Annals of Botany **93**: 379–398, 2004
doi:10.1093/aob/mch052, available online at www.aob.oupjournals.org

Phylogenetic Relationships in *Bupleurum* (Apiaceae) Based on Nuclear Ribosomal DNA ITS Sequence Data

SUSANA S. NEVES* and MARK F. WATSON

Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR, UK

Received: 25 June 2002 Returned for revision: 20 February 2003 Accepted: 8 January 2004 Published electronically: 23 February 2004

• *Backgroud and Aims* The genus *Bupleurum* has long been recognized as a natural group, but its infrageneric classification is controversial and has not yet been studied in the light of sequence data.
• *Methods* Phylogenetic relationships among 32 species (35 taxa) of the genus *Bupleurum* were investigated by comparative sequencing of the ITS region of the 18–26S nuclear ribosomal DNA repeat. Exemplar taxa from all currently accepted sections and subsections of the genus were included, along with outgroups from four other early branching Apioideae genera (*Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*).
• *Key Results* Phylogenies generated by maximum parsimony, maximum likelihood, and neighbour-joining methods show similar topologies, demonstrating monophyly of *Bupleurum* and the division of the genus into two major clades. This division is also supported by analysis of the 5.8S coding sequence alone. The first branching clade is formed by all the species of the genus with pinnate-reticulate veined leaves and *B. rigidum* with a unique type of leaf venation. The other major clade includes the remaining species studied, all of which have more or less parallel-veined leaves.
• *Conclusions* These phylogenetic results do not agree with any previous classifications of the genus. Molecular data also suggest that the endemic Macaronesian species *B. salicifolium* is a neoendemic, as the sequence divergence between the populations in Madeira and Canary Islands, and closer mainland relatives in north-west Africa is small. All endemic north-west African taxa are included in a single unresolved but well-supported clade, and the low nucleotide variation of ITS suggests a recent radiation within this group. The only southern hemisphere species, *B. mundii* (southern Africa), is shown to be a neoendemic, apparently closely related to *B. falcatum*, a Eurasian species.

Key words: *Anginon*, Apiaceae, Apioideae, *Bupleurum*, *Heteromorpha*, ITS, molecular phylogeny, nuclear ribosomal DNA, *Penninervia*, subgenus nov., systematics, Umbelliferae.

INTRODUCTION

Bupleurum L., with around 150 species, is one of the largest genera of the family Apiaceae (Umbelliferae). The genus includes annual and perennial species, from small herbs a few centimetres tall (e.g. *B. semicompositum* L., *B. ranunculoides* L.), to shrubs up to 3 m high (*B. fruticosum* L.). Woodiness in Apiaceae is rare, as woody species are found in only ten of the 400 or so genera in the family (Burtt, 1991). *Bupleurum* is morphologically diverse, but is easily distinguished within the family because of the almost unique feature of the leaves, which are simple and entire (*Hohenackeria* Fisch. & C.A.Mey. and *Nirarathamnos* Balf.f. also have simple and entire leaves, but are otherwise morphologically distinct from *Bupleurum*). All species in *Bupleurum* are totally glabrous; some have pinnate-reticulate veined leaves, but most have parallel-veined leaves (another unusual character in the family); flowers are generally yellow, sometimes purplish (only *B. album* Maire, from north-west Africa, has white flowers); involucral bracts are generally present; involucel bracts (bracteoles) are always present and sometimes conspicuous; mericarps are isodiametric or slightly laterally compressed, and are generally smooth, rarely tuberculate, with five thin or narrowly winged ridges.

Bupleurum is essentially a northern hemisphere genus, occurring in Europe, North Africa, Macaronesia (Canary Islands and Madeira), Asia and North America (a single native species, *B. americanum* Coult. & Rose, from the Rocky Mountains). There is a notable exception to this northern distribution: the southern African endemic *B. mundii* Cham. & Schltdl. Despite this broad distribution, most of the species are rather rare and restricted to small areas. In terms of morphology and taxon number, the highest diversity is found in the Mediterranean, where there are many endemics. *Bupleurum* spp. grow in a great variety of habitats: from sea level (*B. tenuissimum* L.) up to 4900 m (the Himalayan *B. longicaule* Wall. ex DC.); through saline marshlands, calcareous, granitic or basaltic soils; in arid or mesophytic areas; from open areas to dense forests; as weeds or ruderals; and from equatorial to nearly arctic latitudes (Nasir, 1955; Tutin, 1968; Heywood, 1971; Snogerup, 1972; Cauwet-Marc, 1976; Hara and Williams, 1979; Rechinger and Snogerup, 1987; Snogerup and Snogerup, 2001).

Bupleurum has for long been recognized as a natural group. In the 17th century, Tournefort (1694) had a concept of the genus that basically corresponds to its present delimitation. He included in *Bupleurum* species from all

* For correspondence. Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal. Fax (+351) 21 441 1277, e-mail sneves@itqb.unl.pt

TABLE 1. *Infrageneric classification of the genus* Bupleurum

Authors	*Bupleurum* infrageneric classification
Grenier and Godron (1848)	Sect. *Perfoliata*, sect. *Reticulata*, sect. *Aristata*, sect. *Nervosa*, sect. *Marginata*, sect. *Coriacea*. [The first use of section names with *Bupleurum*]
Boissier (1872)	Sect. *Perfoliata*, sect. *Glumacea*, sect. *Graminea*, sect. *Coriacea*
Briquet (1897)	Sect. *Perfoliata* (subsections: *Laevia*, *Rugosa*), sect. *Reticulata*, sect. *Eubupleura* (subsections: *Aristata*, *Juncea*, *Trachycarpa*, *Nervosa*)
Drude (1898)	Sect. *Perfoliata*, sect. *Reticulata*, sect. *Eubupleura* (subsections: *Aristata*, *Juncea*, *Trachycarpa*, *Nervosa*), sect. *Rigida*, sect. *Coriacea*
Calestani (1905)	Genus *Bupleurum*: sect. *Perfoliata*, sect. *Reticulata*, sect. *Glumacea*, sect. *Juncea*, sect. *Trachycarpa*, sect. *Nervosa*, sect. *Rigida*, sect. *Frutescentia*, sect. *Spinosa*, sect. *Coriacea*. Genus *Trachypleurum*
Wolff (1910)	Sect. *Perfoliata* (subsections: *Laevia*, *Rugosa*, *Lophocarpa*), sect. *Reticulata*, sect. *Longifolia*, sect. *Eubupleura* (subsections: *Glumacea*, *Juncea*, *Trachycarpa*, *Nervosa*, *Marginata*, *Rigida*), sect. *Coriacea*
Koso-Poljanski (1913)	Subgenus *Diatropa*: sect. *Laevia* (subsections: *Alophia*, *Lophocarpa*), sect. *Rugosa* (subsections: *Eurugosa*, *Granulata*) Subgenus *Bupleurotypus*: sect. *Eubupleurotypus* (subsections: *Plurivittata*, *Legitima*), sect. *Vittijugata*, sect. *Tenoria* (subsections: *Rigida*, *Coriacea*) Subgenus *Agostana*: sect. *Glumacea*, sect. *Graminea* (subsections: *Lejocarpa*, *Trachycarpa*)
Cearceau-Larrival (1962)	Section I, *Graminifolia*, section II [*Bupleurum*]
Tutin (1968)	Sect. *Bupleurum*, sect. *Reticulata*, sect. *Diaphyllum*, sect. *Isophyllum* (subsections: *Aristata*, *Juncea*, *Trachycarpa*, *Nervosa*, *Marginata*, *Rigida*), sect. *Coriacea*. [following Wolff (1910) but with nomenclatural changes]
Cauwet-Mark (1976)	Subgenus *Bupleurum*: sect. *Bupleurum* (subsections: *Laevia*, *Rugosa*), sect. *Bupleurotypus* (subsections: *Longiradiata*, *Nervosa*), sect. *Vittaejugata*, sect. *Agostana* (subsections: *Glumacea*, *Juncea*, *Trachycarpa*) Subgenus *Tenoria*: sect. *Tenoria* (subsections: *Tenoria*, *Rectinervia*), sect. *Tyrrhenaica* (subsections: *Mesatlantica*, *Acutifolia*)

currently accepted sections (*Bupleurum*, *Coriacea* Gren. & Godr., *Diaphyllum* (Hoffm.) Dumort., *Isophyllum* (Hoffm.) Dumort. and *Reticulata* Gren. & Godr.), and none from different genera (Neves, 2000). Later authors, including Linnaeus (1753) and Sprengel (1820), included species from other genera in *Bupleurum*, or split the genus into segregate genera such as *Buprestis* Spreng., *Diaphyllum* Hoffm., *Odontites* Spreng., *Perfoliata* Fourr., *Tenoria* Spreng., and others (see Pimenov and Leonov, 1993). The current circumscription of *Bupleurum* has remained constant for more than 150 years, with the exception of Calestani (1905) who resurrected the genus *Trachypleurum* Rchb., a clearly artificial group that included the few *Bupleurum* spp. with tuberculate fruits.

Traditionally *Bupleurum* has been placed in subfamily Apioideae, tribe Apieae, subtribe Apiinae (Heywood, 1971). However, Downie *et al*. (2000*b*) found strong molecular evidence showing the isolated position of *Bupleurum* within Apiaceae and concluded that *Bupleurum* should be included within the monogeneric tribe Bupleureae Spreng.

Table 1 summarizes the various infrageneric classifications proposed for *Bupleurum* since Grenier and Godron (1848) first used sections in the genus. Wolff (1910) published the first comprehensive revision of *Bupleurum*, and his classification is the most widely used today. Cauwet-Marc (1976) revised *Bupleurum* using phenetic and cladistic analyses of morphological characters (see also Roux *et al*., 1978). She proposed the subdivision of the genus into two subgenera: *Bupleurum* and *Tenoria* (Spreng.) Cauwet. However, the reliability of these results has been questioned as only a small number of characters were used and no characters were given to support the groups. In summary, there has been almost no agreement on the status or rank of the infrageneric groups within *Bupleurum*, with only sections *Bupleurum* ('*Perfoliata*') and *Coriacea* having some stability in terms of circumscription. The classifications of Koso-Poljansky (1913), Cerceau-Larrival (1962) and Cauwet-Marc (1976) are the most discordant, with Koso-Poljansky and Cauwet-Marc placing great emphasis on vegetative characters.

Systematic data available for Bupleurum

Many data have been gathered for *Bupleurum*, particularly in karyology (mainly chromosome numbers), pollen morphology, anatomy, phytochemistry and more lately molecular studies. Most of the karyological work in the genus was carried out by Cauwet-Marc (1976, 1979*a*, *b*); she recognized five basic chromosome numbers in *Bupleurum*: $x = 4$, 6, 7, 8 and 11; the most common being $x = 8$, followed by $x = 7$ ($x = 7$ or 8 are uncommon in Apiaceae, where the most frequent number is $x = 11$). Some species exhibit various levels of ploidy, e.g. *B. ranunculoides*, with diploid ($2n = 14$), triploid ($2n = 21$), tetraploid ($2n = 28$), and hexaploid ($2n = 42$) populations (Küpfer, 1969; Cauwet-Marc, 1970, 1979*b*). Cases of aneuploidy and dysploidy have been recorded, especially in north-west African taxa (Cauwet-Marc, 1979*b*) and in the Eurasian *B. falcatum* L. *sensu lato* (Ohta, 1991). Unfortunately, chromosome numbers alone have limited taxonomic value, especially when variation in number caused by aneuploidy and dysploidy appears common in certain species.

Pollen in *Bupleurum* is generally characterized as subrhomboidal, and of a 'primitive type' (Cerceau-Larrival,

1962, 1971; Cerceau-Larrival and Roland-Heydacker, 1978; Punt, 1984; Leonardis *et al*., 1997), and exhibits only minor variation across the many species studied.

Some anatomical work has been carried out in the genus, particularly on stems and fruits (Briquet, 1897; Panelatti, 1959; Cauwet-Marc, 1976; Arenas and García, 1993). In some cases, additional features for species delimitation were found, but further data are needed to understand the value of these characters in the study of species relationships.

Some *Bupleurum* species have been included in several chemical studies, in particular concerning the distribution of secondary metabolites (see Carbonnier and Cauwet-Marc, 1979, 1981). However, chemical information can be problematic as uncontrolled environmental factors can affect chemical production, as Berenbaum (1990) demonstrated for furacoumarins in Apiaceae.

Cauwet-Marc *et al*. (1978) undertook a multidisciplinary systematic study in *Bupleurum*, with data from morphology, anatomy, phytochemistry, karyology, palynology and phytodermology. However, the results are problematic as data interpretation was biased by Cauwet-Marc's classification (Cauwet-Marc, 1976), the study lacked a detailed account of the methods, and reasons for the proposed taxon associations were not discussed.

In recent years a few *Bupleurum* spp. have been included in molecular phylogenetic studies carried out on Apiaceae, using sequences of plastid genes (*rbcL*, *matK*), introns, *rpo*C1 and *rpl*16 (Kondo *et al*., 1996; Plunkett *et al*., 1996*a*, *b*, 1997; Downie *et al*., 1998), and chloroplast restriction site analysis (Plunkett and Downie, 1999, 2000). In all of these studies, *Bupleurum* consistently appears as an early branching clade within subfamily Apioideae. Choi *et al*. (1996) published the first ITS (internal transcribed spacers) sequences in *Bupleurum* for *B. euphorbiodes* Nakai, *B. komarovianum* O.A.Lincz., *B. longiradiatum* Turcz. and *B. scorzonerifolium* Willd. They also carried out an RFLP (restriction fragment length polymorphism) analysis of the same samples sequenced for ITS, and found similar taxon relationships. ITS sequences have also been obtained for *B. falcatum* (Lee and Rasmussen, 1998; Valiejo-Roman *et al*., 1998). The three *Bupleurum* spp. in the analysis of Lee and Rasmussen (1998) form a separated clade, but appear in a larger group including genera such as *Aciphylla* J.R.Forst. & G.Forst., *Daucus* L., *Cuminum* L., *Laserpitium* L. and *Thapsia* L. This contradicts the plastid DNA tree for the *rpo*C1 intron (Downie *et al*., 1998). In contrast Valiejo-Roman *et al*. (1998) showed *B. falcatum* in an early branching position within Apioideae, which agrees with the signal from plastid DNA. They also noted that the sequence of *B. falcatum* is highly divergent and that it could not be aligned unambiguously with the rest of the taxa examined. These studies have helped place *Bupleurum* within Apioideae; however, the number of taxa sequenced to date is small and not representative of the taxonomic, morphological, and geographical diversity in the genus. Therefore, a more extended sampling of the species is necessary to achieve a better understanding of the phylogenetic relationships in *Bupleurum*.

The present study sets out to provide more detailed phylogenetic resolution within the genus *Bupleurum*, with particular emphasis on taxa from south-west Europe and north-west Africa (a possible centre of origin for the genus). The increased sampling within the genus would also further test monophyly of *Bupleurum*. The ITS region of nuclear ribosomal DNA was chosen as it has been proved a very valuable source of phylogenetic information, particularly at the genus level and among related genera (Baldwin, 1992; Baldwin *et al*., 1995). The ITS region has also been successfully used in various studies in subfamily Apioideae (Downie and Katz-Downie, 1996; Downie *et al*., 1998, 2000*a*, *c*; Valiejo-Roman *et al*., 1998; Katz-Downie *et al*., 1999).

MATERIALS AND METHODS

Sampling and molecular methods

Bupleurum taxa (the ingroup), representing all currently accepted sections and subsections of the genus, were examined for sequence variation in the ITS region of nuclear rDNA (see Table 2). Authorities for taxa are also given in Table 2. Complete ITS and 5.8S sequences for 68 accessions representing 32 species (35 taxa) of *Bupleurum* and two species of *Anginon* Raf. are reported here for the first time. Details of origin of material, GenBank accession numbers, and location of herbarium vouchers are provided in Table 2. All material used was collected and identified, either from living plants in the wild (18 accessions) or cultivated (eight), or from herbarium specimens (42). The *Anginon* specimens used were identified following Allison and Van Wyk (1997). All fresh material was dried in silica gel upon collection following the protocol by Chase and Hills (1991).

Relationships of *Bupleurum* to other early branching genera in Apioideae are uncertain and a sister group is currently unknown. The ITS sequences of *Bupleurum* are highly divergent and difficult to align with other members of the family (Downie *et al*., 1998; Valiejo-Roman *et al*., 1998; Katz-Downie *et al*., 1999). As choice of outgroups used for tree rooting is critical in the generation of a tree topology (Watrous and Wheeler, 1981; Maddison *et al*., 1984), multiple taxa were selected as outgroups, including four other early branching Apioideae genera that have appeared more closely related to *Bupleurum* in previous molecular phylogenetic studies (Plunkett *et al*., 1996*a*, *b*; Downie *et al*., 1998). The outgroup taxa included: *Anginon difforme*, *A. paniculatum*, *Heteromorpha arborescens* (ITS1 and ITS2 sequences from Downie and Katz-Downie, 1996), *Physospermum cornubiense* (Downie *et al*., 1998) and *Pleurospermum foetens* (Katz-Downie *et al*., 1999). For analysis of the 5.8S coding region alone, the two *Anginon* spp. were used as the outgroup (5.8S has not been sequenced for the other outgroup taxa). In spite of possible close relationships, all the outgroup genera used here are clearly distinct from *Bupleurum*, in both morphological and molecular characters, and so there seems to be no risk that any one of them is nested within *Bupleurum*.

Fresh or herbarium material (generally leaves, sometimes stems, flowers or fruits) were used for total DNA extraction using a modified CTAB (cetyltrimethyl-ammonium brom-

TABLE 2. *Accessions of* Bupleurum *and outgroup taxa examined for nuclear rDNA ITS sequence variation*

Taxon*	Section/subsection of *Bupleurum*†	Source and voucher	GenBank accession number
Anginon difforme (L.) B.L.Burtt	n/a	South Africa, Calvinia, Hantam Mountains, Farm Vanrhynshoek, 21.viii.1990, All Batten AB 1018 (E)	AF459742
Anginon paniculatum (Thunb.) B.L.Burtt	n/a	South Africa, 'Central ridge' above Clanwilliam, dam 5 km from Clanwilliam, 18.ii.1985, H.C. Taylor 11271 (E)	AF467922
Bupleurum acutifolium Boiss. [1]	*Isophyllum/Rigida*	Spain, Málaga, Sierra Bermeja, Peñas Blancas to Los Reales, 05.ix.1997, S.S. Neves 64 (E)	AF467925
B. acutifolium [2]	*Isophyllum/Rigida*	Spain, Málaga, Sierra Bermeja, Estepona to Puerto de Peñas Blancas, 05.ix.1997, S.S. Neves 65 (E)	AF467926
B. acutifolium [3]	*Isophyllum/Rigida*	Spain, Málaga, Tolóx, Sierra Parda, Majada Redonda, 06.v.1994, A. Pérez-Latorre *et al.* (MGC 37708)	AF467927
B. acutifolium [4]	*Isophyllum/Rigida*	Portugal, Baixo Alentejo, Serra do Cercal, Vila Nova de Milfontes to Cercal, 16.ix.1996, S.S. Neves 24 (E)	AF467923
B. acutifolium [5]	*Isophyllum/Rigida*	Portugal, Baixo Alentejo, S. Luis, Serra de S. Domingos, 08.viii.1997, S.S. Neves 27 (E)	AF467924
B. album Maire	(*Isophyllum/Rigida*)	Morocco, Anti-Atlas, 4 km from Igherm, road to Taliouine, 10.vi.1974, Reading University/BM Expedition 532 (RNG)	AF467928
B. angulosum L.	*Reticulata*	Pyrenees. Cultivated, Royal Botanic Garden Edinburgh, UK; RBGE Acc. No. 19861043, 20.viii.1999, S.S. Neves (E)	AF469008
B. balansae Boiss. & Reut. [1]	*Isophyllum/Rigida*	Morocco, Oujda, Al'Youn, Machra, Homadi, Oued Senara, 09.vi.1993, J. Molero *et al.* JMM-3198/5 (RNG)	AF469680
B. balansae [2]	*Isophyllum/Rigida*	Morocco, c. 35 km S of Tetouan, along main road to Chechaouen, near Souk-el-Arba-des-Beni-Hassan, 18.vi.1987, S.L. Jury *et al.* 8338 (RNG)	AF469681
B. baldense Turra	*Isophyllum/Aristata*	Spain, Guadalajara, Checa, river Cabrillas, road from Checa to Orea, 21.vi.1995, M.A. Carrasco *et al.* (MA 558704)	AF469682
B. barceloi Coss. ex Willk.	*Isophyllum/Rigida*	Balearic Islands (Spain), Mallorca, Sóller, 'Barranco' [cliff] 25.vii.1989, J. Orell Casasnovas (MA 474781)	AF477023
B. benoistii Litard. & Maire [1]	(*Isophyllum/Nervosa*)	Morocco, High Atlas, S from Marrakech, N end of Jbel Oikaïmeden near azib in ski resort, 29.vii.1997, S.L. Jury *et al.* 18375 (E)	AF477024
B. benoistii [2]	(*Isophyllum/ Nervosa*)	Morocco, 72 km S from Marrakech, Oukaïmeden, 03.vii.1987, S.L. Jury *et al.* 8858 (RNG)	AF477025
B. benoistii [3]	(*Isophyllum/Nervosa*)	Morocco, prov. of Ksar el Souk, N High Atlas, Tizi n'Tirrecht, N of Ari n'Ayachi, near Midelt, 21.vii.1966, R.M. & A.M. Harley 766 (BM)	AF477026
B. canescens Schousb.	*Isophyllum/Rigida*	Morocco, Immouzer Valley, N of Agadir, 28.iii.1972, D. Bramwell *et al.* 265 (RNG)	AF477027
B. canescens var. *handiense* Bolle [1] [syn. *B. handiense* (Bolle) G.Kunkel]	*Isophyllum/Rigida*	Canary Islands (Spain). Cultivated, Jardim Botânico de Coimbra, Portugal; seed received from the Jardín Botánico 'Viera y Clavijo', Canary Islands, from wild sources; 05.viii.2000, S.S. Neves Acc. No. 28 (COI)	AF477028
B. canescens var. *handiense* [2]	*Isophyllum/Rigida*	Canary Islands (Spain), Lanzarote, Peñas de Chache, cliffs above Famara, 15.v.1969, D. Bramwell 1631 (E)	AF477029
B. dumosum Coss.	*Isophyllum/Rigida*	Morocco, c. 9·5 km NNE of Asni, 5 km SSW of Tahanaoute, Gorge de Moulay Brahim, 15.iii.1994, S.L. Jury *et al.* 14157 (RNG)	AF477030
B. falcatum L.	*Isophyllum/Nervosa*	Spain, Alava, Lagrán, Sierra de Cantabria, rocky crests of Recilla, 15.viii.1992, J.A. Alejandre 733/92 (MA 534085)	AF479290
B. fruticescens L. subsp. *fruticescens* [1]	*Isophyllum/Rigida*	Spain, Huesca, between Baldellou and Camporrels, 30.ix.1987, G. Montserrat (MA 515853)	AF479291
B. fruticescens subsp. *fruticescens* [2]	*Isophyllum/Rigida*	Spain, Murcia, Sierra de Espuña, on road to 'Centro de Interpretación', 24.viii.1997, S.S. Neves 52 (E)	AF479292
B. fruticescens L. subsp. *spinosum* (Gouan) O.Bolòs & Vigo [1]	*Isophyllum/Rigida*	Spain, Granada, Sierra Nevada, Monachil valley, high mountain, 16.viii.1997, S.S. Neves 42 (E)	AF479293
B. fruticescens subsp. *spinosum* [2]	*Isophyllum/Rigida*	Spain, Cádiz, Zahara to Grazalema, margins of road CA-531, 22.viii.1997, S.S. Neves 47 (E)	AF479294
B. fruticescens subsp. *spinosum* [3]	*Isophyllum/Rigida*	Morocco, High Atlas, S from Marrakech, ski resort of Oukaïmeden, 27.vii.1997, S.L. Jury *et al.* 18297 (E)	AF479295
B. fruticescens subsp. *spinosum* [4]	*Isophyllum/Rigida*	Morocco, W Rif, Chefchaouene, Djebel Bouhalla to Djebel Lakraa, 23.vii.1995, M.A. Mateos *et al.* 6988/95 (RNG)	AF479296
B. fruticosum L. [1]	*Coriacea*	Portugal, Estremadura, Serra da Arrábida, on road from Aldeia de Irmaõs to Casais da Serra, 10.viii.1997, S.S. Neves 33 (E)	AF479297
B. fruticosum [2]	*Coriacea*	Spain, Málaga, Sierra Bermeja, road Ronda to Puerto de Alijar, 15.viii.1997, S.S. Neves 41 (E)	AF479298

TABLE 2. *Continued*

Taxon*	Section/subsection of *Bupleurum*†	Source and voucher	GenBank accession number
B.gerardii All. [1]	*Isophyllum/Juncea*	Europe. Cultivated, Jardim Botânico de Coimbra, Portugal; seeds obtained from the Jardin Botanique National de Belgique, Meise; 03.viii.1994, S.S. Neves Acc. No. 17a (E)	AF479847
B. gerardii [2]	*Isophyllum/Juncea*	Portugal, Beira Litoral, Fátima, Valinhos, area of 'Via Sacra', 26.vi.1994, S.S. Neves 1 (E)	AF479848
B. gerardii [3]	*Isophyllum/Juncea*	Spain, Madrid, 'embalse' [dam] of Santillana, Cerro Casal, 10.vii.1981, G. Navarro *et al.* (MA 310732)	AF479849
B. gerardii [4]	*Isophyllum/Juncea*	Spain, Granada, Lobras, Barranco de los Lagartos, 07.v.1980, J. Molero Mesa (MA 214590)	AF479850
B. gibraltarium Lam. [1]	*Coriacea*	Spain, Sevilla, near Coripe, road to Algodonales, slopes bordering river Guadalporcún, 15.viii.1997, S.S. Neves 35 (E)	AF479851
B. gibraltarium [2]	*Coriacea*	Spain, Murcia, Sierra de Espuña, on road to 'Centro de Interpretación', 24.viii.1997, S.S. Neves 51 (E)	AF479852
B. lancifolium Hornem.	*Bupleurum/Bupleurum*	Morocco, Tanger, SW of Chefchaouen, 2·3 km up road to Mokrissèt from Pont du Loukos, 21.iv.1995, S.L. Jury *et al.* 16552 (RNG)	AF479853
B. lateriflorum Coss. ex H.Wolff [1]	*Isophyllum/Rigida*	Morocco, High Atlas, S from Marrakech, 4 km below Oukaïmeden, on road to Vallée de l'Ourika, 28.vii.1997, S.L. Jury *et al.* 18323 (E)	AF479854
B. lateriflorum [2]	*Isophyllum/Rigida*	Morocco, High Atlas, Tizi-n-Test Pass, 02.x.1991, M. Ait Lafkih *et al.* 4939 (E)	AF479855
B. longifolium L.	*Diaphyllum*	Germany, Baviera, Oberpfalz, Kreis Regensburg, 0·3 km WSW from Pentling, N of road from Pentling to Weichsmühl, margins of river Donau, 17.vii.1993, H. Förther 7503 (MAF 149194)	AF479856
B. montanum Coss. [1]	*Isophyllum/Rigida*	Morocco, Chefchaouen (area 7), between Ketama and Bab-Berret, 03.xi.1993, P. García Murillo *et al.* ST 251/93 (SEV)	AF479857
B. montanum [2]	*Isophyllum/Rigida*	Morocco, High Atlas, just above El-Ksiba, along road to Imilchil, 05.vii.1997, S.L. Jury 17456a (E)	AF479858
B. montanum [3]	*Isophyllum/Rigida*	Morocco, Checfchaouen (area 2), Djebel Tassaot, 22.vii.1995, M.A. Mateo*s et al.* 6914/95 (SEV)	AF479859
B. mundii Cham. & Schltdl.	*Isophyllum/Nervosa*	South Africa, Natal. Cultivated, Royal Botanic Garden Edinburgh, UK; RBGE Acc. No. 19972669; 29.xi.1999, S.S. Neves (E)	AF479860
B. odontites L.	*Isophyllum/Aristata*	Tunisia, Kroumirie, c. 14 km N from Jendouba (Souk el Arba) to Ain Draham, S of Tabarka, 11.v.1975, Davis & Lamond D57628 (RNG)	AF479861
B. oligactis Boiss. [1] [syn. *B. atlanticum* Murb.]	*Isophyllum/Rigida*	Morocco, High Atlas, 111 km N from Errachidia (Ksar-es-Souk) along P21 road to Midelt, a few kms S of Tizi-m-Tairhemt, 12.vii.1987, S.L. Jury *et al.* 9240 (SEV 127166)	AF479862
B. oligactis [2]	*Isophyllum/Rigida*	Morocco, High Atlas, about 3 km above Imilchil, on road to lake Tizlite, along El-Ksiba to Imilchil road, 07.vii.1997, S.L. Jury 17603 (E)	AF479864
B. oligactis [3]	*Isophyllum/Rigida*	Morocco, Middle Atlas, road from El-Ksiba to Imilchil, c. 9 km N from Tizi-n-Islay, 05.vii.1997, S.L. Jury 17516 (E)	AF479863
B. plantagineum Desf.	*Isophyllum/Rigida*	Algeria, K2, Cap Carbon, near Bejaïa (Bougie), 29.v.1991, Davis 52959 (RNG)	AF479865
B. praealtum L. [1]	*Isophyllum/Juncea*	Spain, Lérida, Valle de Boí, 2 km from Barruera to Pont de Suert, 23.viii.1987, C. Aedo *et al.* 286–87 ML (MA 449973)	AF480938
B. praealtum [2]	*Isophyllum/Juncea*	Spain, Huesca, San Juan de Plan, near 'ermita' [hermitage] of San Mamés, 01.viii.1981, P. Montserrat *et al.* (JACA 191581)	AF480939
B. praealtum [3]	*Isophyllum/Juncea*	Spain, Teruel, Loscos, Piedrahita, river Noguera, 13.viii.1995, C. Fabregat & Lopez Udias (JACA 683295)	AF481390
B. praealtum [4]	*Isophyllum/Juncea*	Spain, Salamanca, Montemayor del Rio, 02.viii.1983, J.L. Fernández Alonso & A. Guillen (MA 518939)	AF481391
B. ranunculoides L. [1]	*Isophyllum Nervosa*	Europe. Cultivated, Jardim Botânico de Coimbra, Portugal; seed obtained from the Botanischer Garten Tübingen, Germany; 11.iv.1995, S.S. Neves Acc. No. 43 (E – photo)	AF481392
B. ranunculoides [2]	*Isophyllum/Nervosa*	Europe. Cultivated, Royal Botanic Garden Edinburgh, UK; seed obtained from the Botanischer Garten der Universität Postdam, Germany; RBGE No. 19972161, 20.vii.1999, S.S. Neves (E)	AF481393
B. ranunculoides [3]	*Isophyllum/Nervosa*	Spain, Burgos, Rebolledo de la Torre, Peña Castro, 24.vi.1990, J.A. Alejandre 1078/90 (MA 493699)	AF481394
B. ranunculoides [4] [syn. *B. bourgaei* Boiss. & Reut.]	*Isophyllum/Nervosa*	Spain, Jaén, Pontones, Sierra de Banderillas, 10.viii.1982, C. Soriano (MA 462383)	AF481395

TABLE 2. *Continued*

Taxon*	Section/subsection of *Bupleurum*†	Source and voucher	GenBank accession number
B. rigidum L. subsp. *rigidum* [1]	*Isophyllum/Marginata*	Spain, Murcia, Sierra de Espuña, picnic area near 'Centro de Interpretación', 24.viii.1997, S.S. Neves 53 (E)	AF481396
B. rigidum subsp. *rigidum* [2]	*Isophyllum/Marginata*	Spain, Málaga, Sierra Bermeja, on road from Estepona to Puerto de Peñas Blancas, 05.ix.1997, S.S. Neves 63 (E).	AF481397
B. rigidum L. subsp. *paniculatum* (Brot.) H.Wolff [1]	*Isophyllum/Marginata*	Portugal, Beira Litoral, Coimbra, Quinta da Sapata, near Santa Clara, 27.vi.1994, F. Sales & S.S. Neves 3a (E)	AF481398
B. rigidum subsp. *paniculatum* [2]	*Isophyllum/Marginata*	Portugal, Estremadura, Serra da Arrábida, Vale da Rasca, on road to the 'Secil' factory, 10.viii.1997, S.S. Neves 31 (E)	AF481399
B. rotundifolium L.	*Bupleurum/ Bupleurum*	Europe. Cultivated, Jardim Botânico de Coimbra, Portugal; seed obtained from the Botanischer Garten St. Gallen, Switzerland, 03.viii.1994, S.S. Neves Acc. No. 4 (E)	AF481400
B. salicifolium R.Br. ex Buch [1]	*Isophyllum/Rigida*	Madeira (Portugal), between Pico do Arieiro and Pico Ruivo, 29.xi.1989, L. Chilton & N.J. Turland 135 (BM)	AF481926
B. salicifolium [2]	*Isophyllum/Rigida*	Canary Islands (Spain). Cultivated, Jardim Botânico de Coimbra, Portugal; seed received from the Jardín Botánico 'Viera y Clavijo', Canary Islands, from wild sources; 05.viii.2000, S.S. Neves Acc. No. 29 (COI)	AF481927
B. salicifolium [3]	*Isophyllum/Rigida*	Canary Islands (Spain), Gran Canaria, Cruz de Tejeda, between Tejeda and Roque Rublo, 19.vi.1995, M.F. Gardner & S.G. Knees 5750 (E)	AF481928
B. semicompositum L.	*Isophyllum/Trachycarpa*	Spain, Ciudad Real, Daimiel, Tablas de Daimiel, Isla del Morenillo, 12.v.1992, S. Cirujano (MA 552469)	AF481929
B. stellatum L.	*Reticulata*	Switzerland, Canton du Valais, Col du Simplon, 'sous la statue de l'Aigle', 10.viii.1988, B. de Retz 88690 (MAF 145370)	AF481930
B. subspinosum Maire & Weiller	(*Isophyllum/Rigida*)	Morocco, High Atlas, S slope of Jbel Angour, 21.vii.1976, C.J. & A.R. Humphries 99 (BM)	AF481931
B. tenuissimum L.	*Isophyllum/Trachycarpa*	Portugal, Beira Litoral, Figueira da Foz, Vila Verde, 16.ix.1994, S.S. Neves 22 (E)	AF481932
Heteromorpha arborescens (Spreng.) Cham. & Schltdl.	n/a	S/C Africa. Cultivated, University of Illinois at Urbana-Champaign, USA, S. Downie 42 (ILL) – Sequences from Downie and Katz-Downie (1996)	U27578 (ITS1) U30314 (ITS2)
Physospermum cornubiense (L.) DC.	n/a	Ukraine, Crimea, Alikat-Bogaz Pass. Cultivated, Moscow State University Botanical Garden, Russia; Pimenov & Tomkovich *s.n.* (MW) – Sequences from Downie *et al.* (1998)	U78382 (ITS1) U78442 (ITS2)
Pleurospermum foetens Franch.	n/a	China, Yunnan. Cultivated, Royal Botanic Garden Edinburgh, UK; RBGE Acc. No. 19910914, Chungtien, Lijiang & Dali Expedition (1990) 1181 (E) – Sequences from Katz-Downie *et al.* (1999)	AF008604 (ITS1) AF009083 (ITS2)

Sequences of *Heteromorpha*, *Physospermum* and *Pleurospermum* were obtained from the literature. Herbarium acronyms provided according to Holmgren *et al.* (1990). Some herbaria have their own accession numbers of specimens; in such cases this number is given after herbarium abbreviation. Author name abbreviation follows Brummit and Powell (1992).

* Numbers in square brackets are used in Figs 1–3 and correspond to the different samples of a single species or subspecies.

† Classification essentially follows Wolff (1910), with minor corrections (nomenclature of sections) introduced by Tutin (1968). For species described after Wolff's publication, section and subsection names (placed in brackets) are those suggested by the species authors.

ide) method from Doyle and Doyle (1987) with no additional cleaning. In a few cases where DNA extraction or PCR (polymerase chain reaction) amplification was problematic, the DNeasy Plant Mini Kit (QIAGEN Ltd, Crawley, West Sussex, UK) was used.

Double-stranded DNA of the entire ITS region (covering ITS1, 5.8S and ITS2) was amplified with primers 'ITS 5P' (Möller and Cronk, 1997; the same as 'modified ITS 5' in Downie and Katz-Downie, 1996) and 'ITS 4' (White *et al.*, 1990) in an equimolar ratio and 1·5 mM $MgCl_2$. The PCR profile followed Möller and Cronk (1997). In a few cases when PCR product was in low concentration, PCR amplification from original DNA extract was repeated with 40 cycles. Successful PCR amplification produced a single DNA band of approx. 700 bp long on a 1·5 % agarose gel. PCR products were subsequently purified using the QIAquick PCR Purification Kit (QIAGEN). DNA concentration of purified PCR products was estimated on gel by comparison to a ladder of known concentration (we used the 123 bp DNA ladder from Sigma, St Louis, MO, USA).

Purified PCR products were sequenced using the primer strategy of Möller and Cronk (1997) and the dRhodamine Terminator Cycle Sequencing Kit (ABI Prism™; Perkin Elmer Applied Biosystems, Warrington, Cheshire, UK), with Ampli Taq® DNA polymerase FS. Sequence fragments were analysed on an ABI Prism 377 DNA automated sequencer, following the manufacturer's instructions. For each sampled specimen, forward and reverse sequencing reactions were performed for sequence confirmation.

Sequence fragments obtained for each sample were inspected and edited using Factura™, version 1.2.Or6 (Applied Biosystems, Foster City, CA, USA), and later

assembled using the Clustal option of multiple alignment in Sequence Navigator™, version 1.0.1 (Applied Biosystems). Ambiguous positions were verified against the original electropherograms. The complete sequence of the ITS region for each sampled specimen was then stored as a separated text file, and later deposited with GenBank (see accession numbers in Table 2). Sequence boundaries of ITS1, 5.8S and ITS2 were determined following Yokota *et al*. (1989).

Clustal X, version 1.8 (Thompson *et al*., 1997), was used for multiple alignment of complete sequences. SeqPup (version 0.6f, 1995, D. G. Gilbert, University of Indiana, USA) was used for final manual editing on the alignment and for storage of multiple sequence files; aligned sequences can be obtained from the authors.

Pairwise distances (sequence divergence) between taxa and base frequencies (G + C content) were determined using PAUP* (Swofford, 2000). Transition : transversion ratios (Ti : Tv) over a subset of the maximally parsimonious trees were calculated using MacClade version 3.05 (Maddison and Maddison, 1992).

Phylogenetic analysis

Phylogenetic analyses were performed using PAUP*, versions 4.0b4 and 4.0b8. Characters were unordered and equally weighted ('Fitch parsimony'; Fitch, 1971). Gaps were treated as missing data and multistate data interpreted as uncertainty. Maximally parsimonious (MP) trees were sought using the heuristic search option with starting tree obtained by stepwise addition, 1000 random-addition replicates, TBR (tree bisection–reconnection) branch-swapping, 'MultTrees' (= MultPars) and steepest descent options in effect, with ACCTRAN optimization. Independent parsimony analysis of the 5.8S coding region was also performed. To evaluate clade support, bootstrap (Felsenstein, 1985) and jackknife (Lanyon, 1985) analyses were performed in PAUP* using a full heuristic search with 1000 replicates, simple addition sequence, and TBR branch-swapping. To reduce computational time, no more than 10 000 or 1000 trees were saved per replicate for bootstrap or jackknife, respectively. As a measure of clade support based on the original data without perturbation, decay indices (Bremer, 1988, 1994; Donoghue *et al*., 1992) were obtained using AutoDecay (Eriksson, 1999), version 4.0.2, and the reverse constrain option in PAUP*, performing a heuristic search for each node of the MP strict consensus tree, with random-addition of 100 replicates, TBR branch-swapping, and a limit of 10 000 trees per replicate. Measures of character fit to the phylogenetic trees, such as the consistency index (CI) (Kluge and Farris, 1969), the retention index (RI) and the rescaled consistency index (RC) (Farris, 1989), were also calculated using PAUP*.

Maximum likelihood (ML) analyses were performed using PAUP*, with settings corresponding to the Hasegawa–Kishino–Yano model, HKY85 (Hasegawa *et al*., 1985), and using gamma distribution (Yang, 1993, 1996). The Rogers–Swofford approximation method (Rogers and Swofford, 1998) was used to calculate initial branch lengths. Starting likelihood parameters (Ti : Tv ratio, nucleotide frequencies, proportion of invariable sites, and shape parameter of gamma distribution) were estimated from an initial set of trees from the MP analysis; empirical values of nucleotide frequencies were also alternatively used. Likelihood scores were used to perform ML heuristic searches with stepwise addition, addition sequence 'as is' or random (ten replicates), TBR branch-swapping, and 'MultTrees' option in effect. Likelihood scores were estimated for each of the ML trees found and these new parameters used for further searches.

Distance trees were obtained from neighbour-joining (NJ) analyses (Saitou and Nei, 1987) using three of the distance measures available in PAUP*: Jukes and Cantor (1969), Kimura's two parameter (K2P) (Kimura, 1980), and HKY85 models. For K2P and HKY85, substitution rates were also assumed to follow gamma distribution (shape parameter set at various values). Neighbour-joining bootstrap values were calculated from 10 000 replicates.

RESULTS

Sequence analysis

Of the 71 accessions examined (68 new sequences), only 61 were included in the final analyses, because some of the sequences obtained from the same taxon/population were identical, and thus only one sequence was used for each of these. This is represented in Figs 1–3 as more than one number appearing after the species names in the trees. Identical sequences were obtained from different samples in *B. benoistii* (two identical out of three), *B. fruticosum*, *B. gibraltarium*, *B. oligactis* (two out of three), and in the two distinct populations of *B. acutifolium* from Spain (three samples with identical sequences) and Portugal (two identical sequences). However, the Spanish and Portuguese populations of *B. acutifolium* produced considerably different sequences (15 mutations), the latter population including a 35 bp deletion in ITS1 (37 bp indel when aligned with all sequences). Some other samples for a single species showed little variation (1–3 bp), but all of these sequences were used in the analyses (e.g. the three samples of *B. montanum*). Sequences obtained for *B. fruticescens* subsp. *fruticescens* (endemic to Spain) and subsp. *spinosum* (Spanish samples) were all identical (north-west African samples of '*B. spinosum*' showed several differences). Identical sequences were also obtained for taxa that are morphologically distinct species (e.g. *B. benoistii* and *B. lateriflorum*), and little variation was found between several of the north-west African endemic taxa (e.g. *B. benoistii*, *B. lateriflorum*, *B. montanum* and *B. plantagineum*).

The primers used were perfect matches to the sequences in *Bupleurum*, with the exception of primer 'ITS 2G', which sequence should have been: 5′-GTG ACA CCC AGG CAG ACG T-3′. Alignment of all sequences resulted in a matrix of 646 positions, including ITS1, 5.8S and ITS2. Characteristics of these sequences, including length, G + C content, number of constant, autapomorphic and parsimony-informative characters are summarized in Table 3. ITS1 ranged from 172 bp (*Anginon difforme* and

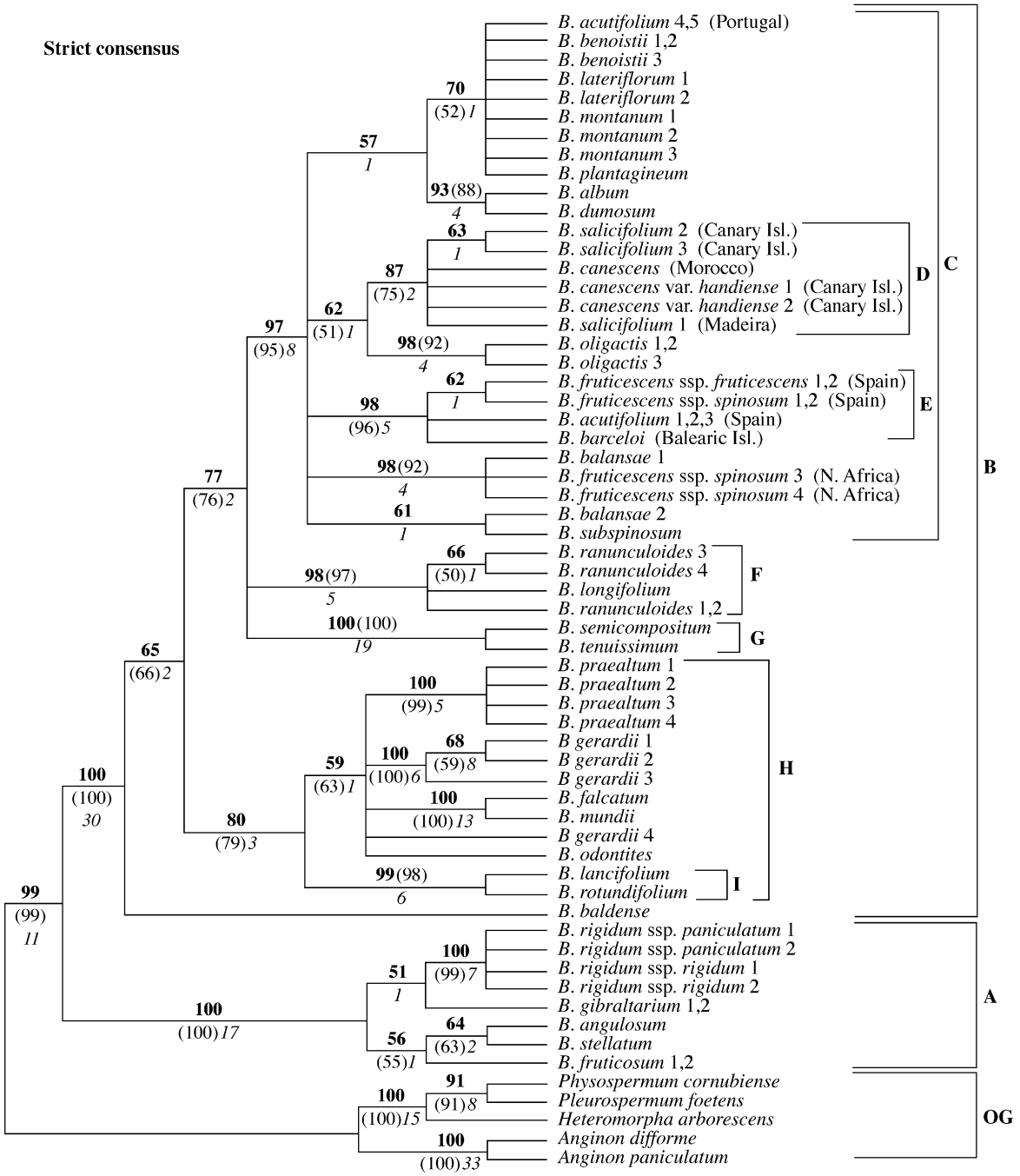

FIG. 1. Strict consensus tree of 128 most-parsimonious trees of 772 steps derived from equally weighted maximum parsimony analysis of sequences of the ITS region of *Bupleurum* and outgroup (OG) taxa (CI = 0·58; RI = 0·85). Bootstrap percentages appear above the branches (boldface); jackknife percentages are given in brackets; values <50 % are not indicated. Decay indices are below the branches in italics. Clade A = subgenus *Penninervia*; clade B = subgenus *Bupleurum*; clade C = the 'NW African' group; clade D = 'Macaronesian' group; clade E = the 'Iberian and Balearic' group; clade F = the '*Ranunculoides*' group; clade G = the '*Trachycarpa*' group; clade I = the '*Perfoliata*' group.

A. paniculatum) to 217 bp (*Bupleurum rigidum* and *Physospermum cornubiense*), and ITS2 from 215 bp (*Pleurospermum foetens*) to 234 bp (*Anginon paniculatum*). The total length of ITS1 + ITS2 ranges from 404 bp (*Bupleurum acutifolium*, Portuguese population, and *Anginon difforme*) to 447 bp (*Bupleurum angulosum*). The 5.8S is 164 bp long in all taxa. ITS1 is slightly shorter and more variable in length than ITS2. Major gaps (indels (insertions/deletions): a 37 bp long indel in *B. acutifolium*, from Portugal, and a 44 bp indel in *Anginon*) occur in a GC-rich region that is located between two broadly conserved sequence motifs ('c1' and 'c2') of ITS1 (Hershkovitz *et al.*,

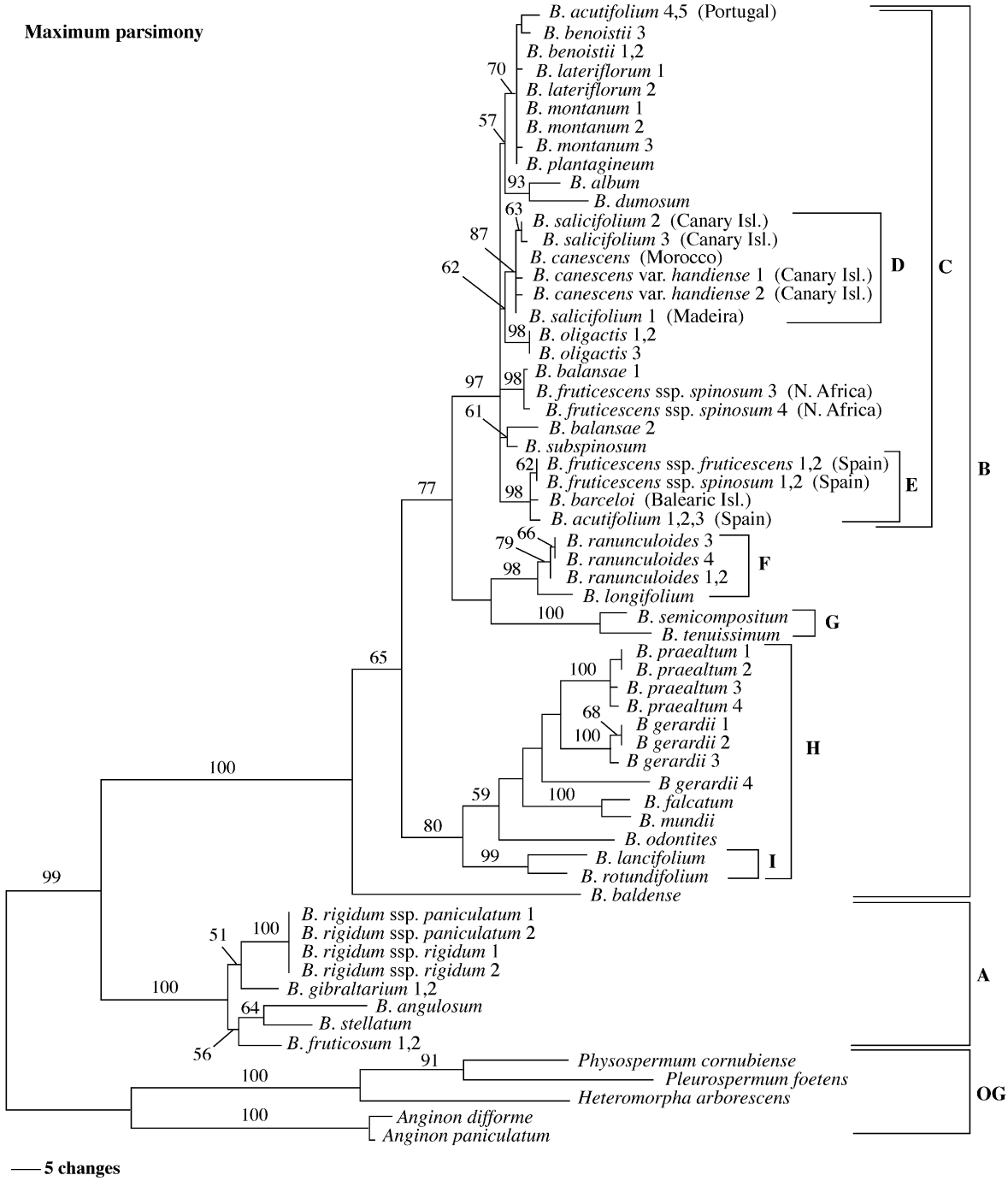

FIG. 2. One of 128 most-parsimonious trees of 772 steps derived from equally weighted maximum parsimony analysis of sequences of the ITS region of *Bupleurum* and outgroup (OG) taxa (CI = 0·58; RI = 0·85). Branch lengths are proportional to the number of substitutions (see scale bar). Numbers represent bootstrap values; values <50 % are not indicated. The letters A–I represent the same *Bupleurum* groups referred to in Fig. 1.

1999; Hershkovitz and Zimmer, 2000). A region of 46 bp at the start of ITS2 was excluded from further analysis, because an unambiguous alignment could not be obtained. As expected, the 5.8S gene showed little variation, with a maximum of 6·1 % divergence across all taxa (4·9 % in *Bupleurum*). The overall of ITS divergence was approx. 29 % within *Bupleurum.*

Phylogenetic analysis

Parsimony analysis of 600 characters, including ITS1 and ITS2 (46 characters excluded in the latter) and the 5.8S region, resulted in 128 most-parsimonious (MP) trees, each 772 steps long, consistency index (CI) 0·58 (excluding uninformative characters) and retention index (RI) of 0·85.

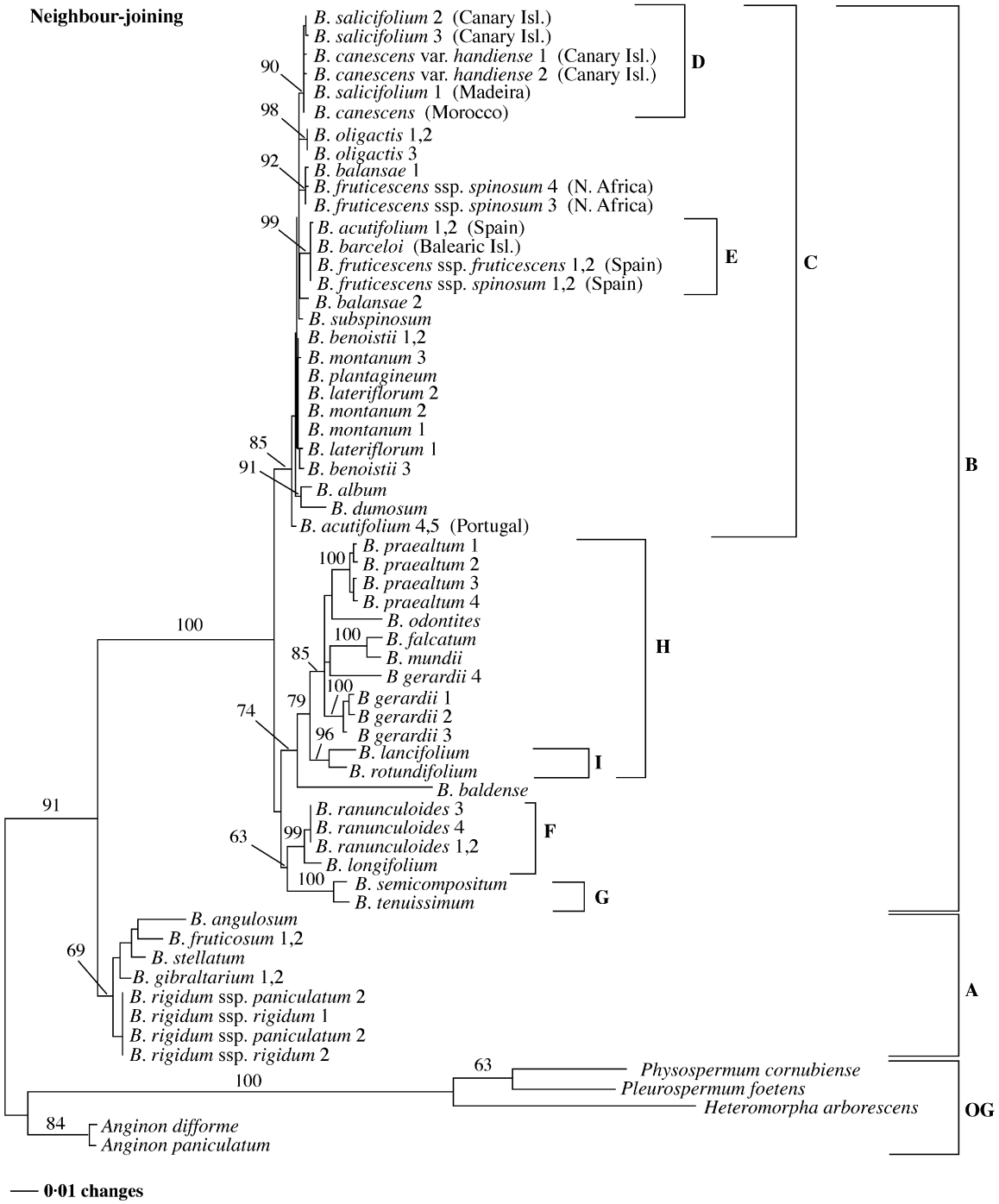

FIG. 3. Single tree obtained from neighbour-joining (NJ) analysis of substitution rates on sequences of the ITS region of *Bupleurum* and outgroup (OG) taxa. Branch lengths are proportional to distances (see scale bar) estimated from the two-parameter method of Kimura (1980), with substitution rates assumed to follow gamma distribution ($\propto$ = 0·5). Numbers above branches are NJ bootstrap estimates; values <50 % are not indicated. The letters A–I represent the same *Bupleurum* groups referred to in Fig. 1.

The strict consensus of these trees, with accompanying bootstrap, jackknife and decay values, is shown in Fig. 1. One of the most-parsimonious trees is presented in Fig. 2. In all these trees, *Bupleurum* forms a strongly supported clade (A plus B), branch length 31 steps, with 99 % bootstrap and jackknife support, and a decay value of 11. The *Bupleurum* clade is then subdivided into two major clades (A and B), both with 100 % bootstrap and jackknife support and high decay values (17 for clade A, and 30 for clade B); there are 27 and 48 bp substitutions supporting, respectively, clades A and B. The relationships within the two major *Bupleurum* clades are not fully resolved, but some of the clades are

TABLE 3. *Sequence characteristics of the internal transcribed spacers, ITS1 and ITS2, and of the 5.8S subunit of nuclear rDNA of* Bupleurum *and outgroup genera (*Anginon, Heteromorpha, Physospermum *and* Pleurospermum*)*

Sequence characteristics	ITS1	ITS2	5.8S*
Length range in all taxa (bp)	172–217	215–234	164
Length range in *Bupleurum* (bp)	180–217	222–231	164
Mean length in *Bupleurum* (bp)	213·9	225·5	164
Length range in outgroup (bp)	172–217	215–234	164
Mean length in outgroup (bp)	198	224·6	164
Aligned length (bp)	229	253	164
G + C content range (mean) in *Bupleurum* (%)	53·9–65·4 (60·7)	53·9–69·4 (61·4)	53·1–54·9 (54·1)
G + C content range (mean) in all taxa (%)	50·2–69·2 (60·5)	53·9–73·9 (61·5)	53·1–56·7 (54·2)
Sequence divergence in *Bupleurum* (%)†	0–29·7	0–29·3	0–4·9
Sequence divergence in all taxa (%)†	0–37·7	0–37·9	0–6·1
Size of indels in *Bupleurum* (bp)†	1–37	1–5	0
Size of indels in all taxa (bp)†	1–44	1–5	0
Number of excluded sites	0	46	0
Number of sites after exclusion	229	207	164
Number (and %) of constant sites†	73 (31·9)	83 (40·1)	148 (90·2)
Number (and %) of variable sites†	156 (68·1)	124 (59·9)	16 (9·8)
Number (and %) of parsimony informative sites†	128 (55·9)	99 (47·8)	11 (6·7)
Number (and %) of autapomorphic sites†	28 (12·2)	25 (12·1)	5 (3·1)
Transitions (minimum)†	217	166	17
Transversions (minimum)†	176	150	6
Transitions : transversions† (Ti : Tv)	1·23	1·11	2·83

* 5.8S sequence data refers only to *Bupleurum* and *Anginon*.
† Based on alignment excluding ambiguous sites.

strongly supported and, in two cases, correspond to taxonomically delimited groups in previous classifications: clade I corresponds to section *Bupleurum*, and clade G to subsection *Trachycarpa* of section *Isophyllum*.

Although final parsimony analyses used five taxa as the outgroups, analyses were also performed using different outgroup combinations, which included several other Apioideae genera (sequences from GenBank). The exclusion of all outgroups except *Heteromorpha* resulted in 32 MP trees, whose strict consensus was virtually identical to the tree given in Fig. 1. Tree statistics were also similar, although slightly higher (CI = 0·67, RI = 0·87), due to the reduction of the number of taxa and so of possibly conflicting characters. Similar results were also obtained when using *Physospermum* and *Pleurospermum* as the outgroup (96 MP trees; CI = 0·66, RI = 0·87). The use of the two species of *Anginon* as the outgroup resulted in 16 MP (CI = 0·67, RI = 0·88), whose strict consensus was a tree similar in topology to that presented in Fig. 3 (NJ tree), with the exception of the position of *B. baldense*, which appeared as the first-branching taxon in clade B, as happened in the MP trees (Figs 1 and 2). In summary, the use of alternative outgroups did not affect the main clades of the *Bupleurum* ITS phylogeny. Only two clades changed position, and both of these had an unresolved relationship in the MP strict consensus tree (clades F and G).

While this paper has been in press, an ITS sequence for *Hohenackeria exscapa* (Steven) Koso-Pol. has become available (GenBank Accession Nos AF337178 and AF337186) from the work of Valiejo-Roman *et al*. (2002). Preliminary results of an analysis of this sequence in our dataset shows that this species is included within clade B (subgenus *Bupleurum*). This shows that *Hohenackeria* would not be an appropriate outgroup for *Bupleurum*, as it appears to belong to the ingroup, and that our choice of outgroup remains acceptable. Preliminary results also suggest that the inclusion of *Hohenackeria* in the dataset does not affect the main clades of *Bupleurum*, although it does reduce the resolution within clade B.

Parsimony analysis of the 5.8S sequences (11 informative characters) produced 16 MP trees, each 21 steps long, with three substitutions separating *Bupleurum* from the outgroup (*Anginon*). The dichotomy of *Bupleurum* was also shown by analysis of the 5.8S, with four substitutions supporting the separation of clade A from clade B (one substitution supporting clade A and three for clade B). The tree statistics of the 5.8S MP trees were high (CI = 0·91, RI = 0·98) indicating that despite the small number of variable characters (16 out of 164), homoplasy was low.

The likelihood scores of the MP trees were similar regarding estimated base frequencies and Ti : Tv ratios (1·48–1·49), but showed larger ranges of values for the proportion of invariable sites (0·07–0·21), and shape parameter of gamma distribution ($\propto$ = 0·59–0·94). Values used were: proportion of invariable sites = 0·08, 0·12 and 0·2, and $\propto$ = 0·5, 0·6 and 0·8. The ML tree (length 774 steps, CI = 0·62, RI = 0·85) found in all analyses was highly congruent with the MP trees and so is not shown.

One NJ tree is presented in Fig. 3. The NJ tree topology was not affected by different models of sequence evolution (Jukes-Cantor, K2P or HKY85); however, slight differences were produced with higher gamma distribution or if substitution rates were set as equal. The NJ tree produced

when assuming equal rates of substitution is similar to the MP trees (e.g. Fig. 2), and this tree is also obtained for values of gamma distribution larger than two.

In all the MP, ML and NJ analyses, *Bupleurum* appears as a strongly supported monophyletic group. The division of the genus into two major clades (A and B in Figs 1–3) is consistent among all trees. The separation of these two clades is also supported by three indels: a 3 bp insertion in ITS1, a 4 bp insertion in ITS2, and a 1 bp deletion at the start of the 26S coding gene. These are all synapomorphies of clade A. Indels were not included in the various analyses and therefore provide further support to the results obtained by analysis of nucleotide substitutions alone.

Other clades that have high statistical support are also present in the trees of all analyses: clades C (mostly north-west African endemic species), D ('Macaronesian' group), E ('Iberian and Balearic' group), F (*B. ranunculoides* and *B. longifolium*), G (*Trachycarpa* group) and I (*Perfoliata* group). The main difference between the MP, ML and NJ trees is the position of *B. baldense*. This species appears as the first-branching *Bupleurum* species in the MP trees (Figs 1 and 2), but as sister of clade H in some of the NJ trees (e.g. Fig. 3) and in the ML tree. As *B. baldense* appeared in conflicting positions in the various analyses, searches were also performed excluding this species, but overall the resulting trees were not affected.

DISCUSSION

ITS characteristics

The length and levels of variability of ITS1 and ITS2 were in line with those reported in other studies of Apiaceae (Downie *et al*., 1998; Katz-Downie *et al*., 1999). The region of ITS2 that was excluded due to difficulty in alignment has been found in the sequences of ITS2 in several plant families (e.g. Apiaceae, see Downie and Katz-Downie, 1996; Gesneriaceae, see Möller and Cronk, 1997), and it corresponds to the variable region 'v1' as referred in Hershkovitz and Zimmer (1996), and also discussed by Mai and Coleman (1997) and Denduangboripant and Cronk (2001). ITS divergence (approx. 29 %) in *Bupleurum* was higher than that found in many other genera (usually under 16 %, although divergence up to 30 % has been recorded in a few other genera; Hershkovitz *et al*., 1999). This high level of divergence is more often found in taxa of higher rank (e.g. Lee and Downie, 1999; Downie *et al*. 2000*a*).

Monophyly of Bupleurum

Regardless of method (parsimony, likelihood or neighbour-joining) or outgroup, all phylogenetic analyses of ITS confirmed that *Bupleurum* is monophyletic, and considerably divergent from the other early branching genera of Apioideae. However, the recently published ITS sequence of *Hohenackeria exscapa* raises the possibility that this genus should be included within *Bupleurum*. *Hohenackeria* is a genus of two species distributed in the western Mediterranean, the Caucasus and south-west Asia. It is traditionally classified alongside *Bupleurum*, differentiated by its white flowers, sessile umbels, and conspicuous calyx teeth, and the lack of bracteoles and of a carpophore (mericarps do not separate). It is expected that further work on this genus of simple-leaved plants will show that these species should be included within *Bupleurum*.

Bupleurum has long been recognized as a natural group, mainly due to its characteristic simple and entire leaves, a synapomorphy of the genus. Another synapomorphy of *Bupleurum* seems to be its characteristic sub-rhomboidal pollen type (Cerceau-Larrival, 1962, 1971; Punt, 1984), found in all the species studied, with the exception of two Chinese species (*B. sibiricum* Vest ex Roem. & Schult. and *B. chinense* DC.), which have suboval and sub-rectangular pollen types (Merrgen, 1994). The typical seedlings of *Bupleurum*, with linear, single-veined and glabrous cotyledons, appear to be unique in the family (Cerceau-Larrival, 1962), and thus are probably also synapomorphic to the genus. Cerceau-Larrival recorded hairy cotyledons in seedlings of some species in *Bupleurum*, but this has not been confirmed in our work with the same species, where all seedlings were found to be glabrous (Neves, 2000).

Relationships of Bupleurum *with other genera*

As mentioned above, some difficulties were experienced unambiguously aligning the *Bupleurum* ITS sequences to those from other Apioideae taxa, including other early branching genera in the subfamily (Downie *et al*., 1998; Valiejo-Roman *et al*., 1998; Katz-Downie *et al*., 1999). However, sequences from clade B members (e.g. *B. fruticosum*) are not so disparate from those of other early branching Apioideae genera, such as *Heteromorpha*, and unambiguous alignment is possible for most of ITS. The only sequence region excluded in the present analysis (46 characters in ITS2) was difficult to align not only between the different genera, but also within *Bupleurum*.

ITS sequences of *Anginon* are distinct from *Bupleurum* (24–35 % sequence divergence) and from *Heteromorpha* (approx. 30 % sequence divergence between *Anginon* and *Heteromorpha*). Previous molecular studies have suggested that *Anginon* and *Heteromorpha* may be closely related (Plunkett *et al*., 1996*a*, *b*, 1997; Downie *et al*., 1998, 2000*b*, *c*; Downie and Katz-Downie, 1999; Plunkett and Downie, 1999, 2000), but the ITS results indicate that they are considerably divergent.

Published molecular studies, mainly using the plastid genome, have demonstrated that *Bupleurum* is an early-branching genus in subfamily Apioideae (Plunkett *et al*., 1996*a*, *b*, 1997; Downie *et al*., 1998, 2000*b*, *c*; Downie and Katz-Downie, 1999; Plunkett and Downie, 1999, 2000). This confirms the long-held belief that *Bupleurum* is among the 'primitive' or 'relict' groups in the family (Drude, 1898; Wolff, 1910; Cerceau-Larrival, 1962, 1971; Cauwet-Marc, 1976). *Bupleurum* has been considered an ancestral group in Apiaceae mainly because it includes woody species and some of the earliest pollen fossil records known for the family (Gruas-Cavagnetto and Cerceau-Larrival, 1978). Woody habit is sometimes regarded as ancestral in the family, as inferred by the close relationship to the mostly woody Araliaceae (Plunkett *et al*., 1996*a*, *b*). Secondary

woodiness is known to occur in Apiaceae (Oskolski, 2001), but the wood in *Bupleurum* exhibits some ancestral characteristics (Rodriguez, 1957; A. Oskolski, Botanical Museum, St Petersburg, pers. comm.). It also appears that the annual habit has evolved at least twice within *Bupleurum*: in clade H, which includes mostly annuals, the only perennial herbs being *B. falcatum* and *B. mundii* (*B. baldense*, an annual, may also belong to this clade); and in clade G (*Trachycarpa*), which includes only annuals.

Bupleurum diverged early in the history of Apioideae, but its relationships to other genera remain uncertain. In the present study, none of the genera used as the outgroup (*Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*) seemed to be closely related, and all are clearly divergent from *Bupleurum*. There are no clear morphological or anatomical characters associating the genus to these or other early-branching genera in Apioideae. The present ITS data are in agreement with previous molecular studies that have found *Bupleurum* to be an isolated group in the family treated as a monogeneric tribe Bupleureae (Downie *et al*., 2000*b*). Inclusion of clade A members of *Bupleurum* in future molecular studies may help to clarify the relationships of the genus to other early branching genera in Apioideae.

Infrageneric classification

The phylogenetic results of the current study do not agree with any published infrageneric classifications of *Bupleurum* (see Table 1). No previous classifications have suggested the subdivision of the genus into the two main groups shown by analysis of ITS. Of the five sections in Wolff's classification (Wolff, 1910), only two are likely to be monophyletic: section *Bupleurum* (the species with perfoliate leaves: clade I; Figs 1–3) and section *Reticulata* (*B. angulosum* and *B. stellatum*). The most species-rich group, section *Isophyllum*, is paraphyletic, with sections *Bupleurum* (clade I) and *Diaphyllum* (*B. longifolium*) nested within this large group (clade B). Section *Coriacea* (*B. fruticosum*, *B. gibraltarium*) may also be paraphyletic, but its status is uncertain in the present study, and *B. foliosum* (the only species not sequenced from section *Coriacea*), should be included in future studies. The status of section *Diaphyllum* could not be assessed, because only one of its two species was included in the analysis (*B. longiradiatum* was not sampled).

Cauwet-Marc's classification (Cauwet-Marc, 1976) also does not agree with the present phylogenetic analysis, with her subgenera *Bupleurum* and *Tenoria* being paraphyletic. Her subgenus *Tenoria* includes the species of section *Coriacea* (in clade A) and many species from section *Isophyllum* (clade B), and her subgenus *Bupleurum* includes species in section *Bupleurum* (clade B), section *Reticulata* (clade A), section *Diaphyllum* (clade B) and the remaining species of section *Isophyllum* (the majority from clade B, and *B. rigidum* from clade A).

A major finding of the current study is that *Bupleurum* shows an early division into two strongly supported groups (clades A and B, Figs 1–3). Maximum likelihood and distance analyses (NJ) also confirm this main dichotomy within the genus, as did analysis of the 5.8S sequences alone.

Although the number of species sampled in the genus is small (32 species out of approx. 150), the taxa studied represent a good sample of the morphological variation seen within *Bupleurum*. Representatives of all the sections and subsections of the currently accepted classification are included in the study (Table 2), and coverage is also good when considering other alternative classifications, such as that of Cauwet-Marc (1976). Although western Mediterranean and Macaronesian species are particularly well represented (only one species missing), many of the species sequenced have distributions that extend into eastern Europe and Asia, and three of the species are from outside the main area of study: *B. longifolium* (from central Europe extending to eastern Russia), *B. mundii* (southern Africa) and *B. stellatum* (European Alps and Corsica). We are, therefore, confident that the implications on the classification of the genus can be extrapolated beyond the taxa studied.

There has been considerable discussion on the need to use several molecular markers in phylogenetic investigations, criticizing the interpretation of single-gene trees as species trees (Doyle, 1992, 1997; Maddison, 1997). Our study is limited in this sense, but with the exception of the study of Choi *et al*. (1996), who sampled four species, the present study is the first systematic investigation within the genus using molecular data. Previous researchers have included a small number of DNA sequences from *Bupleurum*, but their aim was to investigate generic and suprageneric relationships in Apiaceae (e.g. Plunkett *et al*., 1996*a*, *b*, 1997; Downie *et al*., 1998; Valiejo-Roman *et al*., 1998). Although, there is no doubt that more evidence from other genes is needed, the degree of confidence in the two major clades obtained from the current study is strong. Therefore, we formally propose the subdivision of *Bupleurum* into two subgenera, as follows.

Subgenus Penninervia *S.S.Neves & M.F.Watson* subgenus nov. *Subgenus specierum foliis penninervis aut ± parallelinervis diende nervis marginalibus et costa valde crassa, frutices vel herbae perennes, distributionis mediterraneae.*

Type species: *Bupleurum fruticosum* L., *Sp. Pl.*: 238 (1753).

Other included species: *B. angulosum* L., *B. gibraltarium* Lam., *B. rigidum* L. and *B. stellatum* L.

This early branching clade (Clade A) is sister to a larger clade that includes the vast majority of the species in *Bupleurum*. *Penninervia* is formed, as the name suggests, by all the species in the genus with pinnate-reticulate veined leaves, plus *B. rigidum* (which itself has a unique type of leaf venation). This subgenus includes shrubs and perennial herbs, all native to the Mediterranean region. Subgenus *Penninervia* includes the species previously placed in sections *Coriacea* (*B. foliosum*, *B. fruticosum* and *B. gibraltarium*), and *Reticulata* (*B. angulosum* and *B. stellatum*), and one species from section *Isophyllum* subsect. *Marginata* (*B. rigidum*).

No single morphological character has yet been found that is common to all the species of subgenus *Penninervia*, but the molecular signal from ITS is strong, not only by analysis of the nucleotide substitution pattern, but also by the presence of three indels (described above) that are synapomorphic to this clade.

The close relationship between the species of section *Coriacea* and section *Reticulata* was suspected, as they share some morphological characters (pinnate-reticulate leaves, and large fruits with narrowly winged ridges). However, the inclusion of *B. rigidum* in this group was unexpected because this species is morphologically distinct from the other members of the subgenus: *B. rigidum* has more or less parallel-veined leaves, with thick and prominent veins, delicate and diffusely branching inflorescences, small and narrow bracteoles, and fruits with filiform ridges, while the other members of the subgenus have pinnate leaves with a thick midrib (but other veins are delicate), thicker flowering branches, larger bracts and bracteoles, and narrowly winged fruits. Nevertheless, the traditional association based on inflorescence structure of *B. rigidum* to species of section *Isophyllum* (all of them included in clade B) has been controversial, and some classifications treat it in a subsection of its own (subsection *Marginata*). The leaves of *B. rigidum* are by far the longest in the genus (up to 45 cm), and its prominent leaf veins are unique in *Bupleurum* with no obvious affinities with other species. The new placement of this enigmatic taxon is well supported by molecular data for each of the four different accessions, representing the two subspecies of *B. rigidum*, collected from different natural populations. These produced virtually identical ITS sequences (note branch length in Fig. 2). It is possible that the similar morphologies between *B. rigidum* and some species of clade B may be due to ancient hybridization between members of the two subgenera (clades A and B), but due to the mechanisms of concerted evolution (Dover, 1982; Elder and Turner, 1995), only one of the parental sequences was kept in the evolutionary lineage of *B. rigidum*. However, this is a complex explanation with no other evidence to support it. It is simpler to accept that there is only apparent morphological similarity (convergence or parallelism) between the inflorescence of *B. rigidum* and those of some other perennial herbs of subgenus *Bupleurum*, such as *B. acutifolium* or *B. oligactis*.

The basic chromosome number for subgenus *Penninervia* appears to be $x = 7$, with all species being $2n = 14$, with the exception of *B. rigidum*, which also has records of $2n = 16$. Cauwet-Marc (1979*b*) assumed that the basic number for *B. rigidum* was $x = 8$, but when counts of $2n = 14$ were found for the species, she interpreted this as dysploidy, with change from eight to seven. Considering the present molecular evidence, it appears that $x = 7$ was ancestral.

The clades within subgenus *Penninervia* have low support and further research is needed to clarify the relationships between these taxa. If the main subdivision of subgenus *Penninervia* shown in the strict consensus tree is confirmed (Fig. 1), a group defined by the presence of pinnate-reticulate leaves would be paraphyletic: *B. gibraltarium* appears to be more closely related to *B. rigidum* than to *B. fruticosum*, *B. angulosum* and *B. stellatum*. Pinnate venation is probably plesiomorphic, and a classification based on this character would be artificial.

Despite the low statistical support (64 % bootstrap, 63 % jackknife; Fig. 1) of the clade formed by *B. angulosum* and *B. stellatum* (sect. *Reticulata*), the group is probably monophyletic. *Bupleurum angulosum* (endemic to the Pyrenees) and *B. stellatum* (endemic to the Alps and Corsica) are both perennial herbaceous species that are morphologically similar. The only fundamental difference between them is the degree of fusion of the bracteoles in the involucel (free in *B. angulosum*, and fused at least in two-thirds of the total length in *B. stellatum*). The morphological congruence of these species is such that it seems unlikely that it would have arisen independently.

We were unable to sequence *B. foliosum* (the only species missing from sect. *Coriacea*). The DNA extracts were degraded and amplification failed in all attempts. However, *B. foliosum* is expected to be a member of subgenus *Penninervia* as this species is morphologically close to *B. gibraltarium*. Future sequencing of ITS is needed to confirm the inclusion of the species in this subgenus.

Subgenus Bupleurum. This large group (Clade B) is formed by most of the species of the genus (including the type species, *B. rotundifolium*), which typically have ± parallel-veined leaves. The plants of this subgenus are shrubs, subshrubs, perennial or annual herbs, and have a worldwide distribution.

The conservation of *B. rotundifolium* as the type species of *Bupleurum* [*Taxon* 41: 572 (1992); *Taxon* 44: 611 (1995)] is still awaiting confirmation [*Taxon* 48: 373–374, 398 (1999)]. We follow the current usage of *B. rotundifolium* as the generitype, because enforcing the use of the earlier 'mechanical' type designation (*B. rigidum*) would be disruptive both to the traditional and to our new infrageneric classification.

Most of the species in this subgenus appear to have $x = 8$ as the basic chromosome number, but $x = 7$ occurs in some species (*B. ranunculoides*: $2n = 14, 28, 42, 56$), as do other numbers, such as $x = 6$ (*B. barceloi*: $2n = 24$). Dysploidy and aneuploidy are also common in some of the species, such as *B. falcatum* and *B. oligactis* (syn. *B. atlanticum*), which further complicates the understanding of chromosome number evolution in this group.

The phylogeny of subgenus *Bupleurum* is not fully resolved (Fig. 1). In the MP trees, *B. baldense* is sister to all remaining species of the subgenus, and terminates a long branch (43 or 44 steps), suggesting that this clade may be an artefact of sampling. Statistical support for the dichotomy is also low (65 % bootstrap and 66 % jackknife). Moreover, the position of *B. baldense* changes when the ITS data is analysed by ML or NJ (Fig. 3) methods. We have found no conspicuous morphological evidence to suggest that *B. baldense* is evolutionarily isolated within the genus. This problem may be resolved by adding more taxa, as there are some eastern Mediterranean annual species that may be more closely related to *B. baldense*.

Although incompletely resolved, subgenus *Bupleurum* shows some clades that have strong statistical support and are likely to be monophyletic. We withhold attributing formal taxonomic rank to these groups because the relationships of the various clades need further clarification.

The 'NW African' group. Clade C is strongly supported (97 % bootstrap, 95 % jackknife, decay index 8) and includes all the endemic species in north-west Africa and also two Iberian species (*B. acutifolium* and *B. fruticescens*), a species endemic to the Balearic Islands (*B. barceloi*) and the endemic Macaronesian taxa (*B. salicifolium* and *B. canescens* var. *handiense*). This is referred to as the 'NW African' group. The taxa from outside north-west Africa are morphologically close to some of the endemics in the area: *B. acutifolium* and *B. barceloi* are clear allies of *B. oligactis*; *B. fruticescens* subsp. *spinosum* also occurs in north-west Africa, and *B. salicifolium* is undoubtedly close to *B. canescens*. *Bupleurum dianthifolium* Guss. (a subshrub endemic to the island of Marettimo, near Sicily) was not included in the current study, but has morphological affinities to some of the taxa in the group (such as *B. acutifolium* and *B. barceloi*), and is therefore included in this group.

The variation in ITS nucleotide sequence within the 'NW African' group is generally low (in Fig. 2 branch lengths are proportional to the number of substitutions), with some morphologically distinct species having identical sequences (e.g. *B. benoistii* and *B. lateriflorum*). This could indicate that the group has radiated recently. Bateman (1999) has shown that in recently evolved groups, sequences may show little variation and could be unsuitable for delimiting species. This low rate of variation could also be explained by biased gene conversion (Hillis *et al*., 1991) against new variants of a sequence, but generally the ITS region tends to diverge between isolated species, as has been shown in many studies, including those in Apioideae (e.g. Downie *et al*., 2000*a*).

The 'Macaronesian' group. The Macaronesian endemics, *B. salicifolium* (Madeira and Canary Islands) and *B. canescens* var. *handiense* (syn. *B. handiense*: eastern Canary Islands) appear together in Clade D with *B. canescens* sensu stricto (endemic to south Morocco). These taxa are also morphologically similar and are herein referred to as the 'Macaronesian' group. *Bupleurum canescens sensu stricto* occurs in an area of Morocco that is recognized by some authors as 'the Macaronesian enclave' in north-west Africa (Sunding, 1979). ITS sequence data show a 'Macaronesian clade' with good branch support (bootstrap = 87%), but a branch that is only two steps long.

The 'Iberian and Balearic' group. Clade E includes *B. acutifolium* (Spanish population), *B. fruticescens* and '*B. spinosum*' (Spanish populations), and *B. barceloi* (endemic to the Balearic Islands). It is strongly supported (98 % bootstrap; 96 % jackknife), and has a fairly reliable branch length (6 steps; 5 decay index). We refer to this clade as the 'Iberian and Balearic' group. This clade is one of five unresolved clades within clade C (Fig. 1), which is predominantly formed by north-west African species, and may have a north-west African origin.

The 'Trachycarpa' group. Clade G is strongly supported (100 % bootstrap and jackknife support; 19 decay index), and it is traditionally classified as subsection *Trachycarpa* of section *Isophyllum*. It includes annual herbs, with linear to linear-lanceolate leaves, and papillose fruits. The affinities of the '*Trachycarpa*' are not clear from the current study. In the various analyses, it appears either as a sister to the 'NW African' group or as sister to the '*Ranunculoides*' group.

The 'Ranunculoides' group. The group formed by *B. ranunculoides* and *B. longifolium* (clade F) is strongly supported in the analyses (98 % bootstrap; 97 % jackknife; 5 decay index). There are undoubtedly morphological affinities between these species, but more taxa need to be included to confirm the monophyly of the group, in particular perennial herbs from Asia, such as *B. longicaule* Wall. ex DC., *B. candollei* Wall. ex DC., and taxa of the complex '*B. falcatum*' group.

The 'Perfoliata' group. Clade I corresponds to section *Bupleurum* of previous classifications (Tutin, 1968). It is characterized by the absence of involucral bracts and the presence of perfoliate upper leaves; both synapomorphies of the group. Phylogenetic analysis confirms that this is a monophyletic group (99 % bootstrap, 98 % jackknife, 6 decay index), as has long been regarded by taxonomists. There is also a 5 bp deletion in ITS1 synapomorphic to these taxa, which provides further support for the group. The other four species with perfoliate leaves (*B. croceum* Fenzl, *B. heldreichii* Boiss. & Balansa, *B. lophocarpum* Boiss. & Balansa, and *B. schistosum* Woronow: all endemic to Turkey; Snogerup, 1972) should be sequenced in future studies to verify if they also fall within this group.

Species delimitation in Bupleurum

Bolòs and Vigo (1974) treated *B. fruticescens* and *B. spinosum* as a single species, by recognizing *B. spinosum* as a subspecies of *B. fruticescens*. At the time they did not explain the basis for this taxonomic decision, but having studied morphological variation in the herbarium and in the field (Spain), intermediate plants are easily found and we agree with their treatment. The ITS analyses in the current study further endorses their treatment as a single species, with all sequences from Iberian material being invariant. However, the sequences obtained from the North African '*B. spinosum*' showed several differences (12 substitutions and three indels of 1 bp each) in relation to those of the Iberian material of *B. fruticescens*. Morphologically, the Spanish and African populations of '*B. spinosum*' are almost identical and should be considered part of the same species. The divergence in ITS sequences may have arisen

because of the long geographical separation of the populations. However, the African populations of '*B. spinosum*' have an ITS sequence that is much closer to that of another North African species, *B. balansae*, than to its Iberian relatives. This could be an indication of gene introgression (i.e. that hybridization may have occurred between *B. balansae* and the North African '*B. spinosum*'). Both species are shrubs and are found in the same geographical area. If this was the case, we may have only detected the 'balansae' ITS type, which could have replaced, or be more common in the genome, than the 'spinosum' ITS. In our research, only one ITS type of sequence was found in each species, but only extensive sequencing of ITS repeats by cloning could demonstrate that there is a single type of sequence in the genome. Distinct ITS copies (paralogues) in a single genome have been found in several plant groups, in some cases clearly as a result of interspecific hybridization (e.g. Sang *et al*., 1995).

The low bootstrap value (62 %) shown for the clade of the Spanish *B. fruticescens* (part of clade E in Fig. 1), which have identical sequences, reflects the fact that there is little difference between the sequences of these taxa and those of *B. acutifolium* (Spain) and *B. barceloi*.

Bupleurum bourgaei has traditionally been considered a distinct species, endemic to the mountains of south-east Spain. From a morphological study of herbarium material (Neves, 2000) we considered that this taxon was not sufficiently distinct from *B. ranunculoides* and should be included as a synonym within it. The sample of *B. ranunculoides* No. 4 (province of Jaén) corresponds to material previously attributable to *B. bourgaei*, but the sequence of this sample differs in only one ambiguous nucleotide to that of *B. ranunculoides* No. 3, from the province of Burgos in north-east Spain. ITS sequence data thus support the decision based on morphology alone.

The two populations of *B. acutifolium* (Portuguese and Spanish) show minor morphological differences, but these cannot be relied upon, as they are considerably plastic. However, the ITS sequences obtained for the two populations are considerably different. The sequence of the Portuguese material shows a fairly large deletion in ITS1 (35 bp), which is likely to result from a single mutation, but there are also 14 other single nucleotide substitutions. In addition, the two *B. acutifolium* types of sequences arise in different parts of the trees. The geographic separation of the two populations is also quite large, and interbreeding has probably not occurred for some considerable time. ITS data indicates that the two populations of *B. acutifolium* have diverged significantly and could now be considered different species, even though the morphological differences are not so clear cut (this will form the subject of future work). There is also the possibility that hybridization may have occurred between the Spanish population of *B. acutifolium* and *B. fruticescens* (a Spanish 'neighbour', which is morphologically a distinct species). However, the two species are not sympatric, have distinct ecological preferences (the Spanish *B. acutifolium* grows on serpentine soil, whereas *B. fruticescens* is found on calcareous soil), and there is no evidence of morphological introgression between them. Hybridization is rare in the family and these two species merit further study.

Bupleurum balansae is another taxon with considerable variation in ITS sequence data between populations, but in this case the morphological characters used to delimit the taxon may be inadequate. The main characters used to delimit this species are the sessile or subsessile flowers and fruits, and the slightly prominent veins in both surfaces of the leaves. However, there is a great deal of variation in shape and length of leaves, and even in prominence of veins. Future work may reveal that it is possible to distinguish more than one species in these populations of 'shrubs with sessile flowers'. Again, the possibility of reticulate evolution in some of the North African taxa needs to be investigated.

One of the sequences obtained for *B. gerardii* (No. 4) does not group with the others, indicating a problem in species delimitation. Morphological delimitation of *B. gerardii* and *B. praealtum* is also problematic, and difficulties are often experienced in distinguishing them. In Fig. 2, *B. gerardii* No. 4 appears as sister to the clade including *B. gerardii* and *B. praealtum*. One of the possibilities is that hybridization has occurred between *B. gerardii* and *B. praealtum*, which would explain the problems of morphological characterization of the taxa. A second possibility is that we are including two different species under the name *B. gerardii*, which is the opinion of other authors (Snogerup and Snogerup, 2001). A third possibility is that *B. gerardii* and *B. praealtum* are a single species. Therefore, a more detailed study of *B. gerardii* and *B. praealtum* is necessary, including material from the rest of the area of distribution (Europe and west Asia), and also other allied species, such as *B. commutatum* Boiss. & Balansa, and *B. trichopodum* Boiss. & Spruner.

Phylogeny and biogeography

Several clades in the ITS analysis in *Bupleurum* are congruent with the geographical distribution of the taxa. The clearest case is the large group designated here as 'NW African', a clade in which all the endemics to north-west Africa are included. Some of these endemics are morphologically quite distinct, and they have not previously been classified into a single group. As discussed above, two clades that are included within the NW African group, the 'Macaronesian' and the 'Iberian and Balearic' clades also reflect geographical areas.

Two clades of subgenus *Bupleurum* include species that have essentially a Eurasian distribution: (1) the clade including '*Perfoliata*' and the groups of *B. falcatum* and *B. gerardii*; and (2) the clade of *B. ranunculoides* and *B. longifolium*. *Bupleurum lancifolium* and *B. odontites* (included in clade B) occur in North Africa, but their original (native) area of distribution is not clear as both species have been introduced and are ruderals or weeds in cultivated land. The only exception to this Eurasian distribution is *B. mundii*, a species endemic to southern Africa, whose closest relative appears to be *B. falcatum* (Figs 1–3). There are also morphological affinities between these two species.

Evidence from the current study suggests that the genus *Bupleurum* originated somewhere in the western Mediterranean. First, the first branching clade in *Bupleurum* is formed by species that fundamentally only occur in the western Mediterranean. *Bupleurum fruticosum* is the only species with a distribution that extends across the Mediterranean, but this can be explained as subsequent dispersal. Also, the shrubby plants of the genus are restricted to the western Mediterranean (the only exception, again, is *B. fruticosum*). This is particularly relevant if woodiness is regarded as an ancestral state (Plunkett *et al*., 1996*b*), which appears to be supported by wood anatomy in *Bupleurum*. Also, the western Mediterranean is undoubtedly richer in the 'basic' morphological patterns found in *Bupleurum* (Neves, 2000).

The eastern Mediterranean has high species diversity in annuals (e.g. Snogerup, 1972), but it is likely that these have evolved more recently: ITS analysis indicates that the annual habit is a derived state (possibly having evolved more than once in the genus). A shorter life cycle increases the chances of evolutionary change, so a shorter period of time is required to explain increased diversity (molecular and morphological) in annual plants. This could explain why in the ITS phylogeny (Figs 2 and 3) the groups including only herbs and mainly annuals (such as clades G, H and I) appear to have longer branches than those comprised by perennials only (e.g. clade C).

There is little difference between the ITS sequences of *B. salicifolium* (endemic to Madeira and Canary Islands) and those of its closer continental relatives, in particular *B. canescens*. Therefore, *B. salicifolium* is clearly a neoendemic species, with a recent arrival in the islands.

Another neoendemic is the disjunct species *B. mundii* (endemic to southern Africa). Our data refute the notion that this is a remnant species from a past larger distribution of *Bupleurum* in Africa. This has been questioned before, in particular considering the relationships of 'primitive' woody genera of Apiaceae, most of which only occur in central and southern Africa (Cerceau-Larrival, 1971; Burtt, 1991; Downie and Katz-Downie, 1999).

CONCLUSIONS

The results of the present study have confirmed the monophyly of *Bupleurum* and provided resolution of major groups within the genus. Light has also been shed on the origins of this widespread genus, and on the biogeography of some of the enigmatic species and species groups. Inevitably the analyses have given rise to new ideas and hypotheses that will require further work. The phylogenetic signal from the taxa studied will be strengthened by the use of other genes (nuclear and plastid), and the inclusion of new taxa from the eastern Mediterranean and Asia will give a more comprehensive sampling. *Bupleurum foliosum* and *B. dianthifolium* need to be sequenced for verification of their putative placement in subgenus *Penninervia* and the 'NW African' group, respectively. The use of genes with slower rates of mutation than ITS may help to resolve some of the clades in *Bupleurum* (e.g. the plastid genes *trnL-trnF* and *matK* have also been useful at generic and infrageneric levels; Soltis and Soltis, 1998).

The 'NW African' group (clade C) showed very low nucleotide variation in the ITS region. At present, the ITS spacers are two of the fastest evolving regions sequenced for phylogenetic analysis. Other loci with rapid rates of evolution are known, but sequence homology and alignment have been problematic. Therefore, to resolve the relationships of the taxa in this group, we will need methods of analysis that can detect molecular variation at lower levels, e.g. isozymes, AFLPs and microsatellites that have been particularly useful in detecting molecular polymorphism at the species level and below.

ACKNOWLEDGEMENTS

This work was funded by a doctoral scholarship to Susana Neves (BD/4057/94) from the program Praxis XXI, Fundação para a Ciência e a Tecnologia, Lisbon (Portugal). We thank the Royal Botanic Garden Edinburgh (RBGE) and the Institute of Cell and Molecular Biology (ICMB), University of Edinburgh, UK, for ample use of their research facilities and technical assistance. We are especially grateful to Jill Preston and Caroline Guihal (RBGE) and Nicola Preston (ICMB) for help with sequencing, Michael Möller and James Richardson (RBGE) for advice on phylogenetic analysis, and Fátima Sales (Departamento de Botânica, Universidade de Coimbra, Portugal) and Ian Hedge (RBGE) for many valuable discussions during the development of this project. Robert Mill is thanked for checking the Latin diagnosis, and Charlie Jarvis for his advice on the typification of *Bupleurum*. We thank an anonymous reviewer, Mike Fay and particularly Gregory Plunkett, for helpful comments on the manuscript. The Davis Expedition Fund (University of Edinburgh) provided financial support for a field collection trip in Spain. We also thank all the institutions that provided the herbarium and/or living material used in this study: Botanischer Garten Tübingen (Germany); Botanischer Garten der Universität Potsdam (Germany); Botanischer Garten St Gallen (Switzerland); Departamento de Biología Vegetal, Universidad de Málaga (Spain); Departamento de Botânica, Universidade de Coimbra; Departamento de Botánica, Universidad de Sevilla (Spain); Department of Botany, The Natural History Museum, London (UK); Department of Botany, University of Reading (UK); Facultad de Farmacia, Universidad Complutense, Madrid (Spain); Instituto Pirenaico de Ecología, Jaca (Spain); Jardín Botánico 'Viera y Clavijo', Canary Islands; Jardin Botanique National de Belgique, Meise (Belgium); Real Jardín Botánico de Madrid; and Royal Botanic Garden Edinburgh.

LITERATURE CITED

Allison I, Van Wyk BE. 1997. A revision of the genus *Anginon* (Apiaceae). *Nordic Journal of Botany* **17**: 561–577.

Arenas JA, García F. 1993. Atlas carpológico y corológico de la subfamilia Apioidea Drude (Umbelliferae) en España peninsular y Baleares. *Ruizia* **12**.

Baldwin BG. 1992. Phylogenetic utility of the internal transcribed spacers

of nuclear ribosomal DNA in plants: an example from the Compositae. *Molecular Phylogenetics and Evolution* **1**: 3–16.

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**: 247–277.

Bateman RM. 1999. Integrating molecular and morphological evidence of evolutionary radiations. In: Hollingsworth PM, Bateman RM, Gornall RJ, eds. *Molecular systematics and plant evolution. The Systematics Association Special Vol. 57*. London and New York: Taylor & Francis, 432–471.

Berenbaum MR. 1990. Plant consumers and plant secondary chemistry: past, present and future. 4. A case study: furanocoumarins in *Apiaceae*. *Oxford Surveys in Evolutionary Biology* **7**: 285–307.

Boissier E. 1872. *Flora Orientalis. Vol. 2.* Geneva and Basel.

Bolòs O, Vigo J. 1974. Notes on plant taxonomy and nomenclature I. *Butlletí de la Institució Catalana d'Història Natural* **38** [Secció de Botànica, 1]: 83.

Bremer K. 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**: 795–803.

Bremer K. 1994. Branch support and tree stability. *Cladistics* **10**: 295–304.

Briquet J. 1897. Monographie des buplèvres des Alpes Maritimes. In: Burnat E, ed. *Matériaux pour servir à l'histoire de la flore des Alpes Maritimes*. Geneva and Basel: Georg & Co, Libraires-Éditeurs.

Brummit RK, Powell CE. 1992. *Authors of plant names.* Richmond: Royal Botanic Gardens, Kew.

Burtt BL. 1991. Umbelliferae of Southern Africa: an introduction and annotated check-list. *Edinburgh Journal of Botany* **48**: 133–282.

Calestani V. 1905. Contributo alla sistematica delle Ombellifere d'Europa. *Webbia* **1**: 89–170.

Carbonnier J, Cauwet-Marc AM. 1979. Constituants du genre *Bupleurum* (Umbelliferae): mise au point des connaissances actuelles. *Bulletin du Muséum National d'Histoire Naturelle (Paris). Série 4, section B*, **1**: 213–263.

Carbonnier J, Cauwet-Marc AM. 1981. A comparative phytochemical investigation of the genus *Bupleurum* L. (Umbelliferae). *Taxon* **30**: 617–627.

Cauwet-Marc AM. 1970. Races chromosomiques, écologie et biologie du *Bupleurum ranunculoides* L. dans la partie orientale des Pyrénées. *Bulletin de la Société Botanique de France* **117**: 373–384.

Cauwet-Marc AM. 1976. *Biosystématique des Espèces Vivaces de* Bupleurum *L.* (*Umbelliferae*) *du Bassin Méditerranéen Occidental*. PhD Thesis (3 vols). Université des Sciences et Techniques du Languedoc, Perpignan, France.

Cauwet-Marc AM. 1979*a*. Connaissances caryologiques actuelles sur le genre *Bupleurum* L. (Umbelliferae): nombres chromosomiques et nombres de base. *Bulletin du Muséum National d'Histoire Naturelle* (Paris). Série 4, section B, **1**: 191–211.

Cauwet-Marc AM. 1979*b*. Contribution de la caryologie à la connaissance de la systématique et de la phylogénie du genre *Bupleurum* L. *Candollea* **34**: 49–86.

Cauwet-Marc AM, Carbonnier J, Cerceau-Larrival MT, Dodin R, Guyot M. 1978. Contribution à l'étude multidisciplinaire du genre *Bupleurum* L. In: Cauwet-Marc AM, Carbonnier J, eds. **1982.** *Les Ombellifères: contributions pluridisciplinaires à la systématique: actes du 2ème Symposium International sur les Ombellifères, Centre Universitaire de Perpignan, 18–21 Mai* **1977.** St Louis, MO: Missouri Botanical Garden, 623–651.

Cerceau-Larrival MT. 1962. Plantules et pollens d'Ombellifères. *Mémoires du Muséum d'Histoire Naturelle (Paris). Série B, Botanique*, **14**: 1–166.

Cerceau-Larrival MT. 1971. Morphologie pollinique et correlations phylogénétiques chez les Ombellifères. In: Heywood VH, ed. *The biology and chemistry of the Umbelliferae*. London: Academic Press, 112–113.

Cerceau-Larrival MT, Roland-Heydacker F. 1978. Apport de la palynologie à la connaissance des Ombellifères actuelles et fossiles. In: Cauwet-Marc AM, Carbonnier J, eds. **1982.** *Les Ombellifères: contributions pluridisciplinaires à la systématique: actes du 2ème Symposium International sur les Ombellifères, Centre Universitaire de Perpignan, 18–21 Mai* **1977.** St Louis, MO: Missouri Botanical Garden, 213–229.

Chase MW, Hills HG. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon* **40**: 215–220.

Choi HK, Kim HJ, Shin H, Kim Y. 1996. Phylogeny and ribosomal DNA variation of *Bupleurum* (Umbelliferae). *Korean Journal of Plant Taxonomy* **26**: 219–233.

Denduangboripant J, Cronk QCB. 2001. *Evolution* and alignment of the hypervariable arm 1 of *Aeschynanthus* (Gesneriaceae) ITS2 nuclear ribosomal DNA. *Molecular Phylogenetics and Evolution* **20**: 163–172.

Donoghue MJ, Olmstead RG, Smith JF, Palmer JD. 1992. Phylogenetic relationships of Dipsacales based on *rbc*L sequences. *Annals of the Missouri Botanical Garden* **79**: 333–345.

Dover G. 1982. Molecular drive: a cohesive mode of species evolution. *Nature* **299**: 111–117.

Downie SR, Katz-Downie DS. 1996. A molecular phylogeny of Apiaceae subfamily Apioideae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* **83**: 234–251.

Downie SR, Katz-Downie DS. 1999. Phylogenetic analysis of chloroplast *rps*16 intron sequences reveals relationships among woody southern African Apiaceae subfamily Apioideae. *Canadian Journal of Botany* **77**: 1120–1135.

Downie SR, Katz-Downie DS, Spalik K. 2000*a*. A phylogeny of Apiaceae tribe Scandiceae: evidence from nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* **87**: 76–95.

Downie SR, Katz-Downie DS, Watson MF. 2000*b*. A phylogeny of the flowering plant family Apiaceae based on chloroplast DNA *rpl*16 and *rpo*C1 intron sequences: towards a suprageneric classification of subfamily Apioideae. *American Journal of Botany* **87**: 273–292.

Downie SR, Ramanath S, Katz-Downie DS, Llanas E. 1998. Molecular systematics of Apiaceae subfamily Apioideae: phylogenetic analyses of nuclear ribosomal DNA internal transcribed spacer and plastid *rpo*C1 intron sequences. *American Journal of Botany* **85**: 563–591.

Downie SR, Watson MF, Spalik K, Katz-Downie DS. 2000*c*. Molecular systematics of Old World Apioideae (Apiaceae): relationships among some members of tribe Peucedaneae sensu lato, the placement of several island-endemic species, and resolution within the apioid superclade. *Canadian Journal of Botany* **78**: 506–528.

Doyle JJ. 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. *Systematic Botany* **17**: 144–163.

Doyle JJ. 1997. Trees within trees: genes and species, molecules and morphology. *Systematic Biology* **46**: 537–553.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Drude O. 1898. Umbelliferae. In: Engler A, Prantl K, eds. *Die natürlichen Pflanzenfamilien. Vol. 3, Part 8*. Leipzig: Wilhelm Engelman, 63–250.

Elder JF, Turner BJ. 1995. Concerted evolution of repetitive DNA sequences in eukaryotes. *Quarterly Review of Biology* **70**: 297–320.

Eriksson T. 1999. *AutoDecay 4·0* [program distributed by the author]. Bergius Foundation, Royal Swedish Academy of Sciences, Stockholm.

Farris JS. 1989. The retention index and the rescaled consistency index. *Cladistics* **5**: 417–419.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fitch WM. 1971. Toward defining the course of evolution: minimal change for a specific tree topology. *Systematic Zoology* **20**: 406–416.

Grenier M, Godron M. 1848. *Flore de France. Vol. 1.* Paris.

Gruas-Cavagnetto C, Cerceau-Larrival MT. 1978. Presence de pollens d'Ombellifères fossiles dans le Paleogene du Bassin Anglo-Parisien: premiers resultats. In: Cauwet-Marc AM, Carbonnier J, eds. **1982.** *Les Ombellifères: contributions pluridisciplinaires à la systématique: actes du 2ème Symposium International sur les Ombellifères, Centre Universitaire de Perpignan, 18–21 Mai* **1977.** St Louis, MO: Missouri Botanical Garden, 255–267.

Hara H, Williams HJ. 1979. *An Enumeration of the Flowering Plants of Nepal, Vol. 2*. London: Trustees of the British Museum (Natural History), 184–185.

Hasegawa M, Kishino H, Yano T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**: 160–174.

Hershkovitz MA, Zimmer EA. 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. *Nucleic Acids Research* **24**: 2857–2867.

Hershkovitz MA, Zimmer EA. 2000. Ribosomal DNA evidence and disjunctions of Western American Portulacaceae. *Molecular Phylogenetics and Evolution* **15**: 419–439.

Hershkovitz MA, Zimmer EA, Hahn WJ. 1999. Ribosomal DNA sequences and angiosperm systematics. In: Hollingsworth PM, Bateman RM, Gornall RJ, eds. *Molecular Systematics and Plant Evolution. The Systematics Association Special Vol. 57*. London and New York: Taylor & Francis, 268–326.

Heywood VH. 1971. Systematic survey of Old World Umbelliferae. In: Heywood VH, ed. *The biology and chemistry of the Umbelliferae*. London: Academic Press, 31–41.

Hillis DM, Moritz C, Porter CA, Baker RJ. 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. *Science* **251**: 308–310.

Holmgren PK, Holmgren NH, Barnett LC. 1990. *Index herbariorum. Part I. The herbaria of the world, 8th edn*. New York: New York Botanical Garden.

Jukes TH, Cantor CR. 1969. *Evolution* of protein molecules. In: Munro HN, ed**.** *Mammalian protein metabolism. Vol. 3.* New York: Academic Press, 21–132.

Katz-Downie DS, Valiejo-Roman CM, Terentieva EI, Troitsky AV, Pimenov MG, Lee B, Downie SR. 1999. Towards a molecular phylogeny of Apiaceae subfamily Apioideae: additional information from nuclear ribosomal DNA ITS sequences. *Plant Systematics and Evolution* **216**: 167–195.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.

Kluge AG, Farris JS. 1969. Quantitative phyletics and the evolution of anurans. *Systematic Zoology* **18**: 1–32.

Kondo K, Terabayashi S, Okada M, Yuan C, He S. 1996. Phylogenetic relationship of medicinally important *Cnidium officinale* and Japanese Apiaceae based on *rbc*L sequences. *Journal of Plant Research* **109**: 21–27.

Koso-Poljansky BM. 1913. Epitome Bupleurorum Rossiae. *Acta Horti Petropolitani* **30**: 135–333.

Küpfer P. 1969. Recherches cytotaxinomiques sur la flore des montagnes de la Péninsule Ibérique. *Bulletin de la Société Neuchateloise des Sciences Naturelles* **92**: 31–48.

Lanyon SM. 1985. Detecting internal inconsistencies in distance data. *Systematic Zoology* **34**: 397–403.

Lee BY, Downie SR. 1999. A molecular phylogeny of Apiaceae tribe Caucalideae and related taxa: inferences based on ITS sequence data. *Systematic Botany* **24**: 461–479.

Lee SB, Rasmussen SK. 1998. *Phylogenetic analysis of rDNA internal transcribed spacer of* Angelica*,* Bupleurum *and* Peucedanum *species*. Risø-R-1091(EN) [internal report]. Roskilde, Denmark: Riso National Laboratory, 5–13.

Leonardis W, Fichera G, Longhitano N, Marletta G, Fiumara PMR, Zizza A. 1997. Indagine palinologica su alcune specie di *Bupleurum* L. e *Smyrnium* L. (Umbelliferae) in relazione alla posizione sistematica. *Allionia* **35**: 219–223.

Linnaeus C. 1753. *Species Plantarum. Vol. 1*. Stockholm [*Bupleurum* in pp. 236–239].

Maddison WP. 1997. Gene trees in species trees. *Systematic Biology* **46**: 523–536.

Maddison WP, Maddison, DR. 1992. *MacClade*: *analysis of phylogeny and character evolution*, *Version 3*. Sunderland, MA: Sinauer Associates.

Maddison WP, Donoghue MJ, Maddison DR. 1984. Outgroup analysis and parsimony. *Systematic Zoology* **33**: 83–103.

Mai JC, Coleman AW. 1997. The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. *Journal of Molecular Evolution* **44**: 258–271.

Merrgen WQ. 1994. On the pollen morphology of *Bupleurum* L. from Nei Menggu and its classification. *Acta Scientiarium Naturalium Universitatis Neimenggu* **25**: 554–560.

Möller M, Cronk QCB. 1997. Origin and relationships of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA internal transcribed spacer (ITS) sequences. *American Journal of Botany* **84**: 956–965.

Nasir E. 1955. The bupleura (Umbelliferae) of North-West Himalaya. *University of California Publications in Botany* **27**: 417–444.

Neves SS. 2000. *Taxonomy and evolution in* Bupleurum *L. (Umbelliferae) in the W Mediterranean and Macaronesia*. PhD Thesis, University of Edinburgh, UK.

Ohta S. 1991. Cytogenetical study on the speciation of *Bupleurum falcatum* L. (Umbelliferae). *Journal of Science of Hiroshima University, Ser. B, Div. 2 (Botany)* **23**: 273–348.

Oskolski AA. 2001. Phylogenetic relationships within Apiales: evidence from wood anatomy. *Edinburgh Journal of Botany* **58**: 201–206.

Panelatti J. 1959. Contribution a l'étude anatomique du genre *Bupleurum* L. au Maroc. *Travaux de l'Institut Scientifique Chérifien, Série Botanique* **15**.

Pimenov MG, Leonov MV. 1993. *The genera of the Umbelliferae*: *a nomenclator*. Richmond and Moscow: Royal Botanic Gardens Kew and Botanical Garden of Moscow State University.

Plunkett GM, Downie SR. 1999. Major lineages within Apiaceae subfamily Apioideae: a comparison of chloroplast restriction site and DNA sequence data. *American Journal of Botany* **86**: 1014–1026.

Plunkett GM, Downie SR. 2000. Expansion and contraction of the chloroplast inverted repeat in Apiaceae subfamily Apioideae. *Systematic Botany* **25**: 648–667.

Plunkett GM, Soltis DE, Soltis PS. 1996*a*. Higher level relationships of Apiales (Apiaceae and Araliaceae) based on phylogenetic analysis of *rbc*L sequences. *American Journal of Botany* **83**: 499–515.

Plunkett GM, Soltis DE, Soltis PS. 1996*b*. *Evolution*ary patterns in Apiaceae: inferences based on *mat*K sequence data. *Systematic Botany* **21**: 477–495.

Plunkett GM, Soltis DE, Soltis PS. 1997. Clarification of the relationship between Apiaceae and Araliaceae based on *mat*K and *rbc*L sequence data. *American Journal of Botany* **84**: 565–580.

Punt W. 1984. Umbelliferae. In: Punt W, Clarke GC, eds. *The Northwest European Pollen Flora*, *Vol. 4 (37)*. Amsterdam: Elsevier Science, 155–363.

Rechinger KH, Snogerup S. 1987. *Bupleurum*. In: Hedge IC, Lamond JM, Rechinger KH *et al***.** eds. *Umbelliferae*. In: Rechinger KH, ed. *Flora Iranica, Vol. 162*. Graz, Austria: Akademische Druck- u. Verlagsanstalt, 269–297.

Rodriguez RL. 1957. Systematic anatomical studies on *Myrrhidendron* and other woody Umbellales. *University of California Publications in Botany* **29**: 145–318.

Rogers JS, Swofford DL. 1998. A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Systematic Biology* **47**: 77–89.

Roux M, Carbonnier J, Cauwet-Marc AM. 1978. Un exemple d'analyse cladistique: Le genre *Bupleurum* L. (Umbelliferae). In: Cauwet-Marc AM, Carbonnier J, eds. **1982.** *Les Ombellifères: contributions pluridisciplinaires à la systématique: actes du 2ème Symposium International sur les Ombellifères, Centre Universitaire de Perpignan, 18–21 Mai* **1977.** St Louis, MO: Missouri Botanical Garden, 575–592.

Sang T, Crawford DJ, Stuessy TF. 1995. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. *Proceedings of the National Academy of Sciences USA* **92**: 6813–6817.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

Snogerup S. 1972. *Bupleurum*. In: Davis PH, ed. *Flora of Turkey*, *Vol. 4*. Edinburgh: Edinburgh University Press, 393–416.

Snogerup S, Snogerup B. 2001. *Bupleurum* L. (Umbelliferae) in Europe. 1. The annuals, *B.* sect. *Bupleurum* and sect. *Aristata*. *Willdenowia* **31**: 205–308.

Soltis DE, Soltis PS. 1998. Choosing an approach and an appropriate gene for phylogenetic analysis. In: Soltis DE, Soltis PS, Doyle JJ, eds. *Molecular systematics of plants II*: *DNA sequencing*. Boston: Kluwer Academic Publishers, 1–42.

Sprengel K. 1820. Umbelliferae. In: Roemer JJ, Schultes JA, eds. *Systema vegetabilium, Vol. 6*. Sttutgart, Germany.

Sunding P. 1979. Origins of the Macaronesian flora. In: Bramwell, D. ed. *Plants and islands*. London: Academic Press, 13–40.

Swofford DL. 2000. *PAUP*: Phylogenetic analysis using parsimony (*and other methods), Version 4*. Sunderland, MA: Sinauer Associates.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876–4882.

Tournefort JP. 1694. *Élémens de botanique, Vol 1*. Paris: L'Imprimerie Royale.

Tutin TG. 1968. *Bupleurum.* In: Tutin TG, Heywood VG, Burges NA, Moore DM, Valentine DH, Walter SM, Webb DA, eds. *Flora Europaea, Vol. 2*. Cambridge: Cambridge University Press, 345–350.

Valiejo-Roman CM, Pimenov MG, Terentieva EI, Downie SR, Katz-Downie DS, Troitsky AV. 1998. Molecular systematics of the Umbelliferae: using nuclear ribosomal DNA internal transcribed spacer sequences to resolve issues of evolutionary relationships. *Botanicheskii Zhurnal* **83**: 1–22.

Valiejo-Roman CM, Terentieva EI, Samigullin TH, Pimenov MG. 2002. Relationships among genera in Saniculoideae and selected Apioideae (Umbelliferae) inferred from nrITS sequences. *Taxon* **51**: 91–101.

Watrous LE, Wheeler QD. 1981. The out-group comparison method of character analysis. *Systematic Zoology* **30**: 1–11.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols, a guide to methods and applications*. San Diego, London: Academic Press, 315–322.

Wolff H. 1910. *Bupleurum.* In: Engler A, ed. *Das Pflanzenreich Regnis Vegetabilis Conspectus*, *IV.228*. Leipzig, Germany: Verlag von Wilhelm Engelmann, 36–173.

Yang Z. 1993. Maximum-likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. *Molecular Biology and Evolution* **10**: 1396–1401.

Yang Z. 1996. Among-site rate variation and its impact on phylogenetic analyses. *Trends in Ecology and Evolution* **11**: 367–372.

Yokota Y, Kawata T, Iida Y, Kato A, Tanifuji S. 1989. Nucleotide sequences of the 5·8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. *Journal of Molecular Evolution* **29**: 294–301.