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Evaluation of the taxonomic status of water dropwort (*Oenanthe*, Apiaceae) accessions from East Asia based on nuclear rDNA internal transcribed spacer sequences

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ABSTRACT. *Oenanthe* L. is a taxonomically complex genus, several species of which have long been used as vegetables and traditional medicines in East Asia. In order to clarify the taxonomic status of *Oenanthe* accessions and provide baseline data for the sustainable use of its genetic resources, we examined sequence variations in the internal transcribed spacer (ITS) region of *Oenanthe* accessions collected from a wide geographical area in China and its neighboring countries. For comparison, ITS sequences in GenBank for almost all currently reported species of *Oenanthe* were also included in our analyses. Both phylogenetic tree construction methods (Bayesian inference and maximum likelihood) revealed that the accessions tended to cluster

into two groups, which were closely related to *O. mildbraedii* and *O. sarmentosa*. However, these two species have never been recorded in China or its neighboring countries. Therefore, it seems probable that in our sampled locations, *Oenanthe* accessions have been given an incorrect name, such as *O. javanica*. Future studies should carefully check the morphological characteristics of other *Oenanthe* species and sequence their ITS regions in order to clarify the taxonomic status of the genus.

Key words: East Asia; nrDNA ITS; *Oenanthe*; Taxonomy; Water dropwort

INTRODUCTION

The genus *Oenanthe* L. ("water dropwort", Apiaceae) includes about 30 species that are widely distributed in Eurasia, North America, and Africa (Fu and Watson, 2005). *Oenanthe* is a taxonomically complex genus due to its diverse morphology and widespread dispersal (Fu and Watson, 2005; Zhao, 2010; Spalik et al., 2014). In the last decade, great progress has been made in elucidating high-level phylogenetic relationships within the family Apiaceae, including *Oenanthe*, such as the tribe Oenantheae (Downie et al., 2008; Spalik et al., 2009, 2014) and the subfamily Apioideae (Downie and Katz-Downie, 1999; Zhou et al., 2008, 2009) by polymerase chain reaction (PCR) and direct DNA sequencing. The phylogenetic position of *Oenanthe* in Apiaceae has been studied extensively in these studies. However, they only used a few representative species of *Oenanthe* from a limited geographical region. Only a few studies have investigated the phylogenetic relationships between species (Spalik et al., 2014) and genetic variation within species (Huh et al., 2002; Zhao, 2010) in *Oenanthe*; therefore, the taxonomic status of *Oenanthe* species remains unclear.

Oenanthe species have long been used in East Asia as vegetables and as traditional medicines (Fu and Watson, 2005; Zhao, 2010; Su et al., 2011). The fresh leaves and petioles of *Oenanthe* plants contain high levels of proteins, amino acids, calcium, Vitamin C, iron, and flavonoids (Liu et al., 2007; Zhao, 2010). One of the most widely utilized species is *O. javanica*, which has been cultivated and used in China, Japan, Korea, and Southeast Asia for thousands of years (Huh et al., 2002; Zhao, 2010). Assessing the taxonomic status of germplasm resources is essential for the sustainable use of crops and medicinal materials. However, due to the morphological variability of these germplasms, the evaluation of their taxonomic status is still limited in many geographical regions.

In the Chinese version of the "Flora of China", nine species and one variety of *Oenanthe* are recorded (China Flora Editorial Board of CAS, 1985). In a later revision of this genus in an English version of the "Flora of China", Fu and Watson (2005) treated these taxa as only five species (*O. javanica*, *O. benghalensis*, *O. hookeri*, *O. linearis*, and *O. thomsonii*) and six subspecies, based on a re-examination of their morphological characteristics. Although several species, such as *O. benghalensis*, *O. hookeri*, and *O. linearis*, might have been cultivated and used as vegetables and medicines in different regions of China, in our field investigations we found that these germplasm resources were still regarded as *O. javanica* by local people and even the researchers. To date, only a few phylogenetic studies have included *Oenanthe* accessions from China (Zhou et al., 2008; Zhao, 2010). For example, Zhou et al.

(2008) sampled only six accessions, representing four species of *Oenanthe*, in their molecular phylogenetic study of the Chinese Apiaceae subfamily Apioideae; Zhao (2010) studied the genetic diversity and phylogenetic relationships of 20 cultivated accessions of the species "*O. stolonifera*", which is a synonym of *O. javanica*, using random amplified polymorphic DNA (RAPD) molecular markers. As suggested by Fu and Watson (2005), studies over a wide geographical area are still needed to resolve the classification.

The nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) region has been suggested to be the core barcode for the species discrimination of seed plants (Li et al., 2011). The ITS region has also been successfully used for low-level phylogenetic analyses in the Apiaceae (Downie et al., 2001, 2008; Kadereit and Kadereit, 2005; Zhou et al., 2008, 2009; Spalik et al., 2009). The numerous ITS sequences available in GenBank for almost all currently reported species of *Oenanthe* provide a reference for species discrimination. In the present study, we used the ITS region as a molecular marker to examine the sequence variation of *Oenanthe* accessions collected from a wide geographical area in China and its neighboring countries, i.e., North Korea, Japan, and Vietnam. Our main objectives were to evaluate the taxonomic status of these accessions and provide baseline data for the sustainable use of these plant genetic resources.

MATERIAL AND METHODS

Sampling

In total, 170 accessions of *Oenanthe* from East Asia were sequenced and examined for ITS sequence variation, including 165 accessions from 15 provinces of China, two accessions from North Korea, one from Japan, and two from Vietnam (Table 1). Each accession was collected from a different location in each country (Figure 1). Leaf material was collected from each accession, and leaf samples were stored in silica gel until DNA extraction. Vouchers for all of the sampled accessions were deposited in the Herbarium of Wuhan Vegetable Scientific Research Institute, Wuhan, China. To infer phylogenetic relationships between the accessions sampled in this study and other species within the *Oenanthe*, we downloaded the ITS sequences of 43 *Oenanthe* accessions of 29 species from GenBank, which represented nearly all currently recorded species of this genus (Table 2). In addition, two species (*Cicuta virosa*, GenBank accession No. AY524767 and *C. maculate*, AY524733) from the genus *Cicuta* were selected as outgroups in phylogenetic tree reconstructions, according to previous phylogenetic studies (Downie et al., 2008; Zhou et al., 2008; Spalik et al., 2009, 2014).

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from dry leaf material using a Plant Genomic DNA Kit (Tiangen, Beijing, China) following the manufacturer instructions. DNA concentrations were estimated and standardized on 2.0% (w/v) agarose gels.

The ITS regions, including ITS-1, 5.8S rDNA, and ITS-2, were amplified by PCR using the primers "ITS4" and "ITS5" (for the primer sequences, see White et al., 1990). PCR amplifications in 50-μL reactions were conducted with the following reagents: 0.25 mM of each dNTP, 5 μL 10X *Taq* buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 50 mM KCl), 1 mM of each primer, 2 U *Taq* Polymerase (Sangon Biotech, Shanghai, China); a total of 60-80 ng genomic DNA was added to each reaction. The amplifications were conducted in an

Table 1. Locations and haplotypes of 170 *Oenanthe* accessions (V11E-01-V11E-170).

Region	Accession, location, and haplotype	GenBank accession No.
China		Hap1: KT362845
Anhui	V11E-01 (XC, Hap1), V11E-03 (HN, Hap16), V11E-06 (TC1, Hap1), V11E-10 (QS, Hap17), V11E-12 (SX, Hap19), V11E-13 (QY, Hap16), V11E-17 (LJ1, Hap1), V11E-18 (CZ, Hap17), V11E-21 (CH, Hap1), V11E-36 (AQ, Hap24), V11E-38 (NL, Hap1), V11E-42 (YL, Hap26), V11E-44 (LJ2, Hap1), V11E-60 (SC1, Hap1), V11E-67 (HF1, Hap1), V11E-69 (SC2, Hap1), V11E-70 (SC3, Hap1), V11E-72 (HF2, Hap1), V11E-73 (LJ3, Hap31), V11E-74 (LJ4, Hap1), V11E-76 (WH1, Hap1), V11E-100 (TC2, Hap1), V11E-101 (TC3, Hap1), V11E-108 (WH2, Hap1), V11E-121 (WH3, Hap9), V11E-133 (LJ5, Hap39), V11E-138 (LJ6, Hap1), V11E-145 (TC4, Hap43)	Hap2: KT362846 Hap3: KT362847 Hap4: KT362848 Hap5 KT362849 Hap6: KT362850
Fujian	V11E-09 (WYS1, Hap1), V11E-89 (WYS2, Hap1)	Hap7: KT362851
Guangxi	V11E-65 (LZ, Hap1), V11E-87 (GL, Hap2)	Hap8: KT362852
Guizhou	V11E-02 (BJ1, Hap12), V11E-11 (AS1, Hap18), V11E-14 (GY1, Hap1), V11E-15 (QX, Hap1), V11E-16 (ZY1, Hap12), V11E-19 (BJ2, Hap12), V11E-20 (MT, Hap1), V11E-88 (AS2, Hap1), V11E-92 (ZY2, Hap24), V11E-103 (ZY3, Hap1), V11E-124 (AS3, Hap1), V11E-146 (GY2, Hap1), V11E-158 (AS4, Hap44)	Hap9: KT362853 Hap10: KT362854 Hap11: KT362855
Hainan	V11E-71 (QH, Hap30)	Hap12: KT362856
Henan	V11E-78 (XY1, Hap33), V11E-125 (NY, Hap1), V11E- 126 (XY2, Hap10), V11E-141 (WY, Hap12), V11E-142 (PDS, Hap12), V11E-163 (HY, Hap48), V11E-52 (SQ, Hap1)	Hap13: KT362857 Hap14: KT362858
Hubei	V11E-04 (QC, Hap17), V11E-24 (GA, Hap21), V11E-37 (LF, Hap1), V11E-40 (LC, Hap24), V11E-43 (ES1, Hap1), V11E-47 (WUH, Hap1), V11E-48 (JL, Hap1), V11E-50 (LC1, Hap1), V11E-53 (LC2, Hap27), V11E-55 (DH, Hap28), V11E-6 (ES2, Hap1), V11E-77 (YM, Hap32), V11E-85 (LC3, Hap1), V11E-91 (SS, Hap1), V11E-119 (ES3, Hap24), V11E-136 (FBS, Hap1), V11E-140 (HS1, Hap1), V11E-143 (ES4, Hap41), V11E-144 (TM, Hap42), V11E-148 (ES5, Hap1), V11E-149 (SIS, Hap1), V11E-162 (MLX, Hap47), V11E-164 (TSC, Hap49), V11E-165 (JSX, Hap1), V11E-166 (WQ1, Hap24), V11E-167 (WQ2, Hap1), V11E-168 (XF, Hap50),V11E-75 (HS2, Hap16), 79-V11E0128 (BK, Hap34)	Hap15: KT362859 Hap16: KT362860 Hap17: KT362861 Hap18: KT362862 Hap19: KT362863 Hap20: KT362864
Hunan	V11E-25 (RJ1, Hap1), V11E-31 (WL, Hap53), V11E-117 (CD1, Hap7), V11E-134 (YY, Hap40), V11E-151 (CD2, Hap1), V11E-152 (RJ2, Hap1), V11E-153 (SY1, Hap1), V11E-155 (CD3, Hap15), V11E-26 (RJ3, Hap1), V11E-27 (SY2, Hap1), V11E-32 (RJ4, Hap24), V11E-97 (NY, Hap36), V11E-111 (CD4, Hap1), V11E-112 (CD5, Hap1)	Hap21: KT362865 Hap22: KT362866 Hap23: KT362867
Jiangsu	V11E-05 (CZ, Hap1), V11E-30 (LH1, Hap23), V11E-33 (HA1, Hap1), V11E-34 (HZE1, Hap25), V11E-35 (HA2, Hap1), V11E-41 (LY, Hap1), V11E-45 (WX1, Hap1), V11E-49 (NT, Hap1), V11E-56 (YZ, Hap52), V11E-58 (WX2, Hap1), V11E-59 (YQ1, Hap1), V11E-80 (LYG, Hap24), V11E-81 (DY, Hap35), V11E-84 (SZ1, Hap1), V11E-86 (SZ2, Hap1), V11E-102 (YQ2, Hap37), V11E-113 (YX, Hap53), V11E-118 (NJ, H8), V11E-123 (CZ2, Hap1), V11E-137 (SZ3, Hap1), V11E-150 (HZE2, Hap1), V11E-154 (LH2, Hap1), V11E-156 (HA3, Hap1), V11E-159 (SUY, Hap1)	Hap24: KT362868 Hap25: KT362869 Hap26: KT362870 Hap27: KT362871 Hap28: KT362872
Jiangxi	V11E-46 (JJ, Hap1), V11E-62 (LP1, Hap1), V11E-66 (JDZ, Hap1), V11E-83 (LP2, Hap1), V11E-122 (YG, Hap1), V11E-139 (SR, Hap1)	Hap29: KT362873
Liaoning	V11E-07 (PLD, Hap1)	Hap30: KT362874
Shandong	V11E-96 (QF, Hap5)	Hap31: KT362875
Shaanxi	V11E-90 (HZ, Hap12), V11E-114 (XA, Hap1), V11E-157 (CA, Hap1)	Hap32: KT362876
Sichuan	V11E-57 (QL, Hap12), V11E-61 (CQ1, Hap29), V11E-63 (CQ2, Hap1), V11E-64 (CQ3, Hap1), V11E-82 (MS, Hap12), V11E-105 (WX, Hap1), V11E-106 (ZX, Hap1), V11E-107 (NQ, Hap1), V11E-109 (DX, Hap2), V11E-110 (FD, Hap1)	Hap33: KT362877 Hap34: KT362878
Yunnan	V11E-08 (HQ1, Hap12), V11E-22 (HQ2, Hap20), V11E-23 (HQ3, Hap1), V11E-28 (YX1, Hap1), V11E-29 (CG1, Hap22), V11E-51 (CG2, Hap1), V11E-93 (ML, Hap1), V11E-94 (YJ1, Hap3), V11E-95 (SM, Hap4), V11E-98 (JS, Hap51), V11E-115 (KM, Hap6), V11E-116 (AL, Hap24), V11E-120 (CG3, Hap53), V11E-127 (YX2, Hap1), V11E-128 (MJ1, Hap1), V11E-1294 (PER1, Hap1), V11E-130 (PER2, Hap11), V11E-131 (JH, Hap12), V11E-132 (MH, Hap38), V11E-135 (MJ2, Hap13), V11E-147 (YJ2, Hap14), V11E-160 (HLT1, Hap45), V11E-161 (HLT2, Hap46)	Hap35: KT362879 Hap36: KT362880 Hap37: KT362881 Hap38: KT362882 Hap39: KT362883
Chongqing	V11E-104 (CQ4, Hap12)	Hap40: KT362884
		Hap41: KT362885
North Korea		Hap42: KT362886
Pyongyang	V11E-169 (PR1, Hap1), V11E-170 (PR2, Hap1)	Hap43: KT362887
		Hap44: KT362888
Japan		Hap45: KT362889
Oita-ken	V11E-39 (DF, Hap1)	Hap46: KT362890
		Hap47: KT362891
Vietnam		Hap48: KT362892
Hanoi	V11E-54 (HN1, Hap1), V11E-99 (HN2, Hap1)	Hap49: KT362893
		Hap50: KT362894
		Hap51: KT362895
		Hap52: KT362896
		Hap53: KT362897

XC, Xuancheng; HN, Huaining; TC, Tongcheng; QS, Qianshan; SX, Sheqian; QY, Qingyang; LJ, Lujiang; CZ, Chizhou; CH, Caohu; AQ, Anqing; NL, Nanling; YL, Yangliu; SC1, Shucheng; HF, Hefei; WH, Wuhu; PR, Pyongyang; DF, Oita-ken; WYS, Wuyishan; LZ, Liuzhou; GL, Guiling; BJ, Bijie; AS, Anshun; GY, Guiyang; QX, Qianxi; ZY, Zunyi; MT, Meitan; QH, Qionghai; XY, Xinyang; NY, Nanyang; WY, Wuyang; PDS, Pingdingshan; HY, Huaiyuan; SQ, Shenqiu; HN, Hanoi; QC, Qichun; GA, Gongan; LF, Laifeng; LC, Lichuan; ES, Enshi; WUH, Wuhan; JL, Jiangling; DH, Donghu; YM, Yunmeng; SS, Shashi; FBS, Fubaoshan; HS, Huangshi; TM, Tianmen; SIS, Shishou; MLX, Maliuxi; TSC, Tusicheng; JSX, Jianshanxiang; WQ, Wenquan; XF, Xianfeng; BK, Baokang; RJ, Ruanjiang; WL, Wuling; CD, Changde; YY, Yiyang; SY, Shaoyang; NY, Ningyuan; CZ, Changzhou; LH, Liuhe; HA, Huaian; HZE, Hongze; LY, Liyang; WX, Wuxi; NT, Nantong; YZ, Yangzhou; YQ, Yuqi; LYG, Lianyungang; DY, Danyang; SZ, Suzhou; YX, Yixing; NJ, Nanjing; SUY, Suyu; JJ, Jiujiang; LP, Leping; JDZ, Jingdezhen; YG, Yugan; SR, Shangrao; PLD, Pulandian; QF, Qufu; HZ, Hanzhong; XA, Xian; CA, Changan; QL, Qionglai; CQ, Chongqing; MS, Minshan; WX, Wanxian; ZX, Zhongxian; NQ, Nanquan; DX, Daxian; FD, Fengdu; HQ, Heqing; YX, Yuxi; CG, Chenggong; ML, Mengla; YJ, Yuanjiang; SM, Simao; JS, Jianshui; KM, Kunming; AL, Anlong; MJ1, Mojiang; PER, Puer; JH, Jinghong; MH, Menghai; HLT, Heilongtan. Abbreviations for each location and haplotype name (Hap1-Hap53) are shown in parentheses following the accession number.

Eppendorf AG 22331 Hamburg Thermocycler (Eppendorf, Germany), and the PCR conditions were as according to Wang and Li (1998).

Following amplification, the size of each PCR product was determined electrophoretically on 2.0% (w/v) agarose gel run at 100 V in 0.5X TBE (Tris-boric acid-EDTA), and visualized by staining with ethidium bromide. All of the PCR products were purified using a TIANquick Midi Purification Kit following the protocols provided by the manufacturer (Tiangen). Sequences were generated on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) by Sangon Biotech using the same primers as for the amplifications.

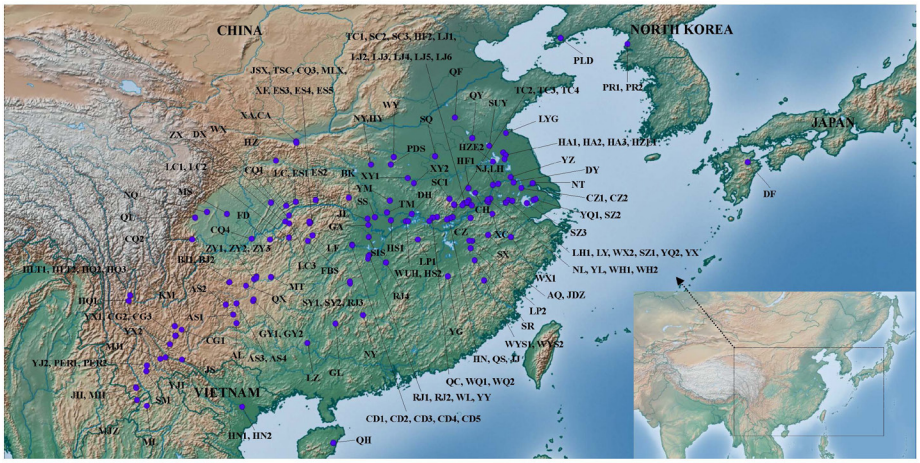

Figure 1. Sampling locations of 170 *Oenanthe* accessions in East Asia (Location codes as in Table 1).

Table 2. GenBank accession numbers of *Oenanthe* internal transcribed spacer sequences from previous studies.

Species	GenBank accession No.	Reference
O. javanica	AY691944	Kadereit and Kadereit, 2005
O. sinensis	AY691943	Kadereit and Kadereit, 2005
O. sarmentosa	AY691942	Kadereit and Kadereit, 2005
O. prolifera	AY691941	Kadereit and Kadereit, 2005
O. peucedanifolia	AY691940	Kadereit and Kadereit, 2005
O. lachenalii	AY691939	Kadereit and Kadereit, 2005
O. fistulosa	AY691938	Kadereit and Kadereit, 2005
O. silaifolia	AY691937	Kadereit and Kadereit, 2005
O. pimpinelloides	AY691935	Kadereit and Kadereit, 2005
O. montis-khortiati	AY691934	Kadereit and Kadereit, 2005
O. divaricata	AY691932	Kadereit and Kadereit, 2005
O. fluviatilis	AY691930	Kadereit and Kadereit, 2005
O. aquatica isolate 2	AY691925	Kadereit and Kadereit, 2005
O. aquatica isolate 1	AY691924	Kadereit and Kadereit, 2005
O. conioides isolate 3	AY691922	Kadereit and Kadereit, 2005
O. linearis subsp *rivularis*	JX962352	Spalik et al., 2014
O. procumbens	JX962351	Spalik et al., 2014
O. lisae	JX962350	Spalik et al., 2014
O. linearis subsp *linearis*	JX962349	Spalik et al., 2014
O. javanica subsp *javanica*	JX962348	Spalik et al., 2014
O. hookeri	JX962347	Spalik et al., 2014
O. prolifera	GQ379319	Spalik et al., 2009
O. thomsonii	EU236186	Zhou et al., 2008
O. linearis subsp *rivularis*	EU236185	Zhou et al., 2008
O. benghalensis	EU236181	Zhou et al., 2008
O. virgata	EU233944	Zhou et al., 2008
O. silaifolia	EU233943	Zhou et al., 2008
O. sarmentosa	EU233942	Zhou et al., 2008
O. palustris	EU233941	Zhou et al., 2008
O. millefolia	EU233940	Zhou et al., 2008
O. mildbraedii	EU233939	Zhou et al., 2008
O. lachenalii	EU233938	Zhou et al., 2008
O. javanica subsp *stolonifera*	EU233937	Zhou et al., 2008
O. foucaudii	EU233936	Zhou et al., 2008
O. divaricata	EU233935	Zhou et al., 2008
O. aquatica	EF177732	Downie et al., 2008
O. sarmentosa	AY360252	Hardway et al., 2004
O. pimpinelloides	AY360251	Hardway et al., 2004
O. peucedanifolia	AY360250	Hardway et al., 2004
O. fistulosa	AY360249	Hardway et al., 2004
O. divaricata	AY360248	Hardway et al., 2004
O. crocata	AY360246	Hardway et al., 2004
O. banatica	AY360245	Hardway et al., 2004

Sequence analysis and phylogenetic reconstruction

Sequences were aligned using CLUSTAL W (Thompson et al., 1994). The aligned sequences were manually inspected prior to analysis, and gaps were inserted to insure positional homology. Insertions/deletions (indels) were treated as point mutations and equally weighted with other mutations. ITS haplotypes were determined from nucleotide substitutions and indels of the aligned sequences.

Bayesian inference (BI) and maximum likelihood (ML) analyses were conducted for the ITS sequence dataset, including sequences of the ITS haplotypes generated from this study and sequences from GenBank. An analysis was conducted for each of the following edited ITS sequence datasets: 1) ITS dataset without indels; 2) ITS dataset with indels, and each indel was treated as one point mutation. The *Oenanthe* cladogram was rooted using *C. viros*a and *C*. *maculate* var. *maculate*.

The BI analysis was conducted using the BEAST v. 1.7.4 program (Drummond and Rambaut, 2007) on each of the two datasets. The best-fit models (GTR + I + G) were selected according to the Akaike information criterion (Akaike, 1974) using MrModeltest 2.3 (Nylander, 2004) in conjunction with PAUP 4.0 (Swofford, 1998) to generate model scores. A starting tree was randomly generated and a Yule process was performed. Two separate Markov chain Monte Carlo analyses were run for 10,000,000 generations, with sampling at every 1000 generations to ensure that all of the effective sample size values were greater than 200. Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) was used to check the parameters, and the first 10% of generations was discarded as burn-in. Bayesian trees were annotated in TreeAnnotator 1.6.0 (http://beast.bio.ed.ac.uk/TreeAnnotator) and edited in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

ML phylogenetic analyses were performed on each of the two edited datasets using the PhyML v.3.0 software (Guindon et al., 2010) using the "GTR + I + G" nucleotide substitution model. Clade stability was estimated by non-parametric bootstrapping with 1000 replicates in PhyML.

RESULTS

Sequence variation

Among the 170 ITS sequences generated in this study, the complete ITS region varied in length from 619 to 622 bp. The total length of the alignment was 624 bp. A total of 101 polymorphic sites (97 substitutions and four indels) of the ITS region resulted in the resolution of 53 haplotypes (Hap1-Hap53) across the 170 accessions (from 170 locations) (Table 1). GenBank accession numbers (Nos. KT362845 to KT362897) for each haplotype are listed in Table 1. Among the 53 ITS haplotypes revealed in this study, three haplotypes (Hap1, Hap12, and Hap24) were widely distributed in the sampled region. Among the 170 *Oenanthe* accessions, 94 were assigned to Hap1, 11 to Hap12, and 8 to Hap24 (Table 1). In addition, 46 of the 53 haplotypes were unique.

The aligned ITS sequence matrix of the 53 haplotypes and the 43 accessions of 29 species was 559 bp in length after removing the indels. When treating each indel as one point mutation, the total length of the ITS sequence matrix was 585 bp. These two data matrices were used in the subsequent phylogenetic analyses.

Phylogenetic analyses

Based on the ITS sequence matrix that contained 98 aligned sequences without indels (53 from the haplotypes revealed in this study, 43 from GenBank representing 29 species, and two from the outgroup), the BI analysis revealed two clades (Clade I and Clade II) of the 53 haplotypes (Figure 2): Clade I contained 16 haplotypes (Bayesian posterior probability [PP] < 0.5), which was closely related to *O*. *mildbraedii*; Clade II contained 37 haplotypes (PP < 0.5), with three accessions of *O*. *sarmentosa* embedded within this clade. For the phylogeny of the whole *Oenanthe* genus, *O*. *procumbens* and *O*. *palustris* were strongly supported (PP = 1) as sisters to the remainder of the ingroup taxa. Accessions from previous studies formed at least five well-supported clades (PP = 0.97-1.00) (Figure 2). Based on the same dataset, the ML analysis revealed a generally similar phylogenetic topology (data not shown) as the Bayesian tree, but with relatively weak bootstrap values supporting the monophyly of several clades. Two groups of *Oenanthe* haplotypes revealed by the ML analysis were the same as those in the BI analysis. The two groups were also shown to be closely related to *O*. *mildbraedii* and *O*. *sarmentosa*.

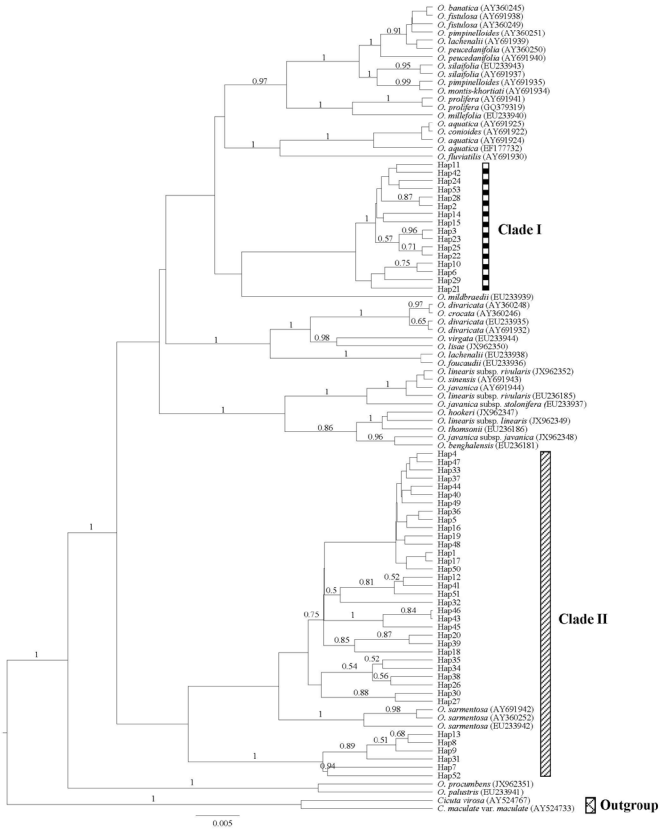

Figure 2. Majority-rule consensus tree obtained from Bayesian analyses of internal transcribed spacer (ITS) sequence data for 53 haplotypes (Hap1-Hap53) generated in the present study and 43 *Oenanthe* accessions of 29 species from GenBank. Posterior probabilities are indicated above branches. The indels in the ITS sequence dataset were removed.

Based on the ITS sequence matrix that contained 98 aligned sequences with indels, the BI analysis also revealed two clades (Clade A and Clade B) of the 53 haplotypes (Figure 3). Clade A (PP = 0.78) and Clade B (PP = 0.97) were shown to be the sister group, with three accessions of *O*. *sarmentosa* basal to these two clades. Clade A contained a different haplotype number to Clade I, and Clade B contained a different number to Clade II. Based on the same dataset with indels, the ML analysis revealed an identical phylogenetic topology (data not shown) as the Bayesian tree.

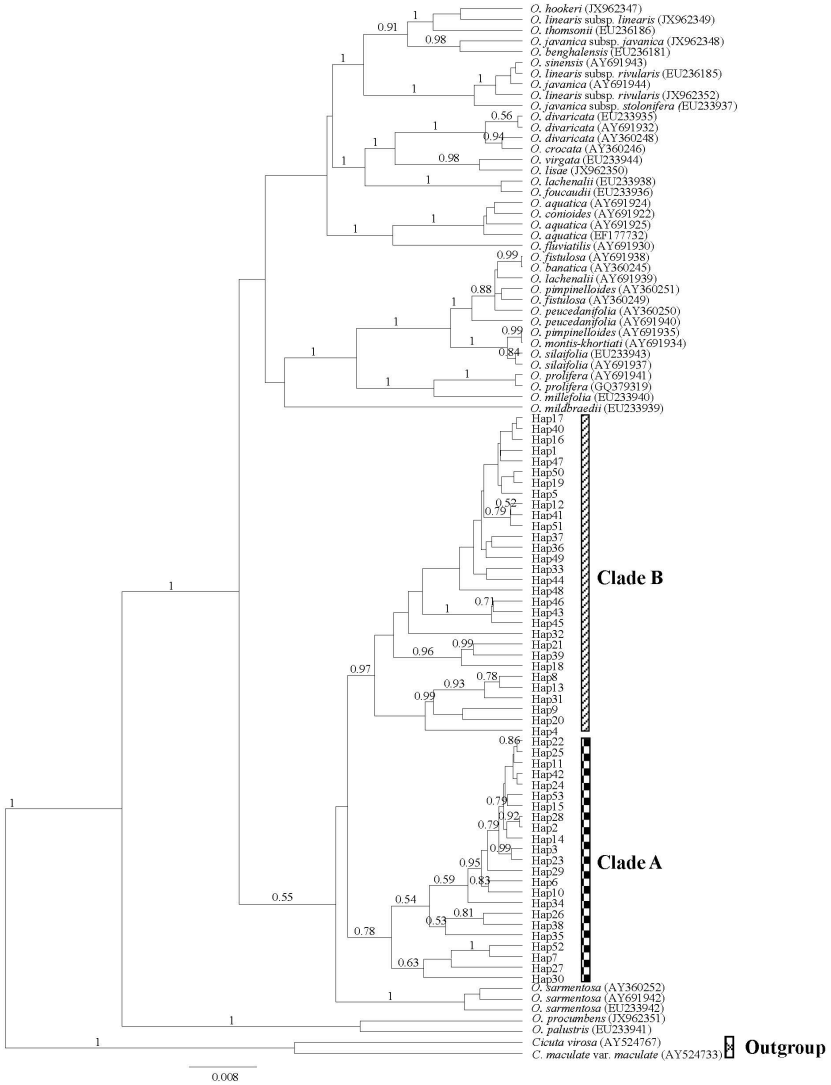

Figure 3. Majority-rule consensus tree obtained from Bayesian analyses of the internal transcribed spacer (ITS) sequence data for 53 haplotypes (Hap1-Hap53) generated in the present study and 43 *Oenanthe* accessions of 29 species from GenBank. Posterior probabilities are indicated above branches. The indels in the ITS sequence dataset were included.

DISCUSSION

In this study, we compared the nrDNA ITS region sequence variation of 170 *Oenanthe* accessions collected from a wide geographical region of China and its neighboring countries with accessions reported in previous studies (Hardway et al., 2004; Kadereit and Kadereit, 2005; Downie et al., 2008; Zhou et al., 2008; Spalik et al., 2014). Using two phylogenetic tree construction methods (BI and ML) and two kinds of dataset (with and without indels), we found that the accessions collected in this study tended to cluster into two groups. From the phylogenetic trees, we also found that one of the two haplotype groups was close to *O. mildbraedii* and the other to *O. sarmentosa*.

To date, many gene markers have been employed in the phylogenetic study of the Apiaceae (Downie et al., 2001, 2008; Kadereit and Kadereit, 2005; Zhou et al., 2008, 2009; Spalik et al., 2009, 2014), such as nrDNA ITS region, chloroplast DNA gene (*rbc*L and *mat*K), intergenic spacer (*psbI*-5'*trnK* and *rps16*-5'*trnK*), and intron (*rpl*16, *rps*16, and *rpo*C1) sequences. Among these, the ITS region is the most frequently used marker. The ITS region of nearly all *Oenanthe* species has been sequenced. Although the utility of the ITS region for high-level phylogenetic estimation in the Apiaceae has been questioned due to high rates of ITS sequence divergence among the lineages (Downie et al., 2008), at present it is the best marker for lower-level phylogenetic analyses (Downie et al., 2001; Zhou et al., 2008). In this study, we used ITS sequences from GenBank that represented all species that have been sequenced. Both the BI and ML analyses demonstrated that there are different accessions for the same species, but those sequenced in different studies were often clustered together: three accessions of *O. sarmentosa* (AY691942, AY360252, and EU233942), three accessions of *O. divaricata* (AY360248, AY691932, and EU233935), two accessions of *O. silaifolia* (EU233943 and AY691937), and two accessions of *O. prolifera* (AY691941 and GQ379319), which shows that the ITS region is a powerful tool for species identification in *Oenanthe*.

Unexpectedly, none of the 53 ITS haplotypes identified in the 170 *Oenanthe* accessions sampled in this study were close to *O. javanica*, which is regarded as the most widely cultivated and used vegetable in the genus in East Asia (Huh et al., 2002; Zhao, 2010). Instead, the two haplotype groups revealed in our phylogenetic analyses were close to *O. mildbraedii* and *O. sarmentosa*. These two species have never been recorded in China (China Flora Editorial Board of CAS, 1985; Fu and Watson, 2005) or its neighboring countries (Huh et al., 2002); therefore, it seems probable that in these sampled locations, *Oenanthe* accessions have been given the incorrect name, *O. javanica*. The morphology of *O. javanica* is highly variable, and the size and shape of the leaves of the *O. javanica* subspecies *javanica* converge with those of the *O. javanica* subspecies *rosthornii* in Malaysia and adjoining areas; in addition, *O. benghalensis* and *O. linearis* have been placed within the broader species concept for *O. javanica* by some workers (Fu and Watson, 2005).

It should be noted that although our sampling range covered a large proportion of the geographical range of *Oenanthe* species in East Asia, in each location we only sampled one individual, and other important *Oenanthe* areas were not sampled; therefore, it is possible that locations where *O. javanica* is present in East Asia were not sampled. In addition, only a few accessions of most of the recorded species in *Oenanthe*, including *O. javanica*, *O. mildbraedii*, and *O. sarmentosa*, were sequenced for the ITS region. Small sample sizes and a lack of morphological information of species outside East Asia are limitations of our comparative taxonomic study. Future studies should carefully check the morphological characteristics of

other *Oenanthe* species and sequence their ITS regions in order to clarify the taxonomic status of the genus.

Conflicts of interest

The authors declare no conflict of interest.

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