

## Evolutionary lineages of nickel hyperaccumulation and systematics in European Alyseae (Brassicaceae): evidence from nrDNA sequence data

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- **Background and Aims** Nickel (Ni) hyperaccumulation is a rare form of physiological specialization shared by a small number of angiosperms growing on ultramafic soils. The evolutionary patterns of this feature among European members of tribe Alyseae (Brassicaceae) are investigated using a phylogenetic approach to assess relationships among Ni hyperaccumulators at the genus, species and below-species level.
- **Methods** Internal transcribed spacer (ITS) sequences were generated for multiple accessions of Alyseae. Phylogenetic trees were obtained for the genera of the tribe and *Alyssum* sect. *Odontarrhena*. All accessions and additional herbarium material were tested for Ni hyperaccumulation with the dimethylglyoxime colorimetric method.
- **Key Results** Molecular data strongly support the poorly known hyperaccumulator endemic *Leptoplax* (*Peltaria*) *emarginata* as sister to hyperaccumulator species of *Bornmuellera* within Alyseae. This is contrary to current assumptions of affinity between *L. emarginata* and the non-hyperaccumulator *Peltaria* in Thlaspeidae. The lineage *Bornmuellera*–*Leptoplax* is, in turn, sister to the two non-hyperaccumulator Mediterranean endemics *Ptilotrichum rupestre* and *P. cyclocarpum*. Low ITS sequence variation was found within the monophyletic *Alyssum* sect. *Odontarrhena* and especially in *A. murale sensu lato*. Nickel hyperaccumulation was not monophyletic in any of three main clades retrieved, each consisting of hyperaccumulators and non-hyperaccumulators of different geographical origin.
- **Conclusions** Nickel hyperaccumulation in Alyseae has a double origin, but it did not evolve in Thlaspeidae. In *Bornmuellera*–*Leptoplax* it represents an early synapomorphy inherited from an ancestor shared with the calcicolous, sister clade of Mediterranean *Ptilotrichum*. In *Alyssum* sect. *Odontarrhena* it has multiple origins even within the three European clades recognized. Lack of geographical cohesion suggests that accumulation ability has been lost or gained over the different serpentine areas of south Europe through independent events of microevolutionary adaptation and selection. Genetic continuity and strong phenotypic plasticity in the *A. murale* complex call for a reduction of the number of Ni hyperaccumulator taxa formally recognized.

**Key words:** Alyseae, *Alyssum* sect, *Odontarrhena*, *Bornmuellera*, Brassicaceae, *Leptoplax*, molecular phylogeny, Ni hyperaccumulation.

### INTRODUCTION

Nickel (Ni) hyperaccumulation is the ability to uptake and store  $>1000 \mu\text{g}$  of Ni  $\text{g}^{-1}$  of leaf dry weight without toxicity symptoms, a relatively rare physiological trait shared by 340–390 plant taxa growing on metalliferous soils (Brooks *et al.*, 1977; Reeves and Baker, 2000; Reeves, 2003; Reeves *et al.*, 2007; Reeves and Adigüzel, 2008; Verbruggen *et al.*, 2009; Krämer, 2010). This ability occurs in approx. 42 unrelated angiosperm families and is certainly of polyphyletic origin (Macnair, 2003; Krämer, 2010). Although most hyperaccumulators are endemic to Ni-rich serpentine soils, some species include both serpentine and non-serpentine populations, suggesting the lack of a strict Ni dependence but, rather, the capacity to take competitive advantage over other species within the local community (Reeves and Baker, 1984; Brooks, 1987). The ability for Ni accumulation is in fact

mostly interpreted as an adaptive trait that serves as a herbivore deterrent against insects and pathogens and an allelopathic factor (Boyd and Martens, 1998; Boyd, 2004; Brady *et al.*, 2005; Kazakou *et al.*, 2008).

Metal hyperaccumulators have recently gained considerable interest because of their potential use in phytoremediation (Chaney *et al.*, 2005; reviewed by Pilon-Smits, 2005), phytomining (Li *et al.*, 2003) and food crop biofortification (Broadley *et al.*, 2007; Palmgren *et al.*, 2008). They also constitute an exceptional biological material and gene reservoir that can be exploited to understand metal homeostasis and adaptation to extremely hostile environments. Classical physiological studies have recently been complemented by molecular research aiming at the identification, expression and transcription of functional genes in hyperaccumulators as compared with related control species (reviewed by Verbruggen *et al.*, 2009). Such an approach implies the importance of

phylogenetic evidence in selecting appropriate model systems including accumulating and non-accumulating taxa of clear evolutionary affinity. Indeed, mechanisms for the transport of metals across cell membranes and for their chelation in cell sap can be both specific and generic and thus subject to evolutionary constraints. Also, the differential expression of transport or chelating proteins may account for phylogenetic differences in metal accumulation between clades (Broadley *et al.*, 2001). According to Krämer (2010), the clarification of the phylogenetic position and taxonomy of metal hyperaccumulator taxa will be necessary for comparative genomics and to prioritize among possible future model metal hyperaccumulators. However, only a few studies have explored the phylogenetic patterns of metal accumulation at the macroevolutionary scale, i.e. the species level or above (Broadley *et al.*, 2001; Jansen *et al.*, 2002, 2004; Mengoni *et al.*, 2003).

Most molecular research is currently carried out using a few members of the family Brassicaceae (order Brassicales, malvids clade of APG III, 2009) as model systems, especially *Arabidopsis halleri* and *Noccaea* ('*Thlaspi*') *caerulescens* (Verbruggen *et al.*, 2009; Krämer, 2010). The remarkable proportion (approx. 2%) of hyperaccumulators in the family (Table 1), however, offers still unexploited opportunities to investigate the expression, regulation and evolution of the genetic traits underlying metal accumulation. The search for new model systems in Brassicaceae consisting of taxa of clear phylogenetic affinity is one of the crucial aspects in current research on metal accumulation (Assunção *et al.*, 2003; Peer *et al.*, 2003, 2006). In spite of this, the evolutionary patterns of this ability in the family are still incompletely known.

According to recent molecular evidence, Mediterranean hyperaccumulators in Brassicaceae originated in only five out of the 35 monophyletic tribes that have been recognized (Reeves and Baker, 2000; Koch and Al-Shehbaz, 2009): Aethionemeae, Alyseae, Cardamineae, Noccaeeae and, putatively, Thlaspidae *sensu stricto* (s.s.) (Table 1). Alyseae include a number of hyperaccumulators (Table 1) and more or less closely related non-hyperaccumulators, therefore representing an ideal system for investigating the evolutionary dynamics of this ability. Previous investigations in this tribe have focused either on single genera (Mengoni *et al.*, 2003) or on relationships between genera (Warwick *et al.*, 2008). However, the limited taxon sampling of both studies left our knowledge incomplete. A powerful hyperaccumulator whose affinities remain enigmatic is *Peltaria emarginata* (synonym: *Leptoplax emarginata*), a serpentine endemic restricted to northern continental Greece (Reeves *et al.*, 1980; Psaras *et al.*, 2000; Chardot *et al.*, 2005; Krämer, 2010; Redjala *et al.*, 2010). Included by some taxonomists in *Peltaria* based on silicle morphology (Ball, 1993; Jalas and Suominen, 1996; Appel and Al-Shehbaz, 2003; Warwick *et al.*, 2008), other authors have placed it in the monotypic genus *Leptoplax* due to its emarginate silicles, malpighiaceae pubescence and distinctive floral morphology (Greuter *et al.*, 1986; Hartvig, 1986, 2002b; Stefanović *et al.*, 2003). The problem remains unresolved and becomes even more relevant in the light of recent phylogenetic studies (Koch and Mummenhof, 2001; Appel and Al-Shehbaz, 2003; Al-Shehbaz *et al.*, 2006; Koch and Al-Shehbaz, 2009) which support the removal of *Peltaria* from Alyseae and its

TABLE 1. List of known Ni hyperaccumulator tribes and genera of Brassicaceae, based on the most recent comprehensive treatment of the family (Koch and Al-Shehbaz, 2009)

Taxa	Phytogeographical region	Literature sources*
Tribe Aethionemeae (1)		
<i>Aethionema</i> (1)	Mediterranean	16
Tribe Alyseae (approx. 55)		
<i>Alyssum</i> (approx. 50)	Circumboreal, Mediterranean, Irano-Turanian	1–5, 7, 16–18
<i>Bornmuellera</i> (5)	Euro-Mediterranean, Irano-Turanian	11, 19
Tribe Cardamineae (1)		
<i>Cardamine</i> (1)	European	6
Tribe Noccaeeae (24)		
<i>Masmenia</i> (1)	Irano-Turanian	12
<i>Microthlaspi</i> (1)	Euro-Mediterranean, Irano-Turanian	15
<i>Noccaea</i> (17) <sup>†</sup>	Circumboreal, Irano-turanian, Eastern Asian, Mediterranean, Rocky Mountains, Madrean	10–15
<i>Pseudosempervivum</i> (2)	Irano-Turanian	12
<i>Raparia</i> (1)	European	10
<i>Thlaspiceras</i> (2)	Mediterranean, Irano-Turanian	12
Tribe Schizopetaleae (1)		
<i>Streptanthus</i> (1)	Madrean	9
Incertae sedis (Thlaspidae ?) (1)		
<i>Leptoplax</i> (1)	Mediterranean	8

Numbers of Ni hyperaccumulator species for tribes and genera are indicated in parentheses.

\*The first report of hyperaccumulation for the species concerned: (1) Minguzzi and Vergnano (1948); (2) Doksoopulo (1961); (3) Menezes de Sequeira (1969); (4) Brooks and Radford (1978); (5) Brooks *et al.* (1979); (6) Vergnano Gambi and Gabbrielli (1979); (7) Vergnano Gambi *et al.* (1979); (8) Reeves *et al.* (1980); (9) Reeves *et al.* (1981); (10) Reeves and Brooks (1983); (11) Reeves *et al.* (1983); (12) Reeves (1988); see Koch and Mummenhof (2001) for placement of *Pseudosempervivum* and *Masmenia* in Noccaeeae rather than in Cochleariaceae as in Krämer (2010); (13) Mizuno *et al.* (2001); (14) Palmer *et al.* (2001); (15) Reeves *et al.* (2001); (16) Adigüzel and Reeves (2002); (17) Reeves and Adigüzel (2004); (18) Ghaderian *et al.* (2007); (19) Reeves and Adigüzel (2008).

<sup>†</sup>Including the two Ni hyperaccumulator species *Thlaspi japonicum* and *T. jaubertii* still not analysed phylogenetically; according to Koch and Al-Shehbaz (2009), *Noccaea* should include also *Masmenia*, *Pseudosempervivum*, *Raparia* and *Thlaspiceras*.

placement in Thlaspidaceae. Thus, assessment of the ancestry and relationships of this species is crucial for understanding the evolution of Ni hyperaccumulation at deep taxonomic levels.

Alyseae also include one of the largest groups of Ni hyperaccumulators in Brassicaceae, *Alyssum* sect. *Odontarrhena* (Brooks *et al.*, 1979). According to Mengoni *et al.* (2003) the capacity for Ni hyperaccumulation in this group has multiple origins, and it might have been gained or lost several times. Implementing the taxon sampling and the quality of DNA sequences used for that study (see Warwick *et al.*, 2008), however, was relevant to gain a broader view of relationships at the inter- and infraspecific level and the geographical structure of the clades. Taxon sampling in *Alyssum* sect. *Odontarrhena* is not straightforward since species-level relationships and limits are still unclear as a consequence of an exceedingly narrow species concept and a redundant nomenclature (Nyárady, 1928, 1929a, b, 1949; Dudley, 1964b). As argued by Hartvig (2002a), such an approach overlooks the strong phenotypic polymorphism especially in *A. murale sensu lato* (s.l.), a complex of serpentine and non-serpentine populations from south-east Europe which was split into some 12 species and subspecies, mainly based on slight variations in silicle and trichome morphology (Nyárady, 1928; Dudley, 1964a, b). Consequently, different taxonomic concepts for this group led to different estimates of the number and identity of hyperaccumulators.

The goal of the present study is to shed further light on the evolutionary lineages of Ni hyperaccumulation in Brassicaceae, focusing on tribe Alyseae, through a phylogenetic approach based on non-coding nuclear ribosomal DNA (nrDNA) sequences. These were obtained from a broad taxonomic and geographical sampling of European species, which were also tested for hyperaccumulation using a standard method. Inclusion of serpentine and non-serpentine accessions of potential hyperaccumulators and non-hyperaccumulators allowed us to test previous hypotheses on the multiple origins of this trait at the genus, species and below-species level. Ultimately, the data emerging from this analysis may contribute to: (a) a deeper understanding of the evolutionary dynamics of Ni hyperaccumulation in Brassicaceae; (b) a clearer and more practical taxonomy of European hyperaccumulators in Alyseae; and (c) the identification of new model systems consisting of phylogenetically related taxa for comparative studies of the molecular mechanisms of metal hyperaccumulation and practical applications in the fields of phytoremediation, phytomining and food crop biofortification.

## MATERIALS AND METHODS

### Plant material

Samples and voucher specimens from wild populations of *Bornmuellera*, *Leptoplax*, *Ptilotrichum*, *Alyssum* sect. *Alyssum*, *Alyssum* sect. *Odontarrhena*, *Aurinia* and *Aethionema* were collected by the author L. Cecchi and F. Selvi on serpentine outcrops and other soil types in Italy, Albania and Greece. Additional material was obtained from herbarium specimens borrowed from European herbaria (CAT, C, FI, SOF, SOM; Table 2).

TABLE 2. List of the investigated accessions, with vouchers (herbarium acronyms are according to Index Herbariorum), GenBank accession numbers and soil type

Taxon	Voucher specimen	Soil type	GenBank accession no.
<i>Aethionema arabicum</i> (L.) Andr. ex O.E.Schulz	Unknown	-	AY254539
<i>Aethionema saxatile</i> (L.) R.Br. subsp. <i>gracum</i> (Boiss. & Sprun.) Hayek	Greece, Sterea Ellas, Mt. Kallidhromon; Cecchi & Selvi 08-11; FI	Serpentine	GQ284853
<i>Alyssoides utriculata</i> (L.) Medik.	Swiss; Vautier 189; MO	Limestone	EF514593
<i>Alyssum alpestre</i> L.	France, Hautes-Alpes, Col du Lautaret; Bruneau 1474	Limestone	AY237957
<i>Alyssum argenteum</i> All.	1. Italy, Piedmont, Vallanta; Selvi 08-31; FI	Limestone	GQ284855
	2. Italy, Piedmont, Molette; Siniscalco s.n.; TO	Serpentine	GQ284854
<i>Alyssum aureum</i> (Fenzl) Boiss.	Israel; Samuelsson s.n.; MO	-	EF514601
<i>Alyssum baldacii</i> Nyár. (= <i>A. fallacinum</i> auct. non Hausskn.)	1. Greece, Sterea Ellas, Fourka pass; Bigazzi & Selvi 01-02; FI	Serpentine	GQ284858
	2. Greece, Sterea Ellas, Kedrhos; Cecchi & Selvi 08-08; FI	Serpentine	GQ284856
	3. Greece, Sterea Ellas, Mt. Kallidhromon; Cecchi & Selvi 08-12; FI	Serpentine	GQ284857
	4. Greece, Crete, Goni; Baker s.n.	Serpentine	AY237946
<i>Alyssum bertolonii</i> Desv. subsp. <i>bertolonii</i>	Italy, Tuscany, Mt. Ferrato; Cecchi & Cecchi 08-35; FI	Serpentine	GQ284859
<i>Alyssum bertolonii</i> Desv. subsp. <i>scutarium</i> Nyár.	1. Albania, Shkodër, Mt. Bardanjolt; Cecchi <i>et al.</i> 06-13; FI (= <i>A. janchenii</i> Nyár.)	Serpentine	GQ284867
	2. Yugoslavia, Kosovo, E of Prizren; Mellesics s.n. (= <i>A. punctatum</i> Nyár.)	Serpentine	AY237930
<i>Alyssum borzaeanum</i> Nyár.	Ukraine; Vakarenko & Mosyakin s.n.; MO	Serpentine	EF514603
<i>Alyssum corymbosoides</i> Form.	1. Greece, Western Macedonia, Mt. Vourinos; Cecchi & Selvi 08-25; FI	Schist	GQ284878
	2. Republic of Macedonia, Prilep; Cernoch 27287	Limestone	GQ284892
<i>Alyssum desertorum</i> Stapf	Albania, Korçë, Bitincka; Hasko 05-07; FI	Serpentine	GQ284881
<i>Alyssum densistellatum</i> T.R.Dudley	1. Greece, Sterea Ellas, Evia; Cecchi & Selvi 08-17; FI	Serpentine	GQ284879

Continued

TABLE 2. *Continued*

Taxon	Voucher specimen	Soil type	GenBank accession no.
<i>Alyssum euboicum</i> Halácsy	2. Greece, Sterea Ellas, Mt. Kallidhromon; <i>Cecchi &amp; Selvi 08-14</i> ; FI	Serpentine	GQ284880
<i>Alyssum fragillimum</i> (Baldacci) Rech.f.	Greece, Sterea Ellas, Evia; <i>Cecchi &amp; Selvi 08-20</i> ; FI	Serpentine	GQ284882
<i>Alyssum heldreichii</i> Hausskn.	Greece, Crete, Lefka Ori; <i>Bergmeier &amp; Matthäs 3254</i> ; C	–	GQ284883
<i>Alyssum klimesii</i> Al-Shehbaz	Greece, Western Macedonia, Mt. Vourinos; <i>Cecchi &amp; Selvi 08-26</i> ; FI	Serpentine	GQ284884
<i>Alyssum menioides</i> Boiss.	India, Kashmir, Ladak; <i>Klimes 38361</i> ; MO	–	EF514608
<i>Alyssum montanum</i> L.	Syria; <i>Samuelsson s.n.</i> ; MO	–	EF514612
<i>Alyssum murale</i> Waldst. & Kit. <i>s.l.</i>	Italy, Tuscany, Monterufoli; FI	Serpentine	AY237938
	1. Albania, Berat; <i>Hasko 07-24</i> ; FI (= <i>A. chalcidicum</i> Janka)	Serpentine	GQ284860
	2. Albania, Elbasan, Librazhd; <i>Hasko 06-19</i> ; FI (= <i>A. markgrafii</i> O.E.Schulz)	Serpentine	GQ284864
	3. Albania, Elbasan, Librazhd; <i>Cecchi et al. 07-21</i> ; FI (= <i>A. markgrafii</i> )	Serpentine	GQ284863
	4. Albania, Elbasan, Librazhd; <i>Hasko 3303</i> ; FI (= <i>A. markgrafii</i> )	Serpentine	GQ284865
	5. Albania, Elbasan, Librazhd; <i>Hasko 3306</i> ; FI (= <i>A. markgrafii</i> )	Serpentine	GQ284866
	6. Albania, Elbasan, Perrënjës; <i>Hasko 06-20</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284871
	7. Albania, Elbasan, Perrënjës; <i>Cecchi et al. 07-22</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284869
	8. Albania, Elbasan, Perrënjës; <i>Hasko 3334</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284870
	9. Albania, Korçë, Bitincka; <i>Hasko 04-01</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284861
	10. Albania, Korçë, Pogradec; <i>Hasko 06-21</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284875
	11. Albania, Korçë, Pogradec; <i>Cecchi et al. 07-23</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284872
	12. Albania, Korçë, Pogradec; <i>Hasko 04-02</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284873
	13. Albania, Korçë, Pogradec; <i>Hasko 05-10</i> ; FI (= <i>A. chalcidicum</i> )	Serpentine	GQ284874
	14. Albania, Kukës, Mt. Paštrik; <i>Cecchi et al. 06-17</i> ; FI (= <i>A. kosaninum</i> Nyár.)	Serpentine	GQ284868
	15. Albania, Lezhë, Rubik; <i>Hasko 3355</i> ; FI (= <i>A. chalcidicum</i> )	Copper mine	GQ284862
	16. Bulgaria, Blagoevgrad, Mt. Belasica; <i>Pavlova et al.</i> ; SOF 104630	Limestone	GQ284885
	17. Greece, Anatolic Macedonia, Mt. Pangeon; <i>Univ. Copenhagen Excurs. 226</i> ; C	Limestone	GQ284886
	18. Greece, Epirus, Metsovo; <i>Cecchi &amp; Selvi 08-04</i> ; FI (= <i>A. chlorocarpum</i> Hausskn.)	Schist	GQ284877
	19. Greece, Central Macedonia, Drosia; <i>Bigazzi &amp; Selvi 01-01</i> ; FI (= <i>A. subvirescens</i> Form.)	Serpentine	GQ284876
<i>Alyssum nebrodense</i> Tineo	20. Italy, Piedmont, naturalized near Casteldelfino; <i>Selvi 08-32</i> ; FI	Schist	GQ284887
<i>Alyssum obovatum</i> (C.A.Mey.) Turcz.	Italy, Sicily, Piano della Battaglia; FI	Limestone	AY237935
<i>Alyssum orbelicum</i> Ančev & Uzunov	Russia, Amur, <i>Boyko &amp; Starchenko s.n.</i> ; MO	–	EF514617
<i>Alyssum robertianum</i> Bernard ex Gren. & Godr.	L	Limestone	GQ284888
<i>Alyssum serpyllifolium</i> Desf.	Italy, Sardinia, Monte Novo San Giovanni; Charpin <i>et al.</i> ; FI	Limestone	GQ284889
<i>Alyssum sibiricum</i> Willd.	Portugal, <i>Auriault 13088</i> ; MO	–	EF514623
<i>Alyssum smolikanum</i> Nyár.	Greece, Sterea Ellas, Evia; <i>Cecchi &amp; Selvi 08-21</i> ; FI	Serpentine	GQ284890
<i>Alyssum tenium</i> Halácsy	Greece, Epirus, Mt. Smolikas; <i>Cecchi &amp; Selvi 08-28</i> ; FI	Serpentine	GQ284891
<i>Alyssum tortuosum</i> Waldst. & Kit. ex Willd.	Greece, Cyclades, Tinos; <i>Baker s.n.</i>	Serpentine	AY237926
<i>Alyssum troodi</i> Boiss. ex Boiss.	Turkmenistan; <i>Kurbanov 391</i> ; MO	–	EF514625
<i>Aurinia saxatilis</i> (L.) Desv.	Cyprus, Mt. Troodos, Mt. Khionistra; <i>Brullo et al. s.n.</i> ; CAT	Serpentine	GQ284893
<i>Aurinia saxatilis</i> (L.) Desv. subsp. <i>orientalis</i> (Ard.) T.R.Dudley	1. <i>Unknown</i>	–	AF401115
<i>Berteroa orbiculata</i> DC.	2. Greece, Sterea Ellas, Aliartos; <i>Cecchi &amp; Selvi 08-15</i> ; FI	Limestone	GQ284894
<i>Bornmuellera baldaccii</i> (Degen) Heywood	Greece; <i>Rechinger 22481</i> ; MO	–	EF514634
	1. Greece, Epirus, Mt. Smolikas; <i>Cecchi &amp; Selvi 08-29</i> ; FI	Serpentine	GQ284895
	2. Greece; <i>Rechinger 20986</i> ; MO	Serpentine	EF514635
	Greece; <i>Rechinger 18407</i> ; MO	Serpentine	EF514640
<i>Bornmuellera tymphaea</i> (Hausskn.) Hausskn.	Iran; <i>Rechinger 6461</i> ; MO	–	EF514641
<i>Clastophus vestitus</i> (Desv.) Boiss.	Turkmenistan; <i>Kurbanov 1887</i> ; MO	–	EF514644
<i>Clypeola jonthlaspi</i> L.	Croatia, Dalmatia	Limestone	DQ249857
<i>Degenia velebitica</i> (Degen) Hayek	Mauritania; <i>Adam 13237</i> ; MO	–	EF514649
<i>Farsetia aegyptia</i> Turra	<i>Unknown</i>	–	DQ249852
<i>Fibigia clypeata</i> (L.) Medik.	Mongolia; <i>Guricheva &amp; Rachkovskaya 2499</i> ; MO	–	EF514655
<i>Galitzkya macrocarpa</i> (Ikonn.-Gal.) V.V.Botschantz.	Spain; <i>Charpin et al. 2504</i> ; MO	–	EF514659
<i>Hormathophylla longicaulis</i> (Boiss.) Cullen & T.R.Dudley			



<i>Leptoplax emarginata</i> (Boiss.) O.E.Schulz	1. Greece, Thessaly, Kedrhos; Cecchi & Selvi 08-10; FI	Serpentine	GQ284897
<i>Lobularia maritima</i> (L.) Desv.	2. Greece, Sterea Ellas, Evia; Cecchi & Selvi 08-18; FI	Serpentine	GQ284896
<i>Peltaria alliacea</i> Jacq.	1. Italy, Tuscany	-	AY237920
<i>Physopychis caspica</i> (Habititz) V.V.Botschantz.	2. Spain; Borgen 3607; O	-	EF514681
<i>Ptilotrichum canescens</i> (DC.) C.A.Mey	Unknown	-	DQ289855
<i>Ptilotrichum cyclocarpum</i> Boiss. subsp. <i>pindicum</i> Hartvig	Iraq; Rechinger 11159; MO	-	EF514682
<i>Ptilotrichum rupestre</i> (Ten.) Boiss.	China, Xinjiang; Bartholomew et al. 8657; MO	-	EF514683
	Greece, Epirus, Mt. Timfi; Cecchi & Selvi 08-30; FI	Limestone	GQ284851
	Italy, Abruzzo, Mt. Cavallo; Conti et al. s.n.; APP	Limestone	GQ284852

The genera of Alyseae are according to Warwick et al. (2008), except for *Leptoplax* and *Ptilotrichum* (Hartvig, 2002).

Complete sequences from the internal transcribed spacer region of nuclear DNA (ITS) were generated for two accessions of *L. emarginata*, *Ptilotrichum rupestre*, *P. cyclocarpum* and a few other taxa of Brassicaceae, in order to examine their position within the framework of the recently published phylogenetic analysis of Alyseae (Warwick et al., 2008).

Sampling in *Alyssum* was more intensive, and included three outgroup accessions of *Alyssum* sect. *Alyssum* and 45 ingroup accessions, of which 37 were original. Together these represent 17 of 19 species of *Alyssum* sect. *Odontarrhena* native to the Euro-Mediterranean region as currently accepted in *Flora Europaea* (Ball and Dudley, 1993) (all except *A. obtusifolium* and *A. caliacrae*). Special emphasis was given to the *A. murale* complex, represented by 22 samples and 14 different geographical accessions, from both ultramafic and non-ultramafic soils. Some of these accessions can be referred to doubtful taxa of *Alyssum* described from southeast Europe, and were included to examine their position in the ITS phylogenetic analysis.

#### Test of Ni hyperaccumulation

All accessions of *Alyssum*, *Leptoplax* and *Bornmuellera* that were included in the phylogenetic analysis were tested for Ni hyperaccumulation in leaf dry weight using the standard colorimetric method proposed by Tschugaeff (1905). In spite of some shortcomings, the dimethylglyoxime (DMG) method is widely utilized and accepted in the literature as a useful screen for Ni, especially in the field or on herbarium material (Brooks, 1998; Reeves et al., 1999; Whiting et al., 2004). Three leaves for each dry collection were screened with DMG (Tschugaeff reagent), a selective reagent turning red when forming nickel complexes. Filter paper slices were firstly impregnated with a 1 % DMG solution in absolute ethanol, then dried at room temperature, rehydrated with sterile water and rubbed against leaf fragments. In such a semi-quantitative test, a negative result generally indicates an Ni concentration of  $<1000 \mu\text{g g}^{-1}$  in dry leaf mass (Reeves et al., 1999). The individual plant collections for which a clear change of colour was obtained were considered Ni hyperaccumulators.

To test the constancy of Ni hyperaccumulation in different accessions of serpentine-tolerant species, the same test was also applied to an additional sample of 90 herbarium specimens representing 18 taxa of *Alyssum* sect. *Odontarrhena*, *Bornmuellera* and *Leptoplax* (Table 3, Appendix).

#### DNA extraction and amplification

Genomic DNA was extracted following a modified 2 × CTAB (cetyltrimethylammonium bromide) protocol (Doyle and Doyle, 1990) using silica-gel dried samples of leaf tissue or herbarium material. The extracted DNA was quantified by spectrophotometric analysis (Biophotometer, Eppendorf).

For amplification of the ITS region, including ITS1, 5-8S and ITS2, the primers ITS4 and ITS5 of White et al. (1990) were used.

PCRs were performed in a total volume of 25  $\mu\text{L}$  containing 2.5  $\mu\text{L}$  of reaction buffer (Dynazyme II, Finnzyme, Espoo, Finland), 1.5 mM  $\text{MgCl}_2$ , 10 pmol of each primer, 200  $\mu\text{M}$  dNTPs, 1 U of *Taq* DNA polymerase (Dynazyme II, Finnzyme, Espoo, Finland) and 10 ng of template DNA.

TABLE 3. Results of Tschugaeff reactive (dimethylglyoxime) test of Ni hyperaccumulation performed on 90 herbarium accessions from ultramafic substrates of European (including Cyprus) serpentine-tolerant taxa of *Alyssum* sect. *Odontarrhena*, *Bornmuellera* and *Leptoplax*

Genus, section and clade	Species	No. of accessions*	Origin†
<i>Alyssum</i> sect. <i>Odontarrhena</i>			
Clade O1	<i>A. euboicum</i>	2/2	Greece (Evia)
Clade O2	<i>A. argenteum</i>	11/11	Italy
	<i>A. tenium</i>	3/3	Greece (Tinos)
	<i>A. serpyllifolium</i>	5/5	Portugal, Spain
	<i>A. baldaccii</i>	5/5	Greece (including Crete)
	<i>A. murale</i>	35/35	Bosnia-Herzegovina, Albania, Kosovo, Macedonia, Greece
Clade O3	<i>A. bertolonii</i>	11/11	Italy
	<i>A. sibiricum</i>	0/1	Greece (Evia)
	<i>A. robertianum</i>	2/2	France (Corsica)
	<i>A. heldreichii</i>	4/4	Greece
	<i>A. smolikanum</i>	1/1	Greece
	<i>A. troodi</i>	2/2	Cyprus
Incertae sedis	<i>A. akamasicum</i>	2/2	Cyprus
	<i>A. cypricum</i>	1/1	Cyprus
	<i>A. lesbiacum</i>	1/1	Greece (Lesbos)
<i>Bornmuellera</i> (Alyseae clade C)	<i>B. baldaccii</i>	2/2	Greece
	<i>B. tymphaea</i>	1/1	Greece
<i>Leptoplax</i> (Alyseae clade C)	<i>L. emarginata</i>	2/2	Greece (including Evia)

Data are shown as in Jansen *et al.* (2004).

\*Ni hyperaccumulator accessions/total accessions.

†See Appendix for details on vouchers and localities.

Reactions were performed in an MJ PTC-100 thermocycler (Peltier Thermal Cycler, MJ Research). Subsequently, 5 µL of each amplification mixture was analysed by agarose gel electrophoresis in TAE buffer (1.5 % w/v) containing 1 µg mL<sup>-1</sup> ethidium bromide, by comparison with a known mass standard. The PCR products were purified of excess salts and primers with a PCR Purification Kit (Roche, Mannheim, Germany) and quantified by spectrophotometry (Biophotometer, Eppendorf). Automated DNA sequencing was performed directly from the purified PCR products using BigDye Terminator v.2 chemistry and an ABI310 sequencer (PE-Applied Biosystems, Norwalk, CT, USA). Sequences are deposited in EMBL (accession numbers are given in Table 2).

#### Sequence alignment and phylogenetic analyses

New sequences of *Leptoplax*, *Alyssum* sect. *Alyssum*, *Alyssum* sect. *Odontarrhena* and other members of Alyseae and *Aethionema* were checked for orthology to the sequences of *Bornmuellera baldaccii* and *Alyssum montanum*. Multiple alignments of the ITS data sets of Alyseae and *Alyssum* sect. *Odontarrhena* were performed separately with Multalin (Corpet, 1988), then carefully examined and slightly corrected by eye. Using separate alignments allowed us to explore and describe Ni hyperaccumulation in two steps representing different phylogenetic depths, the genus and the species level, and to avoid problems with treatments of gaps. For analyses in Alyseae, these were coded as separate characters according to the 'simple gap coding' method (Simmons and Ochoterena, 2000) as implemented in FastGap (Borchsenius, 2009), and appended to the end of the matrices. Because insertions/deletions in the aligned data set of *Alyssum* sect. *Odontarrhena* were introduced only as a result of the accessions retrieved from

GenBank, gaps were treated as missing data in order not to give excessive weight to possible sequencing artefacts. For the phylogenetic analysis of Alyseae, members of Aethionemeae, Camelinae, Thlaspidiae, Arabideae and Malcolmieae were used as outgroups based on their position in the recent study by Warwick *et al.* (2008). The type species of the genera *Aurinia*, *Ptilotrichum* and *Peltaria* were included to evaluate without ambiguity the position of *Leptoplax* and the other critical taxa analysed for the first time here. Representatives of *Alyssum* sect. *Alyssum* were used as outgroups in the analysis of *Alyssum* sect. *Odontarrhena*, based on evidence from the previous analysis of Alyseae. Most of the ITS sequences of section *Odontarrhena* already available from GenBank were not used for phylogenetic reconstructions, as these were judged of 'poor quality' (Bailey *et al.*, 2006; Warwick *et al.*, 2008).

Phylogenetic trees for both data sets were first generated using maximum parsimony (MP) as performed in PAUP 4.0 (Swofford, 2000). Heuristic searches were run with 'tree-bisection-reconnection' (TBR) branch-swapping with accelerated transformation (ACCTRAN) optimization to infer branch (edge) lengths; MULTREES option on, ADDSEQ = random, ten randomized replicates. All characters were weighted equally, and character state transitions were treated as unordered. Most-parsimonious trees were summarized in a strict consensus, that was viewed and edited with TreeView (Page, 1996). Bootstrap support (BS) for clades was obtained, performing the analysis with 1000 replicates, using TBR branch-swapping, ten random taxon entries per replicate and MULTREES option on; search = FASTSTEP.

Both data sets were also analysed using Bayesian inference (BI) with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), with a General Time Reversible (GTR) model and  $\gamma$ -distributed rate variation across sites. Analyses were performed using four

incrementally heated Markov chains (one cold, three heated) simultaneously started from random trees, and run for 1 million cycles sampling a tree every 100 generations. The stationary phase was reached when the average standard deviation of split frequencies approached 0.01. Trees that preceded the stabilization of the likelihood value (the burn-in) were discarded, and the remaining trees were used to calculate a 50 % majority-rule consensus phylogram that was viewed and edited with TreeView, with Bayesian posterior probability (PP) values for the internal tree nodes.

## RESULTS

### *Ni hyperaccumulation test*

All leaf samples of each specimen gave the same result in the DMG test, i.e. they consistently either produced or did not produce a change to red in the reagent. With the exception of *A. sibiricum*, all serpentine accessions of 18 species of *Alyssum* sect. *Odontarrhena*, *Leptoplax* and *Bornmuellera* gave a positive result and showed the potential for Ni hyperaccumulation (Table 3).

The DMG results for 35 specimens of *A. murale* s.l. were mapped together with several non-serpentine sites (Fig. 5). Ni hyperaccumulators and non-hyperaccumulator accessions were basically mixed with each other, depending on the geography of serpentine outcrops. Due to the higher frequency and extent in the southern Balkans, hyperaccumulator populations mainly occur in Albania and Greece, and correspond to the ‘*chalcidicum*’ morphotype. The typical form of *A. murale*, instead, is restricted to non-serpentine soils and is mainly concentrated in the central Balkans.

### *Phylogenetics of Alysseae*

The aligned ITS matrix for Alysseae (TreeBASE ID: 10525; Supplementary Data 1, available online) was 794 bp long, including coded gaps that were appended to the end of the matrix (120 positions). Variation within the ingroup (Alysseae) was relatively high, with 38.8 % of variable positions in at least one accession. The mean pairwise genetic distance (Kimura 2-parameters) between accessions was 0.079.

In the MP analysis, 318 characters were constant, 166 parsimony uninformative and 310 (39 %) potentially informative. The two most-parsimonious trees retrieved were 1251 steps long, with consistency index (CI) = 0.57 and retention index (RI) = 0.63; the first tree is shown in Fig. 1. Bayesian analysis (BI) resulted in a 50 % majority-rule consensus phylogram (Fig. 2) largely congruent with the MP trees.

Monophyly of Alysseae, including *Leptoplax*, was strongly supported (94 % BS; 1.00 PP). *Alyssum klimesii* (Camelineae), *Peltaria alliacea* (Thlaspidaceae), *Ptilotrichum canescens* (Arabiaceae) and the *Lobularia/Farsetia* clade (Malcolmieae) were confirmed to fall outside this tribe. Four main clades were retrieved in Alysseae, here indicated as A, B, C and D. The deep nodes between these clades did not receive statistical support, leaving the relationships between these lineages basically unresolved. Clade A (56 % BS; 0.99 PP) included members of *Alyssum* sect. *Alyssum*, *Alyssum* sect. *Odontarrhena* and *Alyssum* sect. *Meniocus*, together with a clearly nested

*Clypeola*. The relationship between *Hormathophylla* and the other genera of clade B did not receive bootstrap support and was only suggested in the BI analysis (0.51 PP) where *Hormathophylla longicaulis* was sister to a sub-clade of *Aurinia*, *Berteroa* and *Galitzkya* (95 % BS; 1.00 PP). In clade C (83 % BS; 1.00 PP), *Ptilotrichum cyclocarpum* and *P. rupestre* clustered together with 94 BS % and 1.00 PP; they were retrieved as sister to a strongly supported sub-clade (98 % BS; 1.00 PP) formed by the three accessions of *Bornmuellera baldaccii* and *B. tymphaea* (77 % BS; 0.79 PP) sister to *Leptoplax emarginata* (86 % BS; 1.00 PP). *Leptoplax* and *Bornmuellera* share 14 exclusive single nucleotide polymorphisms (SNPs), seven in ITS1 and seven in ITS2; the mean pairwise genetic distances (Kimura 2-parameters) between accessions of the two genera is 0.037. The two accessions of *Leptoplax* from Evia and Thessaly differed in 27 SNPs, accounting for their different branch lengths in the BI and MP analyses. Clade D (99 % BS; 1.00 PP) included *Fibigia* sister to a group formed by *Alyssoides*, *Degenia*, *Clastopus* and *Physoptychis* (55 % BS; 0.65 PP).

### *Phylogenetics of Alyssum sect. Odontarrhena*

The aligned matrix was 651 bp long (TreeBASE ID: 10525; Supplementary Data 2, available online); nucleotide variation within the ingroup was low (89 variable positions = 13.7 %, of which 57 are in ITS1, eight in 5.8S and 24 in ITS2); as expected, the mean pairwise genetic distance was considerably lower (0.02) than that found among members of Alysseae.

In the MP analysis, 527 characters were constant, 46 were non-informative and 78 potentially informative. Heuristic search yielded 202 most-parsimonious trees with length (L) = 199, CI = 0.71, RI = 0.79; though poorly resolved, the resulting strict consensus (not shown) showed no conflict with the more resolved phylogram from the Bayesian analysis (Fig. 3), which is described here.

*Alyssum* sect. *Odontarrhena* was retrieved as a monophyletic group (1.00 PP) including three major clades, here named O1, O2 and O3, with unresolved relationships. Clade O1 (1.00 PP) contained the Greek island endemics *A. euboicum*, an Ni hyperaccumulator from serpentine of Evia, and *A. fragillimum*, a non-hyperaccumulator from calcareous mountains on Crete. Clade O2 (0.85 PP) includes the non-hyperaccumulator *A. corymbosoides* as sister to a broad and largely unresolved group including all accessions of the predominantly Ni-hyperaccumulating taxa *A. argenteum*, *A. baldaccii*, *A. murale*, *A. serpyllifolium* and *A. tenium* mixed with the non-hyperaccumulators *A. orbelicum* and *A. tortuosum* (type species of section *Odontarrhena*). Clade O3 (0.84 PP) consists of four non-hyperaccumulator species with unresolved relationships and five mainly hyperaccumulator taxa. Of these, the four European species *A. heldreichii*, *A. smolikanum*, *A. bertolonii* and *A. robertianum* were retrieved as a monophyletic group (0.98 PP), with a clear affinity (0.96 PP) between the two latter taxa.

## DISCUSSION

### *Double origin of Ni hyperaccumulation in tribe Alysseae*

According to Warwick *et al.* (2008), Alysseae consist of 12 genera in addition to *Straussiiella* which was not included in

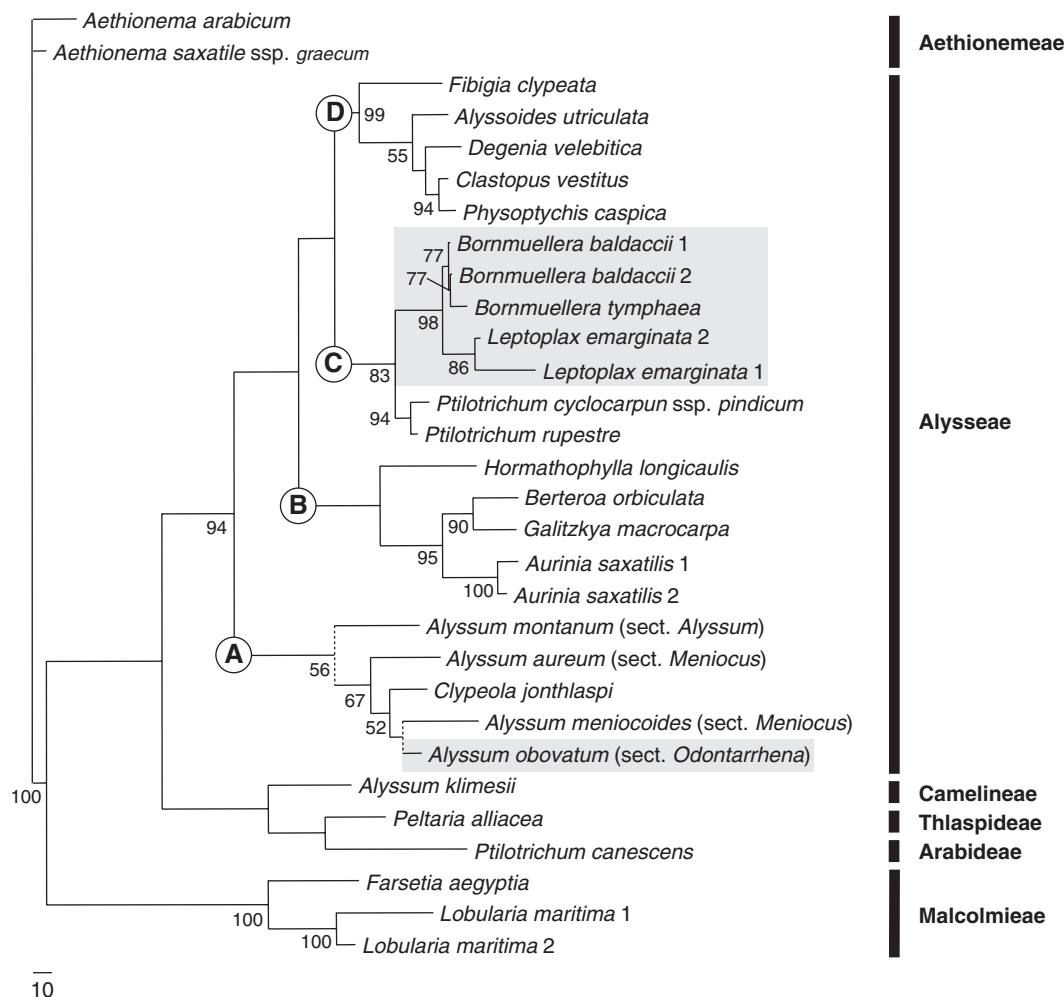


FIG. 1. One of the two most-parsimonious trees of tribe Alyseae generated by ITS 5-8S sequences with branch lengths ( $L = 1251$ ,  $CI = 0.57$ ,  $RI = 0.63$ ). The main clades discussed in the text are indicated by upper case letters (A–D). Bootstrap values  $>50\%$  are indicated at the nodes; nodes collapsing in the strict consensus are indicated by dotted lines. Grey boxes indicate clades including Ni hyperaccumulators.

their molecular study. However, adding accessions of *Leptoplax* and two Mediterranean species of *Ptilotrichum* to the phylogenetic analysis provided results that shed new light on the number and relationships of Ni hyperaccumulator lineages in this group. *Leptoplax emarginata* has no affinity with *Peltaria*, in which it was included by some recent taxonomists (Ball, 1993; Jalas and Suominen, 1996; Appel and Al-Shehbaz, 2003; Warwick *et al.*, 2008). Fruit characters shared by two taxa, such as the pendent, indehiscent, strongly compressed latiseptate silicles, are most probably homoplasious and due to parallel evolution. In contrast, our results support *L. emarginata* as a separate lineage, in agreement with the views of other taxonomists (Greuter *et al.*, 1986; Hartvig, 1986, 2002b) who stressed its placement in a monotypic genus of Alyseae. Molecular data clearly demonstrate that *Leptoplax* is sister to the genus *Bornmuellera* (clade C, 96% BS, 1.0 PP), as represented by the two Ni hyperaccumulator species *B. tymphaea* and *B. baldaccii*, endemic to the serpentine outcrops of continental Greece (Stefanović *et al.*, 2003). The mean pairwise genetic distance within the *Bornmuellera*–*Leptoplax* clade is closer to that found among species of *Alyssum* sampled here than that among

genera of Alyseae, corroborating the nearly congeneric status of the two taxa. In addition, *L. emarginata* and *Bornmuellera* are diploids with a similar genome size (Peer *et al.*, 2006) and chromosome complements based on  $x = 8$  (Constantinidis *et al.*, 2002), unlike *Peltaria* with  $x = 7$ . Given their broadly sympatric distribution (Fig. 4) and identical specialization for serpentine soils, this condition may have contributed to the formation of the intergeneric hybrids and introgressive populations involving *L. emarginata* and *Bornmuellera tymphaea* reported from northern Greece (Hartvig, 1986, 2002b; Ball, 1993). Shoot concentrations of  $\leq 34\,000$  and  $31\,000\,\mu\text{g g}^{-1}$  (Chardot *et al.*, 2005; see also Bani *et al.*, 2009; Reeves *et al.*, 2009; Redjala *et al.*, 2010), respectively, show that *Leptoplax* and *Bornmuellera* have evolved a striking ability for Ni hyperaccumulation, representing an early synapomorphy due to common ancestry. Coupled with high biomass production (Chardot *et al.*, 2005) and broad tolerance to different climatic constraints (from Mediterranean to sub-alpine habitats; Hartvig, 2002; pers. obs.), *L. emarginata* is an ideal model for phytoremediation and phytomining in different environments.



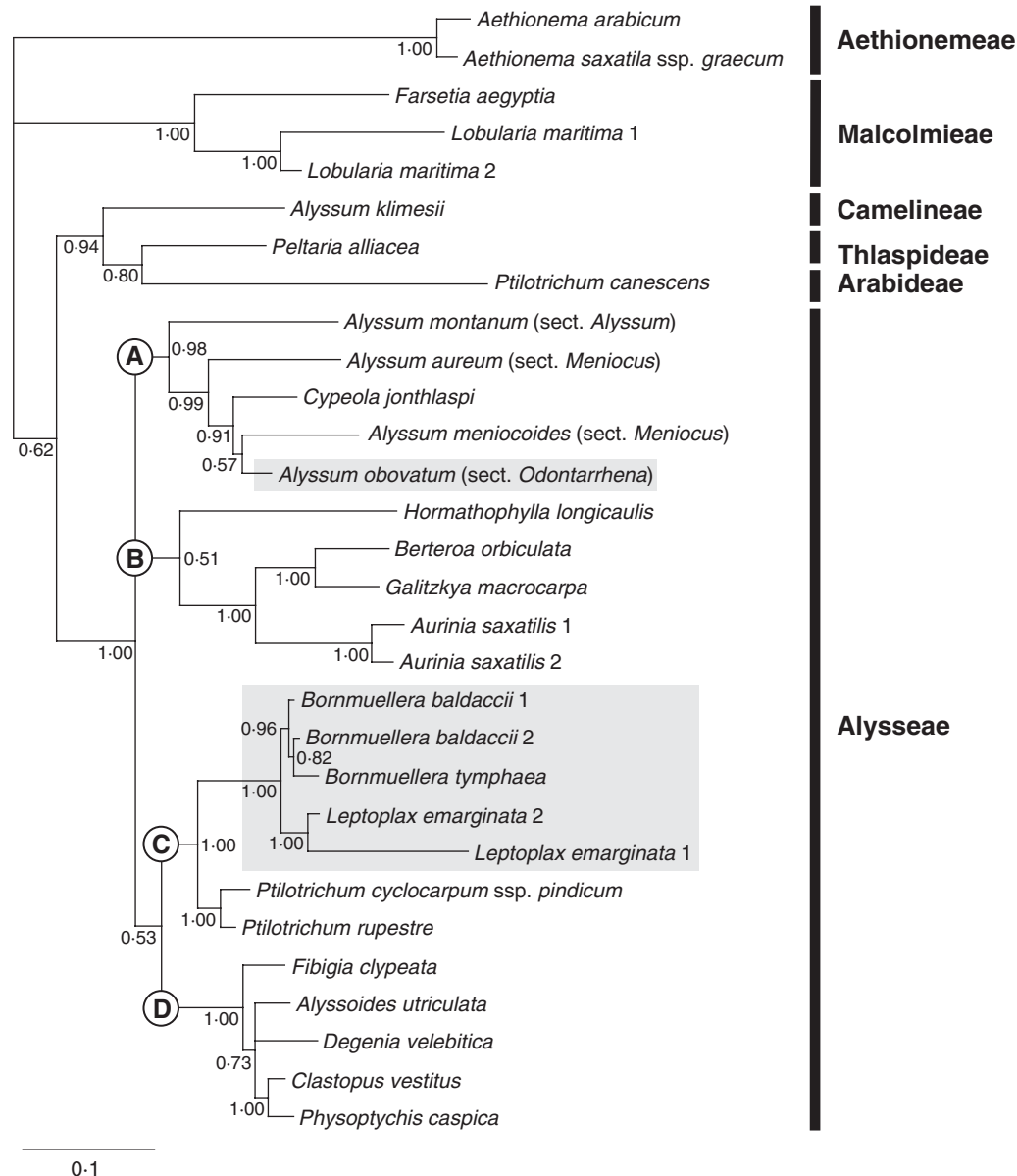


FIG. 2. Bayesian phylogram (consensus) of tribe Alyseae generated by ITS 5-8S sequences. The main clades discussed in the text are indicated by upper case letters (A–D). Posterior probabilities are indicated at the nodes. Grey boxes indicate clades including Ni hyperaccumulators.

Removing *Leptoplax* from *Peltaria*, as advocated here, implies that the ability for Ni hyperaccumulation did not originate in Thlaspidaceae, since the approx. 15 hyperaccumulators formerly included in *Thlaspi* are firmly nested in tribe Noccaeae (Koch and Mummenhof, 2001; Koch and Al-Shehbaz, 2009). The recent phylogenetic scheme of metal hyperaccumulation in Brassicaceae (Krämer, 2010) should be amended by placing '*Peltaria*' *emarginata* in Alyseae under *Leptoplax*, and removing Thlaspidaceae from the list of tribes of Brassicaceae accumulating nickel.

Given their distant phylogenetic position, different mechanisms of hyperaccumulation and tolerance may have evolved in the *Noccaeae* and *Bornmuellera*–*Leptoplax* clades, suggesting the latter as a model for further physiological and molecular investigations. Based on molecular evidence, the latter

lineage is sister to the well supported group formed by *Ptilotrichum rupestre* and *P. cyclocarpum*, indicating a common origin from an ancestor distributed in montane areas of the central-eastern Mediterranean. In addition, ITS sequences reveal no affinity between these two species and the Asian type species of *Ptilotrichum*, *P. canescens* (tribe Arabideae), suggesting the transfer of the two Mediterranean taxa to a separate genus of Alyseae.

*Ptilotrichum rupestre* and *P. cyclocarpum* are strictly calcicolous species endemic to the central Apennines and the Balkan peninsula (Fig. 4), respectively, where they grow in rocky habitats such as cliffs and screes of calcareous and/or dolomitic nature (Pignatti, 1982; Hartvig, 2002b). The edaphic requirements of *Bornmuellera*–*Leptoplax* and *Ptilotrichum* corroborate the evidence that Brassicaceae are

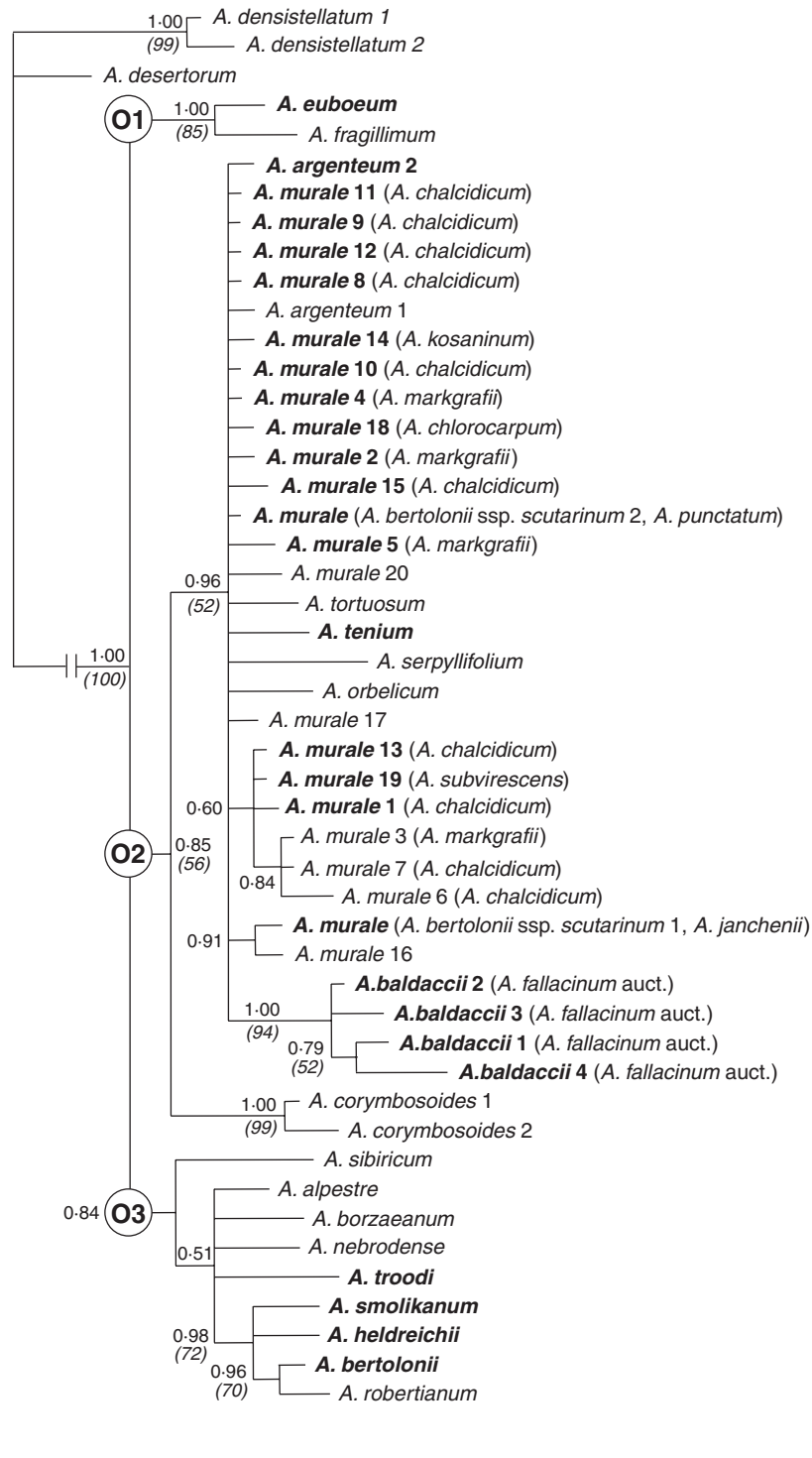


FIG. 3. Bayesian phylogram of *Alyssum* section *Odontarrhena* generated by ITS 5-8S sequences. The main clades discussed in text are indicated as O1–O3. Posterior probabilities are shown at the nodes; corresponding bootstrap values >50 % of MP strict consensus tree (not shown) are reported in parentheses. Ni hyperaccumulator accessions as resulting from the DMG test are in bold; species names are based on evidence of the present study (see text) and taxonomic synonyms are given in parentheses.

often a dominant group in the floras of Mg-rich substrates such as dolomites and serpentines (Proctor, 1999; Mota *et al.*, 2008). From a phylogenetic viewpoint, the capacity for Ni hyperaccumulation in clade C may be seen as a symplesiomorphy

secondarily lost in the *Ptilotrichum rupestre*–*cyclocarpum* lineage or, more probably, as a synapomorphy which originated in *Bornmuellera* and *Leptoplax* from a non-hyperaccumulator ancestor under the selective pressure of serpentine soils in the

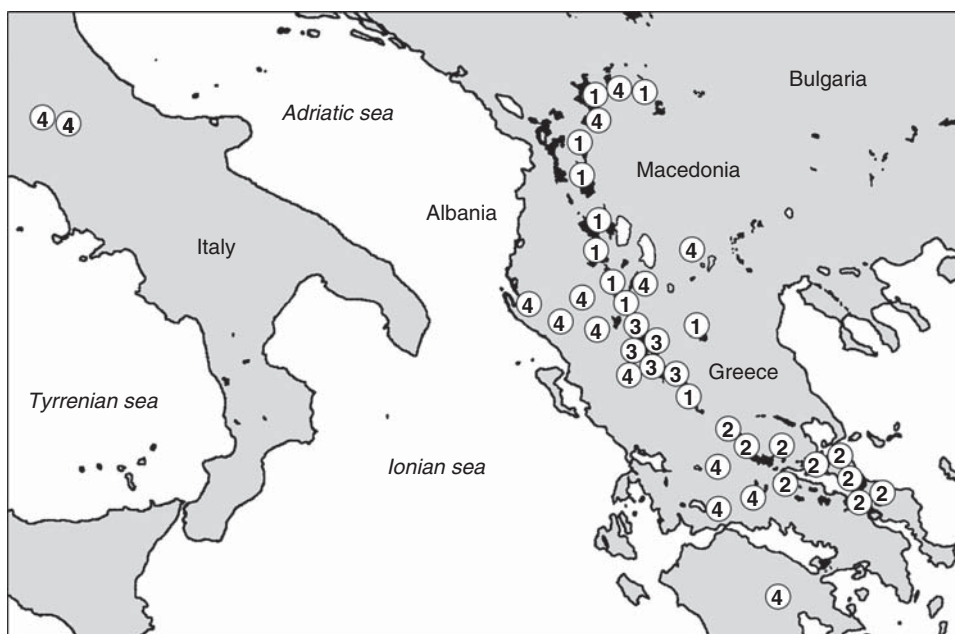


FIG. 4. Chorological relationships among *Bornmuellera* (1), *Leptoplax* (2), *Bornmuellera* plus *Leptoplax* (overlapping area 3), *Ptilotrichum cyclocarpum* and *P. rupestre* (4); black spots indicate the main serpentine areas in SE Europe.



FIG. 5. Distribution of *Alyssum murale* in the Balkan peninsula. White and black circles indicate, respectively, non-Ni-hyperaccumulating and Ni-hyperaccumulating populations as resulting from DMG tests; black spots indicate the main serpentine areas in SE Europe.

southern Balkans. The basophilous character of this ancestor may have favoured the evolution of new lineages with a stronger edaphic specialization, enhancing their competitive ability on ultrabasic serpentine outcrops. Recent phylogenetic analyses of Boraginaceae tribe Lithospermeae (Thomas *et al.*, 2008; Cecchi and Selvi, 2009) revealed a similar relationship in the two serpentine endemics *Halacsya sendtneri* and *Paramoltkia doerfleri*, which are sister to a group of strictly basophilous species of *Lithodora* growing on limestone. This provides support that most serpentine endemics in the Balkan flora have as their closest relatives basophilous taxa linked to calcareous substrates (Stefanović *et al.*, 2003). In addition, the ability to cope with high soil Mg concentration of many serpentine plants is often considered a key component of adaptation to ultramafics (Brady *et al.*, 2005; Kazakou *et al.*, 2008), suggesting that the possibly elevated amounts of this element in the substrates of Mediterranean *Ptilotrichum* may have been a prerequisite for the evolution of obligate serpentinophytism in *Bornmuellera* and *Leptoplax*.

*Lack of geographical cohesion and polyphyly in Ni hyperaccumulators of Alyssum sect. Odontarrhena*

Molecular data from our expanded taxonomic sample strongly support *Alyssum* section *Odontarrhena* as a monophyletic lineage characterized by a single ovule per locule, the two-seeded silicles and the usually branched inflorescence. Accordingly, this could also be treated as a genus separate from *Alyssum* s.s. (Warwick *et al.*, 2008). Available data and DMG tests show that hyperaccumulator species of *Alyssum* are exclusively found in this section, corroborating its phylogenetic distinctiveness. Although other species from different *Alyssum* sections are also able to colonize serpentine soils (e.g. *A. montanum*, *A. densistellatum*, *A. desertorum* and *A. mouradicum* of section *Alyssum*), they cannot hyperaccumulate nickel, showing that this capacity did not evolve, or it was lost, outside section *Odontarrhena*. With the exception of *A. sibiricum* (Reeves and Adigüzel, 2004, 2008; pers. obs.), all serpentine taxa of this group hyperaccumulate Ni when growing on ultramafic soils, suggesting that this ability may itself be an adaptive strategy for metal tolerance. On the other hand, serpentinophytism is not an obligate condition in *Odontarrhena*, as shown by calcicolous species such as *A. nebrodense*, *A. fragillimum* and *A. orbelicum*.

Low ITS variation in this group does not allow a complete resolution of relationships among species and, coupled with weak phenotypic differentiation, suggests a relatively recent radiation in southern Europe. Our data show that the evolutionary distribution of hyperaccumulation is even more patchy than previously known (Mengoni *et al.*, 2003), and that this capacity does not represent a synapomorphy for any of the three major clades that were retrieved. Hyperaccumulators and non-hyperaccumulators are mixed in each of these species groups, providing evidence for the multiple origins or losses of this ability from distinct ancestors. In addition, no clear correlation with geographical distribution can be found within the three clades, two of which include accessions from the western, central and eastern Mediterranean regions. A striking example is the western Alpine hyperaccumulator *A. argenteum* that is more closely related to the Balkan

complex of *A. murale* than to the geographically closer *A. bertolonii* from central Italy. The latter, in turn, was closer to the Corso-Sardinian *A. robertianum* and, to a lesser extent, the Greek hyperaccumulators *A. smolikanum* and *A. heldreichii*. This corroborates the genetic continuity of members of section *Odontarrhena* and the likely polytopic origin of hyperaccumulators through repeated events over the different serpentine 'islands' of southern Europe, without involving strong genetic divergence events. A form of genetic pre-adaptation allowing the rapid development of this ability in populations that come into contact with ultramafic soils is therefore a remarkable synapomorphy for this group.

Our results are in line with other studies describing repeated evolution of metal accumulation ability and/or associated serpentine tolerance within single genera or species complexes. For example, Nyberg Berglund *et al.* (2001) showed that metal tolerance in serpentine populations of the *Cerastium alpinum* complex in Fennoscandia probably evolved two or more times independently. Enhanced Zn tolerance in metallicolous populations of *Arabidopsis halleri* were found to be of polyphyletic origin, resulting from independent local microevolutionary adaptation (Pauwels *et al.*, 2006; Jiménez-Ambríz *et al.*, 2007). In this species and *Nocca caerulea*, the contributions of colonization of metalliferous sites from local populations on non-metalliferous sites and from distant populations on other metalliferous sites are currently under investigation (Krämer, 2010). Rajakaruna *et al.* (2003) provided compelling evidence that a pair of cryptic species in the *Lasthenia californica* complex have undergone parallel evolution of serpentine tolerance based on differences in their ability to absorb various cations. Similar evidence was obtained for the genus *Calochortus*, in which DNA sequences revealed that ultramafic specialization evolved through seven events at least (Patterson and Givnish, 2004). All such findings suggest that metal tolerance and/or hyperaccumulation can be rapidly lost or gained within groups of closely related taxa through a selection process affecting the expression and regulation of genes that are not species specific or novel (Verbruggen *et al.*, 2009).

Genetic variation within the *A. murale* clade is extremely low, with most geographically distinct accessions referred to *A. murale* or other related taxa having identical or similar ITS sequences. Considering the broadly overlapping ranges of these taxa and the high phenotypic plasticity that often obscures their weak morphological boundaries, we advocate a reduction in the number of species recognized. Molecular and morphological evidence from native populations (pers. obs.) indicate that *A. bertolonii* subsp. *scutarium*, *A. chlorocarpum*, *A. jankenii*, *A. markgrafii* and *A. chalcidicum*, previously regarded as endemic hyperaccumulators (Brooks *et al.*, 1979; Hasko and Çullaj, 2001; Vinterhalter and Vinterhalter, 2005; Bani *et al.*, 2009), can hardly be recognized as distinct from *A. murale*. On the other hand, field observations and evidence from DMG tests show that most populations of this species growing in Albania and Greece have a clear preference for serpentine soils and an associated ability for Ni hyperaccumulation (Table 3, Fig. 5). The slight differentiation in trichome density and inflorescence morphology of these populations (Hartvig, 2002a) may support, at most, their recognition at the varietal rank. Accordingly, literature reports of hyperaccumulation in



*A. murale* (Brooks and Radford, 1978; Reeves *et al.*, 2001) are likely to be referred to this race or to closely related taxa from Anatolian serpentine areas. Finally, ITS sequences corroborate morphology in the recognition of Balkan endemics such as *A. baldaccii* (hyperaccumulator) and *A. corymbosoides* (non-hyperaccumulator), whereas there is no molecular support for the Alpine endemic *A. argenteum*, in spite of its distinctiveness in fruit characters. Summing up, the balance between molecular data, morphology, distribution and ecology should lead to the recognition of only 11 European Ni hyperaccumulator species of *Alyssum*, as summarized in Table 4.

## CONCLUSIONS

To date, only a few studies have explored the phylogenetic patterns of metal accumulation at the macroevolutionary scale, i.e. the species level or above (Broadley *et al.*, 2001; Jansen *et al.*, 2002, 2004; Mengoni *et al.*, 2003). Our data are basically consistent with these studies in suggesting that this ability and serpentine tolerance are usually associated with given plant groups but not monophyletic within them. Reassessing the evolutionary patterns of Ni hyperaccumulation in Alysseae demonstrated this ability to be confined to the phylogenetically distant clades of *Bornmuellera*–*Leptoplax* and

TABLE 4. List of European species of *Alyssum* sect. *Odontarrhena* with indication of range and edaphic habitat based on personal observations and literature data (Ball *et al.*, 1993; Küpfer and Nieto Feliner, 1993; Hartvig, 2002a)

Taxon	Geographic range	Edaphic preferences
<i>Alyssum alpestre</i> L.	W Alps	Limestone
<i>Alyssum argenteum</i> All.	W Alps	Mainly serpentine
<i>Alyssum baldaccii</i> Vierh. ex Nyár. = <i>Alyssum fallacinum</i> auct. non Hausskn.	S and C Greece, Crete	Serpentine
<i>Alyssum bertolonii</i> Desv.	C Italy	Serpentine
<i>Alyssum corymbosoides</i> Form. = <i>Alyssum rechingeri</i> Nyár. = <i>Alyssum rhodopense</i> Form. = <i>Alyssum vranjanum</i> Nyár.	SE Balkans	Limestone or other basic soils
<i>Alyssum degenianum</i> Nyár.	N Aegean sea (Samothraki)	Limestone or schist
<i>Alyssum euboeum</i> Halácsy	E Greece (Evia)	Serpentine
<i>Alyssum fragillimum</i> (Baldacci) Rech.f.	Crete	Limestone
<i>Alyssum heldreichii</i> Hausskn.	N Greece	Serpentine
<i>Alyssum lesbiacum</i> (P.Candargy) Rech.f.	Eastern Aegean (Lesbos)	Serpentine
<i>Alyssum murale</i> Waldst. & Kit. = <i>Alyssum balkanicum</i> Nyár. = <i>Alyssum bertolonii</i> Desv. subsp. <i>rigidum</i> Nyár. = <i>Alyssum bertolonii</i> Desv. subsp. <i>scutarinum</i> Nyár. = <i>Alyssum chalcidicum</i> Janka = <i>Alyssum chlorocarpum</i> Hausskn. = <i>Alyssum jancheni</i> Nyár. ex Novák = <i>Alyssum kosaninum</i> Nyár. = <i>Alyssum markgrafii</i> O.E.Schulz* = <i>Alyssum orphanidis</i> Nyár. = <i>Alyssum pichleri</i> Velen. = <i>Alyssum punctatum</i> Nyár. = <i>Alyssum subvirescens</i> Form.	C and S Balkans, Anatolia	All soil types
<i>Alyssum nebrodense</i> Tineo	Sicily and C Greece	Limestone
<i>Alyssum orbelicum</i> Ančev & Uzunov	SW Bulgaria	Limestone
<i>Alyssum robertianum</i> Bernard ex Gren. & Godr. = <i>A. tavolarae</i> Briq.	Corsica and Sardinia	Limestone and serpentine
<i>Alyssum samium</i> T.R.Dudley & Christod.	Eastern Aegean (Samos)	Limestone
<i>Alyssum serpyllifolium</i> Desf. = <i>A. malacitanum</i> T.R.Dudley = <i>A. pintodasilvae</i> T.R.Dudley	Iberian peninsula	Limestone and serpentine
<i>Alyssum sibiricum</i> Willd. = <i>A. borzaeanum</i> Nyár. = <i>A. caliacrae</i> Nyár. = <i>A. epirotum</i> (Halácsy) Nyár. = <i>A. halacsyi</i> Nyár. = <i>A. lepidulum</i> Nyár. = <i>A. obtusifolium</i> Steven ex DC. subsp. <i>helioscopioides</i> Nyár. = <i>A. suffrutescens</i> (Boiss.) Halácsy	E Europe, Anatolia and Siberia	All soil types
<i>Alyssum smolikanum</i> Nyár.	S Albania and N Greece	Serpentine
<i>Alyssum tenium</i> Halácsy	Cyclades (Tinos)	Serpentine
<i>A. tortuosum</i> Waldst. & Kit. = <i>A. grintescui</i> Nyár.	Continental Europe and CE Asia	Not serpentine

Species with European Ni-hyperaccumulating populations are in bold.

\*No morphological or molecular evidence emerges from the present study for keeping *A. markgrafii* as distinct from *A. murale* as in *Flora Europaea* (Ball and Dudley, 1993).

*Alyssum* sect. *Odontarrhena*. Unquestionable lack of affinity between *Leptoplax* and *Peltaria* implies that it did not originate within Thlaspidaceae. Ni hyperaccumulation is not monophyletic for either *Alyssum* sect. *Odontarrhena* or any of its three European clades. Lack of geographical cohesion within these clades possibly results from a recent radiation and polytopic origin of Ni hyperaccumulation through local and independent events of microevolutionary adaptation. It appears that such an ability can be gained or lost multiple times in basically pre-adapted plant groups, through selection and adaptation processes involving structural or regulatory changes in the genome.

Elucidating relationships and affinities in Alyseae may have implications for further research on the molecular mechanisms and genetic bases of Ni hyperaccumulation. The latter have been investigated less with respect to other metals, especially Zn and Cd. Species of *Bornmuellera* and *Leptoplax* and hyperaccumulators in *Alyssum* sect. *Odontarrhena* may be screened and compared with closely related non-hyperaccumulators through genome sequencing, microarray analysis or quantitative trait loci analysis to look for candidate genes involved in the uptake, transport and accumulation of nickel. The role of molecules such as histidine, organic acids, nicotianamine or glutathione (Montargé-Pelletier *et al.*, 2008; Verbruggen *et al.*, 2009) could also be compared in these species. Using closely related control species, new insights may be obtained on the genetic or epigenetic basis of Ni physiology. To date, lack of solid phylogenetic evidence has led to the use of model systems including only distantly related taxa, possibly reducing the information content of the results obtained. Examples are the systems *A. lesbiacum*/*A. montanum* used for the role of histidine (Krämer *et al.*, 1996; Kerkeb and Krämer, 2003; Ingle *et al.*, 2005) and the assimilation of *Peltaria turkmena* into *Leptoplax emarginata* when evaluating genetic similarity with *Arabidopsis thaliana* (Peer *et al.*, 2006). The present data suggest new models at three evolutionary levels, such as Mediterranean *Ptilotrichum* vs. *Bornmuellera*–*Leptoplax* at the genus level (clade C), *Alyssum euboicum* vs. *A. fragillimum* (clade O1) or *A. baldaccii* vs. *A. corymbosoides* at the species level (clade O2) and *A. robertianum* or *A. murale* populations at the infraspecific level.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of annotated NEXUS files (in .txt format) for the ITS alignment for Alyseae (data file 1) and for *Alyssum* (data file 2).

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## APPENDIX

List of 90 herbarium accessions of *Alyssum* section *Odontarrhena*, *Bornmuellera* and *Leptoplax* from European ultramafic outcrops tested for Ni hyperaccumulation.

*Alyssum akamasicum* Burt.: CYPRUS, Paphos, Akamas, 1941, Davis 3308 (type); 1999, Van Buggenhout Soc. éch. Eur. Méd. 18981, FI. *A. argenteum* All.: ITALY, Aosta Valley, Chatillon, 1997, Baker; St. Vincent, 1902, Ferrari 274, FI; Valtournenche (type locality), 1900, Vaccari, FI; Emilia Romagna, Bobbio, 1977, Arrigoni et al., FI; 1978 (Mengoni et al., 2003); Mt. Prinzer, 1958, Minio, FI; Lombardia, Brallo pass, 1950, Viola, FI; Piedmont, Molette, 1996, Siniscalco, TO; Mt. Musiné, 1860, Chabert, FI; Piosasco, 1998, Siniscalco, TO; Sacra di S. Michele, 1905, Ferrari, FI. *A. baldaccii* Vierh. ex Nyár.: GREECE, Crete, Gonies, 1995, Baker; Mylopotamos, 1899, Baldacci It. Cret. Alt. 52, FI (type); Sterea Ellas, Fourka pass, 2001, Bigazzi & Selvi 01-02, FI; Mt. Kallidhromon, 2008, Cecchi & Selvi 08-12, FI; Thessaly, Kedrhos, 2008, Cecchi & Selvi 08-08, FI. *A. bertolonii* Desv.: ITALY, Emilia Romagna, Mt. Prinzer, 1858, Parlato, FI; Liguria, Sarzana, 1992, Ferrarini, FI; Tuscany, Impruneta, 1842, Scaffai, FI; Mt. Gabbro, 1864, Beccari, FI; Mt. Massi, 1843, Ricasoli, FI; Monterufoli, 2006, Cecchi 06-06, H.Cecchi; Mt. Ferrato, 2008, 1884, Sommier, FI; L.Cecchi & M.Cecchi 08-35, H.Cecchi; Pomarance, 1987, Gabbriellini et al., FI; Italy, Tuscany, Riparbella, 1868, Amidei, FI; Upper Tibery Valley, 1979, Pignatti, FI. *A. cypricum* Nyár.: CYPRUS, Limassol, Mt. Troodos, 1991, Alziar et al., FI. *A. euboeum* Hálácsy: GREECE, Evi, (Mengoni et al., 2003); Limni, 2008, Cecchi & Selvi 08-20, FI. *A. heldreichii* Hausskn.: GREECE, Thessaly, Doliana, 2008, Cecchi & Selvi 08-06, FI; Malakasi, 1896, Sintenis It. Thess. 574, FI; Witomo, 1896, Sintenis It. Thess. 218, FI; Western Macedonia, Mt. Vourinos, 2008, Cecchi & Selvi 08-26, FI. *A. lesbiacum* (Candargy) Rech.f.: GREECE, North Aegean Islands, Lesbos, 1992, Baker. *A. murale* Waldst. & Kit. (= *A. chalcidicum* Janka): ALBANIA, sine loco, Hasko 07-24, FI; Berat, Perisnake, 1892, Baldacci It. Alb. 181, FI; Devoli, Bitincka, Hasko 04-01, FI; Has, Mt. Paštrik (= *A. kosaninum* Nyár.), Cecchi et al. 06-17, FI; Librazhd, Librazhd (= *A. markgrafii* O.E.Schulz), Cecchi et al. 07-21, FI; Hasko 05-05, FI; Hasko 05-06, FI; Hasko 06-19, FI; Perrënjas, Cecchi et al. 07-22, FI; Hasko 05-09, FI; Hasko 06-20, FI; Mirditë, Rubik, Hasko 05-08, FI; Pogradec, Piskupat, Cecchi et al. 07-23, FI; Hasko 04-02, FI; Hasko 05-10, FI; Hasko 06-21, FI; Pukë, Mt. Prenkollit, Cecchi et al. 06-18, FI; Shkodër, Mt. Bardanjolt (= *A. janchenii* Nyár., = *A. bertolonii* Desv. subsp. *scutarium* Nyár.), Cecchi et al. 06-13, FI; BOSNIA ERZEGOVINA, Srpska Republic, Višegrad, 1897, Fiala, FI; GREECE, Central Macedonia, Drosia (= *A. subvirescens* Form.), 2001, Bigazzi & Selvi 01-01, FI; Ierissos-Gomation, 1871, Janka, FI (type of *A. chalcidicum* Janka); Thessaloniki, 1857, Orphanides Fl. Graec. Exs. 644, FI (type of *A. orphanidis*); 1997, Baker; Evia, Mt. Dyrphys, 1876, Heldreich, FI; Steni, 1901, Leonis, FI; Evia, Limni, 2008, Cecchi & Selvi 08-19, FI; Epirus, Mt. Smolikas, 1896, Baldacci It. Alb. IV 206, FI; Sterea Ellas, Mt. Parnes, ?, Heldreich, FI; Thessaly, Kalambaka (=



- A. chlorocarpum* Janka), 2008, *Cecchi & Selvi* 08-05, FI; Kedrhos, 2008, *Cecchi & Selvi* 08-09, FI; Western Macedonia, Palaioastron, 2008, *Cecchi & Selvi* 08-23, FI; Macedonia, Pindus Mts., 1896, *Sintenis It. Thessal.* 488, FI; Samarina, 2008, *Cecchi & Selvi* 08-22, FI; KOSOVO, Prizren (= *A. punctatum* Nyár.), 1962, *Metlesics*; MACEDONIA, Radušc (= *A. chlorocarpum*), 1936, *O.Behr & E.Behr*, FI. *A. robertianum* Bernard ex Gren. & Godr.: FRANCE, Corsica, Mt. Fosco, 1881, *Chabert*, FI; Mt. S. Leonardo, 1881, *Chabert*, FI. *A. serpyllifolium* Desf.: PORTUGAL, Bragança, Bragança (= *A. pintodasilvae* T.R.Dudley), 1995, ?; Campo Redondo (= *A. pintodasilvae* T.R.Dudley), 1884, *Moller*; SPAIN, Andalusia, Sierra Bermeja [= *A. malacitanum* (Rivas Goday) T.R.Dudley], 1994, *Baker*; Galicia, Puente Basandre, 1996, *Baker*; Malaga, Sierra de Carratraca, 1982, *Losa Quintana Soc. éch. Eur. Méd.* 11242. *A. smolikanum* Nyár.: GREECE, Epirus, Mt. Smolikas, 2008, *Cecchi & Selvi* 08-28, FI. *A. tenium* Halácsy: GREECE, Cyclades, Tinos, 1901, *Heldreich Heldr. Herb. Gr. Norm.* 1608, FI; 1978 ([Mengoni et al., 2003](#)); 1997, *Baker*. *A. troodi* Boiss.: CYPRUS, Limassol, Mt. Troodos, 1880, *Sintenis & Rigo* 844, FI (type); Cyprus, Limassol, Mt. Troodos, 1948, *Mavromoustakis*. *Bornmuellera baldaccii* (Degen) Heywood: GREECE, Epirus, Mt. Smolikas, 1896, *Baldacci It. Alb.* IV 211, FI; 2008, *Cecchi & Selvi* 08-29, FI. *B. tymphaea* (Hausskn.) Hausskn.: GREECE, Western Macedonia, Mt. Vourinos, 2008, *Cecchi & Selvi* 08-27, H.Cecchi. *Leptoplax emarginata* (Boiss.) O.E.Schulz: GREECE, Evia, Mt. Dirphys, 2008, *Cecchi & Selvi* 08-18, FI; Thessaly, Kedrhos, 2008, *Cecchi & Selvi* 08-10, FI.