

PHYLOGENY AND CLASSIFICATION OF CHINESE *BUPLEURUM* BASED ON NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER AND *rps16*

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Separate and combined analyses of nuclear ribosomal DNA internal transcribed spacer (ITS) and cpDNA *rps16* provided phylogenetic hypotheses and molecular data for the taxonomy of Chinese *Bupleurum* species. The phylogenetic results derived from Bayesian and maximum parsimony analyses supported the monophyly of the *Bupleurum* with strong evidence. The origin of Chinese *Bupleurum* is likely to be through the Middle East and the Caucasus from the Mediterranean region with the basic chromosome number (8→7→6), and polyploidization. Our molecular data are not consistent with other earlier Chinese *Bupleurum* classifications and are consistent with the molecular classification proposed by Neves and Watson. The analyses also provide molecular data to elucidate the taxonomic treatments for *Bupleurum falcatum* from China and Europe.

Key words: Chinese *Bupleurum*, phylogeny, taxonomy, ITS, *rps16*.

INTRODUCTION

Bupleurum is a large and identifiable group in Apiaceae, with up to 180–190 species distributed in the North Temperate Zone (mainly in Eurasia, the Mediterranean and North Africa) (Liu et al., 2003; Neves and Watson, 2004). *Bupleurum* is traditionally treated in the subfamily Apioideae, tribe Apieae, subtribe Apiinae (Heywood, 1971). Earlier work by Downie and collaborators (Downie et al., 2000a) included several species of *Bupleurum*, which was treated as the monogeneric tribe Bupleureae Spreng. Pan et al. (2002) argued that chemical components differ between *Bupleurum* and other taxa in Apiaceae, and that *Bupleurum* should be segregated from subfamily Apioideae as subfamily Bupleuroideae S. L. Pan.

As one of the centers of the frequency and diversity of *Bupleurum*, China harbors 42 species, half of which are native species (Sheh and Watson, 2005). Most of the Chinese *Bupleurum* are used in traditional medicine for common cold with fever, influenza, hepatitis, malaria and menoxenia (Pan, 2006). Chinese *Bupleurum* species present the primitive pollen type (from subrhomboid or subrotund to subellipsoid) which appeared in the early

Palaeocene; this may indicate that China is one of the regions of origin for *Bupleurum* (Shu and Sheh, 2001).

Extensive taxonomic research on the Chinese species of *Bupleurum* has been carried out, but many *Bupleurum* species are difficult to characterize, as is evident from the complex classifications where all possible taxonomic ranks have been used. Much research examines the karyology of Chinese *Bupleurum* (mainly reporting chromosome numbers) (Pan and Qin, 1981; Pan et al., 1985; Qin et al., 1989; Jiang et al., 1994; Jiang et al., 2002). A few attempts at infrageneric classification of Chinese *Bupleurum* have been made. According to the numerical taxonomy of 14 medical *Bupleurum* species, Shu (1998) proposed a classification system for Chinese *Bupleurum*, including two subgenera: Longifolia (Wolff) Yuan and Eubupleura (Briq.) Yuan. The pollen morphology of Chinese *Bupleurum* has been studied since the 1960s (Pollen morphology group in plant research of Chinese Academy, 1960; Mo and Wu, 1994; Wang et al., 1995; Wang and Fu, 1995), but only minor variations have been found in the surveyed species. Liu et al. (2003) studied the anatomy of eight species in the genus *Bupleurum* from northeastern China, but more data

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is needed to understand its value for the study of species relationships. Only several recent papers included molecular analyses of Chinese *Bupleurum* (Neves and Watson, 2004; Wu et al., 2005; Xie et al., 2006; Yang et al., 2007; Wang et al., 2008).

The nrDNA ITS region is the best marker for lower-level phylogenetic analyses of Apiaceae (Baldwin, 1992; Baldwin et al., 1995; Tian and Li, 2002). Moreover, the numerous ITS sequences available in the GenBank enable immediate comparative analyses. The chloroplast marker of the *rps16* intron is also a useful marker for reconstruction of phylogeny (Downie and Katz-Downie, 1999).

A broad sampling of Chinese *Bupleurum* is indispensable for a comprehensive classification and phylogeny of these species based on molecular data. Our study has the following goals: (1) to evaluate the monophyly of *Bupleurum* and to ascertain its phylogenetic placement; (2) to discover the origin of Chinese *Bupleurum*; (3) to resolve the infrageneric taxonomy of Chinese *Bupleurum*; and (4) to find new information on the relationship of *Bupleurum falcatum* L. from China and Europe.

MATERIALS AND METHODS

PLANT MATERIAL

Species were selected to represent the Chinese *Bupleurum* as completely as possible. We also included other genera from Apiaceae as outgroups. We followed the classification of Apiaceae in *Flora of China* (Wu and Peter, 2005). In this study we used a total of 73 GenBank accessions (ITS) representing 72 *Bupleurum* species and varieties (Tab. 1). Of these, *rps16* of 27 species (including varieties) and ITS of 10 species (including varieties) from China have not been studied in previous phylogenetic work. We also used 60 GenBank accessions, including both ITS (not including 5.8s) and the *rps16* intron, representing 29 genera (Tab. 1).

The six outgroups used in this study included three members in Araliaceae, *Schefflera minutistellata*, *Hedera nepalensis* var. *sinensis* and *Fatsia japonica*, for discussion of the relationships between *Bupleurum* and another genus in Apiaceae. *Anginon difforme*, *Anginon paniculatum* and *Heteromorpha arborescens* were chosen to infer relationships among *Bupleurum* species; previous molecular phylogenetic studies have shown that they may be used as outgroups for *Bupleurum* (Neves and Watson, 2004).

MOLECULAR DATA

Fresh young leaves were collected and dried with silica gel during fieldwork (Tab. 1). Total genomic DNA

was extracted following the protocol by Doyle and Doyle (1987), or with the DNasecure plant Kit (Tiangen Biotech, Beijing, China). The whole ITS region was amplified using primers ITS4 and ITS5 (White et al., 1990). The primers for amplifying *rps16* were *rps16*-F: ATA GAC GGC TCA TTG GGA TA and *rps16*-R: CGT GCG ACT TGA AGG ACA. They were designed using primer 5 (Clarke and Gorley, 2001), from three completely sequenced *rps16* introns (*Daucus carota*, *Nicotiana tabacum*, and *Oryza sativa*) without self-dimer, hairpin, and pair-dimer formation. Details of the PCR amplification reactions and sequencing strategies are the same as both ITS described in Wang et al. (2008) and for the *rps16* intron.

The PCR products were subcloned into pGM-T Easy vectors (Tiangen Biotech, Beijing, China) and subsequently transformed to competent cells with the ligation products following the manufacturer's protocol. The transformed cells were cultured overnight at 37°C on Luria-Bertoni (LB) ampicillin plates. The colonies were identified by colors (blue/white), and white colonies were picked and grown in 3 ml LB liquid medium overnight. Inserts with the correct size of DNA were verified by PCR of the bacteria colonies. Lastly, the products were sent to Invitrogen Biotech Co. Ltd. (Shanghai, China) for sequencing. Sequencing was done using an ABI-3730XL DNA sequencer. For each sampled specimen, forward and reverse sequencing reactions were performed for confirmation. The boundaries of ITS1, 5.8S and ITS2 regions were determined following the published sequences from GenBank. All newly procured sequences were edited manually in SeqMan of DNASTar ver. 4.0 (DNASTAR, 2001). All sequences obtained from this study were deposited in the GenBank (Tab. 1).

PHYLOGENETIC ANALYSES

The DNA sequences were aligned using CLUSTAL W ver. 1.8 (Thompson et al., 1994). Alignment gaps were treated as missing data for all taxa.

In total, three matrices were constructed. Two matrices included the individual datasets (ITS and *rps16*) for *Bupleurum* and other species from Apiaceae. One combined matrix was constructed based on *rps16* plus ITS (Tab. 1). Phylogenetic analyses of relationships were carried out using maximum parsimony (MP) and Bayesian inference (BI) methods.

The incongruence length difference (ILD) test was used to evaluate the congruence among the combined data of ITS (not including 5.8s) and *rps16*. The ILD test was conducted with PAUP* 4.0b10 (Swofford, 2003) with 1000 replicates, and tree bisection reconnection (TBR) branch swapping heuristic searches. Gaps were treated as missing data.

TABLE 1. Taxa included in the molecular analyses, with voucher specimens and GenBank accession numbers. New sequences are asterisked

Taxon	Origin and voucher	GenBank accession number	
		ITS/ITS1/ITS2	<i>rps16</i>
<i>Ammi majus</i> L.	Downie et al. (1998)	U78386/U78446	AF164814
<i>Anethum graveolens</i> L.	Downie et al. (1998)	U30550/U30551	AF110542
<i>Angelica ampla</i> A. Nelson	Downie et al. (1998)	U79597/U79598	AF358598
<i>A. archangelica</i> L. subsp. <i>archangelica</i>	Downie et al. (1998)	U30576/U30577	AF110536
<i>A. breweri</i> A. Gray	Downie et al. (1998)	U78396/U78456	AF358599
<i>A. pinnata</i> S. Watson	Downie et al. (2002), U.S.A.: Hartman 41500 (RM)	AF358465/AF358532	AF358600
<i>Anthriscus caucalis</i> M. Bieb.	Downie et al. (1998)	U79601/U79602	AF110549
<i>Anginon difforme</i> (L.) B. L. Burt	Neves and Watson (2004), South Africa: All Batten AB 1018 (E)	AF459742	
<i>A. paniculatum</i> (Thunb.) B. L. Burt	Neves and Watson (2004), South Africa: H. C. Taylor 11271 (E)	AF467922	
<i>Apium graveolens</i> L.	Downie et al. (1998)	U30552/U30553	AF110545
<i>Berula erecta</i> (Huds.) Coville	Downie et al. (1998)	U79605	AF164819
<i>Bupleurum acutifolium</i> Boiss.	Neves and Watson (2004), Spain: S. S. Neves 64 (E)	AF467925	
<i>B. album</i> Maire	Neves and Watson (2004), Morocco: BM Expedition 532 (RNG)	AF467928	
<i>B. angulosum</i> L.	Neves and Watson (2004), UK: S. S. Neves RBGE 19861043 (E)	AF469008	AF110568
<i>B. angustissimum</i> (Franch.) Kitagawa	Xie et al. (2006), China: S. L. Pan 0506 (SHMU)	DQ285464	
<i>B. aureum</i> Fisch. ex Hoffmann	Yang et al. (2007), China: 20060815 (SHMU)	EF101158	
<i>B. balansae</i> Boiss. & Reut.	Neves and Watson (2004), Morocco: S. L. Jury et al. 8338 (RNG)	AF469681	
<i>B. baldense</i> Turra	Neves and Watson (2004), Spain: M. A. Carrasco et al. (MA 558704)	AF469682	
<i>B. barceloi</i> Coss. ex Willk.	Neves and Watson (2004), Spain: J. Orell Casasnovas (MA 474781)	AF477023	
<i>B. benoistii</i> Litard. & Maire	Neves and Watson (2004), Morocco: S. L. Jury et al. 18375 (E)	AF477024	
<i>B. boissieuanum</i> H. Wolff	China: Q. Z. Wang 362549 (SZ)	EU220931*	EU220939*
<i>B. candollei</i> var. <i>atropurpureum</i> C. Y. Wu	China: Q. Z. Wang 356541 (SZ)	EU220928*	EU220940*
<i>B. candollei</i> Wallich ex DC.	Xie et al. (2006), China: H. Xie 040517002 (SHMU)	DQ285471	
<i>B. canescens</i> Schousb.	Neves and Watson (2004), Morocco: D. Bramwell et al. 265 (RNG)	AF477027	
<i>B. canescens</i> Schousb. var. <i>handiense</i> Bolle	Neves and Watson (2004), Spain: D. Bramwell 1631 (E)	AF477029	
<i>B. chaishoui</i> R. H. Shan & M. L. Sheh	China: Q. Z. Wang & F. D. Pu 361536 (SZ)	EU001333	EU220941*
<i>B. chinense</i> DC.	China: C. Q. Feng 200508 (Institute of Natural Medicine and Chinese Medicine Resources)	EU001334	EU220942*
<i>B. commelynoideum</i> H. Boissieu	China: Q. Z. Wang & F. D. Pu 374627 (SZ)	EU001335	EU220933*
<i>B. commelynoideum</i> var. <i>flaviflorum</i> R. H. Shan & Yin Li	China: Q. Z. Wang & F. D. Pu 350064 (SZ)	EU220929*	EU220943*

TABLE 1. Continuation

<i>B. dielsianum</i> H. Wolff	China: Q. Z. Wang 362551 (SZ)	EU220932	EU220944
<i>B. dumosum</i> Coss.	Neves and Watson (2004), Morocco: S. L. Jury et al. 14157 (RNG)	AF477030	
<i>B. exaltatum</i> M. Bieb.	China: Q. Z. Wang 2005388 (SZ)	EU220923	EU220945
<i>B. falcatum</i> L.	China: J. Y. H	EU001336	
<i>B. falcatum</i> L.	Neves and Watson (2004), Spain: J. A. Alexandre 733/92 534085 (MA)	AF479290	
<i>B. frutescens</i> L. subsp. <i>frutescens</i>	Neves and Watson (2004), Spain: G. Montserrat (MA 515853)	AF479291	
<i>B. frutescens</i> L. subsp. <i>spinosum</i> (Gouan) O. Bolos & Vigo	Neves and Watson (2004), Spain: S. S. Neves 42 (E)	AF479293	
<i>B. fruticosum</i> L.	Neves and Watson (2004), Spain: S. S. Neves 41 (E)	AF479298	
<i>B. gerardii</i> All.	Neves and Watson (2004), Portugal: S. S. Neves Acc. No. 17a (E)	AF479847	
<i>B. gibraltarium</i> Lam.	Neves and Watson (2004), Spain: S. S. Neves 51 (E)	AF479852	
<i>B. gracilipes</i> Diels	China: Q. Z. Wang & F. D. Pu 361529 (SZ)	EU001337	EU220946
<i>B. hamiltonii</i> N. P. Balakrishnan	China: Q. Z. Wang & F. D. Pu 348618 (SZ)	EU001338	EU220934
<i>B. hamiltonii</i> var. <i>humile</i> (Franchet) R. H. Shan & M. L. Sheh	China: Q. Z. Wang & F. D. Pu 366440 (SZ)	EU001339	EU220937
<i>B. krylovianum</i> Schischkin ex Krylov	China: Nurbay 88-0383 (SZ)	EU220924	EU220947
<i>B. kweichowense</i> R. H. Shan	China: Q. Z. Wang 200706 (Guangxi Medicinal Botanical Garden)	EU220925	EU220948
<i>B. lancifolium</i> Hornem.	Neves and Watson (2004), Morocco: S. L. Jury et al. 16552 (RNG)	AF479853	
<i>B. lateriflorum</i> Coss. ex H. Wolff	Neves and Watson (2004), Morocco: S. L. Jury et al. 18323 (E)	AF479854	
<i>B. latissimum</i> Nakai	South Korea	AY551292	
<i>B. longicaule</i> var. <i>amplexicaule</i> C. Y. Wu ex R. H. Shan & Yin Li	China: Q. Z. Wang & F. D. Pu 366442 (SZ)	EU001340	EU220949
<i>B. longicaule</i> var. <i>franchetii</i> H. Boissieu	China: Q. Z. Wang & F. D. Pu 374629 (SZ)	EU001342	EU220950
<i>B. longicaule</i> var. <i>giraldii</i> H. Wolff	China: Q. Z. Wang 362552 (SZ)	EU220930	EU220951
<i>B. longifolium</i> L.	Neves and Watson (2004), Germany: H. Forther 7503 (MAF 149194)	AF479856	
<i>B. longiradiatum</i> Turcz.	China: C. B. Wang 200607 (Jia Mu Si University)	EU001341	EU220935
<i>B. longiradiatum</i> f. <i>australe</i> R. H. Shan & Yin Li	Xie et al. (2006), China: Z. Chao & D. G. Zhang 0307003 (SHMU)	DQ285460	
<i>B. malconense</i> R. H. Shan & Yin Li	China: Q. Z. Wang 357520 (SZ)	EU220926	EU220952
<i>B. marginatum</i> var. <i>stenophyllum</i> (H. Wolff) R. H. Shan & Yin Li	China: Q. Z. Wang & F. D. Pu 374614 (SZ)	EU001344	EU220938
<i>B. marginatum</i> Wallich ex DC.	China: Q. Z. Wang & F. D. Pu 361530 (SZ)	EU001343	EU220953
<i>B. microcephalum</i> Diels	China: Q. Z. Wang & F. D. Pu 356516 (SZ)	EU001345	EU220954
<i>B. montanum</i> Coss.	Neves and Watson (2004), Morocco: S. L. Jury 17456a (E)	AF479858	
<i>B. mundii</i> Cham. & Schldl.	Neves and Watson (2004), South Africa: S. S. Neves RBGE Acc. No. 19972669(E)	AF479860	

TABLE 1. Continuation

<i>B. odontites</i> L.	Neves and Watson (2004), Tunisia: Davis & Lamond D57628 (RNG)	AF479861	
<i>B. oligactis</i> Boiss.	Neves and Watson (2004), Morocco: S. L. Jury et al. 17516 (E)	AF479863	
<i>B. petiolulatum</i> Franch.	China: Q. Z. Wang & F. D. Pu 374622 (SZ)	EU001332	EU220955
<i>B. petiolulatum</i> var. <i>tenerum</i> R. H. Shan & Yin Li	Xie et al. (2006), China: Z. Chao & D. G. Zhang 0308010(SHMU)	DQ285459	
<i>B. plantagineum</i> Desf.	Neves and Watson (2004), Algeria: Davis 52959 (RNG)	AF479865	
<i>B. polyclonum</i> Yin Li & S. L. Pan	Xie et al. (2006), China: H. Xie 040517001 (SHMU)	DQ285463	
<i>B. praealtum</i> L.	Neves and Watson (2004), Spain: P. Montserrat et al. 191581 (JACA)	AF480939	
<i>B. ranunculoides</i> L.	Neves and Watson (2004), Germany: S. S. Neves Acc. No. 43	AF481392	
<i>B. rigidum</i> L. subsp. <i>paniculatum</i> (Brot.) H. Wolff	Neves and Watson (2004), Portugal: F. Sales & S. S. Neves 3a (E)	AF481398	
<i>B. rigidum</i> L. subsp. <i>Rigidum</i>	Neves and Watson (2004), Spain: S. S. Neves 53 (E)	AF481396	
<i>B. rockii</i> H. Wolff	China: Q. Z. Wang & F. D. Pu 361540 (SZ)	EU001346	EU220956
<i>B. rotundifolium</i> L.	Neves and Watson (2004), Switzerland: S. S. Neves Acc. No. 4 (E)	AF481400	
<i>B. salicifolium</i> R. Br. ex Buch	Neves and Watson (2004), Spain: S. S. Neves Acc. No. 29 (COI)	AF481927	
<i>B. scorzonerifolium</i> Willd.	China: C. B. Wang 200607 (Jia Mu Si University)	EU001347	EU220936
<i>B. semicompositum</i> L.	Neves and Watson (2004), Spain: S. Cirujano 552469 (MA)	AF481929	
<i>B. sibiricum</i> Vest	Xie et al. (2006), China: 0519 (SHMU)	DQ285457	
<i>B. sichuanense</i> S. L. Pan & P. S. Hsu.	Xie et al. (2006), China: 0308052 (SHMU)	DQ285447	
<i>B. smithii</i> H. Wolff	Xie et al. (2006), China: H. Xie 040727001 (SHMU)	DQ285455	
<i>B. stellatum</i> L.	Neves and Watson (2004), Switzerland: B. de Retz 88690 (MAF 145370)	AF481930	
<i>B. subspinosum</i> Maire & Weiller	Neves and Watson (2004), Morocco: C. J. & A. R. Humphries 99 (BM)	AF481931	
<i>B. tenuissimum</i> L.	Neves and Watson (2004), Portugal: S. S. Neves 22 (E)	AF481932	
<i>B. thianschanicum</i> Freyn	China: F. Wang 04-0111(SZ)	EU220927	EU220957
<i>B. wenchuanense</i> R. H. Shan & Yin Li	China: Q. Z. Wang & F. D. Pu 361535 (SZ)	EU001348	EU220958
<i>B. yinchowense</i> R. H. Shan & Yin Li	Xie et al. (2006), China: H. Xie & F. L. Liu 040723001(SHMU)	DQ285454	
<i>B. yunnanense</i> Franch.	China: Q. Z. Wang & F. D. Pu 374574 (SZ)	EU001349	EU220959
<i>Cicuta maculata</i> L. var. <i>angustifolia</i> Hook.	USA: Nelson et al. 33517 (RM)	AY360235	AF358601
<i>Conium maculatum</i> L.	Downie et al. (1998)	U30588/U30589	AF110546
<i>Cryptotaenia canadensis</i> (L.) DC.	Downie et al. (1998)	U79613	AF358602
<i>Daucus pusillus</i> Michx.	Lee and Downie (1999)	AF077788/AF077103	AF123729
<i>Eryngium billardierei</i> Delaroche	Sn: J. HELLWIG 040442 (MJG)	EU168971	EU168949
<i>Fatsia japonica</i> (Thunb.) Decne. & planch.	China: Q. Z. Wang 364224 (SZ)	EU786158	EU786161
<i>Foeniculum vulgare</i> Mill.	Downie et al. (1998)	U78385/U78445	AF110543

TABLE 1. Continuation

<i>Hedera nepalensis</i> K. Koch var. <i>Sinensis</i> (Tobl.) Rehd.	China: Q. Z. Wang 364223 (SZ)	EU786157	EU786160
<i>Heracleum sphondylium</i> L.	Downie et al. (1998)	U30544/U30545	AF164800
<i>Heteromorpha arborescens</i> (Spreng.) Cham. & Schtdl. var. <i>abyssinica</i> (A. Rich.) H. Wolff	South Africa: Hilliard & Burt 10191 (UC)	DQ368848	
<i>Ligusticum scoticum</i> L.	Downie et al. (1998)	U78357/U78417	AF123756
<i>Oenanthe pimpinelloides</i> L.	Downie et al. (1998)	U78371	AF110553
<i>Osmorhiza longistylis</i> (Torr.) DC.	Downie et al. (1998)	U79617/U79618	AF123754
<i>Pastinaca sativa</i> L.	Downie et al. (1998)	U30546/U30547	AF110538
<i>Petroselinum crispum</i> (Mill.) A.W. Hill	Downie et al. (1998)	U78387/U78447	AF110544
<i>Pleurospermum foetens</i> Franch.	Katz-Downie et al. (1999)	AF008639/AF009118	AF110559
<i>Sanicula arctopoides</i> Hook. & Arn.	Gould 868 (MO)	EU070743	DQ832375
<i>Scandix pecten-veneris</i> L.	Downie et al. (1998)	U30538/U30539	AF123753
<i>Schefflera minutistellata</i> Merr. ex Li	China: Q. Z. Wang 364222 (SZ)	EU786156	EU786159
<i>Seseli mairei</i> H. Wolff	China: F. D. Pu & Q. Z. Wang (SZ)	EF555727	EU220960
<i>S. squarulosm</i> R. H. Shan & M. L. Sheh	China: F. D. Pu & Q. Z. Wang 361539 (SZ)	EU001350	EU220961
<i>Sium suave</i> Walter	Cultivation	AF358523/AF358590	AF358643
<i>Torilis arvensis</i> (Huds.) Link	Downie et al. (2000b)	AF164844/AF164869	AF110548
<i>Turgenia latifolia</i> (L.) Hoffm.	Lee and Downie (1999)	AF077810/AF077125	AF123743

The Bayesian analysis was conducted using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001). The settings appropriated for the best fit GTR+G model of nucleotide substitution, as selected by MrModeltest version 2.2 (Nylander, 2004) under the Akaike Information Criterion (AIC), were used as the text editor of PAUP* to prepare a Nexus file that was subsequently run in MrBayes (statefreqpr = dirichlet(1, 1, 1, 1); nst = 6; rates = invgamma). The priors on state frequency rates and variation across sites were estimated automatically by the program. Four Markov chains starting with a random tree were run simultaneously for one million generations, sampling trees at every 100th generation. Trees from the first 200,000 generations were regarded as "burn in" and discarded, and the posterior probability values were determined from the remaining 8,000 trees.

Maximum parsimony analysis was performed using PAUP* ver. 4.0b10 (Swofford, 2003). Heuristic searches were carried out with 1,000 random addition sequence replicates. TBR was used to swap branches. Characters were unordered and equally weighted. Gaps were treated as missing data, and multistate data were interpreted as uncertainty. Bootstrap values were calculated from 1,000

replicate analyses using TBR branch swapping and simple stepwise-addition.

RESULTS

The strict consensus trees of ITS, *rps16* and ITS + *rps16* are shown in Figures 1 and 2. Chinese *Bupleurum* species (in cladeA) are divided into two subclades (E and F), which appeared to be the sister group of the northwestern African species (Fig. 1, cladeB). The Mediterranean species form a basal group with strong support (Fig. 1, cladeD, PB: 100; PP: 0.98). *Bupleurum* is monophyletic and sister group to other taxa from the subfamily Apioideae with good support (PB: 100; PP: 1.00) in *rps16* and ITS (not including 5.8s) + *rps16* phylogeny analyses (Fig. 2).

ITS CHARACTERISTICS AND PHYLOGENETIC ANALYSES

Internal transcribed spacer sequences (586bp ~610bp) of 73 *Bupleurum* species resulted in a matrix of 638 bp of aligned sequences. Among these, 335 characters were constant, 88 were autapomor-

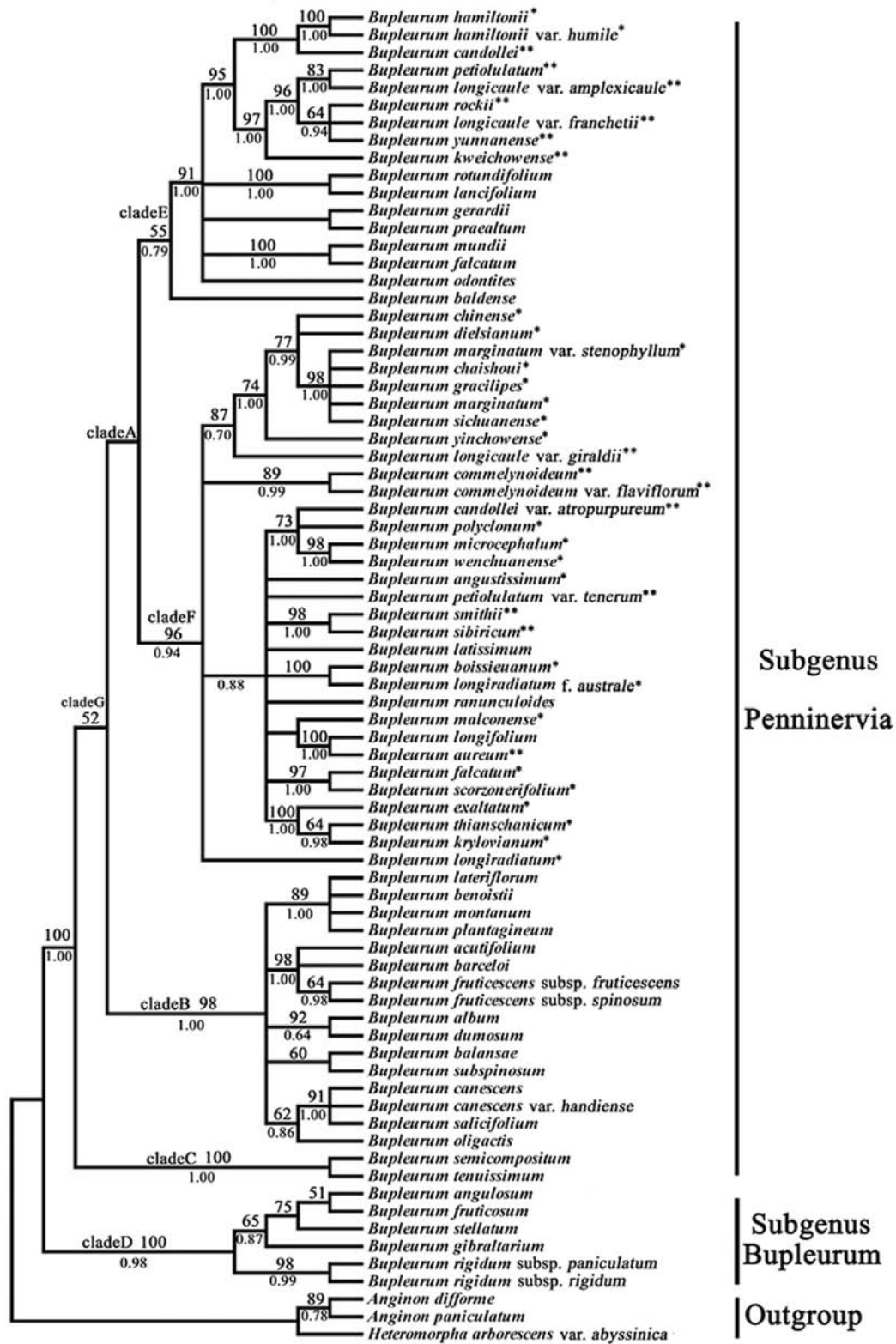


Fig. 1. Strict consensus tree of 100 ITS trees (CI=0.62; RI =0.87) obtained by parsimony analysis of 73 taxa of *Bupleurum*. Parsimony bootstrap values (PB) higher than 50% are indicated above branches. Bayesian posterior probability (PP) is indicated below branch. *species' bracteoletypes are small. **species' bracteoletypes are large.

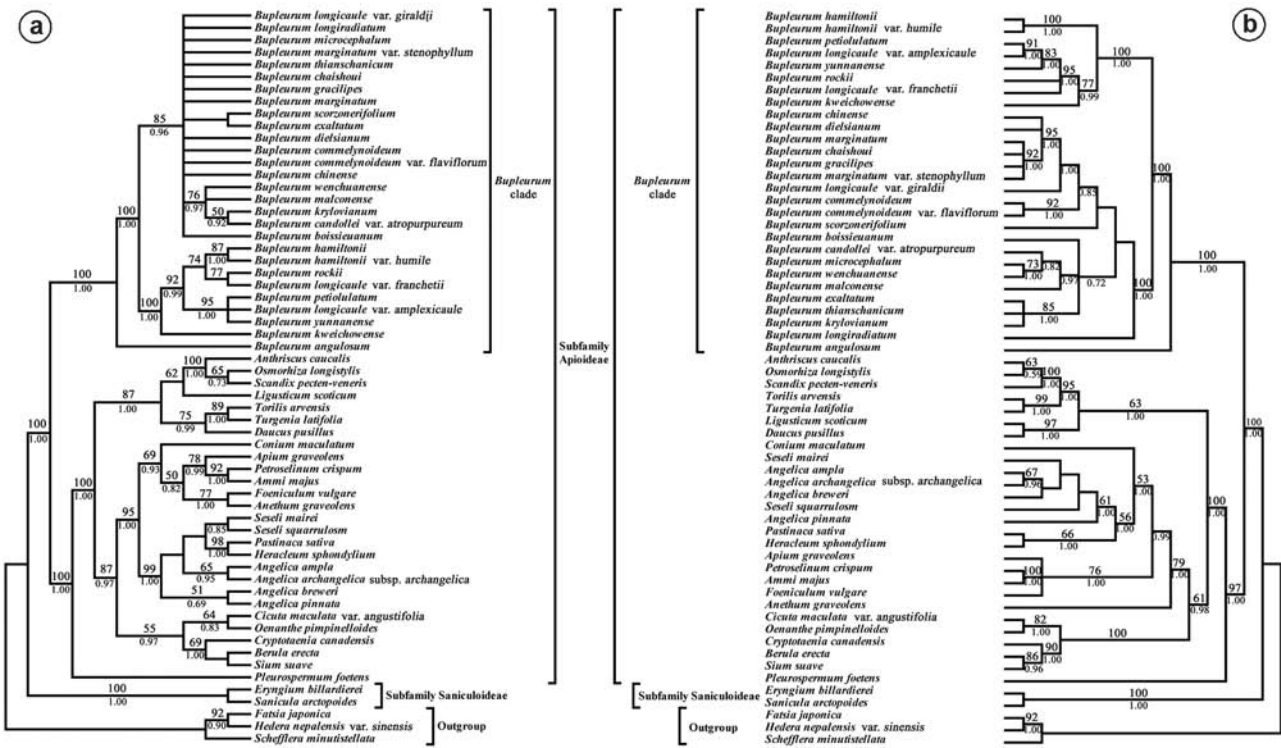


Fig. 2. Strict consensus trees from separate and combined analyses. (a) Strict consensus tree from *rps16* dataset analysis, (b) Strict consensus tree from combined dataset analysis (*rps16* plus ITS, not including 5.8s).

phic and 215 were potentially parsimony-informative. The strict consensus tree of 100 MP trees and the BI phylogeny were highly concordant (Fig. 1). All analyses gave strong support for *Bupleurum* species (in cladeA) falling into two main clades, and for the Mediterranean species (*B. fruticosum*, the only woody species with a distribution in Africa and the Mediterranean) comprising a well-supported basal clade (cladeD) (PB=100; PP=1.00). CladeB is strongly supported (PB=98; PP=1.00) and includes all endemic species from northwest Africa. In all analyses, cladeB is a sister group to a larger cladeA that includes the vast majority of the Chinese species. CladeC includes *B. semicompositum* and *B. tenuissimum* with good support (PB=100; PP=1.00), and it is traditionally classified as subsection *Trachycarpa* of section *Isophyllum* (Tutin, 1968).

CladeA is divided into two clades: cladeE with low support (PB=55; PP=0.79) and cladeF with high support (PB=96; PP=0.94). CladeE and cladeF include species that have essentially a Eurasian distribution: *B. ranunculoides* and *B. longifolium* are included in cladeF. The rest of the species in cladeE include *B. falcatum* from Spain. *B. mundii* is the only species endemic to southern Africa. *B. gerardii* and *B. praealtum*, *B. rotundifolium*, *B. lancifolium*

and *B. odontites* belong to section *Bupleurum* in Tutin's (1968) classifications.

CladeE, the species of which are confined to the southwest of China, forms the sister group to the other non-Chinese species, with strong support (PB=95; PP=1.00). CladeF includes all northern and southern Chinese *Bupleurum* species. The relationship between *B. longiradiatum* from China in the subgenus *Longifolia* and other Chinese species in the subgenus *Eubupleura* from cladeF (Shu et al., 1998) awaits confirmation.

The sequences of *B. falcatum* from Europe and China were obtained. It seems clear that the Chinese *B. falcatum* is not closely related to the exemplar sequences from Europe (Fig. 1). They are considerably different sequences (65 mutations, 10.89% divergence).

PHYLOGENETIC ANALYSES OF *rps16* AND COMBINED *rps16* AND ITS

Based on cpDNA *rps16* data, other species within subfamily Apiaceae (except for *Bupleurum* taxon) form a strongly supported clade (PB: 100; PP: 1.00) in all analyses (Fig. 2a). *Eryngium billardierei* and *Sanicula arctopoides* from subfamily Saniculoideae form the basal grade with strong support in all

analyses (PB: 100; PP: 1.00) of *rps16* phylogeny (Fig. 2a).

The results of the partition homogeneity test ($P=0.001$) showed that the nrDNA ITS and cpDNA *rps16* datasets were not significantly incongruent. Visual inspection of the combined cpDNA *rps16*, nrDNA ITS (not including 5.8s, because of little variation) + cpDNA *rps16* trees revealed *Bupleurum* to be a monophyletic group with strong support (PB: 100, PP: 1.00, Fig. 2). The relationship between *Seseli mairei* and *S. squarulosum* is unresolved (Fig. 2B).

DISCUSSION

SYSTEMATIC POSITION OF *BUPLEURUM*

Bupleurum has long been recognized as a natural group (Tournefort, 1694; Drude, 1898; Wolff, 1910; Tutin, 1968), mainly due to its characteristic simple and entire leaves, a synapomorphy of the group in Apiaceae. There are conspicuous bracts and bracteoles on the inflorescences. Pollen morphology exhibits few variations within the genus. The fruit is oblong to ovoid-oblong or ellipsoid, slightly laterally compressed, and the mericarp is subpentagonal (rarely rounded) in cross section. In addition to its unusual morphology, *Bupleurum* is characterized by a base chromosome number of $x = 8$, a number that is rare in Apiaceae ($x = 11$) and is found in only ~0.1% of apioid species ($x = 4$ in *Bupleurum*) (Moore, 1971).

The combination of its unusual morphological and cytological features have led specialists to treat *Bupleurum* as a natural group in Apiaceae, and traditionally *Bupleurum* has been placed in subfamily Apioideae, tribe Apieae, subtribe Apiinae (Heywood, 1971). However, studies found *Bupleurum* to be the basal lineage in Apiaceae (Plunkett et al., 1996a, b, 1997; Downie and Katz-Downie, 1996, 1999; Downie et al., 1998, 2000a, b; Plunkett and Downie, 1999, 2000). Downie et al. (2000a) concluded that *Bupleurum* should be included within the monogeneric tribe Bupleureae Spreng. based on cpDNA *rpl16* sequences of three *Bupleurum* species. Pan et al. (2002) found chemical evidence showing that *Bupleurum* should be a subfamily included in the monogeneric tribe Bupleureae Spreng.

However, phylogenetic estimates of maximum parsimony and Bayesian inference of separate and combined matrices strongly support (PB: 100; PP: 1.00) the monophyly of *Bupleurum* and its sister-group relationship with all other members of the subfamily Apioideae in our study, whereas Hydrocotyloideae represents the basal elements in our phylogenetic trees (Fig. 2).

Consistent with the results of previous research (Downie et al., 2000a), *Bupleurum* is the sister group

of the remaining Apioideae. Our study indicates that ITS sequences can be used for inferring phylogeny among closely related members of Apioideae, given the high rates of nucleotide substitutions. Both the separate and combined datasets improve the resolution of the hierarchical placement of *Bupleurum*, with strong bootstrap-supported values.

THE ORIGIN OF CHINESE *BUPLEURUM* SPECIES

Based on evidence from pollen shape and fossils, it has been hypothesized that China is one of the centers of origin for *Bupleurum* (Shu and Sheh, 2001; Song et al., 2004). Most *Bupleurum* species from southwest China retain the primitive pollen type (from subrhomboid to subrotund or subellipsoid) appearing in the early Palaeocene. Neves and Watson (2004) suggested that *Bupleurum* originated somewhere in the western Mediterranean, which was the early branching clade (including *B. fruticosum*), but Chinese exemplars were omitted from their study.

Our study included a broad sampling of taxa representing all currently accepted ITS sequences in GenBank. The phylogenetic tree (Fig. 1) clearly shows cladeD which includes the only woody species from the western Mediterranean (*B. fruticosum*) as the most basal species with good support (PB: 100; PP: 1.00), except for *B. fruticosum* distributed in African and Mediterranean regions. *Bupleurum rigidum* subsp. *paniculatum* and *B. rigidum* subsp. *rigidum* were in the basal branch of cladeD with strong support (PB: 98; PP: 1.00), with distinct morphology, but the relationship with others remains unresolved. CladeC and cladeG (cladeA and cladeB, including all Chinese species and other *Bupleurum* species) diverge next, though the position of cladeA receives weak bootstrap support. Widely distributed in Europe, Africa and Asia, cladeC comprises *B. semicompositum* and *B. tenuissimum*. Most species in cladeD share a base chromosome number of $x = 8$.

Cauwet-Marc (1977) proposed the base number $x = 8$ as the primitive state in *Bupleurum*, and $x = 6, 7$ as derived characters. The most common number is $x = 6, 8$ in species distributed in China, rarely $x = 4, 7$ (Jiang et al., 2002; Jiang et al., 1994; Pan and Qin, 1981; Pan et al., 1985; Qin et al., 1989; Wang et al., 2008). Different species exhibit various levels of polyploidy. For example, *B. marginatum* is tetraploid ($2n = 24$) based on $x = 6$, and another tetraploid species, *B. bicaule* ($2n = 32$), is based on $x = 8$. *Bupleurum sibiricum* is octoploid ($2n = 64$) also based on $x = 8$. In accordance with previous results (Neves and Watson, 2004), our phylogenetic analyses of ITS sequences indicate that the western Mediterranean *Bupleurum* species with the basic chromosome

number (8→7→6) and then polyploidization may be the ancestral condition in the species in China. They may have migrated northward into China through the Middle East and the Caucasus. This conjecture is contrary to the hypothesis that China is a potential center of origin (Shu and Sheh, 2001).

INFRAGENERIC CLASSIFICATION OF CHINESE *BUPLEURUM* SPECIES

In Wolff's (1910) classification, Chinese *Bupleurum* species are divided into sect. *Isophyllum* and *Bupleurum* with fewer taxa. Other classifications of *Bupleurum* rarely include Chinese *Bupleurum* species.

The Chinese *Bupleurum* are divided into two subgenera and two sections, with insufficient sampling (Shu et al., 1998). The two subgenera comprise the monotypic subgenus *Longifolia* (*B. longiradiatum* only) and subgenus *Eubupleura*. *Eubupleura* is further divided into section *Ranunculoidea* with large and conspicuous bracteoles and section *Falcata* with small and narrow bracteoles. Shu's Chinese *Bupleurum* classification emphasizes numerical traits, so it creates artificial assemblages of species.

The genus *Bupleurum* is monophyletic and splits into two main clades (cladeD and cladeG) corresponding to two recognized subgenera: *Penninervia* and *Bupleurum* (Neves and Watson, 2004), with comprehensive sampling of Chinese taxa in our study (Fig. 1). The phylogenetic results of the study are not in agreement with previous classifications (Wolff, 1910; Shu et al., 1998), except for Neves and Watson's (2004) infrageneric classification of *Bupleurum*.

CladeA (including all Chinese *Bupleurum* species that are subdivided into clades E and F) corresponds to subgenus *Bupleurum* which typically has parallel-veined leaves. Those plants are shrubs, subshrubs, perennial or annual herbs with a worldwide distribution. An exception to all Chinese medicinal plants is *B. longiradiatum* in cladeF, with a thick rhizome containing toxic constituents, broad leaves, base cordate and clasping, pinnately veined, petal apices notched, stylopodium conic and long, mericarp subtrotund in cross section; it has thus been recognized as a distinct species in a monotypic subgenus. However, the relationship between *B. longiradiatum* and other Chinese species is uncertain in our studies. In our study, various species with different bracteole shapes, belonging to small and large types, were intermingled. Bracteole shape was found to be useful as a taxonomic trait (Fig. 1), but according to our work it cannot be used to differentiate sections.

We formally propose that Chinese *Bupleurum* species should be treated as subgenus *Bupleurum*.

The relationship between clades E and F is uncertain because of weak support. A complete reassessment of species relationships is required, through further systematic study of this intractable group.

SPECIES DELIMITATION OF *BUPLEURUM FALCATUM*

Some taxonomists suggested that *B. falcatum* is widely distributed not only in China but also in Central Asia and westwards to Southern Europe (Hiroe, 1952; Ohwi, 1965; Tutin, 1968), and *Bupleurum scorzonerifolium*, *B. gracillimum*, *B. chinense*, *B. komarovianum* and *B. bicaule* are treated as varieties of *B. falcatum* in China (Shan, 1940). Field (1994) treated *B. falcatum* as a single species distributed only in England.

Bupleurum falcatum from Europe possesses microchromosomes ($2n = 16$), but this species from China, Japan and the Korean Peninsula exhibits macrochromosomes and a different base chromosome number ($2n = 8, 12, 20, 24, 26, 32$) (Ohta, 1986, 1991, 2006; Jiang et al., 1994). Japanese researchers (Ohwi, 1965) held that *B. chinense* and *B. scorzonerifolium* from China were a synonym of *B. falcatum* from Japan. However, the dysploidy ($2n = 19\sim 40$) with a bimodal karyotype of *B. falcatum* from Japan ($2n = 26$, similar to the Wu Mu Li population from China) differs from *B. chinense* and *B. scorzonerifolium* (Jiang et al., 1994).

Our results showed considerable divergence of nrDNA ITS sequences (65 mutations, 10.89% divergence) between the European and Chinese populations currently assigned to *B. falcatum*. Their divergence is higher than species pairs. They are all nested in different clades in the phylogenetic trees. *Bupleurum falcatum* is widely distributed from Europe to Eurasia, and new forms in *B. falcatum* have spread across Eurasia. We further suggest that the *B. falcatum* populations from different regions should be separated as different species. There is a clear need for further intensive systematic study so that the distinguishing traits of the cryptic species presently placed under the name "*Bupleurum falcatum*" can be clarified.

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