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Full Length Article

Application of ITS2 Sequences for Species Identification and Phylogeny of Genus *Acer* (Aceraceae)

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Abstract

Acer Linn. is a genus of highly variable species of the family Aceraceae. Currently, it is intricate to conduct species identification and phylogeny investigations using traditional morphological method. This study was applied to assess the applicability of Internal Transcribed Spacer 2 (ITS2) for species identification and evolutionary relatedness of *Acer*. For this purposes, 337 ITS2 sequences from 105 *Acer* species were analyzed. The results showed that ITS2 sequences displayed significant inter-specific divergences, clear DNA barcoding gaps, and relatively high species identification efficiency (>64% for BM, BCM, ASB and BLASTA1 analysis). Cluster analysis based on ITS2 regions largely agreed with the relationships among *Acer* species established by morphological studies. However, the taxonomic status of several sections such as sect. *Acer*, sect. *Goniocarpa*, sect. *Saccharodendron*, sect. *Negundo* and sect. *Oblonga* as well as species such as *A. wardii* and *A. pectinatum* should be further analyzed. Our results propose to merge sect. *Acer*, sect. *Goniocarpa* and sect. *Saccharodendron* into one group, which would contain five series. The results also support to promote *A. wardii* into a single section and assign *A. pectinatum* to ser. *Micranthum*. *A. negundo* should be treated as a separated section rather than a species under sect. *Cissifolia*. The systematic relationship of sect. *Oblonga* warrants future investigations to define clear clarification systems. Our results indicated that ITS2 sequence holds sound applicability in the identification and phylogeny assessment of *Acer*. © 2020 Friends Science Publishers

Keywords: *Acer* Linn.; Molecular Identification; DNA Barcode; ITS2; Phylogeny

Introduction

Acer Linn. of the *Aceraceae* family is one of the largest genera of deciduous forests in the northern hemisphere with over 130 species (Xu 1998; Xu *et al.* 2013). This genus is furnished with comprehensive fossil record and is often used as a model plant to study the origin and evolution of woody plants (Yang and Li 2010). Many of its taxa are also ideal materials for studying the intermittent distribution of plants in East Asia-North America (Chang *et al.* 1991). The *Acer* plants carry unique co-source characteristics such as opposite leaves and samara, which are easy to distinguish from the adjacent genera. However, other morphological characteristics, such as leaf shape, fruit shape and inflorescence are highly variable among species. Thus, classification and phylogenetic study of this genus is comparatively complex (Pojárkova 1933; Tian *et al.* 2002).

In 1885, Pax established the first system of *Acer*, in which the genus was classified into 14 sections, mainly on the basis of the relative position of stamens to discs (Pax 1885). Since then, many researchers have successively studied the system of *Acer* in the fields of morphology (Koidzumi 1911; Pojárkova 1933; Rehder 1936; Ogata 1967; Fang 1981; De Jong 1994; Xu *et al.* 2013), relic fossils (Wolfe and Tanai 1987), palynology (Erdtmen 1952; Tian *et al.* 2001), isozyme (Liu *et al.* 2001; Wang *et al.* 2007), molecular systematics (Suh *et al.* 2000; Tian *et al.* 2002; Grimm *et al.* 2006) and branch taxonomy (Fang 1981; Tian and Li 2004). By now, the system of *Acer* has been fundamentally defined. However, there are still disputes over classification of some sections, which pose complications in species identification of *Acer*. In addition, the infrageneric phylogenetic relationships in this genus are also controversial. Although some evidence including gross morphology, seed proteins, chemical composition, geographic distributions, fossils and molecular information are available, the conclusions are not in consensus (Tian *et al*. 2002).

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DNA barcode technology is a molecular biological technique and is based on the principle of sequencing method (Deef 2019; Afzal *et al*. 2020). It has been widely used in the field of biodiversity assessment, species identification, phylogenetic analysis and ecological studies (Lin *et al.* 2017; Shinwari *et al.* 2018; Mitchell *et al.* 2020). For animals, the mitochondrial cytochrome oxidase I (*COI*) gene has been considered as the standard DNA barcode (CBOL Plant Working Group 2009). However, the choice of DNA barcoding in plants is more complicated compared to animals owing to their uniparentally inherited, nonrecombining and structurally stable genome (Kress *et al.* 2005). In recent years, many gene sequences such as *mat*K, *rbc*L, *psb*A-*trn*H, *rpl*16, *atp*F-*atp*H, *ycf*1 and ITS, have been successively used in distinguishing different taxonomic groups in plants, but no universal DNA barcoding has been found yet (Kress *et al.* 2005; Yao *et al.* 2010; Dong *et al.* 2015; He *et al*. 2019; Prasad *et al.* 2020; Wu *et al.* 2020).

ITS2, a non-coding region of the ribosomal DNA ITS, has been proven as a potential universal DNA barcode to authenticate species (Feng *et al.* 2016; Mbareche *et al.* 2020; Shi *et al.* 2020). For herbs, the identification success rate of ITS2 was up to 92.7% (Chen *et al.* 2010). It also performed well in species-level discrimination of *Physalis* L., *Panax* L. and *Paris* L. (Feng *et al.* 2016; Sun *et al.* 2016). Compared with ITS sequence, ITS2 holds advantages such as shorter sequence length and higher amplification efficiency and is therefore considered as a suitable candidate sequence for standard barcoding for plants (Liu *et al.* 2012; Dong *et al.* 2015; Zhao *et al.* 2015; Timpano *et al.* 2020). Furthermore, ITS2 has been proven to be applicable in plant phylogenetic studies (Li *et al.* 2014; Feng *et al.* 2016; Sun *et al.* 2016). In this study, ITS2 region was used to barcode *Acer* and to reconstruct the phylogenetic relationships of *Acer* species.

Materials and Methods

Experimental material and sampling

A total of 60 samples from 50 *Acer* species were collected for this study (Table 1). In addition, 277 published *Acer* ITS2 sequences from 94 *Acer* species were downloaded from GenBank (Table 2). There were totally 105 species which represent the 23 sections of *Acer* of Xu's system (Xu 1996; Xu *et al.* 2013). All samples were confirmed using the botanical information from Chinese Virtual Herbarium (http://www.cvh.org.cn/). Vouchers and digital images were deposited in the Herbarium of Ningbo Key Laboratory of Landscape Plant Development, Ningbo City College of Vocational Technology.

DNA Extraction, PCR amplification and sequencing

In order to extract DNA, 2 mg of dried leaves were milled with liquid nitrogen. This crushed blend was used to extract genomic DNA as per manufacturer's recommendation (Lifefeng Co., Shanghai, China). Using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA), the quality and quantity of the extracted DNA was determined. All samples were diluted to 100ng. μL^{-1} for later use. PCR amplification was performed using AG 22331 sequence amplification apparatus (Eppendorf Ltd., Hamburg, Germany). Forward primer was ITS4: 5'-TCCTCCGCTTATTGATATG-3' and reverse primer was ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3'. A total of 50 μL PCR reaction mix was prepared containing 1 μL genome DNA, 1 μL forward and reverse primer (concentration is 10 $\mu mol \cdot L^{-1}$), 25 μL 2 × Taq PCR Mix (BioTeke Co., Beijing, China) and 22 μL ddH_2O. The PCR amplification conditions were applied as follow: pre-degeneration for 5 min at 94°C, degeneration for 30 s at 94°C, annealing for 30 s at 55°C, renaturation for 1 min at 72°C and extend for 7 min at 72°C for a total of 35 cycles. The PCR products were visualized in 1% agarose gel electrophoresis purified and recovered by DNA Recovery Kit (Axygen, Hangzhou, China). These PCR products sequenced using PCR primers in both directions by the Shanghai Sunny Biotechnology Co., LTD. Newly acquired sequences were submitted to GenBank (Table 1).

Data analysis

The raw sequences were edited using CodonCode Aligner 5.1 software (CodonCode Co., USA) to remove low-quality fragments, and the sequences less than 150 bp were deprecated. The 5.8 s and 28 s region of all sequences was removed according to the Hidden Markov model (HMM) to retain the complete ITS2 region (Keller *et al.* 2009). Clustal X2.1 software was used for multi-sequence comparison of sequences, and BioEdit V5.0.6 software was used to calculate the length and GC contents (Hu *et al.* 2011). The K2P (kimura-2 parameter) genetic distance between sequences was obtained by MEGA 6.0 software (Tamura *et al.* 2013). DNA barcoding gaps were plotted according to intra- and inter-specific variations of the ITS2 sequences and Wilcoxon signed-rank tests were performed (Slabbinck *et al.* 2008; Lee *et al.* 2016). TaxonDNA 1.0 software was used to evaluate the identification efficiency of ITS2 region (Slabbinck *et al.* 2008). In addition, BLASTA1 method was also applied to assess the discriminatory capability of ITS2 sequence (Gao *et al.* 2010).

Phylogenetic analysis of *Acer* was performed using Bayesian inference (BI) method on MRBayes 3.1 (Huelsenbeck and Ronquist 2001), and the best-fit model (GTR+G) was selected by the Akaike information criterion (AIC) in MrModeltest 2.3 (Nylander 2004). Posterior probabilities (PP) for individual clades were computed with MrBayes. *Dipteronia dyeriana* was selected as outgroup for its close relation to *Acer* species. Furthermore, the Neighbor-Net (NN) splits phylogenetic network of *Acer* was constructed using the SplitsTree 4.13.1 software based on the uncorrected p-distance (Hu *et al.* 2011).

Table 1: Voucher information of the *Acer* plants samples in this study

Section	Species	Voucher No.	Locality information	GenBank/Accession No.
Spicata	*A. ukurunduense* Trautv. et Meyer	LN06	Kuandian, Dandong, Liaoning	KY649425
Palmata	*A. palmatum* Thunb.	FH01	Mt. Siming, Ningbo, Zhejiang	KU902463
	A. linganense Fang et P. L. Chiu	NJ04	Mt. Zhongshan, Nanjing, Jiangsu	KX494348
	A. japonicum Thunb.	SH10	Chengdu, Sichuan	KX494352
	A. pseudosieboldianum Komarov	LN04	Kuandian, Dandong, Liaoning	KX494353
	A. flabellatum Rehd.	HZ07	Hangzhou, Zhejiang	KU902482
	A. elegantulum Fang & Chiu	GL01	Yanshan, Guilin, Guangxi	KU902460
	A. elegantulum Fang & Chiu	KM01	Kunming, Yunnan	KU902461
	A. elegantulum Fang & Chiu	HZ04	Mt. Tianmu, Hangzhou, Zhejiang	KU902487
	A. elegantulum Fang & Chiu	NB01	Mt. Siming, Ningbo, Zhejiang	KU902488
	A. sinense Pax	HZ06	Hangzhou, Zhejiang	KU902493
	A. pubinerve Rehd.	SH12	Chenshan, Shanghai	KX494354
	A. kweilinense Fang & Fang	SC01	Chengdu, Sichuan	KU902496
	A. wilsonii Rehd.	SH04	Chenshan, Shanghai	KU902481
	A. oliverianum Pax	WH06	Wuhan, Hubei	KU902485
	A. fabri Hance	WH04	Wuhan, Hubei	KU902466
	A. fabri Hance	GL03	Yanshan, Guilin, Guangxi	KU902465
	A.laevigatum Wall.	WH03	Wuhan, Hubei	KU902462
Platanoidea	*A. miaotaiense* Tsoong	SH02	Chenshan, Shanghai	KU902468
	A.yangjuechi Fang & Chiu	HZ05	Taoyuanling, Hangzhou, Zhejiang	KU902489
	A.yangjuechi Fang & Chiu	WH07	Shennongjia Forestry District, Hubei	KU902490
	A. campestre L.	HZ15	Taoyuanling, Hangzhou, Zhejiang	KY649427
	A. acutum Fang	HZ02	Mt. Tianmu, Hangzhou, Zhejiang	KU902475
	A. acutum Fang	SH03	Chenshan, Shanghai	KU902473
	A. acutum Fang	BJ01	Fragrance Hill, Beijing	KU902474
	A. truncatum Bunge	WH08	Wuhan, Hubei	KU902494
	A. mono Maxim.	HZ10	Mt. Tianmu, Hangzhou, Zhejiang	KX494362
	A. cappadocicum Gled. var. *sinicum* Rehd.	KM06	Kunming, Yunnan	KU902486
	A. longipes Franch. ex Rehd. var. *weixiense* Fang	KM05	Kunming, Yunnan	KU902484
	A. amplum subsp. *tientaiense* Chen	HZ08	Mt. Tiantai, Taizhou, Zhejiang	KY649428
Ginnala	*A. tataricum* subsp. *ginnala* Maxim.	SH06	Chenshan, Shanghai	KU902495
Oblonga	*A. buergerianum* Miq.	BJ02	Fragrance Hill, Beijing	KU902477
	A. buergerianum Miq.	FH02	Xikou, Fenghua, Zhejiang	KU902478
	A. buergerianum Miq.	LS02	Mt. Lushan, Jiujiang, Jiangxi	KU902479
	A. paxii Franch.	KM02	Kunming, Yunnan	KU902464
	A. cinnamomifolium Hayata	KM08	Kunming, Yunnan	KU902492
	A. oblongum Wall. ex DC.	WH02	Yaowan, Wuhan, Hubei	KU902459
	A. wangchii Fang subsp. *tsinyunense* Fang	CQ01	MT. Jinyun, Chongqing	KU902498
	A. cordatum Pax	SH09	Chenshan, Shanghai	KY649430
Macrantha	*A. davidii* subsp. *grosseri* Pax	KM10	Anning District, Kunming,Yunnan	KX494355
	A. hookeri Miq.	HZ01	Hangzhou, Zhejiang	KU902472
	A. davidii Franch.	KM03	Kunming, Yunnan	KU902471
	A. capillipes Maxim.	SH07	Xuhui, Shanghai	KU902502
	A. pectinatum Wall. ex Nichols.	KM15	Kunming, Yunnan	KX494356
	A. tegmentosum Maxim.	LN01	Kuandian, Dandong, Liaoning	KU902470
	A.komarovii Pojark.	SX01	Xian, Shanxi	KY649429
	A. caudatifolium Hayata	HZ07	Taoyuanling, Hangzhou, Zhejiang	KU902500
Lithocarpa	*A. sinopurpurascens* Cheng	HZ03	Taoyuanling, Hangzhou, Zhejiang	KU902483
	A. tsinglingense Fang & Hsieh	HN05	MT. Funiu, Luanchuan, Henan	KU902469
	A. sterculiaceum subsp. *franchetii* (Pax) Murray	WH01	Wuhan, Hubei	KU902458
	A. kungshanense Fang & Chang	KM11	Kunming, Yunnan	KX494357
Pentaphylla	*A. pentaphyllum* Diels	KM12	Kunming, Yunnan	KX494358
Trifoliata	*A. griseum* (Franch.) Pax	NJ02	Mt. Zhongshan, Nanjing, Jiangsu	KX494359
	A. nikoense (Franch.) Pax	LS01	Mt. Lushan, Jiujiang, Jiangxi	KU902467
	A. triflorum Komarov	LN02	Kuandian, Dandong, Liaoning	KU902476
	A. mandshuricum Maxim.	LN03	Kuandian, Dandong, Liaoning	KX494360
Arguta	*A. barbinerve* Maxim.	LN08	Kuandian, Dandong, Liaoning	KY649432
Rubra	*A. saccharinum* L.	SX02	Xian, Shanxi	KY649431
Cissifolia	*A. henryi* Pax	SH08	Chenshan, Shanghai	KX494361
Negundo	*A. negundo* L.	SH01	Chenshan, Shanghai	KU902456
	A. negundo L.	LN05	Kuandian, Dandong, Liaoning	KY649424

Results

ITS2 sequence properties

The ITS2 sequences from 60 *Acer* samples were obtained under this study, and a total of 337 sequences (277 sequences downloaded from GenBank) were used for analysis. It was noticed that the ITS2 sequence length ranges from 208 to 254 bp with an average length of 234 bp. The GC contents were different among species, with the lowest value of 57.63%, the highest value of 68.60% and the average value of 62.02%. Post-alignment analysis identified that the

Table 2: GenBank accession numbers of *Acer* plants samples and *Dipteronia sinensis* (Outgroup) in this study

Section	Species	GenBank/Accession No.
Parviflora	*A.nipponicum* Hara	AF020380, DQ366140, DQ366141, DQ366143
Distyla	*A.distylum* Sieb. & Zucc.	AF241485, AF401155, DQ238354, DQ238355
Spicata	*A. caudatum* Wall.	AY605432, AY605433
	A. ukurunduense Trautv. et Meyer	AY605434, AY605435
	A. spicatum Lam.	U89911, AF241503, AF401122
Palmata	*A. palmatum* Thunb.	AB683975, JF980312, AB690435
	A. linganense Fang et P. L. Chiu	KX494348
	A. japonicum Thunb.	U57776, AF241489
	A. pseudosieboldianum Komarov	DQ238405, DQ238406
	A. shirasawanum Koidzumi	AY605428, DQ238409, DQ238409, DQ238410, DQ238411
	A. circinatum Pursh	AY605412, AY605413, HM352653
	A. flabellatum Rehd.	AY605417, DQ238394
	A. sinense Pax	HM352663
	A. pubinerve Rehd.	KP093224, AF401125
	A. wilsonii Rehd.	HM352665
	A. oliverianum Pax	AY605422, AY605423, AY605424
	A. tutcheri Duth.	KP093225
	A. miaoshanicum Fang	AF401124
	A. erianthum Sch.	EU720501, DQ238391, DQ238392, DQ238393
	A. tonkinense Lec.	HM352664
	A. fabri Hance	KP096075, KP093223, JF975777
	A. crassum Hu & Cheng	AF401135
Glabra	*A. glabrum* Torrey	DQ238338, AF056017, AF241488, AF401139, DQ238337, DQ238340
Platanoidea	*A. campestre* L.	LK022464, LK022604, LK022459, AF401158
	A. miyabei Maxim.	AY605451, AY605452
	A. truncatum Bunge	AY605459, LK022669
	A. mono Maxim.	U57775, JF980310, AF241491
	A. cappadocicum var. *divergens* (Pax) Murray	LK022629, LK022630, LK022631, LK022632
	A. cappadocicum Gled.	AJ634579, DQ238439, DQ238440, DQ238444, LK022625, LK022626
	A. platanoides L.	AF401136, EF494236, LK022679, LK022672, U57773, DQ238461
Ginnala	*A. tataricum* subsp. *ginnala* Maxim.	AF241487, AF401147
	A. tataricum subsp. *semenovii* (Regel & Herder) Murray	AY605365, AY605366
	A. tataricum L.	AF401146, AM265511, JF975781, AM265512
	A. tataricum subsp. *aidzuense* Franchet	AM113519, AM113520, AM113521
Acer	*A. caesium* Wall. ex Brandis	AY605293, AY605294, DQ366115, DQ366116, DQ366117
	A.caesium Wall. ex Brandis subsp. *giraldii* Murray	AY605296, DQ366121, AY605295, AF406969
	A. pseudoplatanus L.	DQ366132, AY605338, AY605340, AY605346, DQ366131, DQ366133
	A. heldreichii Orphanides ex Boissier	AY605301, AY605302, AM238280, AY605303, AY605304
	A. trautvetteri Medvedev	AY605351, AF401126, AY605355, AM238285
	A. velutinum Boissier	AM238291, AM238294, AY605358, DQ366132, DQ366137
Saccharodendron	*A. saccharum* L.	EU720502, AF401152
	A. saccharum ssp. *skutchii* (Rehd.) Murray	FJ906753, FJ906754, FJ906755
	A. saccharum ssp. *floridanum* (Chap.) Desma.	DQ366138, DQ366139
Pubescentia	*A. pilosum* Maxim.	DQ238344, DQ238345, DQ238346
Oblonga	*A. buergerianum* Miq.	AF401133, U89908, AY605466
	A. buergerianum ssp. *formosanum* Hance	FN651690, FN651694, FN651695
	A. paxii Franch.	AF401132
	A. cinnamomifolium Hayata	DQ238468, DQ238470
	A. oblongum Wall. ex DC.	AF241494
	A. albopurpurascens Hayata	DQ238471, FN651702, FN651712,
	A. poliophyllum Fang	AF401134
	A. cordatum Pax	HM352654
Goniocarpa	*A. monspessulanum* L.	AY605321, AF401127, AM238361, DQ366128
	A. hyrcanum Fisch. & Mey.	DQ366129, DQ366130, AY605305, AY605306
	A. obtusifolium Sibthorp & Smith	AM238327, AM238331, AM238332
	A. opalus Mill.	AF401128, AY605328, AM238302, AY605331, AY605332
	A. sempervirens L.	AY605352, AY605353, DQ366123
Macrantha	*A. davidii* subsp. *grosseri* Pax	HM008383, HM008394, HM008397, AY605396
	A. davidii Franch.	AF401144, HM008393
	A. capillipes Maxim.	DQ238368, DQ238371
	A. laxiflorum Pax	HM008386
	A. crataegifolium Siebold & Zucc.	AY605391, DQ238376, DQ238378, DQ238379
	A. micranthum Siebold & Zucc.	HM008404, HM008407, AF020369
	A. rufinerve Siebold & Zucc.	AY605399, AY605400, DQ238372, DQ238373, DQ238374
	A. komarovii Pojark.	HM008405
	A. maximowiczii Pax	HM008400, HM008401, HM008402
	A. pectinatum Wall. ex Nichols.	KX494356, JF975779
	A. tegmentosum Maxim.	DQ366113, AF241505
	A. caudatifolium Hayata	DQ238380
	A. pensylvanicum L.	AY605398, AF020370, AF241497
	A. wardii Smith	DQ366146, DQ238413, DQ238415, DQ238416, DQ238418
Lithocarpa	*A. sterculiaceum* subsp. *franchetii* (Pax) Murray	DQ366145
	A. kungshanense Fang & Chang	AF401143
	A. diabolicum Blime	AF241484, AY605382, AY605383, AF020366
Marcophylla	*A. macrophyllum* Pursh	AY605387, AY605388, DQ238347, DQ238350, AF401156
Pentaphylla	*A. pentaphyllum* Diels	DQ238477, DQ238478, AF241498, AF401137

Table 2: Continued

Table 2: Continued

Trifoliata	*A. griseum* (Franch.) Pax	DQ238480, DQ238481, AF401131, AY605469
	A. nikoense (Franch.) Pax	DQ238483, DQ238487, AJ698721, AJ698722
	A. triflorum Komarov	AF241506, AJ698128
	A. mandshuricum Maxim.	DQ238473, DQ238474, DQ238476, AF401129
Hyptiocarpa	*A. decandrum* (Merr.) Murray	AF401149
	A. laurinum Hasskarl	DQ366114, AM113541, AM113542, AM113543
Arguta	*A. stachyophyllum* Hiern	AY605373, AY605374, AY605375, AY605376
	A. stachyophyllum subsp. *betulifolium* Maximowicz	AY605373, AY605374
	A. acuminatum Wall.	AY605370, AY605371, AY605372
	A. argutum Maxim.	AF401153, AF241480
	A. barbinerve Maxim.	AJ634569, AJ634571, AJ634573
Rubra	*A. pycnanthum* Koch.	AM113528, AM113529
	A. rubrum L.	AY605461, AF401150, AF020385
	A. saccharinum L.	AF401151, AY605462, AY605463, AM113531
Indivisa	*A. carpinifolium* Siebold & Zucc.	AF401148, AY605377, AY605379, AY605380
Cissifolia	*A. henryi* Pax	AY605404, AY605405, AF401141, AJ634574
	A. cissifolium (Sieb. & Zucc.) Koch.	AY605401, AY605402, AF241483, AF401140
Negundo	*A. negundo* L.	AF401142, U89909, DQ238362, DQ238356
Outgroups	*D. sinensis* Oliv.	AY605290, EU720445, AF401121

Table 3: Interspecific and Intraspecific variation of the ITS2 sequence in 337 samples of 105 *Acer* species

Measurement	K2P value
Average interspecific distance	0.0777±0.0293
Theta prime	0.0766±0.0299
The minimum interspecific distance	0.0728±0.0299
Average intraspecific distance	0.0048±0.0108
Theta	0.0045±0.0096
Coalescent depth	0.0073±0.0129

Table 4: Authentication efficiency for ITS2 by using different methods

Parameter	Correct identification	Ambiguous identification	incorrect identification	No match
All species barcodes	216 (64.09%)	100 (29.67%)	15 (4.45%)	6 (1.78%)
Best match	218 (64.68%)	88 (26.11%)	31 (9.19%)	0
Best close match	216 (64.09%)	88 (26.11%)	27 (8.01%)	6 (1.78%)
BLASTA1	222 (65.87%)	86 (25.52%)	29 (8.61%)	0

sequence length was 296 bp, containing 107 conserved sites, 181 variable sites and 158 reduced information sites. Thus, the ITS2 fragments of *Acer* species displayed considerable variation in the length and GC content.

Genetic variation within and between *Acer* species

The genetic variation of the *Acer* species samples were evaluated by MEGA 6.0 and six parameters (average inter-specific distance, theta prime, the minimum inter-specific distance, average intra-specific distance, theta and coalescent depth) were used to characterize inter- and intra-specific variation. Table 3 exhibited the calculated results of six parameters that the divergence of congeneric was relatively higher than that of conspecific. The average inter-specific genetic distance (0.0766 ± 0.0299) was 15 times of the average intra-specific genetic distance (0.0045 ± 0.0096), and the minimum inter-specific genetic distance (0.0728 ± 0.0299) was significantly higher than the maximum average intra-specific genetic distance (0.0073 ± 0.0129).

Barcoding gap test

The genetic distances of ITS2 sequences were calculated by TaxonDNA 1.0 software, and the barcoding gap of genetic variation distribution within and between *Acer* species was plotted (Fig. 1). There was an obvious barcoding gap in ITS2. These results highlight that ITS2 gene can potentially be applied to identify and differentiate species. Meanwhile, Wilcoxon test was used to further analyze the inter-specific and intra-specific divergence of ITS2 sequences. The analysis showed that the inter-specific divergence of ITS2 sequences was significantly (P<0.001) greater than the intra-specific variation.

Authentication ability of ITS2 region

TaxonDNA 1.0 software was used to evaluate the identification efficiency of ITS2 region, and three criteria (Best Match: BM; Best Close Match: BCM; and All Species Barcodes: ASB) were selected to analyze the authentication ability of ITS2 sequences (Table 4). The results showed that ITS2 region had relatively higher species identification success rates (>64%) and low misidentification rates (<10%) based on the BM, BCM and ASB analysis. For BLASTA1 analysis, similar data were obtained (Table 4). In addition, TaxonDNA 1.0 software was also applied to estimate the discriminatory capability of ITS2 region to sister species. Nearly two-thirds (64.76%) of the ITS2 sequences had considerable inter-specific heterogeneity that were larger than intra-specific variation (Fig. 2), which revealed that the ITS2 sequences had obvious inter-specific boundaries for most species of *Acer*.

Phylogenetic analysis

According to the taxonomic treatment of *Acer* in Xu's system (Xu 1996; Xu *et al.* 2013), all the *Acer* species used in this study belonged to 23 sections (Table 1, 2). By using BI method, a phylogenetic tree was constructed based on the ITS2 sequences, and all the *Acer* species were clustered into

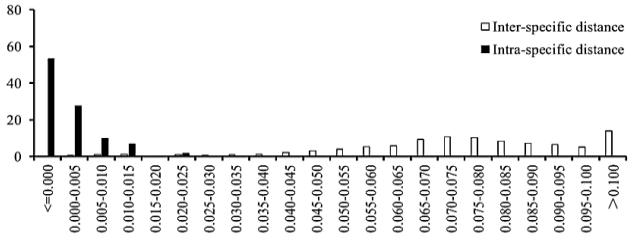

Fig. 1: Relative distribution of inter-specific distance between *Acer* species and intra-specific variation in the ITS2 region using K2P genetic distances

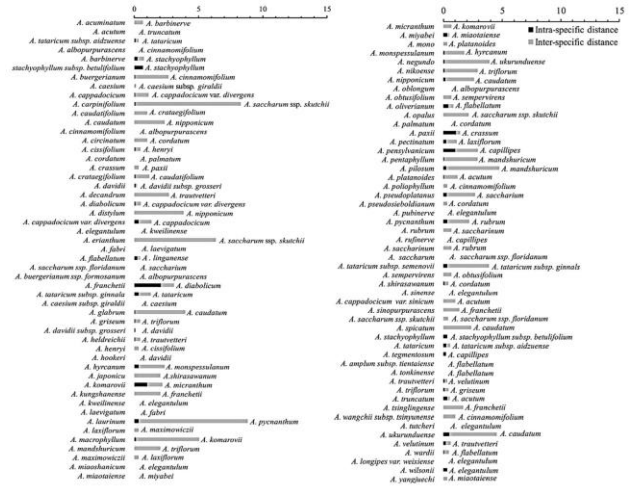

Fig. 2: The heterogeneity and separation for individual taxa of ITS2 based on 105 *Acer* species by TaxonGap

The left side gives the list of *Acer* species used in this study. The right side represents the within species heterogeneity (showed as light gray horizontal bar) and between-species separation (presented as dark gray horizontal bar)

five main groups (Fig. 3). Group I contained 31 *Acer* species from eight sections, and was further classified into three subgroups, among which subgroup I-2 was a monophyletic group formed by sect. *Platanoidea*. Subgroup I-1 comprised 18 species: three from sect. *Lithocarpa*, fourteen from sect. *Macrantha*, and one from sect. *Marcophylla.* Subgroup I-3 included those species from sect. *Parviflora*, sect. *Spicata*, sect. *Distyla* and sect. *Negundo*g. Group II involved 19 species from four sections, was also been further categorized into three subgroups. Subgroup II-1 contained two species (*A. caesium* and *A.caesium* subsp. *giraldii*) belonging to sect. *Acer*. Subgroup II-2 was formed by five species from sect. *Arguta*. All species from sect. *Goniocarp* and sect. *Saccharodendron* and four species (*A. pseudoplatanus, A. heldreichii, A. trautvetteri* and *A. velutinum*) from sect. *Acer* were clusted into subgroup II-3. Group III contained 22 species which clustered into three subgroups. Subgroup III-1 included all species from sect. *Pubescentia*, sect. *Oblong*, sect. *Trifoliata* and sect. *Pentaphylla*. Subgroup III-2 was composed of two species (*A. decandrum* and *A. laurinum*)

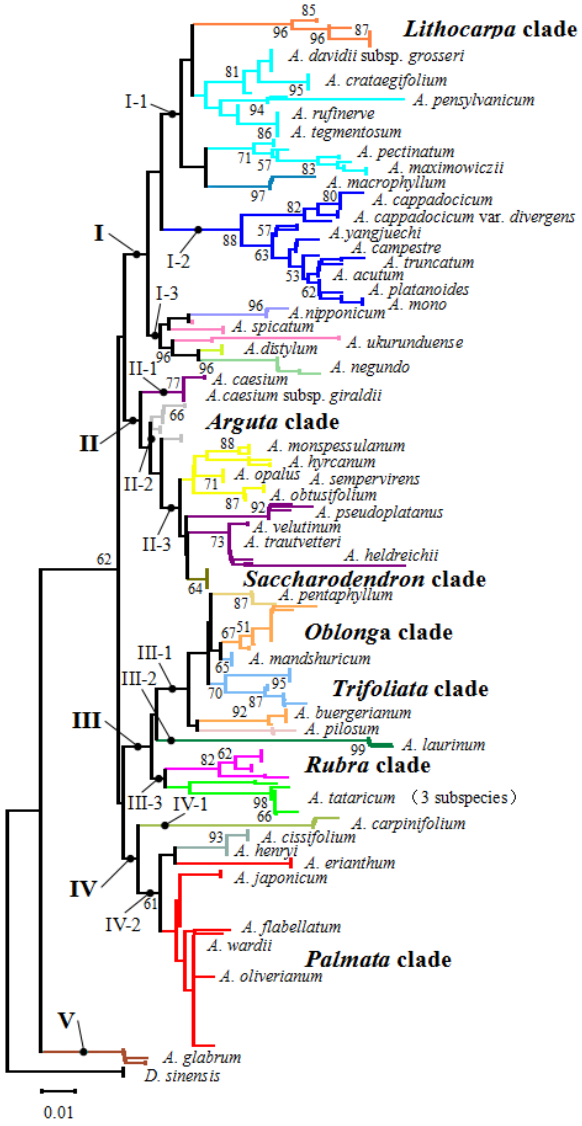

Fig. 3: Bayesian phylogenetic tree based on ITS2 sequences for *Acer* species

Posterior probabilities (PP) ≥50 are shown above/down the branch

from sect. *Hyptiocarpat*. Subgroup III-3 contained the species from sect. *Rubra* and sect. *Ginnala*. A total of 22 species were assigned group IV, which was further subdivided into two subgroups. Subgroup IV-1 included one species (*A. carpinifolium*) from sect. *Indivisa*. Subgroup IV-2 contained 21 species, in addition to the species from sect. *Palmata*, all species (*A. henryi* and *A. cissifolium)* from Sect. *Cissifolia* and the species *A. wardii* from sect. *Macrantha* were clustered in this subgroup. *A. Glabrum*, a species from Sect. *Glabra* was distant from other *Acer* species, and which constituted a separate group V.

In order to further clarify the phylogenetic relationship of *Acer*, a NN splits graph was constructed. Resultant NN splits graph exhibited a similar phylogenetic relationship among *Acer* to *bayesian analysis* (Fig. 4).

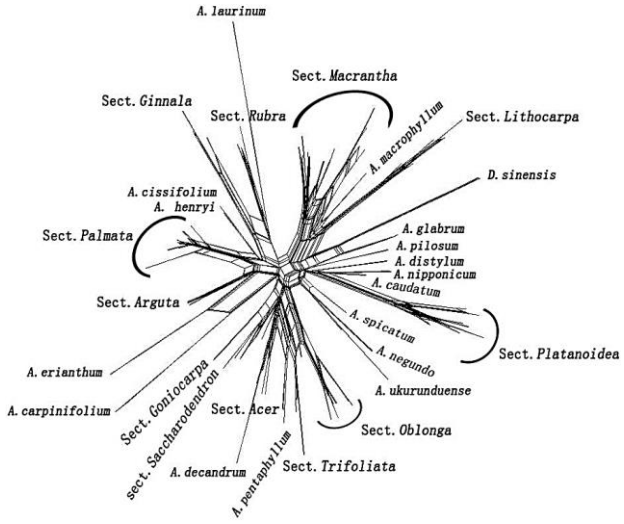

Fig. 4: Neighbor-net splits network for *Acer* species computed with uncorrected p-distances based on ITS2 sequences

Discussion

In previous studies, ITS2 has been proven to have good species-identification capability and therefore been suggested as a standard barcode to identify plant species (Chen *et al.* 2010; Yao *et al.* 2010; Feng *et al.* 2016; Sun *et al.* 2016). In our study, the ITS2 locus exhibited sufficient genetic variability among congeneric *Acer* species and also displayed a relatively high discrimination efficiency (>64% for BM, BCM, ASB and BLASTA1 analysis). For most *Acer* species, they could be successfully identified based on their ITS2 locus. However, the ITS2 locus was less effective to discriminate morphologically similar species of Sect. *Palmata*. For instance, four species (*A. elegantulum*, *A. sinense*, *A. pubinerve and A. kweilinense*) in sect. *Palmata* could not be distinguished due to their identical ITS2 sequences. Thus, some other DNA barcodes should be explored and applied to identify these closely relative sect. *Palmata* species. Here, it should be pointed out that the taxonomy of sect. *Palmata* has always been controversial and many species in this section such as *A. olivaceum*, *A. changhuaense*, *A. schneiderianum* and *A. anhweiense* have been redefined in the Flora of China (Fang 1981; De Jong 1994; Xu *et al.* 2013). Our result implied that there might be more species of this section to be revised.

Many studies have confirmed that ITS2 could not only barcode plant species, but also provided a superior phylogenetic marker for plant systematics and evolutionary research (Chen *et al.* 2010; Liu *et al.* 2012; Zhao *et al.* 2015; Feng *et al.* 2016). In this study, the ITS2 region exhibited sound applicability in the identification of *Acer*, and it also provided a taxonomic signature for *Acer* taxonomy. As shown in the dendrogram generated from ITS2 data, the genus *Acer* was revealed to be monophyletic (Fig. 3). However, monophyletic groups could not be formed in some sections of *Acer*, such as *Macrantha*, *Spicata*, *Acer* and *Oblonga* (Fig. 3). As for sect. *Macrantha*, 14 species were sampled representing three different series (*Micrantha*, *Tegmentosa* and *Crataegifolia*) of this section, and were categorized into two clades. The species from ser. *Tegmentosa* and ser. *Crataegifolia* formed two monophyletic clades, and the species from ser. *Micrantha* were grouped together with the species from sect. *Marcophylla*, it made *Macrantha* a possible paraphyletic section. It should be noted that *A. pectinatum* in ser. *Tegmentosa* was nested within ser. *Micrantha* species (Xu 1996) with relatively high support (PP=83), indicating *A. pectinatum* should be reassigned from ser. *Tegmentosa* to ser. *Micranthum*. In addition, as reported earlier (Grimm *et al*. 2006), *A. wardii* from sect. *Macrantha* was included within group IV (IV-2) together with the species from sect. *Palmata*. Morphologically, *A. wardii* was similar to sect. *Macrantha*, but some characters, such as conspicuous bracts, reflexed sepals and amphistaminal disk, were atypical features of *Macrantha*. Thus, de Jong's treatment of placing this species in a monotypic section was supported here (De Jong 1994). The species from sect. *Spicata* were grouped into subgroup I-3 together with species from sect. *Distyla* and *Parviflora*, indicating a close relationship among the sections, although the internal support was relatively weak (PP=62). This was consistent with Momotani's and de Jong's treatments by placing sect. *Distyla* and sect. *Parviflora* under sect. *Parviflora* as another two series. Actually, these three sections shared similar morphological features of cotyledon, samara, endocarp and pollen (Ogata 1967). *A. negundo* was strongly supported (PP=96) as a sister species to *A. distylum* of sect. *Distyla*. In gross morphology, these two species are obviously different, such as the type of inflorescences, the number of bud-scales and the arrangement of leaves. In Xu's system (2013), *A. negundo* was combined with sect. *Cissifolia* for their compound leaves. Our result indicated that *A. negundo* should be treated as a separate section rather than a species under sect. *Cissifolia*. It was also supported by palynological evidence of *Acer* (Tian *et al*. 2001).

The species from sect. *Acer* (except *A. caesium* and *A.caesium* subsp. *giraldii*) were grouped into subgroup II-3 together with the species from sect. *Goniocarpa* and sect. *Saccharodendron* (Fig. 3), indicating the phylogenetic relationships among the sections were close. In fact, due to the gross morphological similarities, these sections were reduced to the rank of series and put under sect. *Acer* by De Jong (1994). It was also backed by Ogata's study on the wood rays of *Acer* (Ogata 1967). Therefore, we supported de Jong's treatment of merging the three sections into sect. *Acer* (De Jong 1994). However, our finding didn't support de Jong's division of this section into three series (ser. *Acer*, ser. *Monspessulana* and ser. *Saccharodendron*). *A. caesium* and its subspecies failed to be included into subgroup II-3 and formed subgroup II-1. It is possibly caused by the long-

time geographical isolation, as the two species are endemic to Southwest China, while other sect. *Acer* species are distributed in Northern America and Southern Europe—Western Asia. Thus, our results tend to place *A. caesium* and its subspecies into a separate series. In addition, as reported earlier (Ogata 1967; Tian *et al.* 2001), *A. pseudoplatanus* was distant from other de Jong's ser. *Acer* species and formed an independent clade in this study. Morphologically, *A. pseudoplatanu* was obviously different from other ser. *Acer* species, *e.g*. (i) inflorescence long paniculate, (ii) filament hairy and (iii) pollen exine sculpture arranged very irregularly (Ogata 1967; Tian *et al.* 2001). Therefore, it is suggested that *A. pseudoplatanu* should be treated as a monotypic series.

The close relationships among sect. *Oblonga*, sect. *Pentaphyllum* and sect. *Trifoliata* were supported in our study, which were also backed by De Jong (1994) and Tian *et al*. (2002). However, the systematic relationships among the three sections were still controversial. In De Jong (1994) system, sect. *Oblonga* was treated as a series (*i.e*. ser. *Trifida*) under sect. *Pentaphyllum*. It was different from other treatments (Ogata 1967; Fang 1981; Xu *et al.* 2013), but was supported here, as sect. *Pentaphyllum* species formed a sister-clade to sect. *Oblonga* species (except *A. buergerianum*). *A. buergerianum* was strangely placed as a sister species to *A. pilosum* in sect. *Pubescentia* (Fig. 3). Though the two species shared many similar morphological traits, some important taxonomic features were obviously different; for instance, the number of stamens and the type of leaf margin. Additionally, *A. mandshuricum* from sect. *Trifoliata* expressed a closer relationship with sect. *Oblonga*, although there were obvious differences in leaf shapes. Thus, it is necessary to use more methods to address the relationships among the three sections.

Conclusion

The ITS2 sequence carried comparatively high identification efficiency at the section level of *Acer*, and it also proposed reliable identification efficiency for most of the species in genus *Acer*. In addition, the phylogenetic tree constructed based on ITS2 sequences revealed the phylogenetic relationship of *Acer* and highlighted that the ITS2 sequences are potentially applicable in the identification and phylogenetic investigation of *Acer* species.

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Author Contributions

Li Lin performed the experiments, analyzed the data and wrote the manuscript; Zhiyong Zhu, Lejing Lin and Yuan Zhou provided essential reagents and materials; Tao Fu and Feng Liu provide technical assistance in molecular experiments and data analysis; Wen Li and Yulong Ding gave suggestions to revise the manuscript.

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