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Diversification of *Biscutella* ser. *Biscutella* (Brassicaceae) followed post-Miocene geologic and climatic changes in the Mediterranean basin

Mª Ángeles Alonso*, Alicia Vicente, Manuel B. Crespo

Departamento de Ciencias Ambientales y Recursos Naturales (dCARN), Universidad de Alicante, P.O. Box 99, ES-03080 Alicante, Spain

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ABSTRACT

Biscutella ser. *Biscutella* (= ser. *Lyratae* Malin.) is a group of mostly annual or short-lived perennial plants, with petals gradually tapering at the base and lateral intrastaminal nectaries, endemic to the Mediterranean basin and the Middle East. Recent taxonomic work has revealed that a relative morphological homogeneity occurs in Europe and Asia, but a high plasticity is found in N Africa for most of the characters traditionally used for taxonomic arrangements. This fact had generally led to overestimation of the number of taxa, which currently is reduced to ten (namely 7 species and 3 additional varieties), some of them being narrow endemics. In the present contribution, on the basis of a previous detailed morphological study carried out by the authors, the first comprehensive phylogeny based on 47 DNA sequence data including concatenation of two plastid (*rpl32-trnL* and *trnV*) and one nuclear (ITS) regions, together with the first time-calibrated phylogenetic tree, allows re-appraisal of evolutionary and biogeographic relationships among the accepted taxa in the series. According to all evidence gathered in the present study, the current distribution of *B.* ser. *Biscutella*, mostly centred in the southern parts of the Mediterranean basin and the Middle East, suggests that it evolved in relation with the major geological and climatic events occurred in the Mediterranean basin and Eurasia within the last 20 million years. The origin of *Biscutella* is dated ca 18.75 Mya, and the radiation of the series triggered ca 5.87 Mya with the Messinian Salinity Crisis. Rapid diversification occurred coetaneously to the Intensification of Northern Hemisphere Glaciation (ca 2.86 Mya) onwards, with parallel large-amplitude aridity cycles in Africa and southwestern Asia. In recent times, the divergence of lineages became faster in the W Mediterranean (ca 1.54 to 0.43 Mya), mostly related to geographical and ecological patterns of specialisation. In many cases, the distribution of the current species is apparently linked to ancient glacial refuges in S Mediterranean basin.

1. Introduction

The Mediterranean Region (including Macaronesia) is currently considered a global centre of rapid and recent plant diversification (Valente and Vargas, 2013; Rundel et al., 2016). About 24,000 species occur in that territory, ca. 60% of which are endemics (Greuter, 1991). In particular, the W Mediterranean basin is one of the most important plant biodiversity hotspots at a global scale (Médail and Myers, 2004), which includes a high number of taxa with narrow distributions (Thompson, 2005).

Multiple paleoclimatic and paleogeographic events happened in that area through the late Tertiary and the Quaternary favouring varied scenarios that gave rise to an outstanding rich flora (Nieto Feliner, 2014; Simón-Porcar et al., 2015). Furthermore, the Mediterranean basin stands at a biogeographical crossroads among the European, Saharian and Irano-Turanian regions (Quézel, 1985; Médail and Diadema, 2009), and has suffered a continuous human influence from prehistoric times that intensified in the last 5000 years (Abulafia, 2011). All those factors have directly contributed to mould its outstanding plant biota (flora and vegetation).

Biscutella L. (Brassicaceae) is a genus of annual herbs and dwarf shrubs distributed through the Mediterranean basin, C Europe, and SW Asia, which comprises about 45–53 species (cf. Warwick and Al-Shehbaz, 2006; Marhold, 2011; Al-Shehbaz, 2012). It is the type of tribe *Biscutelleae*, in which other morphologically divergent genera such as *Heldreichia* Boiss., *Lunaria* L., *Megadenia* Maxim. and *Ricotia* L. (Özüdoğru et al., 2015, 2017) are also included. *Biscutella* displays a high taxonomic complexity, due to the the relative uniformity of flower and fruit characters which contrasts with a broad inter- and intraspecific morphological plasticity of vegetative features in many taxa (Olowokudejo, 1986; Guinea and Heywood, 1993). As a result, and depending on the criteria adopted and sources used by the different

* Corresponding author.
E-mail address: ma.alonso@ua.es (M.Á. Alonso).

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authors, many contrasting taxonomic treatments are available in regional floras and monographs (cf. Al-Shehbaz, 2012; Appel and Al-Shehbaz, 2003; deCandolle, 1811; Guinea, 1964; Machatschki-Laurich, 1926; Malinowski, 1911; Jordan, 1864; Marhold, 2011; The Plant List, 2013).

Two morphologically well differentiated sections are often recognised in *Biscutella* (Grau and Klingenberg, 1993; Guinea and Heywood, 1993): *B.* sect. *Biscutella* and *B.* sect. *Jondraba* (Medik.) DC. (≡ *Jondraba* Medik.). *Biscutella* ser. *Biscutella* (= ser. *Lyratae* Malin.) belongs to the former section and comprises mostly annual or short-lived perennial plants mainly distributed in N Africa, SE Asia and W Europe, showing petals gradually tapering at the base and lateral intrastaminal nectaries (Malinowski, 1911; Olowokudejo, 1986; Guinea and Heywood, 1993).

The morphological variation of *Biscutella* is however unevenly distributed. Taxa of *B.* ser. *Biscutella* occur exclusively throughout the Mediterranean basin and SW Asia (Malinowski, 1911; Machatschki-Laurich, 1926; Maire, 1967; Hedge, 1968; Olowokudejo, 1986; Guinea and Heywood, 1993). Relatively morphological homogeneity can be observed in C-E Mediterranean basin to SW Asia where *B. didyma* L. is mostly distributed, whereas the highest morphological plasticity and diversity occurs in N Africa with up to five species (cf. Vicente et al., 2016, 2019a, 2019b).

Biscutella didyma is the type of the series (and also the genus) and it has been treated in rather conflicting ways, sometimes including a huge number of infraspecific taxa in several regional floras and monographs (cf. Quézel and Santa, 1963; Guinea and Heywood, 1964, 1993; Maire, 1967; Pottier-Alapetite, 1979; Hedge, 1968, 1984; Raffaelli, 1991; Boulos, 1999; Fennane, 1999). Some names have usually been connected to that species at contrasting taxonomic ranks, such as *B. ciliata* DC., *B. columnae* Ten., *B. depressa* Willd., *B. depressa* var. *applanata* Mach.-Laur., *B. eriocarpa* DC., *B. leiocarpa* DC., *B. didyma* subsp. *apula* Nyman, *B. morisiana* Raffaelli, or *B. didyma* var. *dunensis* Chrtek & B. Slavík, among others. All those taxa were described on the basis of slight variations in leaf and fruit features. The enormous variation of *B.* ser. *Biscutella* found in N Africa was arranged by Maire (1967) in 30 infraspecific taxa within *B. didyma*, and it has been attributed in later floras to the variability of both "*B. didyma* sensu lato" and "*B. microcarpa* DC." (Fennane, 1999) or *B. boetica* (Grau, 2002), the two latter species having been described from the southern Iberian Peninsula. In this respect, the name *B. lyrata* (=*B. microcarpa*) is traditionally applied to plants occurring in S Spain and sometimes cited in N Africa (Desfontaines, 1798; Battandier and Trabut, 1902; Maire, 1967; Pottier-Alapetite, 1979; Guinea and Heywood, 1993; Le Floc'h et al., 2010). However, recent molecular and morphological work (Vicente et al., 2016, 2019a) allowed treating it as a Spanish endemic not present in Africa. Furthermore, similar integrative approaches (cf. Sukhorukov et al., 2017; Vicente et al., 2016) have contributed to clarify the circumscription and distribution of the close central Mediterranean pair *B. maritima* Ten. (Italy, Sicily, Malta, Algeria, and Tunisia) and *B. raphanifolia* Poir. (Algeria and Tunisia), as well as to describe *B. pseudolyrata* A. Vicente, M.Á. Alonso & M.B. Crespo, a remarkable new species growing in sandy Quaternary substrates from Gharb region in NW Morocco (Vicente et al., 2019a).

A recent comprehensive revision of *B.* ser. *Biscutella* (Vicente et al., 2019b), including a reevaluation of morphological characters, has reduced its diversity to ten taxa (7 species and 3 varieties) exhibiting distinct morphologies and well-defined distributions and/or ecology, which in several cases have proved to be monophyletic groups (cf. Vicente et al., 2016, 2019a). That arrangement is accepted here as the starting point of further phylogenetic and evolutionary work.

Patterns of diversification in Mediterranean plant groups have been often connected to geological and climate changes (Blondel et al., 2010; Simón-Porcar et al., 2015; Affenzeller et al., 2018), and glacial refuges in N Africa and S Europe have usually been suggested to play an important role in maintenance of biodiversity during the late Tertiary and the Quaternary (Médail and Diadema, 2009). In this respect, Parisod and Besnard (2007) argued that diversification of *Biscutella* ser. *Laevigatae* Malin. in Central Europe followed recent and rapid processes involving polyploidy and reticulate evolution during the Last Glacial Maximum (LGM; about last 20,000 years). However, no data exist so far on the evolutionary history of *B.* ser. *Biscutella*. The available information concerning long-term evolutionary events in Brassicaceae dates the divergence time of *Biscutelleae* in about 27.4–25.4 Million years ago (Mya) (Huang et al., 2015), 20–17.5 Mya (Hohmann et al., 2015), 27.4–17.9 Mya (Mandáková et al., 2018), or 21.0–14.0 Mya (Huang et al., 2019), when the tribe diverged from other cruciferous lineages.

In this scenario, the aims of the present work are: (i) to generate the first comprehensive phylogeny of *B.* ser. *Biscutella* based on two plastid (*rpl32-trnL, trnV*) and one nuclear (ITS) DNA regions; (ii) to obtain the first time-calibrated phylogenetic tree to estimate the divergence times of lineages within the series; (iii) to relate the diverging lineages and the major Post-Miocene geological and climatic events occurred in the Mediterranean basin and Eurasia for disentangling evolutionary relationships and eventual biogeographical patterns in *B.* ser. *Biscutella*; and (iv) to investigate the role that Mediterranean glacial refuges played in the preservation and subsequent diversification of lineages in the series.

2. Material and methods

2.1. Plant sampling

Fresh material of all ten accepted taxa in *Biscutella* ser. *Biscutella* collected during field work in Algeria, Greece, Italy (including Sicily and Sardinia), Morocco, Spain, Turkey and Tunisia, as well as herbarium specimens conserved at ABH, B, BC, BCN, CAI, COA, COI, COFC, EGE, FI, G, GDA, GZU, HAL, JE, K, MA, MPU, P, RGN, SALA, SEV, VAL and VLA (acronyms according to Thiers, 2019) were used for morphological and molecular studies (see Table 1). The principal Mediterranean floras were consulted for taxonomic identification (Battandier, 1888; Quézel and Santa, 1963; Maire, 1967; Hedge, 1968, 1984; Pottier-Alapetite, 1979; Pignatti, 1982; Grau, 2002; Grau and Klingenberg, 1993; Guinea and Heywood, 1993; Boulos, 1999; Fennane, 1999). Over 800 herbarium vouchers were visually examined (see Vicente et al., 2019b), and both qualitative and quantitative analyses were conducted on over 600 of those specimens. The characters observed or measured (Table 2) were selected from those traditionally used in the literature on the genus (Jordan, 1864; Poiret, 1789; Malinowski, 1911; Guinea, 1964; Maire, 1967). For a more detailed description of materials and methods used for previous morphological studies undertaken on *B.* ser. *Biscutella* see Vicente et al. (2019b). Author names of taxa cited in the text and tables accord with IPNI (2019).

2.2. Molecular analyses

Forty-five samples belonging to 10 taxa of *Biscutella* ser. *Biscutella* were used for phylogenetic reconstruction, using *Lepidium draba* L. [*Cardaria draba* (L.) Desv.] and *Megadenia speluncarum* Vorob., Vorosch. & Gorovoj (sensu Artyukova et al., 2014) as outgroups. Plant source information and GenBank accession numbers are shown in Table 1. The DNA extraction was made from silicagel-dried leaves (Chase and Hill, 1991) or voucher material, according to a modification of the 2× CTAB protocol (Doyle and Doyle, 1987). Total DNA was purified using MOBIO minicolums and kept in 0.1× TE buffer. The study is based on one nrDNA internal transcribed spacer region (ITS) and the cpDNA regions *rpl32-trnL* and *trnV* intron. The PCR amplifications of ITS was obtained using the primers ITS5/ITS4 (White et al., 1990), whereas *rpl32-trnL* and *trnV* intron sequences were obtained using the primer pairs *rpl32*F/*trnL* (Shaw et al., 2007) and *trnV*_F/R (Wang et al., 2003). The amplifications were performed on a reaction volume of 25 µl

Table 1
Samples included in the phylogenetic analyses of *Biscutella* ser. *Biscutella*.

Code	Taxon	Locality	Voucher	GenBank ref. *rpl32-trnL*	GenBank ref. *trnV*	GenBank ref. ITS
L. draba ES	*Lepidium draba* L.	SPAIN. Alicante: San Vicente del Raspeig	ABH71952	KU746330	KU746332	KU746329
M. speluncarum RU	*Megadenia speluncarum* Vorob. & al.	RUSSIA. Primorskii Krai, Lozovy	VLA10454	KX943557	KX943556	KX943555
B. pseudolyrata MO8	*Biscutella pseudolyrata* A. Vicente & al.	MOROCCO. Rabat-Salé-Zemmour, road from Salé to Sidi Allal el Bahraoui	ABH74994	MF521261	MF521296	MF521226
B. pseudolyrata MO9	*B. pseudolyrata*	MOROCCO. Rabat-Salé-Zemmour, road from Sidi Allal el Bahraoui to Kenitra	ABH75002	MF521262	MF521297	MF521227
B. pseudolyrata MO10	*B. pseudolyrata*	MOROCCO. Larache road from Larache to Ksar-el-Kebir	ABH74993	MF521263	MF521298	MF521228
B. erioc. var. *riphaea* DZ1	*B. eriocarpa* DC. var. *riphaea* A. Vicente & al.	ALGERIA. Tlemcem	ABH69561	KU570220	KU574029	KU570210
B. erioc. var. *riphaea* DZ2	*B. eriocarpa* var. *riphaea*	ALGERIA. Tlemcem, Plateau Lalla Setti	ABH59292	MF521239	MF521274	MF521204
B. erioc. var. *riphaea* MO3	*B. eriocarpa* var. *riphaea*	MOROCCO. Taza, Bab-Azhar, Djbel Tazzeka	ABH68350	MF521238	MF521273	MF521203
B. erioc. var. *riphaea* MO4	*B. eriocarpa* var. *riphaea*	MOROCCO. Oujda, Beni Snassen, prox. Taforalt	ABH68371	KU570219	KU574028	KU570209
B. erioc. var. *riphaea* MO5	*B. eriocarpa* var. *riphaea*	MOROCCO. High Atlas, Afourer to Bin-el-Ouidane	MA625029	MF521240	MF521275	MF521205
B. erioc. var. *riphaea* MO6	*B. eriocarpa* var. *riphaea*	MOROCCO. Azilal, Djbel Azourki	ABH68360	MF521241	MF521276	MF521206
B. erioc. var. *riphaea* MO7	*B. eriocarpa* var. *riphaea*	MOROCCO. High Atlas, Tizi'n-Tichka	ABH69882	MF521266	MF521301	MF521231
B. erioc. var. *riphaea* MO11	*B. eriocarpa* var. *riphaea*	MOROCCO. From Marrakech to Ouarzazat	MA799996	MF521268	MF521303	MF521233
B. erioc. var. *riphaea* MO12	*B. eriocarpa* var. *riphaea*	MOROCCO. Chaouia-Ouardigha, road Rabat to Slimane	ABH74996	MF521265	MF521300	MF521230
B. erioc. var. *eriocarpa* MO1	*B. eriocarpa* var. *eriocarpa*	MOROCCO. Fès, Birtam-Tam	ABH68408	MF521237	MF521272	MF521202
B. erioc. var. *eriocarpa* MO2	*B. eriocarpa* var. *eriocarpa*	MOROCCO. Meknés, road Azrou-Meknés, pr. Ito	ABH68336	MF521264	MF521299	MF521229
B. erioc. var. *eriocarpa* MO13	*B. eriocarpa* var. *eriocarpa*	MOROCCO. Between Agadir and Tafraoute	SEV270620	MF521267	MF521302	MF521232
B. erioc. var. *eriocarpa* MO14	*B. eriocarpa* var. *eriocarpa*	MOROCCO. Grand Casablanca	ABH74998	MF521269	MF521304	MF521234
B. erioc. var. *eriocarpa* MO15	*B. eriocarpa* var. *eriocarpa*	MOROCCO. Souss-Massa-Draa, between Ait Mansour and Afella Ighir	ABH74999	MF521270	MF521305	MF521235
B. erioc. var. *eriocarpa* MO16	*B. eriocarpa* var. *eriocarpa*	MOROCCO. Chemaia	SEV203362	MF521271	MF521306	MF521236
B. boetica MO17	*B. boetica* Boiss. & Reut.	MOROCCO. Chefchauen, pr. Aguelman	MA782810	MF521242	MF521277	MF521207
B. boetica MO18	*B. boetica*	MOROCCO. Tánger-Tétouan, Oued Laou	MA807348	MF521244	MF521279	MF521209
B. boetica MO19	*B. boetica*	MOROCCO. Fahs-Anjra, Ksar-es-Seghir	ABH69317	MF521245	MF521280	MF521210
B. boetica MO20	*B. boetica*	MOROCCO. Chauen, Djebel Kalaa	ABH68347	MF521246	MF521281	MF521211
B. boetica MO21	*B. boetica*	MOROCCO. Tayenza, Forêt Bouhachem	ABH69331	MF521243	MF521278	MF521208
B. boetica ES4	*B. boetica*	SPAIN. Cádiz: Alcalá de los Gazules	ABH70652	MF521247	MF521282	MF521212
B. boetica ES5	*B. boetica*	SPAIN. Málaga: Sedella, ruta del Pozancón	ABH 59820	MF521248	MF521283	MF521213
B. raph. var. *algeriensis* DZ29	*B. raphanifolia* Poir. var. *algeriensis* (Jord.) A. Vicente & al.	ALGERIA. Médea, Berrouaghia	ABH72640	KU570216	KU574025	KU570206
B. raph. var. *algeriensis* DZ35	*B. raphanifolia* var. *algeriensis*	ALGERIA. Blida, Chrea National Park	ABH72641	KU570217	KU574026	KU570207
B. raph. var. *raphanifolia* DZ10	*B. raphanifolia* var. *raphanifolia*	ALGERIA. Wilaya de Tizi-Ouzou, l'Akfadou, près de la maison forestière de Tala Kitane	VAL33967	KU570218	KU574027	KU570208
B. maritima IT1	*B. maritima* Ten.	ITALY. Sicilia: Cerami	ABH70575	MF521249	MF521284	MF521214
B. maritima IT2	*B. maritima*	ITALY. Calabria: Punta Stalleti	ABH70571	MF521250	MF521285	MF521215
B. *maritima* TN1	*B. maritima*	TUNISIA. Nabeul, cap Bon	MA797732	KU570214	KU574023	KU570204
B. maritima TN2	*B. maritima*	TUNISIA. Rouhia, 30 km from Rouhia to Maktar	MA724301	KU570215	KU574024	KU570205
B. maritima TN3	*B. maritima*	TUNISIA. Tabarka	Herb. Fac. Sci. Bizerta	MF521251	MF521286	MF521216
B. didyma var. *didyma* GR	*B. didyma* L. var. *didyma*	GREECE. Attikí, Cape Sounion	ABH58628	KU570212	KU574021	KU570202
B. didyma var. *didyma* IT4	*B. didyma* var. *didyma*	ITALY. Sardinia: Siniscola Mte. Albo	ABH70560	MF521252	MF521287	MF521217
B. didyma var. *ciliata* IT5	*B. didyma* var. *ciliata* (DC.) Vis.	ITALY. Sardinia: Jerzu	ABH70563	MF521253	MF521288	MF521218
B. didyma var. *ciliata* IT6	*B. didyma* var. *ciliata*	ITALY. Puglia, Bari	ABH70564	MF521254	MF521289	MF521219
B. didyma var. *ciliata* IT7	*B. didyma* var. *ciliata*	ITALY. Puglia, Martina Franca	ABH70559	KU570213	KU574022	KU570203
B. didyma var. *ciliata* TR1	*B. didyma* var. *ciliata*	TURKEY. Muğla province, Datça	ABH76351	MF521256	MF521291	MF521221
B. didyma var. *didyma* TR2	*B. didyma* var. *didyma*	TURKEY. Balkesir, Ayvalık Şeytan Sofrası	EGE19956	MF521257	MF521292	MF521222
B. didyma var. *didyma* CY	*B. didyma* var. *didyma*	CYPRUS. Paphos, ruines du château byzantin	MA526436	MF521255	MF521290	MF521220
B. didyma var. *ciliata* JO	*B. didyma* var. *ciliata*	JORDAN. Prov. Ammann, Dschebel Siyagha, bei Madaba	GZU00318728	MF521258	MF521293	MF521223
B. lyrata ES1	*B. lyrata* L.	SPAIN. Cádiz: Alcalá de los Gazules, ruta al Picacho	ABH70649	KU570211	KU574020	KU570201
B. lyrata ES2	*B. lyrata*	SPAIN. Cádiz: Alcalá de los Gazules, ruta al Picacho	ABH70648	MF521259	MF521294	MF521224
B. lyrata ES3	*B. lyrata*	SPAIN. Cádiz: Jerez de la Frontera	MA790862	MF521260	MF521295	MF521225

Table 2
Most valuable morphological characters defineng taxa in *B.* ser. *Biscutella*, according to Vicente et al. (2019b).

Character	*B. didyma* var. *didyma*	*B. didyma* var. *ciliata*	*B. raphanifolia* var. *raphanifolia*	*B. raphanifolia* var. *algeriensis*	*B. maritima*	*B. pseudolyrata*	*B. lyrata*	*B. boetica*	*B. eriocarpa* var. *riphaea*	*B. eriocarpa* var. *eriocarpa*
Stem length (cm)	(7–)15–50	(7–)15–50(–60)	30–100	23–40	15–85(–120)	25–45(–60)	15–45	20–60(–70)	(10–)20–50(–65)	13–37
Stem indumentum	hirsute at base	hirsute at base	hirsute to lanate at base	hirsute at base	hirsute at base	hirsute at base	glabrescent	hirsute at base	hirsute at base	glabrescent to hirsute at base
Leaf indumentum	hirsute	hirsute	hirsute to lanate	hirsute	hirsute	hirsute	glabrescent, scarcely hirsute	hirsute	hirsute	hirsute
Basal leaf shape	oblanceolate (pinnatisect)	oblanceolate (pinnatisect)	lyrate-pinnatipartite	lyrate-pinnatipartite	lyrate-spatulate (oblanceolate)	lyrate to oblanceolate	lyrate-pinnatipartite	oblanceolate (pinnatipartite)	pinnatipartite-pinnatisect	dentate to pinnatipartite-pinnatisect
Basal leaf size (cm)	up to 8 × 2	up to 7 × 2	up to 20 × 6	up to 8.5 × 3.5	up to 20 × 6	up to 12 × 3	9 × 3	up to 11 × 3.5	up to 13 × 4.5	up to 7 × 2.5
Cauline leaves	absent (occasionally 1)	present	present	present (occasionally absent)	absent (occasionally 1)	absent (occasionally 1–2)	1–5 (rarely absent)	1–5 (rarely absent)	1–8	0–1(–3)
Cauline leaves insertion	attenuate	attenuate	amplexicaul to auriculate	amplexicaul to attenuate	attenuate-semiamplexicaul	attenuate	attenuate	attenuate-semiamplexicaul	attenuate to amplexicaul	attenuate to semiamplexicaul
Inflorescence branching pattern	B–C +	B–C +	D	D	B–C +	A–C(C +)	A–C(C +)	B–C(C +)	(A)B–C(C +)	(A)B–C
Num. of terminal racemes per branch	1–8(–15)	1–8(–11)	8–20(–30)	8–20(–30)	2–8(–15)	1–8	1–8	2–8(–15)	2–8(–13)	1–8
Raceme length/stem length ratio	0.06–0.25	0.09–0.30	0.05–0.16	0.10–0.25	0.10–0.35	0.19–0.42	0.21–0.5	0.25–0.5(–0.6)	0.15–0.4	0.10–0.33(–0.4)
Pedicel insertion	non-unilateral erect (to erect-patent)	non-unilateral erect (to erect-patent)	erect-patent	erect-patent	non-unilateral, patent to erect-patent	non-unilateral erect-patent	patent to erect-patent	non-unilateral, patent to erect-patent (unilateral)	unilateral or non-unilateral erect to erect-patent	unilateral or non-unilateral, patent to erect-patent
Pedicel length (first 5 basal fruit mean)	2.0–6	2.2–8	5.3–11	5.4–10.4	(5.5–)7–12	6.5–11	5–9	6.5–9(–10)	4.5–8.5(–9.5)	(3.5–)4.7–9.0
Sepal length (mm)	1.3–2.5	1.3–2.5	1.8–3	1.4–2.6	(1.5–)2–3	1.8–3.0	1.3–2.0	1.5–2.5	1.5–2.6	1.5–2.5
Petal length (mm)	(2–)2.5–4.5	2.1–5	3–6	2.9–5(–6)	(3.5–)4–6.5	2.8–5.0(–6.0)	2–4	2.5–5.0	2.3–4.8	2.5–5.0
Staminal filament	not winged	not winged	not winged	not winged	not winged	not winged	broadly membranous wing	not winged	not winged	not winged
Median nectary length	inconspicuous or up to 0.20	inconspicuous or up to 0.20	inconspicuous or up to 0.4 mm	inconspicuous or up to 0.4 mm	(0.4–)0.5–0.7(–0.8) mm	0.4–0.6 mm	up to 0.4 mm	0.2–0.4 mm	up to 0.25 mm	up to 0.25 mm
Raceme density (fruits/cm)	5–9	(2.5–)3–9	(1.5–)2–5	(1.8–)2–4.5	1.5–4.5(–6)	1.3–2.8(–3.4)	1.5–3.5	1.5–3(–3.5)	(2–)3–8	(2.5–) 4–10
Fruit width (mm)	7.0–13.5	7.0–13.5	(8.2–)9–15	4.5–9(–11)	(6.0–)7–13	(6.5–)7–12	4–6	(4.5–)5.0–10	5.5–10(–13)	5–10
Style length	1.5–2.5	1.8–3.0	2.6–4.5(–5.0)	2.0–3.0	2.0–3.5	(1.6–)1.9–2.9	1–2(–2.5)	1.8–3.0(–3.8)	1.5–3.0	1.5–2.5
Style length/fruit width ratio	0.18–0.23	0.19–0.25	0.27–0.40	0.28–0.45	(0.23–)0.25–0.37	(0.18–)0.22–0.33	0.30–0.40	(0.28–)0.30–0.45	0.22–0.40	0.22–0.35
Fruit indumentum type	S1-S6	S1-S6	S6 (S1-S5)	S2 (mostly), S1-S6	S1-S6	S1-S6	S1, S4-S6	S2-S3, S6	S2-S3, S6	S1-S3, S6

containing 22.5× ABGene, 1.1× Master Mix, 2.5 mMT $MgCl_2$ (Thermo Scientific Waltham, MA, U.S.A.), 0.5 µl of 0.4% bovine serum albumin (BSA), 0.5 µl of each primer (10 pmol/µl) and 1 µl of template DNA, on a 9700 GeneAmpl thermocycler (Applied Biosystems). The PCR programmes used were, for ITS: 2 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min and a final extension of 72 °C for 5 min; for *rpl32-trnL*: 2 min at 94 °C, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1.5 min, 72 °C for 10 min and a final extension of 72 °C for 10 min; and for *trnV*: an initialization step of 3 min at 94 °C, followed by 42 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1.5 min and a final extension of 72 °C for 10 min.

Sequencer 4.1 (Gene Codes Corp., Ann Arbor, MI, USA) was used to assemble complementary strands. The DNA regions were aligned using Clustal W, conducted in MEGA X (v. 10.0.5) (Kumar et al., 2018), with subsequent minor manual improvement. Three different data sets were produced, corresponding respectively to: (1) the combined plastid (two regions) data matrix; (2) the nuclear (ITS) data matrix; and (3) the concatenated molecular (plastid plus nuclear) sequence data matrix. The gaps were codified with FastGap 1.2 (Borchsenius, 2009) following the method of Simmons and Ochoterena (2000), and then added to DNA data matrix as separate partitions when appropriate. Maximum parsimony analysis (MP) were conducted in both PAUP (using Heuristic search options with the tree searching strategy based on Nearest Neighbour Interchange, NNI) and MEGA (using Heuristic search options with the tree searching strategy based on Subtree-Pruning-Regrafting –SPR– with search level 1; Nei and Kumar, 2000) for result comparison, with 10,000 replicates. Maximum Likelihood (ML) (Felsenstein, 1981) and Neighbour-Joining (NJ) (Saitou and Nei, 1987) analyses were performed in MEGA. To determine the best model of DNA substitutions for each region, jModelTest 2.1.10 (Darriba et al., 2012) was performed, using the Akaike Information Criterion (AIC; Akaike, 1974); models with the lowest BIC (Bayesian Information Criterion) scores were considered to best describe the substitution pattern for the ML and NJ analyses. Evolutionary distances for NJ and phylogenetic reconstructions for ML were estimated using the T92 model (3-parameter model of Tamura, 1992) for both the combined plastid and concatenated molecular matrices, and the General Time Reversible (GTR) model for the nuclear matrices; the evolutionary rate variation among sites was modelled with a Gamma distribution (*G* parameter = 0.7055), and partial deletion of gaps was applied in all cases (positions with less than 95% site coverage were eliminated). Furthermore, Bayesian inference (BI) analyses were conducted with MrBayes 3.2 (Ronquist et al., 2012). For BI analyses, the Markov chain Monte Carlo (MCMC) runs were performed for 1.0×10^7 generations and sampled every 1000 generations. Two runs were executed. The GTR + proportion of invariant sites (*I*) + gamma distribution (*G*) model was used in the analyses (set nst = 6 rates = invgamma). The first 25% generations (burninfrac = 0.25) were excluded and the remaining trees were used to compile a posterior probability (PP) distribution using a 50% majority-rule consensus. For all methods, support was assessed by the bootstrap (Felsenstein, 1985) with 10,000 replicates, but holding only 10 trees per replicate. Clades showing bootstrap (BS) values of 50–74% were considered as weakly supported, 75–89% moderately supported and 90–100% strongly supported. Because our preliminary analyses of each matrix revealed that the resulting trees prior to and after gap inclusion were identical, gaps were therefore considered only in the MP analyses performed in PAUP, but they were disregarded in the MP, ML and NJ analyses performed in MEGA due to their computing features.

2.3. Congruence between datasets

Topological incongruence between cpDNA (*rpl32-trnL* and *trnV* intron) and nDNA (ITS region) datasets was checked by two methods. Firstly, ILD test (Farris et al., 1994) was performed in PAUP v.4.0.b10 (Swofford, 2002) using heuristic search options, which included 100 random addition replicates and tree-bisection-reconnection (TBR) branch swapping with MulTrees in effect, and keeping 10 trees per replicate.

Secondly, comparison of the ML phylogenetic trees of individual cpDNA and nDNA datasets was explored using MEGA, with the substitution model T92 + *G* (Tamura, 1992) as selected in jModelTest, and with 1000 fast bootstrap replicates. A tanglegram comparing the ML tree of each dataset was computed in Dendroscope 3.6.2 (Huson and Scornavacca, 2012) and checked for topological conflicts on the basis of BP support ≥85% (Norup et al., 2006), but also BP support ≥75% to detect further relationships.

2.4. Dating of phylogenetic trees

Divergence time estimation was conducted in MEGA. Firstly, the molecular clock test was performed by comparing the ML value for the given topology with and without the molecular clock constraints under T92 + *G* model (Tamura, 1992), and the null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level (P = 3.354E-040). Secondly, a timetree was inferred using the Reltime method (Tamura et al., 2012, 2018) and the Tamura 3-parameter model (Tamura, 1992) as implemented in MEGA. Provided that neither fossil records nor taxon-specific substitution rates are available for sequence calibrations within Biscutelleae, one node age available for outgroup taxa was utilised. The *Lepidium-Megadenia* node was calibrated with a minimum age of 17.5 Mya and a maximum age of 20 Mya as the only constraint to compute the timetree, according to the divergence time estimates for tribes Lepidieae and Biscutelleae (cf. Hohmann et al., 2015). The estimated log likelihood value was −6534.97. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; +*G* = 0.1944). The analysis involved 47 nucleotide sequences, with a final dataset including a total of 2254 positions. All positions of the nucleotide sequences with less than 95% site coverage were eliminated (fewer than 5% alignment gaps), missing data and ambiguous bases were allowed at any position (partial deletion option). The obtained MEGA timetree was managed with FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/), for improving the output design.

3. Results

3.1. Phylogenetic results

Concatenation of both plastid regions generated an aligned dataset of 2004 positions (1644 disregarding gaps), of which 46.01% were potentially parsimony informative. The aligned ITS dataset included 634 positions (610 disregarding gaps), of which 15.61% were parsimony informative. When all plastid plus nuclear regions were concatenated, the resulting aligned molecular matrix was of 2634 positions (2254 disregarding gaps), with 8.16% of them being parsimony informative.

Preliminary analyses of each individual matrix (with and without gaps) using NJ, MP, ML and BI methods yielded trees with similar major topologies and almost identical support in most branches (see Supplemental material Fig. S1 for ML tree of cpDNA). Nevertheless, the ITS trees slightly differed by a lower branch support and the unresolved position or very weak support of some branches (see Supplemental material Fig. S2 for ML tree). Bayesian PP and parsimony BS values were well correlated in all cases. Application of ILD test suggested the existence of slight incongruence between plastid and nuclear data sets (P = 0.01), whereas comparison of individual ML trees of cpDNA and nDNA datasets yielded no remarkable conflicts (excepting the weakly supported ambiguous position of *B. raphanifolia* and *B. maritima* with regard to *B. didyma*). Consequently, as no major differences were found in the topologies of all obtained trees and also because some authors (Barker and Lutzoni, 2002) argued that combining heterogeneous data

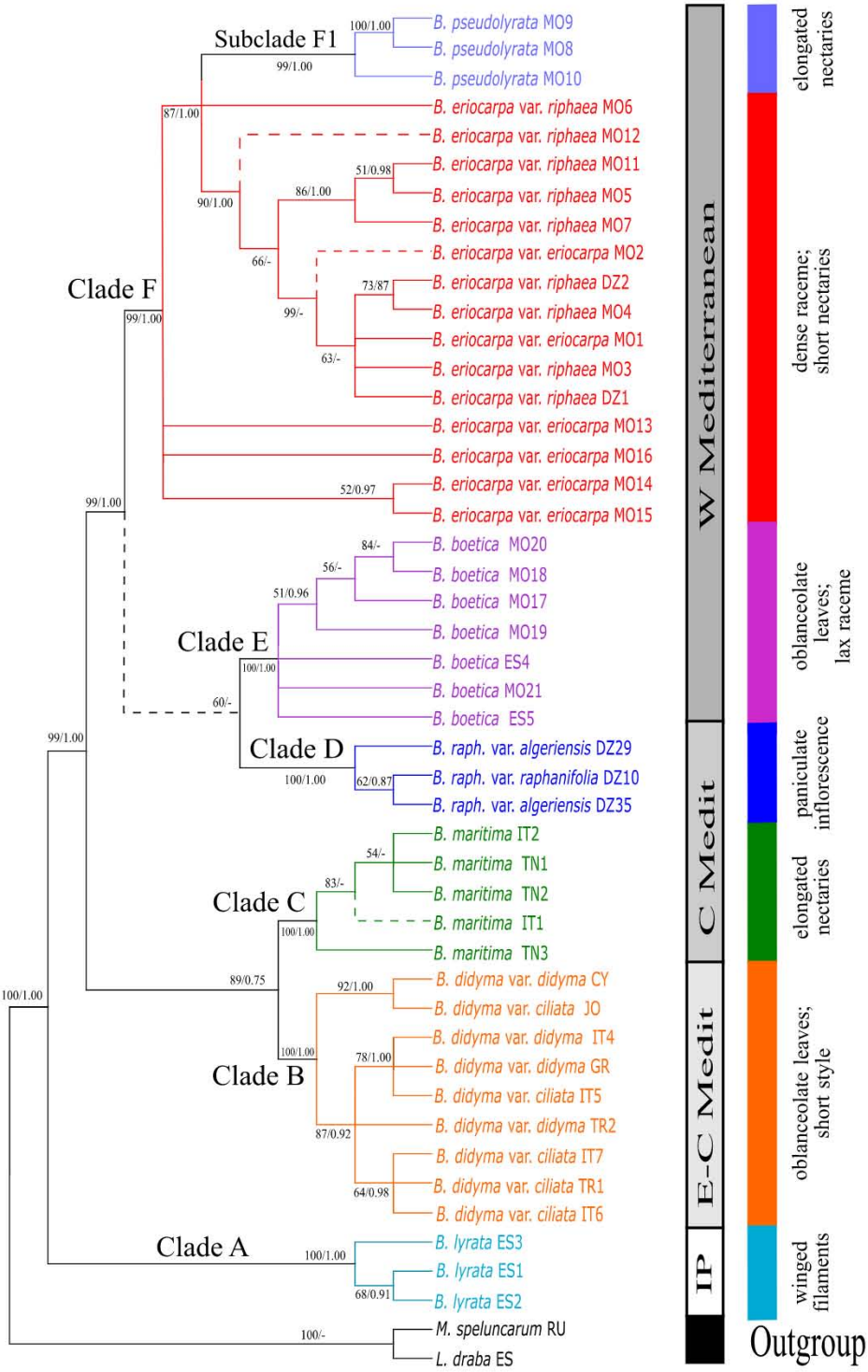

Fig. 1. Phylogenetic tree of *Biscutella* ser. *Biscutella* from the Maximum Likelihood (ML) analysis using Tamura 3-parameter model (log likelihood −6518.31), estimated from concatenated cpDNA (*rpl*32-*trnL* and *trnV*) and nrDNA (ITS) sequences (final dataset of 47 nucleotide sequences, with a total of 2254 positions). A discrete Gamma distribution was used to model evolutionary rate differences among sites (*G* parameter = 0.7055). Dotted branches indicate clades not recovered in the Bayesian analysis. Bootstrap values (BS) and Bayesian posterior probability (PP) are shown above branches.

can also increase accuracy even if ILD analyses do not explicitly incorporate that heterogeneity, we accept the phylogenetic trees obtained from the combined molecular matrix (concatenated plastid plus nuclear regions) as a good reconstruction of the evolutionary history of *B.* ser. *Biscutella*, according to our previous results (Vicente et al., 2016). In fact, some authors have long disregarded ILD as an appropriate tool for testing suitability of dataset concatenation (Yoder et al., 2001; Pirie, 2015).

In Fig. 1, the phylogenetic relationships of taxa of *Biscutella* are shown as recovered in our ML consensus tree of the combined molecular matrix (2254 positions, highest log likelihood = −6518.31), in which BS percentages and PP values are placed above branches (respectively, from the ML and BI analyses). The topology of that tree, like in most trees resulting from all our combined analyses, is congruent with that reported by Vicente et al. (2016) for a more reduced set of taxa, but shows even higher support for some branches.

Biscutella is recovered as monophyletic with a strong support (100% BS, 1.00 PP) in all analyses. The basal clade of the ingroup (Clade A) is formed by the three accessions of *B. lyrata* (100% BS, 1.00 PP), which are strongly supported (99% BS, 1.00 PP) as sister to the remaining taxa in the series. Within this group, *B. didyma* (Clade B; 100% BS, 1.00 PP), including both varieties, and *B. maritima* (Clade C; 100% BS, 1.00 PP) constitute a clade with moderate support (89% BP, 75 PP) that is strongly supported (99% BS, 1.00 PP) as sister to a weakly supported (60% BP, - PP) clade including *B. raphanifolia* (Clade D; 100% BS, 1.00 PP) and *B. boetica* (Clade E; 100% BS, 1.00 PP), which is sister to the "*B. eriocarpa-B. pseudolyrata* clade" with strong support (Clade F; 99% BS, 1.00 PP). In fact, in the NJ combined analysis *B. boetica* is found as sister of the "*B. eriocarpa-B. pseudolyrata* clade", though with weak support (63% BS). The "*B. eriocarpa-B. pseudolyrata* clade" as recovered in the ML analysis includes some polytomies and exhibits in general terms a topology very similar to the one yielded in the plastid tree. Within that latter clade, relationships of the *B. eriocarpa* var. *eriocarpa* samples remain unresolved, but most samples of *B. eriocarpa* var. *riphaea* (90% BS, 1.00 PP) and *B. pseudolyrata* (Clade F1; 99% BS, 1.00 PP) nest as strongly supported (87% BS, 1.00 PP) sister groups.

However, the position of some clades slightly changed in the trees obtained from analyses of some individual databases. In the ITS analyses, *B. raphanifolia* was recovered as moderately supported (74% BS) sister to the *B. didyma-B. maritima* clade. Similarly, in the plastid analyses, *B. maritima* was not sister to *B. didyma* but weakly supported (58% BS) sister to the "*B. boetica-B. raphanifolia* plus *B. eriocarpa-B. pseudolyrata* group", the rest of major clades being identically arranged as in the combined molecular matrix analyses.

3.2. Time estimation of major diversification events

According to the adopted calibration, the obtained timetree (Fig. 2) yields *Biscutella* having diverged from *Megadenia* in the early Miocene (ca 18.75 Mya). The first split in that lineage occurred around the late-Miocene (ca 5.87 Mya), which originated the endemic Iberian clade of *B. lyrata* (Clade A), it being the oldest extant representative of the genus. The second outstanding divergence event took place much later, in the late Pliocene (ca 2.86 Mya), which apparently gave rise to two main lineages. The first one includes most of the extant C and E Mediterranean taxa and quickly, by the early Pleistocene (ca 2.55 Mya) produced the C-E Mediterranean *B. didyma* (Clade B) and the C Mediterranean *B. maritima* (Clade C), both diversifying in successive events throughout their current distribution areas. The second lineage groups mostly the current W Mediterranean taxa and it diversified rather later in parallel to the former, by the mid-Pleistocene (ca 1.54 Mya), to produce the two recentmost lineages. The first one yielded almost coetaneously (ca 1.40 Mya) the mostly C Mediterranean *B. raphanifolia* (Clade D) and the W Mediterranean *B. boetica* (Clade E), which also diverged in parallel in their distributions. Interestingly, the split between the S Iberian and N Moroccan populations of the latter is dated in very recent times (ca 0.19 Mya). The second W Mediterranean lineage includes the complex of *B. eriocarpa* (Clade F), which rather recently (ca 0.76–0.55 Mya) began a rapid diversification process that originated *B. eriocarpa* var. *eriocarpa* mostly in W and S Morocco, from which later (ca 0.43–0.41 Mya) two lineages arose within the complex: i) *B. pseudolyrata* (Clade F1), concealed in the coastal Gharb region (NW Morocco), and ii) *B. eriocarpa* var. *riphaea*, distributed mainly through the Middle Atlas and S Rif Mountains to W Algeria.

4. Discussion

Biscutella ser. *Biscutella* (= ser. *Lyratae*) is a taxonomically complex group, including morphologically variable entities which have been traditionally treated in rather diverging ranks (cf. Cosson, 1887; Maire, 1967; Pottier-Alapetite, 1979; Pignatti, 1982; Grau and Klingenberg, 1993; Fennane, 1999). Most characters often used to arrange the series have revealed not always diagnostic for taxonomy since they are highly variable even in a single population (cf. Vicente et al., 2019b). Combination of both morphological and molecular data reveals promising for new insights on the specific limits and taxonomic arrangement of taxa in the series (cf. Sukhorukov et al., 2017; Vicente et al., 2016). Our results allow recognition of seven monophyletic groups, which can be

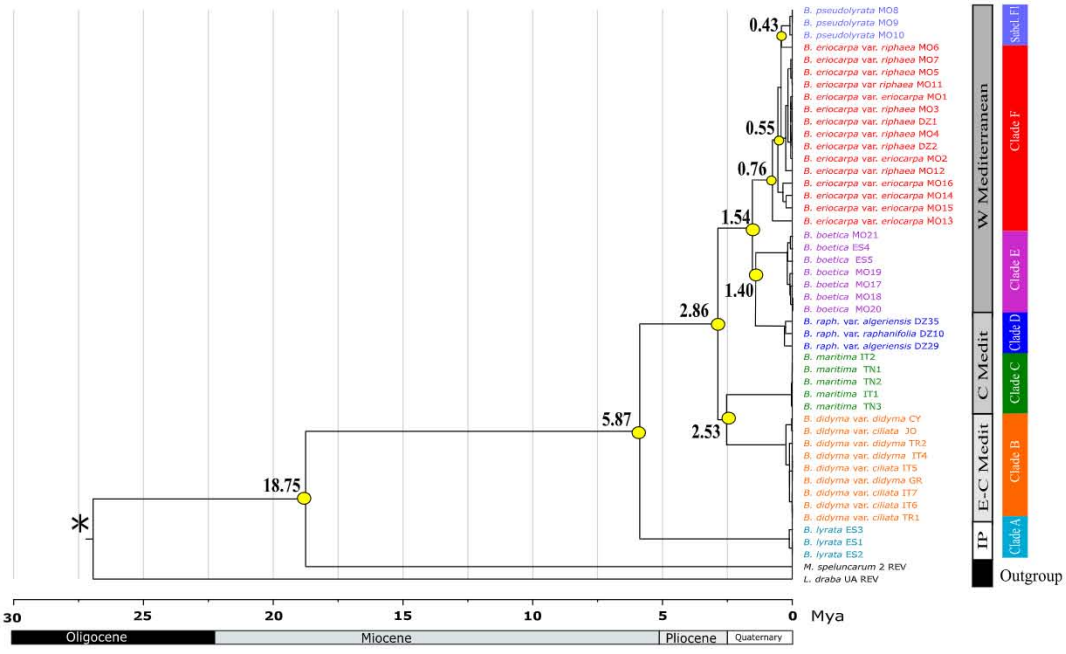

Fig. 2. Timetree inferred using Reltime method and the Tamura 3-parameter method for taxa of *Biscutella* ser. *Biscutella* (final dataset of 47 nucleotide sequences, with a total of 2254 positions). The tree (log likelihood value of −6534.97) was computed using a single calibration constraint at the outgroup node *Lepidium-Megadenia* (17.5–20 million years ago, Mya), which is marked with an asterisk. A discrete Gamma distribution was used to model evolutionary rate differences among sites (*G* parameter = 0.1944). Divergence times of the principal groups are marked on nodes in million years.

rather easily defined by a syndrome of morphological characters (Table 2), and therefore they can be accepted at species rank. Most of these groups arose rapidly in connection to major geological and climatic events occurred in the Mediterranean basin and southwestern Asia, as dated in our timetree (Fig. 2). All this information led Vicente et al. (2019b) to produce a new arrangement including ten taxa (seven species plus three varieties), which is more consistent with the observed morphological and molecular variation in the series, and hence it is followed here.

The concatenated molecular phylogenetic tree (Fig. 1) in combination with the timetree (Fig. 2) obtained in this study for *B.* ser. *Biscutella* are the first comprehensive evolutionary approaches to the genus so far. Our estimation for divergence time of *Biscutella* and *Megadenia* in the early Miocene (ca 18.75 Mya) is fully congruent with recent estimation of Mandáková et al. (2018), who placed it about 24.75–17.9 Mya (with crown at 21.67 Mya).

According to our results, *Biscutella lyrata* (Clade A; 100% BS, 1.00 PP) constitutes the basal and isolated lineage of the series. It is endemic to southern Spain (Cádiz-Algeciras region) and shows a quite constant and unique morphology (e.g. broadly winged staminal filaments and small fruits; Table 2), to which a chromosome number $2n = 12$ (Manton, 1937; Olowokudejo and Heywood, 1984; Santa Bárbara et al., 1994; all three as "*B. microcarpa*") also unique in the whole genus, can be added. All these data suggest *B. lyrata* to be ancestral in *Biscutella* ser. *Biscutella,* with the chromosome basic number $x = 6$ having appeared early in the evolution of the series, very likely following a dysploidy event (the remaining sections and series are $x = 8, 9$). This pattern might parallel the genome evolution shown by Mandáková et al. (2018) for the related genus *Ricotia* L., in which descendant dysploidy played an important role in cladogenesis of that genus. Further research is needed to shed light on that issue. The origin of the Iberian endemic *B. lyrata* is dated in our timetree around the late-Miocene epoch, namely within the Messinian age (ca 5.87 Mya), albeit Mandáková et al. (2018) found it to have diverged earlier (ca 12–7.5 Mya, with crown at 9.64 Mya), perhaps because their analysis was rooted on the estimated divergence time of Brassicaceae (ca 40–20 Mya). In the Messinian and connected with tight closure of the Strait of Gibraltar, the Mediterranean Sea fluctuated and repeatedly experienced pulses of partial or nearly complete desiccation and flooding, which extended throughout the final part of the Messinian, in the so-called Messinian Event or Messinian Salinity Crisis (cf. Hsü et al. 1973, 1978; Bocquet et al., 1978; Krijgsman et al., 1999). The Iberian and African plates remained connected about 0.6 million years through a temporal bridge along the Betic-Rifan Arc (cf. Hsü, 1974; Platt et al., 2013). This scenario likely favoured the arising of divergent lineages of *Biscutella* to colonise eastwards and southwards the newly emerged territories, as well as the persistence of ancient lineages in S Iberia such as *B. lyrata*. The opening of the Strait of Gibraltar in the late-Miocene (ca 5.3 Mya), with the subsequent "Zanclean flood", favoured isolation of the Iberian and African platforms and speciation processes began (Rodríguez-Sánchez et al., 2008), albeit in the case of *Biscutella* they might happen mostly in the southern territories.

In the late Pliocene (ca 2.86 Mya), following the onset of the Mediterranean climatic rhythm with summer drought (ca 3.2 Mya; Suc, 1984) and coetaneously to the Intensification of Northern Hemisphere Glaciation (iNHG, 2.5–3.2 Mya; Maslin et al., 2014), a second important diversification event occurred in *Biscutella* which produced two main lineages. The first one evolved in the C and E Mediterranean basin and soon produced, in the early Pleistocene (ca 2.53 Mya) with the beginning of the climatic fluctuations and the span of glaciation of the Northern Hemisphere, two new lineages whose extant representatives are *B. didyma* and *B. maritima*. First, specimens belonging to *B. didyma* group form a strongly supported clade (Clade B; 100% BS, 1.00 PP) in all trees, in which inner subclades do not match a clear geographical or ecological pattern. This is a species widespread through the C and E Mediterranean basin and SW Asia, not present in the W Mediterranean basin and NW Africa (Morocco and Algeria). Some morphological variations were observed (Table 2), which allow recognition of two mostly coexisting varieties (*B. didyma* var. *didyma* and *B. didyma* var. *ciliata*), showing numerous transitional stages sometimes difficult to assign. Combination of morphological and phylogenetic results do not support a clear ecological or geographical separation between both morphotypes, which suggests treatment at varietal rank. All chromosome counts for *B. didyma* yielded $2n = 16$ (cf. Manton 1937 as "*B. ciliata*"; Al-Shehbaz and Al-Omar 1982; Olowokudejo and Heywood 1984; Raffaelli and Fiorini 1986). It is worth mentioning that the Sardinian plants described as *B. morisiana* by Raffaelli (1991) are embedded among other members of *B. didyma*, without any geographical pattern (Fig. 1, Clade B: samples IT4 and IT5), and consequently *B. morisiana* is here better treated as a synonym of *B. didyma* (cf. Vicente et al., 2019b).

The second C Mediterranean lineage is currently represented by *B. maritima*, a species occurring in Italy, Sicily, N Tunisia and E Algeria. It forms a strongly supported group (Clade C; 100% BS, 1.00 PP) and exhibits a unique combination of very constant morphological characters (Sukhorukov et al., 2017) allowing easy recognition (Table 2). Counts of $2n = 16$ chromosomes have been reported for this species (cf. Manton, 1932 as "*B. lyrata*"; Larsen and Laegaard, 1971 as "*B. radicata*"; Olowokudejo and Heywood, 1984 as "*B. lyrata*"; Fiorini and Raffaelli, 1990 as "*B. lyrata* subsp. *laxiflora*"). The position of *B. maritima* as sister of *B. didyma* is not fully resolved in all analyses and further research is needed to clarify this issue. Samples of *B. maritima* from Tunisia and Sicily merge together in all analyses in a single unresolved clade showing some very recent radiation (ca 0.027 Mya). This fact might be interpreted as the result of dispersal events between both territories without subsequent speciation, since the climate and geological substrates are very similar in both territories and had remained without remarkable change. In fact, most of the territory currently occupied by *B. maritima* does correspond to ancient glacial refuges (see Médail and Diadema, 2009), and perhaps the N African populations might have acted as genetic source for recent northwards migrations. Population variation data will help to clarify this point.

Regarding the W Mediterranean members of the series (Clades D–F), namely "*B. didyma* pro parte maxima" sensu Maire (1967) and Fennane (1999), interesting results can be deduced from our data. The whole group is strongly suppoted (99% BS, 1.00 PP) in the ML tree, and is also recovered in as a monophyletic group diverged in the mid-Pleistocene (ca 1.54 Mya) in our timetree. North African endemics such as *B. eriocarpa*, *B. raphanifolia* and *B. pseudolyrata* together with the Iberian-Moroccan endemic *B. boetica*, are found in that group, a fact emphasizing N Africa as centre of diversity of that group (cf. Grau, 1999; Vicente et al., 2019b) through multiple glacial refugia. This broad N African clade split soon (ca 1.40 Mya) into two groups with rather clear geographical patterns. First, *B. raphanifolia* (Clade D) and *B. boetica* (Clade E) occur mostly in littoral habitats of the SW Mediterranean basin. In all analyses, each of them constituted strongly supported subclades (100% BS, 1.00 PP, in both cases). Only in the ML tree they were recorded as sisters with a very weak support (60% BS, - PP), whereas their position and relationships to other congeners remained unresolved in the rest of analyses. However, both species are morphologically very easy to recognise (Table 2). As remarked by Vicente et al. (2016, 2019b), plants found in Algeria and Tunisia that show lyrate leaves, lack highly elongated median nectaries and produce inflorescences in profusely branched panicle, fit with *B. raphanifolia* (Clade D). Our molecular tree supports results of Vicente et al. (2016), who recognised two extremes of variation within that species at varietal rank. *B. raphanifolia* is rather similar at first glance to *B. maritima*, and sometimes both have been confused. However, our phylogenetic results place both species in distinct lineages. The ambiguous position of *B. raphanifolia* (as sister to the C-E Mediterranean *B. maritima-B. didyma* clade in the ITS tree, but sister to the W Mediterranean *B. boetica* in the plastid tree; see Supplemental material Figs. S1–S2) might be

interpreted as the result of a likely hybridogenous origin from unidentified ancestors close to the current "*B. maritima-B. didyma*" and "*B. boetica-B. eriocarpa*" groups. Although morphological traits would support that hypothesis, cytological and population variation data are needed to elucidate the issue.

With regard to *B. boetica* (Clade E), all Moroccan (mostly from the Tingitana Peninsula and neighbouring Rif areas) and Iberian specimens nested together in a strongly supported clade (100% BS, 1.00 PP). They show a somatic number of $2n = 16$ chromosomes (cf. Chepinoga et al., 2009; Mejías and Luque, 1987; Olowokudejo and Heywood, 1984; Santa Bárbara et al., 1994; Ubera and Ruiz de Clavijo, 1984), and exhibit a rather constant morphology, with oblanceolate and regularly dentate to pinnatifid leaves, and inflorescence usually loose and not unilateral with patent pedicels (Table 2). No clear geographic subclades are found in our trees, both Moroccan and Spanish samples being merged unresolved in all trees. Like in the case of *B. maritima*, diversification of the *B. boetica* clade occurred through the last 0.2 million years, which makes plausible the existence of dispersal events between N Morocco and S Spain without posterior speciation.

As said before, the second mid-Pleistocene (ca 1.54 Mya) lineage includes most of the southwestern Mediterranean populations of *B.* ser. *Biscutella*, which form a strongly supported clade (Clade F; 100% BS, 1.00 PP). They show low variation in the studied DNA regions, and hence some of the obtained subclades are not well-resolved and show a very weak or no support in some cases. This is probably due to a recent diversification of the group through either ecological and geographical drift or ongoing gene flow which may obscure patterns of differentiation at DNA level, and therefore make difficult a clear taxonomic arrangement based solely on the molecular phylogeny. This fact has also been reported for other cruciferous groups (cf. Bleeker et al., 2002; Carlsen et al., 2009; Hurka et al., 2005, 2012). However, the combination of molecular data with both morphological, ecological and distributional evidence allows recognition of several well-defined taxonomic entities (Table 2). Taxa in Clade F are characterised by typically pinnatipartite to pinnatisect leaves (rarely only dentate) and/or long dense fruiting racemes, and they experienced rapid diversification processes by the Mid-Pleistocene (ca 0.76 Mya) onwards, perhaps related to glacial-interglacial periods. According to Vicente et al. (2019b), two morphologically well-defined entities can be recognised, which share the chromosome number $2n = 16$ (cf. Schönfelder, 1968 as "*B. lyrata*"; Ruiz de Clavijo, 1991 as "*B. lyrata*"; Chepinoga et al., 2009 as "*B. baetica*" *p.p.*).

On the one hand, *B. eriocarpa* DC. applies to most of the N African samples, in which two morphotypes (*B. eriocarpa* var. *eriocarpa* and *B. eriocarpa* var. *riphaea*) can be identified (Table 2), according to the broad morphological variation in the leaf division and distribution on stems or pedicel insertion pattern. First, the obtained timetree dates the origin of *B. eriocarpa* (s.l.) by the mid-Pleistocene (ca 0.55 Mya), probably in arid environments like those in which the current *B. eriocarpa* var. *eriocarpa* is found. Secondly, *B. eriocarpa* var. *riphaea* arose later (ca 0.43 Mya), perhaps as a response to more humid climates as those occurring in the area it occupies nowadays. Thirdly, our timetree identifies a further divergence event that took place almost coetaneously (ca 0.41 Mya) to the diversification of *B. eriocarpa* var. *riphaea* (within the "*B. eriocarpa* clade"), giving rise to *B. pseudolyrata* in the Atlantic coastal areas of Gharb (or also Rharb) region. Plants from that area (Subclade F1) are always found on Quaternary deep sandy soils, and they exhibit a very distinct morphology clearly differing from the surrounding populations of both *B. boetica* and *B. eriocarpa* s.l. (Table 2), which at first sight relate them to *B. maritima* (cf. Vicente et al., 2019a). Although in the plastid and combined trees the clade of *B. pseudolyrata* is embedded with other *B. eriocarpa* groups within a bigger clade not well resolved (87% BS, 1.00 PP), its position as the strongly supported Subclade F1 (99% BS, 1.00 PP) does not fully respond to geographical patterns, but ecological ones. Collection sites MO8 and MO9 (both of *B. pseudolyrata*) are much closer to MO14 (*B. eriocarpa* var. *eriocarpa*) than to MO10 (*B. pseudolyrata*), and similarly the site MO8 (*B. pseudolyrata*) keeps the same distance from MO2 (*B. eriocarpa* var. *eriocarpa*) and MO12 (*B. eriocarpa* var. *riphaea*) than from MO10 (*B. pseudolyrata*). Furthermore, as said before populations of *B. pseudolyrata* are restricted to Quaternary red sandy layers of the western Gharb, which were deposited during the last 0.5–1 Mya, an age congruent with the dated origin of that species in our timetree. Accordingly, *B. pseudolyrata* should have arisen as an edaphic specialist (cf. Christian et al., 2013) in sandy substrates, following a rapid and recent ecological diversification as suggested by Vicente et al. (2019a). Furthermore, the current mild Mediterranean climate conditions of N Morocco probably have existed during most of the Pleistocene without remarkable altitudinal variations (Rodríguez-Sánchez et al., 2008), a fact facilitating radiation of recent lineages in relation to substrate characteristics. Actually, the Gharb area is considered an ancient glacial refugium for cork oak (Lumaret et al., 2005), in which speciation processes still appear to be active.

Although morphological patterns are found to easily differentiate between members of the "*B. eriocarpa* clade" (Table 2), our current molecular results indicate that this group arose after recent evolutionary processes started in the mid-Pleistocene (ca 0.76 Mya), but still active. These could include morphological divergence following introgression and/or rapid adaptive radiation, a fact that is also common in other taxonomically complex Mediterranean groups, such as *Limonium* Mill. (Lledó et al., 2005, 2011) or *Helianthemum* Mill. (Parejo-Farnés et al., 2013), in which traditional molecular phylogenies did not get internal resolution of lineages. As suggested by some authors (Sucher et al., 2012; Tonnabel et al., 2014), application of Massive Sequencing techniques will surely render better resolved phylogenetic relationships in non-model groups like *Biscutella*, which will contribute to a more satisfactory taxonomic solution. In the meantime, when no molecular resolution is recovered in our phylogenies, syndromes of remarkable morphological characters are utilised for taxon recognition, usually at lower taxonomic rank. As shown in Fig. 1, all three varieties accepted in *Biscutella* ser. *Biscutella* by Vicente et al. (2019b) are not monophyletic and they represent extremes of variation (morphotypes) of incompletely diverged lineages. This might point at the fact that morphological traits on which such varieties are founded (e.g. stem foliation, basal rosette presence and density, or leaf division) evolved several times in the concerned lineages. In such cases, lower taxonomic ranks (e.g. variety or form) can be safely applied to those morphotypes, a solution that is well assumable since many morphologically intermediate individuals are found that link the observed extremes. Similar patterns of non-monophyletic taxa are also found in other crucifer genera such as *Arabidopsis* Heynh., *Aphragmus* Andrz. ex DC., *Eutrema* R. Br. (incl. *Taphrospermum* C.A. Mey. and *Thellungiella* O.E. Schulz) (Warwick et al., 2006), *Capsella* Medik. (Hurka et al., 2012), *Cardamine* L. (Bleeker et al., 2002) or even *Biscutella* (Tremetsberger et al., 2002), in which recently evolved lineages, often morphologically well-defined, still show not enough molecular differentiation. The taxonomic rank to be applied in each case should be congruent with the degree of morphological divergence displayed by the concerned group.

5. Conclusions

According to our research, *Biscutella* ser. *Biscutella* is a new example to add to other Mediterranean groups recently and rapidly evolved in the Mediterranean basin (cf. Simón-Porcar et al., 2015; Affenzeller et al., 2018). The current distribution of the series, mostly centred in the southern parts of the Mediterranean and the Middle East (Fig. 3), suggests that *Biscutella* radiated in connection with the major geological and climatic events that took place in the Mediterranean basin and Eurasia within the last 20 million years. Similar patterns of diversification were reported for tribe Vellinae (Simón-Porcar et al., 2015), an Iberian-Mahgrebian cruciferous aggregate rich in narrow endemic taxa.

Ancestors of *Biscutella* found multiple glacial refuges in North Africa

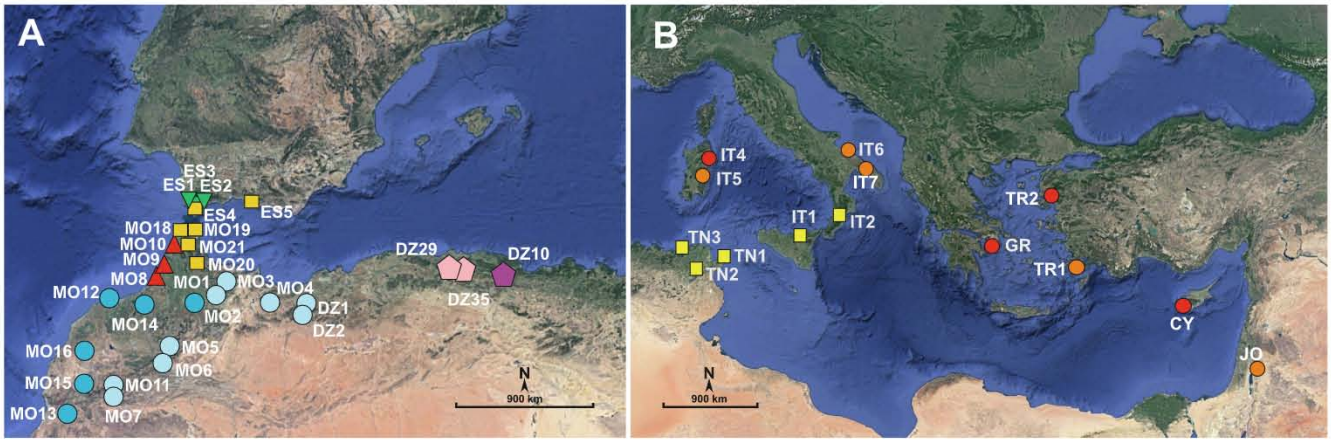

Fig. 3. Provenance of the material used in the phylogenetic analyses of *Biscutella* ser. *Biscutella*; sample codes according to Table 1. **A.** Distribution map of *B. lyrata* (green inverted triangles), *B. boetica* (yellow squares), *B. pseudolyrata* (red triangles), *B. eriocarpa* var. *eriocarpa* (deep blue circles), *B. eriocarpa* var. *riphaea* (light blue circles), *B. raphanifolia* var. *raphanifolia* (violet pentagons), and *B. raphanifolia* var. *algeriensis* (pinkish pentagons) in the western Mediterranean. **B.** Distribution map of *B. didyma* var. *didyma* (red circles), *B. didyma* var. *ciliata* (orange circles), and *B. maritima* (yellow squares) in the central and eastern Mediterranean. Satellite images from Google Earth Pro. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the southernmost areas of Europe during the Miocene (cf. Médail and Diadema, 2009), which acted as genetic reservoirs. Based on the timetree obtained in the present study the origin of *Biscutella* is dated ca. 18.75 Mya, albeit the diversification of the series triggered with the ancestor of *B. lyrata* and the remaining members of the whole series diverging about 5.87 Mya, during the Messinian Salinity Crisis. This fact also points at considering the southern part of the Iberian Peninsula (Cádiz-Algeciras region) as a putative refuge in the late-Miocene (see Médail and Diadema, 2009) from which lineages migrated and diversified. However, the main radiation of the aggregate occurred in connection with the establishment of the Mediterranean climate (ca 3.2 Mya) and the Intensification of Northern Hemisphere Glaciation (iNHG, 2.5–3.2 Ma), which resulted in periodic advances and retreats of ice sheets on a hemispherical scale. The glacial pulses affected the Mediterranean territories in different ways, but the existence of numerous potential glacial refuges with distinct geologic and climatic characteristics favoured maintenance of biodiversity in the series. This process became faster in the last 1.7 Mya, where diversification of *Biscutella* intensified in the Western Mediterranean basin, successively with radiation of the *B. boetica-raphanifolia* clade (1.40 Mya) and the *B. eriocarpa s.l.* clade (0.76 Mya). Remarkably, the latter clade rapidly splitted into several lineages that mostly respond to geographical and ecological patterns, e.g. *B. eriocarpa* var. *eriocarpa* specialised first in drier environments (ca 0.76 Mya) and *B. pseudolyrata* split later (ca 0.41 Mya) as an edaphic specialist paralleling its closest relative *B. eriocarpa* var. *riphaea*, which mostly occurs in more humid mountain areas.

This hypothesis is fully congruent with the widely accepted assumption that changes in aridity at regional (and also local) scale may directly influence on diversification of lineages. In that sense, the onset of large-amplitude African and SW Asian aridity cycles that synchronically occurred in parallel to iNHG by the same geological times (Maslin et al., 2014; deMenocal, 2004, 2011) contributed to shape the genetic, distributional and diversification patterns of numerous plant taxa in the Mediteranean basin and neighbouring areas (e.g. Blondel et al., 2010; Nieto Feliner, 2014; Affenzeller et al., 2018). In the case of *B.* ser. *Laevigatae*, genetic refuges during the Last Glacial Maximum (LGM) together with subsequent processes of polyploidy and reticulate evolution, have been argued as triggers of recent diversification in Central Europe (Parisod and Besnard, 2007). Something similar occurred to *B.* ser. *Biscutella* mainly in the S Mediterranean basin, where the climate remained almost stable and polyploidy apparently was not a decisive factor in speciation. The genus *Biscutella* reveals as a good example of recent and rapid diversification following climatic and geological changes, and further studies including population genetics should contribute to better understand those processes in detail.

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Author contribution

MAA and MBC designed the study. AV performed most of the laboratory analyses. AV and MBC conducted the analyses. MAA and MBC wrote the major part of the manuscript and prepared the illustrations. All authors participated in the field work, collected plant material, and greatly contributed and provided comments on the first drafts.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106644.

References

Abulafia, D., 2011. The Great Sea: A Human History of the Mediterranean. Penguin Books, London.

Affenzeller, M., Kadereit, J.W., Comes, H.P., 2018. Parallel bursts of recent and rapid radiation in the Mediterranean and Eritreo-Arabian biodiversity hotspots as revealed by *Globularia* and *Campylanthus* (Plantaginaceae). J. Biogeogr. 45, 552–566. https://doi.org/10.1111/jbi.13155.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Autom. Control 19, 716–723. https://doi.org/10.1109/TAC.1974.1100705.
Al-Shehbaz, I.A., 2012. A generic and tribal sinopsis of the *Brassicaceae (Cruciferae*). Taxon 61, 931–954. https://doi.org/10.1002/tax.615002.
Al-Shehbaz, I.A., Al-Omar, M.M., 1982. Brassicaceae-chromosome number reports. In: Löve, A (Ed.), IOPB chromosome number reports LXXVI. Taxon 31, 587–589.
Appel, O., Al-Shehbaz, I.A., 2003. Cruciferae. In: In: Kubitzki, K., Bayer, C. (Eds.), The Families and Genera of Vascular Plants, vol. 5. Springer, Berlin & Heidelberg, pp. 75–174.
Artyukova, E.V., Kozyrenko, M.M., Boltenkov, E.V., Gorovoy, P.G., 2014. One or three species in *Megadenia* (*Brassicaceae*): insight from molecular studies. Genetica 142, 337–350. https://doi.org/10.1007/s10709-014-9778-1.
Barker, F.K., Lutzoni, F., 2002. The utility of the incongruence length difference test. Syst. Biol. 51, 625–637. https://doi.org/10.1080/10635150290102302.
Battandier, J.A., 1888. Dicotylédones. In: Battandier, J.A., Trabut, L.C. (Eds.), Flore de l'Algérie, vol. 1(1). A. Jourdan and F. Savy, Alger and Paris.
Battandier, J.A., Trabut, L.C., 1902. Flore analytique & synoptique de l'Algérie et de la Tunisie. Giralt, Alger.
Bleeker, W., Franzke, A., Pollmann, K., Brown, A.H.D., Hurka, H., 2002. Phylogeny and biogeography of Southern Hemisphere high-mountain *Cardamine* species (Brassicaceae). Austral. Syst. Bot. 15, 575–581. https://doi.org/10.1071/SB01026.
Blondel, J., Aronson, J., Bodiou, J.-Y., Boeuf, G., 2010. The Mediterranean Basin – Biological Diversity in Space and Time. Oxford University Press, Oxford.
Bocquet, G., Widler, B., Kiefer, H., 1978. The Messinian model – a new outlook for the floristics and systematics of the Mediterranean area. Candollea 33, 269–287.
Borchsenius, F., 2009. FastGap 1.2. Denmark: Department of Biosciences. Aarhus University.
Boulos, L., 1999. Flora of Egypt, vol. 1 (Azollaceae-Oxalidaceae). Al Hadara Publishing, El Caire.
Candolle, A.P. de, 1811. Monographie des Biscutelles ou Lunetières. Ann. Mus. Natl. Hist. Nat. 18, 292–301.
Carlsen, T., Bleeker, W., Hurka, H., Elven, R., Brochmann, C., 2009. Biogeography and phylogeny of *Cardamine* (Brassicaceae). Ann. Missouri Bot. Gard. 96, 215–236. https://doi.org/10.3417/2007047.
Chase, M.W., Hills, H.G., 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. Taxon 40, 215–220. https://doi.org/10.2307/1222975.
Chepinoga, V.V., Aleksandr, A.G., Enushchenko, I.V., Rosbakh, S.A., Marhold, K., Breitwieser, I., 2009. IAPT/IOPB chromosome data 8. Taxon 58, 1281–1314. https://doi.org/10.1002/tax.2009.58.issue-410.1002/tax.584017.
Christian, A.A., Vleminckx, J., Drouet de la Thibauderie, T., Lejoly, J., Meerts, P., Hardy, O.J., 2013. Species responses to edaphic heterogeneity in SemiDeciduous Forests from the Congo Basin. Greener J. Biol. Sci. 3, 365–374. http://doi.org/10.15580/GJBS.2013.10.112913997.
Cosson, E., 1887. Compendium Florae Atlanticae ou Flore des États Barbaresques, Algérie, Tunisie et Maroc, vol. 2. Imprimerie Nationale, Paris.
Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9 (8), 772. https://doi.org/10.1038/nmeth.2109.
deMenocal, P.B., 2004. African climate change and faunal evolution during the Pliocene-Pleistocene. Earth Planet. Sci. Lett. 220, 3–24. https://doi.org/10.1016/S0012-821X(04)00003-2.
deMenocal, P.B., 2011. Climate and human evolution. Science 331, 540–542. https://doi.org/10.1126/science.1190683.
Desfontaines, R.L., 1798. Flora Atlantica: sive historia plantarum quae in Atlante, agro tunetano et algeriensi crescunt, vol. 2. L.G. Desgranges, Paris.
Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.
Farris, J.S., Källersjö, M., Kluge, A.G., Bult, C., 1994. Testing significance of congruence. Cladistics 10, 315–319. https://doi.org/10.1111/j.1096-0031.1994.tb00181.x.
Felsenstein, J., 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. J. Mol. Evol. 17, 368–376. https://doi.org/10.1007/BF01734359.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791. https://doi.org/10.2307/2408678.
Fiorini, G., Raffaelli, M., 1990. Números cromosomáticos de plantas occidentales, 602–607. Anales Jard. Bot. Madrid 47 (2), 419–423.
Grau, J., 1999. Nota sobre *Biscutella* en el Norte de Marruecos. Lagascalia 21, 244–246.
Fennane, M., 1999. *Biscutella* L. in: Fennane, M., Tattou, M., Mathez, J., Ouyahya, A., Oualidi, J. (Eds.), Flore Pratique du Maroc, vol. 1. Institut Scientifique, Université Mohammed V, Rabat, pp. 401–404.
Grau, J., 2002. *Biscutella* L. In: Valdés, B., Rejdali, M., Achhal El Kadmiri, A., Jury, S.L., Montserrat, J.M. (Eds.), Catalogue des plantes vasculaires du nord du Maroc, vol. 1. Consejo Superior de Investigaciones Científicas, CSIC, Madrid, pp. 259–260.
Grau, J., Klingenberg, L., 1993. *Biscutella* L. In: In: Castroviejo, S., Aedo, C., Cirujano, S., Laínz, M., Montserrat, P., Morales, R., Muñoz Garmendia, F., Navarro, C., Paiva, J., Soriano, C. (Eds.), Flora iberica, vol. 3. Real Jardín Botánico, CSIC, Madrid, pp. 293–311.
Greuter, W., 1991. Botanical diversity, endemism, rarity, and extinction in the Mediterranean area: an analysis based on the published volumes of Med-Checklist. Bot. Chron. 10, 63–79.
Guinea, E., 1964. El género *Biscutella*. Anales Inst. Bot. Cavanilles 21, 387–405.
Guinea, E., Heywood, V.H., 1964. *Biscutella* L. In: In: Tutin, T.G., Heywood, V.H., Burges, N.A., Valentine, D.H., Walters, S.M., Webb, D.A. (Eds.), Flora Europaea, vol. 1. Cambridge University Press, Cambridge, pp. 325–330.
Guinea, E., Heywood, V.H., 1993. *Biscutella* L. In: Tutin, T.G, Burges, N.A., Chater, A.O., Edmondson, J.R., Heywood, V.H., Moore, D.M., Valentine, D.H, Walters, S.M., Webb, D.A. (Eds.), Flora europaea ed. 2, vol. 1. Cambridge University Press, Cambridge, pp. 393–398.
Hedge, J., 1968. *Biscutella* L. In: In: Rechinger, K.H. (Ed.), Flora Iranica 57. Akademische Druck und Verlagsanstalt, Graz, pp. 99–100.
Hedge, I.C., 1984. *Biscutella* L. In: In: Davis, P.H. (Ed.), Flora of Turkey and the East Aegean islands, vol. 1. Edinburgh University Press, Edinburgh, pp. 313–314.
Hohmann, N., Wolf, E.M., Lysak, M.A., Koch, M.A., 2015. A time-calibrated road map of Brassicaceae species radiation and evolutionary history. Plant Cell 27, 2770–2784. https://doi.org/10.1105/tpc.15.00482.
Huang, C.-H., Sun, R., Hu, Y., Zeng, L., Zhang, N., Cai, L., Zhang, Q., Koch, M.A., Al-Shehbaz, I.A., Edger, P.P., Pires, J.C., Tan, D.-Y., Zhong, Y., Ma, H., 2015. Resolution of Brassicaceae phylogeny using nuclear genes uncovers nested radiations and supports convergent morphological evolution. Mol. Biol. Evol. 33, 394–412. https://doi.org/10.1093/molbev/msv226.
Huang, X.-C., German, D.A., Koch, M.A., 2019. Temporal patterns of diversification in Brassicaceae demonstrate decoupling of rate shifts and mesopolyploidization events. Ann. Bot. mcz123. https://doi.org/10.1093/aob/mcz123.
Huson, D.H., Scornavacca, C., 2012. Dendroscope 3: An interactive tool for rooted phylogenetic trees and networks. Syst. Biol. 61, 1061–1067. https://doi.org/10.1093/sysbio/sys062.
Hsü, K.J., 1974. The Miocene desiccation of the Mediterranean and its climatical and zoogeographical implications. Naturwissenschaften 61, 137–145. https://doi.org/10.1007/BF00602586.
Hsü, K.J., Montadert, L., Bernoulli, D., Cita, M.B., Erickson, A., Garrison, R.E., Kidd, R.B., Mélières, F., Müller, C., Wright, R., 1978. History of the Mediterranean salinity crisis. In: Hsü, K.J., Montardet, L., Garrison, R.B., Fabricius, F.H., Kidd, R.B., Müller, C., Cita, M.B., Bizon, G., Wright, R.C., Erickson, A.J., Bernoulli, D., Mélières, F. (Eds.), Initial Reports of the Deep Sea Drilling Project, vol. 42(1). US Government Printing Office, Washington, pp. 1053–1078. https://doi.org/10.2973/dsdp.proc.42-1.1978.
Hsü, K.J., Ryan, W.B.F., Citá, M.B., 1973. Late Miocene desiccation of the Mediterranean. Nature 242, 240–244. https://doi.org/10.1038/242240a0.
Hurka, H., Friesen, N., German, D.A., Franzke, A., Neuffer, B., 2012. 'Missing link' species *Capsella orientalis* and *Capsella thracica* elucidate evolution of model plant genus *Capsella* (Brassicaceae). Mol. Ecol. 21, 1223–1238. https://doi.org/10.1111/j.1365-294X.2012.05460.x.
Hurka, H., Paetsch, M., Bleeker, W., Neuffer, B., 2005. Evolution within the Brassicaceae. In: Endress, P.K., Lüttge, U., Parthier, B. (Eds.), From plant taxonomy to evolutionary biology. Nova Acta Leopoldina NF 92(342), 113–127.
IPNI, 2019 [continuously updated]. The International Plant Names Index. http://www.ipni.org (accessed 21 May 2019).
Jordan, A., 1864. Diagnoses d'espèces nouvelles ou méconnues, pour servir de matériaux à une flore réformée de la France et des contrées voisines, vol 1(1). F. Savy, Paris.
Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652–655. https://doi.org/10.1038/23231.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.
Larsen, K., Laegaard, S., 1971. Chromosome studies of the sicilian flora. Bot. Tidsskr. 66, 249–268.
Le Floc'h, E., Boulos, L., Vela, E., 2010. Catalogue synonymique commenté de la Flore de Tunisie. Ministère de l'Environnement et du Développement durable et Banque Nationale de Gènes, Montpellier et Tunis.
Lledó, M.D., Crespo, M.B., Fay, M.F., Chase, M.W., 2005. Molecular phylogenetics of *Limonium* and related genera (Plumbaginaceae): biogeographical and systematic implications. Amer. J. Bot. 92, 1189–1198.
Lledó, M.D., Karis, P.O., Crespo, M.B., Fay, M.F., Chase, M.W., 2011. Endemism and evolution in Macaronesian and Mediterranean *Limonium* taxa. In: Bramwell, D., Caujapé-Castells, J. (Eds.), The Biology of Island Floras. Cambridge University Press, Cambridge, pp. 325–337.
Lumaret, R., Tryphon-Dionnet, M., Michaud, H., Sanuy, A., Ipotesi, E., Born, C., Mir, C., 2005. Phylogeographical variation of chloroplast DNA in cork oak (*Quercus suber*). Ann. Bot. 96, 853–861. https://doi.org/10.1093/aob/mci237.
Machatschki-Laurich, B., 1926. Die Arten der Gattung *Biscutella* L. Sect. *Thlaspidium* (Med.) DC. Bot. Arch. 13, 1–115.
Maire, R., 1967. Flore de l'Afrique du Nord 13 Paul Lechevalier, Paris.
Malinowski, E., 1911. Monographie du genre *Biscutella* L. I. Classification et distribution géographique. Bull. Int. Acad. Sci. Cracovie, Cl. Sci. Math., Ser. B Sci. Nat. 1910, 111–139.
Mandáková, T., Guo, X., Özüdoğru, B., Mummenhoff, K., Lysak, M.A., 2018. Hybridization-facilitated genome merger and repeated chromosome fusion after 8 million years. Plant J. 96, 748–760. https://doi.org/10.1111/tpj.14065.
Manton, I., 1932. Introduction to the general cytology of the Cruciferae. Ann. Bot. 46, 509–556. https://doi.org/10.1093/oxfordjournals.aob.a090334.
Manton, I., 1937. The problem of *Biscutella laevigata* L. II. The evidence from meisois. Ann. Bot. 1, 439–462. https://doi.org/10.1093/oxfordjournals.aob.a083479.
Marhold, K., 2011 [cotinuously updated]. Brassicaceae. in: Euro+Med PlantBase – the information resource for Euro-Mediterranean plant diversity. http://ww2.bgbm.org/EuroPlusMed (accessed 1 Feb 2016).
Maslin, M.A., Brierley, C.M., Milner, A.M., Shultz, S., Trauth, M.H., Wilson, K.E., 2014. East African climate pulses and early human evolution. Quat. Sci. Rev. 101, 1–17. https://doi.org/10.1016/j.quascirev.2014.06.012.
Médail, F., Diadema, K., 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. J. Biogeogr. 36, 1333–1345. https://doi.org/10.1111/j.1365-2699.2008.02051.x.
Médail, F., Myers, N., 2004. Mediterranean Basin. In: Mittermeier, R.A., Robles Gil, P., Hoffmann, M., Pilgrim, J., Brooks, T., Mittermeier, C.G., Lamoreaux, J., da Fonseca,

G.A.B. (Eds.), Hotspots revisited: Earth's biologically richest and most endangered terrestrial ecoregions. CEMEX, Monterrey, Conservation International, Washington, and Agrupación Sierra Madre, Mexico, pp. 144–147.
Mejías, J.A., Luque, T., 1987. Números cromosomáticos de plantas occidentales, 403–410. Anales Jard. Bot. Madrid 43, 412–416.
Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.
Nieto Feliner, G., 2014. Patterns and processes in plant phylogeography in the Mediterranean Basin. A review. Perspect. Plant Ecol. Evol. Syst. 16, 265–278. https://doi.org/10.1016/j.ppees.2014.07.002.
Norup, M.V., Dransfield, J., Chase, M.W., Barfod, A.S., Fernando, E.S., Baker, W.J., 2006. Homoplasious character combinations and generic delimitation: A case study from the Indo-Pacific arecoid palms (Arecaceae: Areceae). Am. J. Bot. 93, 1065–1080. https://doi.org/10.3732/ajb.93.7.1065.
Olowokudejo, J.D., 1986. The infrageneric classification of *Biscutella (Cruciferae)*. Britonnia 38, 86–88. https://doi.org/10.2307/2807426.
Olowokudejo, J.D., Heywood, V.H., 1984. Cytotaxonomy and breeding system of the genus *Biscutella (Cruciferae*). Pl. Syst. Evol. 145, 291–309. https://doi.org/10.1007/BF00983955.
Özüdoğru, B., Akaydin, G., Erik, S., Al-Shehbaz, I.A., Mummenhoff, K., 2015. Phylogeny, diversification and biogeographic implications of the eastern Mediterranean endemic genus *Ricotia* (*Brassicaceae*). Taxon 64, 727–740. https://doi.org/10.12705/644.5.
Özüdoğru, B., Al-Shehbaz, I.A., Mummenhoff, K., 2017. Tribal assignment of *Heldreichia* Boiss. (Brassicaceae): evidence from nuclear ITS and plastidic ndhF markers. Plant Syst. Evol. 303, 329–335. https://doi.org/10.1007/s00606-016-1374-7.
Parejo-Farnés, C., Albaladejo, R.G., Arroyo, J., Aparicio, A., 2013. A phylogenetic hypothesis for *Helianthemum* (Cistaceae) in the Iberian Peninsula. Bot. Complut. 37, 83–92. https://doi.org/10.5209/rev_BOCM.2013.v37.42272.
Parisod, C., Besnard, G., 2007. Glacial in situ survival in the Western Alps and polytopic autopolyploidy in *Biscutella laevigata* L. (Brassicaceae). Mol. Ecol. 16, 2755–2767. https://doi.org/10.1111/j.1365-294X.2007.03315.x.
Pignatti, S., 1982. Flora d'Italia, vol. 1 Edagricole, Bologna.
Pirie, M., 2015. Phylogenies from concatenated data: Is the end nigh? Taxon 64, 421–423. https://doi.org/10.12705/643.1.
Platt, J.P., Behr, W.M., Johanesen, K., Williams, J.R., 2013. The Betic-Rif arc and its orogenic hinterland: a review. Annu. Rev. Earth Planet. Sci. 41, 313–357. https://doi.org/10.1146/annurev-earth-050212-123951.
Poiret, J.L.M., 1789. Voyage en Barbarie, vol. 2. J.B.F. Née de la Rochelle, Paris.
Pottier-Alapetite, G., 1979. Flore de la Tunisie. Angiospermes-Dicotylédones. Apétales-Dialypétales, part 1. Imprimerie Officielle de la Republique, Tunis.
Quézel, P., 1985. Definition of the Mediterranean region and the origin of its flora In: Gómez-Campo, C. (Ed.), Plant conservation in the Mediterranean area. [Geobotany 7]. W. Junk, Dordrecht, pp. 9–24.
Quézel, P., Santa, S., 1963. New flora of Algeria and the desert regions Meridional. French National Center for Scientific Research, Paris.
Raffaelli, M., 1991. *Biscutella* L. Ser. *Lyratae* Malin. (*Cruciferae*) in Italia. Discussione sui caratteri morfologici e tassonomia. Webbia 45, 1–30. https://doi.org/10.1080/00837792.1991.10670489.
Raffaelli, M., Fiorini, G., 1986. Numeri cromosomici per la Flora Italiana: 1049–1051. Informatore Bot. Ital. 17, 99–101 [front cover: 1985].
Rodríguez-Sánchez, F., Pérez-Barrales, R., Ojeda, F., Vargas, P., Arroyo, J., 2008. The Strait of Gibraltar as a melting pot for plant biodiversity. Quaternary Sci. Rev. 27, 2100–2117. https://doi.org/10.1016/j.quascirev.2008.08.006.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 1–4. https://doi.org/10.1093/sysbio/sys029.
Ruiz de Clavijo, E., 1991. Notas cariológicas sobre algunas especies norteafricanas. Acta Bot. Malacitana 16, 449–454.
Rundel, P.W., Arroyo, M.T.K., Cowling, R.M., Keeley, J.E., Lamont, B.B., Vargas, P., 2016. Mediterranean biomes: Evolution of their vegetation, floras, and climate. Annu. Rev. Ecol. Evol. Syst. 47, 383–407. https://doi.org/10.1146/annurev-ecolsys-121415-032330.
Santa Bárbara, V.J., Juan, R., Pastor, J., Diosdado, J.C., 1994. Números cromosómicos para la flora española, 720–747. Lagascalia 17, 367–391.
Saitou, N., Nei, M., 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
Schönfelder, P., 1968. Chromosomenzahlen einiger Arten der Gattung *Biscutella* L. Österr. Bot. Z. 115, 363–371. https://doi.org/10.1007/BF01373314.
Shaw, J., Lickey, E.B., Schilling, E.E., Small, R.L., 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetics studies in angiosperms: the tortoise and the hare III. Am. J. Bot. 94, 275–288. https://doi.org/10.3732/ajb.94.3.275.
Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49 (2), 369–381. https://doi.org/10.3732/ajb.94.3.275.
Simón-Porcar, V.I., Pérez-Collazos, E., Catalán, P., 2015. Phylogeny and systematics of the western Mediterranean *Vella pseudocytisus-V. aspera* complex (Brassicaceae). Turk. J. Bot. 39, 472–486. https://doi.org/10.3906/bot-1406-87.
Suc, J.P., 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. Nature 307, 429–432. https://doi.org/10.1038/307429a0.
Sucher, N.J., Hennell, J.E., Carles, M.C., 2012. DNA fingerprinting, DNA barcoding, and next generation sequencing technology in plants. Methods Mol. Biol. 862, 13–22. https://doi.org/10.1007/978-1-61779-609-8_2.
Sukhorukov, A.P., Verloove, F., Alonso, M.Á., Belyaeva, I.V., Chapano, C., Crespo, M.B., El Aouni, M.H., El Mokni, R., Maroyi, A., Shekede, M.D., Vicente, A., Dreyer, A., Kushunina, M., 2017. Chorological and taxonomic notes on African plants, 2. Botany Lett. 164 (2), 135–153. https://doi.org/10.1080/23818107.2017.1311281.
Swofford, D.L., 2002. PAUP* Phylogenetic Analysis Using Parsimony (* and other methods), version 4.0b10 for Macintosh. Sinauer Associates, Sunderland.
Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. Mol. Biol. Evol. 9, 678–687. https://doi.org/10.1093/oxfordjournals.molbev.a040752.
Tamura, K., Battistuzzi, F.U., Billing-Ross, P., Murillo, O., Filipski, A., Kumar, S., 2012. Estimating divergence times in large molecular phylogenies. Proc. Natl. Acad. Sci. USA 109, 19333–19338. https://doi.org/10.1073/pnas.1213199109.
Tamura, K., Qiqing, T., Kumar, S., 2018. Theoretical foundation of the RelTime Method for estimating divergence times from variable evolutionary rates. Mol. Biol. Evol. 35, 1770–1782. https://doi.org/10.1093/molbev/msy044.
The Plant List, 2013. The Plant List. Version 1.1 http://www.theplantlist.org/ (accessed 10 May 2017).
Thiers, B., 2019 [continuously updated]. Index Herbariorum: a global directory of public herbaria and associated staff. New York Botanical Garden's virtual herbarium. http://sweetgum.nybg.org/science/ih/ (accessed 29 April 2019).
Thompson, J.D., 2005. Plant Evolution in the Mediterranean. Oxford University Press, Oxford.
Tonnabel, J., Olivieri, I., Mignot, A., Rebelo, A., Justy, F., Santoni, S., Caroli, S., Sauné, L., Bouchez, O., Douzery, E.J.P., 2014. Developing nuclear DNA phylogenetic markers in the angiosperm genus *Leucadendron* (Proteaceae): a next-generation sequencing transcriptomic approach. Mol. Phylogenet. Evol. 70, 37–46. https://doi.org/10.1016/j.ympev.2013.07.027.
Tremetsberger, K., König, C., Samuel, R., Pinsker, W., Stuessy, T., 2002. Infraspecific genetic variation in *Biscutella laevigata* (Brassicaceae): new focus on Irene Manton's hypothesis. Pl. Syst. Evol. 233, 163–181. https://doi.org/10.1007/s00606-002-0189-x.
Ubera, J.L., Ruiz de Clavijo, E., 1984. Números 342–348 [Números cromosómicos para la flora española, 300–364]. Lagascalia 12, 294–296.
Valente, L.M., Vargas, P., 2013. Contrasting evolutionary hypotheses between two mediterranean-climate floristic hotspots: The Cape of southern Africa and the Mediterranean Basin. J. Biogeogr. 40, 2032–2046. https://doi.org/10.1111/jbi.12156.
Vicente, A., Alonso, M.Á., Crespo, M.B., 2016. Taxonomic circumscription of the N African endemic *Biscutella raphanifolia* (*Brassicaceae*) based on morphological and molecular characters. Willdenowia 46, 411–422. https://doi.org/10.3372/wi.46.46309.
Vicente, A., Alonso, M.Á., Crespo, M.B., 2019a. *Biscutella pseudolyrata (Brassicacea*e, *Biscutelle*ae), a new species endemic to NW Morocco based on morphological and molecular evidence. Willdenowia 49, 155–166. https://doi.org/10.3372/wi.49.49204.
Vicente, A., Alonso, M.Á., Crespo, M.B., 2019b. Born in the Mediterranean: Comprehensive taxonomic revision of *Biscutella* ser. *Biscutella* (Brassicaceae) based on morphological and phylogenetic data. Ann. Mo. Bot. Gard. (submitted for publication).
Wang, W.P., Hwang, C.Y., Lin, T.P., Hwang, S.Y., 2003. Historical biogeography and phylogenetic relationships of the genus *Chamaecyparis (Cupressaceae*) inferred from chloroplast DNA polymorphism. Pl. Syst. Evol. 241, 13–28. https://doi.org/10.1007/s00606-003-0031-0.
Warwick, S.I., Al-Shehbaz, I.A., 2006. *Brassicaceae*: Chromosome number index and database on CD-Rom. Pl. Syst. Evol. 259, 237–248. https://doi.org/10.1007/s00606-006-0421-1.
Warwick, S.I., Al-Shehbaz, I.A., Sauder, C., 2006. Phylogenetic position of *Arabis arenicola* and generic limits of *Eutrema* and *Aphragmus* (Brassicaceae) based on sequences of nuclear ribosomal DNA. Canad. J. Bot. 84, 269–281. https://doi.org/10.1139/b05-161.
White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.
Yoder, D.Y., Irwin, J.A., Payseur, B.A., 2001. Failure of the ILD to determine data combinability for slow loris phylogeny. Syst. Biol. 50, 408–424. https://doi.org/10.1080/106351501300318003.