (100)	52/52 (100)

Table 4: Analysis of inter-specific divergence and intra-specific variation of the six candidate barcodes for thewhole samples

Marker	ITS	ITS2	*mat*K	*rbc*L	*psb*A-*trn*H	*trn*L-*trn*F
Theta	0.0016±0.0018	0.0019±0.0030	0.0015±0.0020	0.0005±0.0007	0.0031±0.0055	0.0024±0.0044
Coalescent depth	0.0027±0.0029	0.0033±0.0047	0.0018±0.0033	0.0009±0.0014	0.0038±0.0085	0.0040±0.0071
Average intra-specific distance	0.0014±0.0019	0.0021±0.0067	0.0010±0.0023	0.0008±0.0012	0.0015±0.0048	0.0033±0.0062
Theta prime	0.0621±0.0215	0.0818±0.0309	0.0083±0.0038	0.0039±0.0024	0.0298±0.0146	0.0126±0.0073
Minimum inter-specific distance	0.0611±0.0214	0.0799±0.0307	0.0078±0.0041	0.0036±0.0023	0.0286±0.0162	0.0122±0.0077
Average inter-specific distance	0.0618±0.0228	0.0790±0.0321	0.0087±0.0040	0.0041±0.0027	0.0295±0.0151	0.0120±0.0072

*rbc*L, respectively (Table 3). The *rbcL* region represented the lowest variable (3.67%).

Intra- and Inter-specific Genetic Divergence of *Acer*

For all candidate barcodes, the genetic divergence was lower within species than between species (Table 4). Comparisons of the inter-specific divergence of the six barcode loci, ITS2 exhibited the highest average inter-specific distance and theta prime, followed by ITS, and the four plastid barcodes (*mat*K, *rbc*L, *psb*A-*trn*H and *trn*L-*trn*F) displayed relatively lower divergence among species. In addition, minimum inter-specific distances of ITS and ITS2 regions were larger than their maximum intra-specific distances (Coalescent depth). For other candidates, the differences between minimum inter-specific variation and their maximum intra-specific variation were not so significant (Table 4).

Barcoding Gap Assessment

The barcoding gaps between six inter- and intra-specific candidates were evaluated by graphing the distribution of genetic divergence based on the K2P model. The results indicated that ITS and ITS2 regions exhibited clear barcoding gaps, whereas the inter- and intra-specific distances of *mat*K, *rbc*L, *psb*A-*trn*H and *trn*L-*trn*F were overlapped without barcoding gaps (Fig. 1).

The significance between inter- and intra-specific K2P distances was calculated using Wilcoxon signed-rank tests. The results indicated that the intra-specific distances were lower than inter-specific distances, and ITS2 exhibited the highest variation among congeneric species, followed by ITS, while *rbc*L showed the lowest intra-specific variation (Table 5, 6). At the intra-specific level, the lowest variability was displayed by *rbc*L, and there was no significant difference in divergence between ITS and ITS2 (Table 6).

Species Discrimination Test

Taxon DNA was applied to analyze the species identifications in *Acer* samples. Discrimination success rates varied from 16.66% (*rbc*L) to 77.77% (ITS) according to BM method, varied from 16.66% (*rbc*L) to 74.26% (ITS) according to BCM method, and varied from 25.49% (*rbc*L) to 73.09% (ITS) using the ASB method (Fig. 2). Among the six barcode candidates, ITS region turned out to have the highest identification efficiency, followed by ITS2, and *rbc*L had the lowest success rates. Additionally, *rbc*L sequence provided the highest misidentification rate in the BM and BCM analysis, and the misidentification rates of ITS and ITS2 were both lower than 10% in the BM and BCM analysis, and lower than 5% in ASB analysis.

Table 5: Wilcoxon signed tests for interspecific divergences of candidate sequences

W+	W-	Relative Ranks	Results
ITS	ITS2	W+=30862.5, W−=886472.5 (n =1378, P≤3.121e-194)	ITS <ITS2
ITS	*mat*K	W+=245001, W−=349 (n =703, P≤1.229e-115)	ITS > *mat*K
ITS	*rbc*L	W+=368503.5,W−=7.5 (n =861, P≤4.752e-142)	ITS > *rbc*L
ITS	*psb*A-*trn*H	W+=432508.5,W−=5071.5 (n =946, P≤1.255e-147)	ITS >*psb*A-*trn*H
ITS	*trn*L-*trn*F	W+=686287, W−=2264 (n =1176, P≤6.396e-191)	ITS > *trn*L-*trn*F
ITS2	*mat*K	W+=245026, W−=324 (n =703, P≤1.131e-115)	ITS2> *mat*K
ITS2	*rbc*L	W+=367625,W−=28 (n =861, P≤7.554e-142)	ITS2> *rbc*L
ITS2	*psb*A-*trn*H	W+=440068.5,W−=1261.5 (n =946, P≤1.617e-153)	ITS2>*psb*A-*trn*H
ITS2	*trn*L-*trn*F	W+=686377, W−=2174 (n =1176, P≤5.139e-191)	ITS2> *trn*L-*trn*F
matK	*rbc*L	W+=60661.5, W−=9089.5 (n =861, P≤6.564e-46)	*mat*K > *rbcL*
*mat*K	*psb*A-*trn*H	W+=4055, W−=329281 (n =820, P≤8.599e-129)	*mat*K < *psb*A-*trn*H
*mat*K	*trn*L-*trn*F	W+=66844, W−=264861 (n =820, P≤2.874e-49)	*mat*K < *trn*L-*trn*F
*rbc*L	*psb*A-*trn*H	W+=372, W−=565144 (n =1081, P≤5.014e-175)	*rbc*L< *psb*A-*trn*H
*rbc*L	*trn*L-*trn*F	W+=19811, W−=561770 (n =1081, P≤8.886e-155)	*rbc*L < *trn*L-*trn*F
*psb*A-*trn*H	*trn*L-*trn*F	W+=834765.5,W−=41060.5 (n =1176, P≤2.665e-179)	*psb*A-*trn*H> *trn*L-*trn*F

Table 6: Wilcoxon signed tests for intraspecific variations of candidate sequences

W+	W-	Relative Ranks	Results
ITS	ITS2	W+=144, W−=156 (n =39, P≤0.864)	ITS = ITS2
ITS	*mat*K	W+=40, W−=26 (n =17, P≤0.534)	ITS = *mat*K
ITS	*rbc*L	W+=100, W−=20 (n =23, P≤0.023)	ITS >*rbc*L
ITS	*Psb*A-*trn*H	W+=97, W−=93 (n =28, P≤0.936)	ITS = *Psb*A-*trn*H
ITS	*trn*L-*trn*F	W+=175, W−=176 (n =33, P≤0.990)	ITS = *trn*L-*trn*F
ITS2	*mat*K	W+=29, W−=26 (n =17, P≤0.878)	ITS2 = *mat*K
ITS2	*rbc*L	W+=65, W−=13 (n =23, P≤0.041)	ITS 2 >*rbc*L
ITS2	*psb*A-*trn*H	W+=44, W−=61 (n =28, P≤0.594)	ITS2 = *Psb*A-*trn*H
ITS2	*trn*L-*trn*F	W+=121, W−=132 (n =33, P≤0.858)	ITS2 = *trn*L-*trn*F
*mat*K	*rbc*L	W+=28, W−=17 (n =17, P≤0.514)	*mat*K=*rbc*L
*mat*K	*psb*A-*trn*H	W+=23, W−=55 (n =18, P≤0.209)	*mat*K = *psb*A-*trn*H
*mat*K	*trn*L-*trn*F	W+=18, W−=60 (n =17, P≤0.099)	*mat*K = *trn*L-*trn*F
*rbc*L	*psb*A-*trn*H	W+=21, W−=70 (n =25, P≤0.087)	*rbc*L= *psb*A-*trn*H
*rbc*L	*trn*L-*trn*F	W+=57, W−=48 (n =27, P≤0.778)	*rbc*L = *trn*L-*trn*F
*psb*A-*trn*H	*trn*L-*trn*F	W+=111, W−=79 (n =28, P≤0.520)	*psb*A-*trn*H= *trn*L-*trn*F

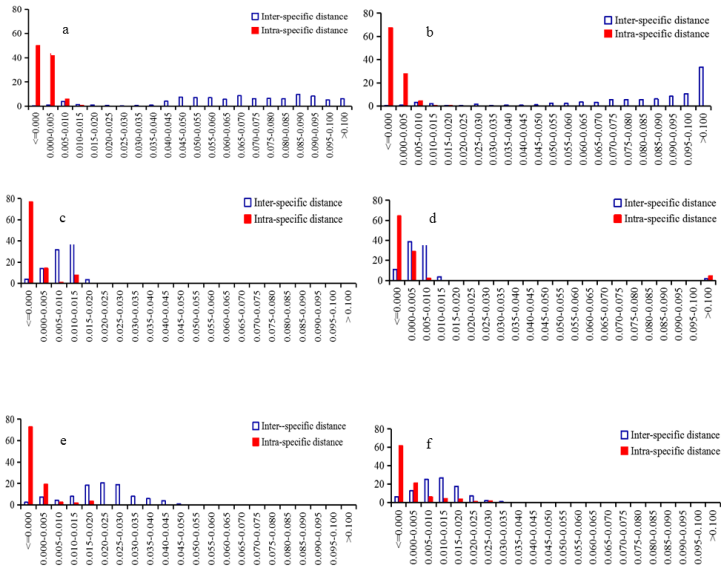

Fig. 1: Relative distribution of inter-specific and intra-specific K2P distances among *Acer* samples of the six barcodes. (a) ITS, (b) ITS2, (c) *matK*, (d) *rbc*L, (e)*psb*A-*trn*H, (f)*trn*L-*trn*F

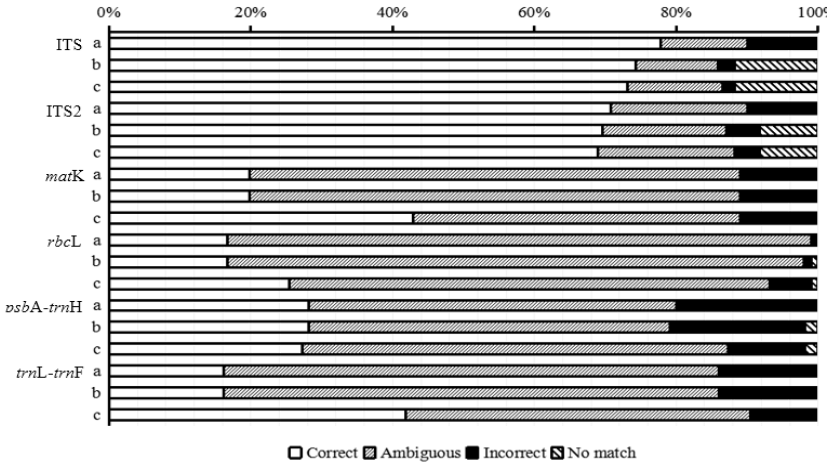

Fig. 2: Performance of the six barcodes based on the analysis of BM (a), BCM (b), and ASB (c). The distance threshold for BCM and ASB was computed from the intra-specific distances at 5% distances cut-off

Phylogenetic Analysis

To put the potential barcodes assessed by Taxon DNA into perspective, ITS region that provided the highest success rate in *Acer* species discrimination (77.77 and 74.26% in the BM and BCM analysis, respectively) (Table S2) was selected for constructing phylogenetic trees. According to the *Acer* species classification system reported in Flora of China (Xu *et al.*, 2013), all *Acer* species were classified into 14 sections, in addition to some species without grouping, such as *A. campestre*, *A. saccharum*, and *A. capillipes.* In the present study, ML tree was constructed based on 171 ITS sequences from 51 *Acer* species, which were grouped into twelve sections (*Palmata*, *Macrantha*, *Arguta*, *Negundo*, *Spicata*, *Platanoidea*, *Lithocarpa*, *Acer*, *Ginnala*, *Pentaphylla*, *Trifoliata*, and *Oblonga*), and all these samples were grouped into four main clusters (Fig. 3). Group I comprised 19 species from five sections, which was further divided into four subgroups. Except for *A. crassum*, all species belonging to sect. *Palmata* were included into subgroup I-1. *A. wardii*, a species from sect. *Macrantha*, was also included into subgroup I-1. Two species (*A. stachyophyllum* and *A. stachyophyllum* ssp. *betulifolium*) in sect. *Spicata* were clustered into subgroup I-2. The species from sect. *Negundo* and *Spicata* were grouped into subgroup I-3 and subgroup I-4, respectively. Group II contained 18 species, all species from sect. *Platanoidea* were classified into subgroup II-1, four species (*A. sinopurpurascens*, *A. tsinglingense*, *A. sterculiaceum* subsp. *franchetii* and *A. kungshanense*) from sect. *Platanoidea* were included into subgroup II-2, and all species in sect. *Macrantha* were grouped into subgroup II-2 except for *A. wardii* in subgroup I-1. Four species were clustered into group III. Subgroup III-1 included two species, one from sect. *Lithocarpa* and another one from sect. *Acer*, subgroup III-2 contained two species (*A. tataricum* subsp. *ginnala* and *A. tataricum* subsp. *semenovii*) from sect. *Ginnala*. In addition to one species (*A. crassum*) from sect. *Palmata*, all species from other three sections were classified into Group IV. *A. pentaphyllum* constituted a separate subgroupIV-1. Three species (*A. griseum*, *A. nikoense*, and *A. triflorum*) from sect. *Trifoliata* were grouped into subgroup IV-2. Subgroup IV-3 contained six species from three sections: one from sect. *Trifoliata*, one from sect. *Palmata*, and other four from sect. *Oblonga*.

Discussion

DNA barcode has been proved as an effective tool for species discrimination, though some experts still have doubts about its utility (Kress *et al.*, 2005; Yu *et al.*, 2011a). In animals, *COI* gene was established as the universal barcode (Hebert *et al.*, 2003a; Luo *et al.*, 2010). In plants, the standard DNA barcode have not yet been available (Yu *et al.*, 2011a). Howerer, several DNA regions have been proved effective for some plant taxa identification (Kress *et al.*, 2005; Chen *et al.*, 2010; Lee *et al.*, 2016). In this study, six DNA sequences (ITS, ITS2, *mat*K, *rbc*L, *psb*A-*trn*H and *trn*L-*trn*F) were assessed as candidate DNA barcodes in *Acer*. Considering the sequencing efficiency and quality, *rbc*L and *mat*K performanced better than other sequences (Table 3), which were also the two most widely accepted potential barcodes (Wang *et al.*, 2016; Asahina *et al.*, 2010), whereas the low substitution rates led to the low discrimination success in *Acer* (Tables 3, S2). Two chloroplast DNA sequences, *trn*H-psbA and *trn*L-*trn*F, provided more variable sites than the coding regions (*rbc*Land *mat*K), and expressed higher identification success rate than them, but the ambiguous identification of which were also high (Fig. 2). Among these candidates, ITS exhibited the most effective discrimination, followed by ITS2, although their sequencing efficiency were relatively lower than others (Fig. 2; Table 3).

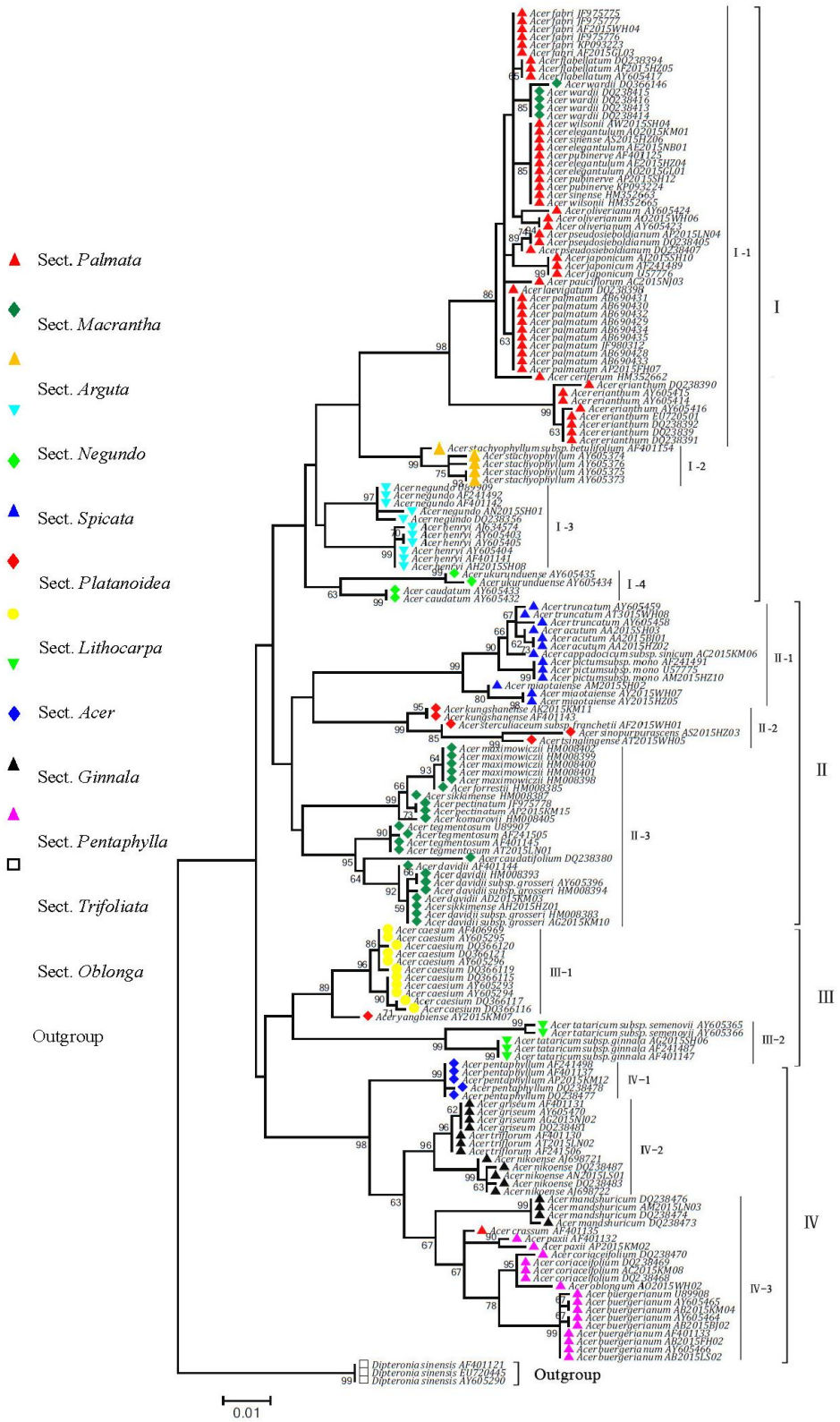

Fig. 3: Maximum likelihood (ML) tree based on ITS sequences for *Acer* species. Numbers above branches indicate bootstrap support (BS ≥ 50)

As in many previous studies (Chen *et al.*, 2008; Gao *et al.*, 2010b; Li *et al.*, 2010), ITS has been proved powerful in taxa discrimination at different levels of taxon samples. Although ITS has sometimes been deemed as an inappropriate DNA barcode for the possible impact of incomplete concerted evolution of nrDNA (Buckler and Holtsford, 1996; Ren *et al.*, 2009), our study demonstrated that the possible impact may not play an important role in *Acer*. By contrast, the ITS sequence is effective in our study for its reliable discrimination capability. The ITS exhibited high genetic difference among congeneric species, which was significantly higher than the intra-specific divergence (Tables 4, 5, 6) and ITS region achieved the highest species solution percentage (in the ASB analysis) at 73.09% in *Acer*.

The ITS region cannot differentiate all the species in *Acer*. For example, *A. elegantulum*, *A. sinense* and *A. pubinerve* with identical ITS sequences were failed to be distinguished. However, more than 70% of *Acer* species could be identified using ITS locus, and the ambiguous and incorrect discrimination of ITS were the lowest, these values were relatively high for the plastid regions (Table S2). Therefore, ITS region was seemed to be the most suitable DNA barcode for *Acer* species. Additionally, some species with only one sample, the sequences of which were almost evaluated as no match; and may somewhat have resulted in a decrease in the value of correct discrimination. Hence, if taken this factor into consideration, discrimination success rates may be higher for *Acer*.

Many studies have proved that DNA barcode can not only be as a powerful tool for species-level discrimination, but also applicable for taxonomic and biodiversity studies (Tian *et al.*, 2002; Feng *et al.*, 2016; Lee *et al.*, 2016). In this study, ITS can be used to differentiate *Acer* species and serve to reconstruct phylogenetic trees in *Acer*, and 12 of Xu *et al*'s 14 sections were supported in the trees. As reported in Fang's system (Fang, 1981), all *Acer* species were classified into two subgenera, 15 sections and 22 series, and *A. wilsonii*, *A. elegantum*, *A. sinense*, *A. olivaceum* and *A. erianthum* were grouped into sect. *Microcarpa*, *A. fabri* and *A. laevigatum* were grouped into sect. *Integrifolia*. Based on similar morphological characters such as typically palmate leaves, 4 paired bud scales, and corymbose inflorescences, De Jong (1994) and Xu *et al.* (2013) combined sect. *Palmata* and sect. *Microcarpa* into one section, sect. *Palmata*, and *A. fabri* and *A. laevigatum* were also been put in this section. Our clustering results support the classification criteria of De Jong (1994) and Xu *et al.* (2013) here. The samples of *A. negundo* and *A. henryi* were grouped together within subgroup I-3, which indicated that these two species have a close genetic relationship and it was reasonable to combined these species into sect. *Negundo* in Xu *et al*'s system (Xu *et al.*, 2013). As shown in the ML tree, *A. yangbie* and *A. caesium* formed a monophyletic clade with high bootstrap support (89%). However, some important morphological differences were existed between the two species, such as bud scales number, inflorescences and branchlets type. In Xu *et al.* (2013) system, *A. yangbie* was classified into sect. *Lithocarpa* rather than sect. *Acer*. Therefore, more evidence is needed for ensuring the classification status of *A. yangbie*. In addition, we found *A. wardii* was nested within species of sect. *Palmata* with relatively high bootstrap support (85%). This was backed by the study of Grimm *et al.* (2006). In the previous studies (Fang, 1996, 1981; Xu, 1996; Xu *et al.*, 2013), *A. wardii* were treated as a species of sect. *Macrantha*; while in de Jong's system, *A. wardii* was divided as a monotypic section for the uncharacteristic inflorescences (bracts, cincinni) and flowers (reflexed sepals), and this was supported by the study of Tian *et al.* (2002). In our opinion, *A. wardii* was a species worthy of further study, for its atypical morphological characteristics of sect. *Macrantha*, and for its close relationship to species of sect. *Palmata*. The species clusters also showed that the taxonomic status of *A. mandshuricum* should be revalued. *A. mandshuricum* exhibited closer relationship to sect. *Oblonga* though belonging to sect. *Trifoliata*, this was not in consensus with previously studies (Fang, 1996, 1981; Xu, 1996; Xu *et al.*, 2013). Interestingly, similar result was also found in the study of Tian *et al.* (2002).

DNA barcodes have been commonly used in species identification as complementary tool for traditional taxonomy, although some doubts on their utility still exist. DNA barcode can help non-professionals discriminate species rapidly and accurately (Luo *et al.*, 2010). In this study, six DNA barcodes for *Acer* were evaluated, and the results showed that ITS region was effective for *Acer* species identification. The ITS sequences of *Acer* species were also found having clear phylogenetic signals to resolve evolutionary relationships, and overall agreement with morphologically defined taxa (Xu *et al.*, 2013).

Conclusion

ITS region was proved the most suitable barcode for evaluation and taxonomic implications in *Acer*, and our classification result overall agreement with morphologically-based taxa of Xu *et al.* (2013) 14 sections.

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