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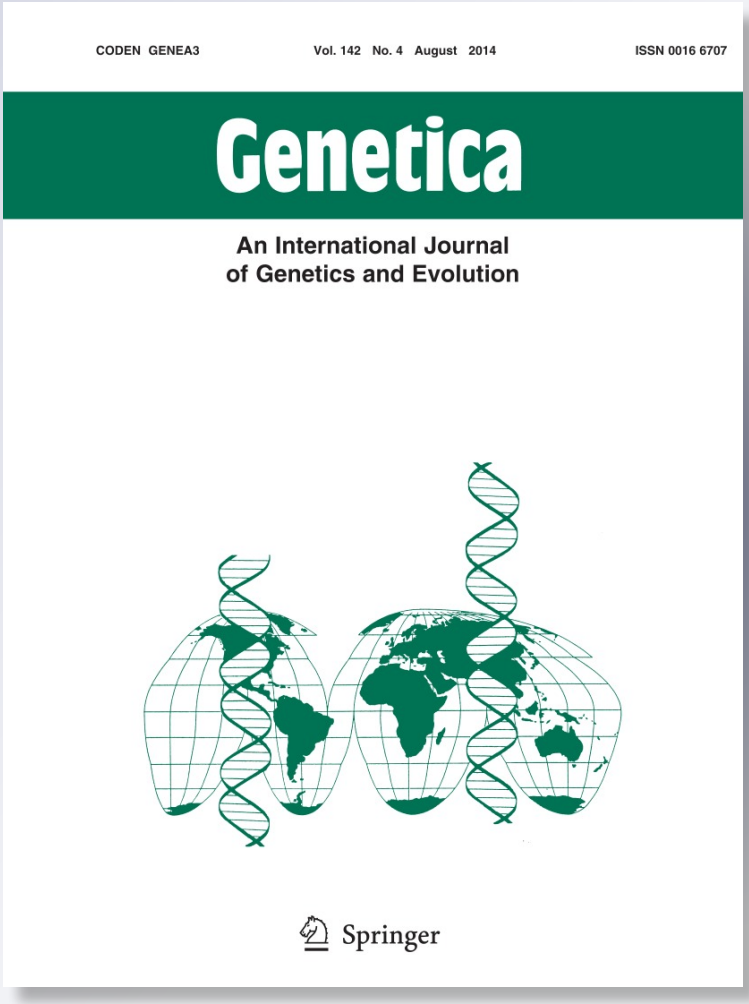

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One or three species in *Megadenia* (Brassicaceae): insight from molecular studies

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Abstract *Megadenia* Maxim. is a small genus of the Brassicaceae endemic to East Asia with three disjunct areas of distribution: the eastern edge of the Qinghai–Tibetan Plateau, the Eastern Sayan Mountains in southern Siberia, and Chandalaz Ridge in the southern Sikhote-Alin Mountains. Although distinct species (*M. pygmaea* Maxim., *M. bardunovii* Popov, and *M. speluncarum* Vorob., Vorosch. and Gorovoj) have been described from each area, they have lately been reduced to synonymy with *M. pygmaea* due to high morphological similarity. Here, we present the first molecular study of *Megadenia*. Using the sequences of 11 noncoding regions from the cytoplasmic (chloroplast and mitochondrial) and nuclear genomes, we assessed divergence within the genus and explored the relationships between *Megadenia* and *Biscutella* L. Although *M. bardunovii*, *M. speluncarum*, and *M. pygmaea* were found to be indiscernible with regard to the nuclear and mitochondrial markers studied, our data on the plastid genome revealed their distinctness and a clear subdivision of the genus into three lineages matching the three described species. All of the phylogenetic analyses of the chloroplast DNA sequences provide strong support for the inclusion of *Megadenia* and *Biscutella* in the tribe Biscutelleae. A dating analysis shows that the genus *Megadenia* is of Miocene origin and diversification within the genus, which has led to the three extant lineages, most likely occurred during the Early–Middle Pleistocene, in agreement with the vicariance pattern. Given the present-day distribution, differences in habitat preferences and in some anatomical traits, and lack of a direct genealogical relationship, *M. pygmaea, M. bardunovii*, and *M. speluncarum* should be treated as distinct species or at least subspecies.

Keywords Brassicaceae · Biscutelleae · *Megadenia* · Molecular markers · Phylogeny · Coalescent analysis

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Introduction

Genus *Megadenia* Maxim. is a poorly known member of the Brassicaceae, and its systematic position within the family has long remained uncertain (Dorofeyev 2004; German and Al-Shehbaz 2008). *Megadenia* plants were first described as *M. pygmaea* and assigned to a new genus of the Brassicaceae by K.I. Maximowicz (1889) based on herbarium specimens of plants collected by N.M. Przhevalsky in the upper reach of the Yellow River (Gansu, China) during his voyage to Tibet (1879–1880). The plants of the genus are herbaceous, short-lived, polycarpic perennials with a thin, deeply buried rhizome, rosellate, monocyclic, monocarpic shoots, tiny white flowers, and didymous fruits resembling a pair of spectacles. *Megadenia* had been recognised as a monotypic genus with a single species, *M. pygmaea*, until the second half of the twentieth century when *Megadenia* plants were found in Russia, firstly in the Eastern Sayan Mountains (Tunka Valley, Buryat Republic, Russia) and later on Chandalaz Ridge (southern Sikhote-Alin Mountains, Primorskii Krai, Russia). The plants from these localities were described as distinct

species, *M. bardunovii* Popov and *M. speluncarum* Vorob., Vorosch. and Gorovoj, respectively (Popov 1954; Vorob'ev et al. 1976). Therefore, the genus exhibits a highly disjunct distribution within East Asia, with distances of approx. 1,800 and 2,900 km between its disjunct populations (Fig. 1).

In China, *M. pygmaea* grows along the eastern edge of the Qinghai–Tibetan Plateau (QTP) and occurs in provinces Xizang, Gansu, Qinghai, and Sichuan (Zhou et al. 2001). Based on data from the Chinese Virtual Herbarium (available at http://pe.ibcas.ac.cn/sptest/syninvok.aspx. Accessed 30 October 2013) and the Global Biodiversity Information Facility Portal (available at http://www.gbif.org. Accessed 30 October 2013), *M. pygmaea* occurs at elevations from 2,100 to 4,160 m above sea level. The species occupies diverse habitats with stony, sandy, or humus-rich soils along the banks of rivers and streams, in alpine meadows, at the base of rocks, on slopes under shrubs and in *Picea-Juniperus* forest understories, and on talus slopes.

M. bardunovii has been described from Tunka Valley (180 km west of Lake Baikal, the left bank of the Irkut River valley, in the vicinity of Turan village), with the plants occupying an area of several tens of square metres along a small mountain stream. However, the habitat in the type locality was destroyed by human activity in 1976, and the species was unknown elsewhere for several decades and thought to be extinct. After intensive fieldwork, three new populations of the species were found 8–14 km from the type locality, and all the new habitats of *M. bardunovii* are similar to that of the type locality (Makry and Kazanovsky 2002). The species grows in the taiga belt of the Eastern Sayan Mountains along small mountain streams on wet soils containing gravel and rubble limestone or on a cushion of the moss *Cratoneuron filicinum* (Hedw.) Spruce and is a local endemic species with a very narrow distribution.

The narrowest distribution is characteristic of *M. speluncarum*. The species occurs in a unique habitat on a small plot of damp limestone soil at the entrance of a single cave on the south slope of Chandalaz (Lozovy) Ridge: stable temperatures and high humidity inside the cave provide favourable environmental conditions for the growth of *M. speluncarum*. Intense searches for new habitats of *M. speluncarum* have not been successful to date (Gorovoy et al. 2011), and the only known population is very small in size, making the species particularly vulnerable to extinction due to anthropogenic environmental changes.

Controversy over the species status of *M. speluncarum* and *M. bardunovii* exists, and some researchers consider the genus to be monotypic (Zhou et al. 2001; Warwick et al. 2006), placing both species in synonymy with *M. pygmaea* due to a lack of conspicuous differences in most morphological characters (Berkutenko 1998). However, it is known that assessing taxon delimitation based solely on morphology can lead to incorrect conclusions due to the limited number and convergence of diagnostic characters used in the Brassicaceae (Al-Shehbaz et al. 2006; Bailey et al. 2006; Mummenhoff et al. 2009; Franzke et al. 2011, and references therein). Certain anatomical traits are often used to clarify taxonomic controversy concerning species with weak morphological differences. Among other characters, petiole anatomy has proven to be useful in delimitating some Brassicaceae taxa that could not be discriminated with the traditionally used diagnostic traits (Olowokudejo 1987; Khalilov and Trifonova 1992). Indeed, our previous study revealed some differences in the anatomical structure of the petiole between *Megadenia* plants from distant parts of the genus distribution area (Gorovoy et al. 2011). Admittedly, comparisons at the DNA level are of great value in estimating relatedness among taxa and are widely used to confirm taxon delimitation in Brassicaceae (Slotte et al. 2006; Jordon-Thaden et al. 2010; Abdelaziz et al. 2011; Liu et al. 2011). Plant cell genomes with different modes of inheritance evolve at different rates (Huang et al. 2012) and can provide better insight into the relationships among taxa and their evolutionary histories (Koch and Kiefer 2006; Puşcaş et al. 2008; Mishiba et al. 2009; Yue et al. 2009; Koch et al. 2010; Winkler et al. 2010).

Here, we use 11 noncoding regions from the nuclear and cytoplasmic (chloroplast and mitochondrial) genomes to assess the relationships within *Megadenia* and between the *Megadenia* and *Biscutella* genera, which have recently been placed in the same tribe based on sequence data of the internal transcribed spacers (ITS) of the nuclear ribosomal DNA (German et al. 2009; Warwick et al. 2010). The main goals of the study were to examine the level of genetic variation in different genomes within *Megadenia*, assess the level of divergence between plants from disjunct parts of the genus range, infer the evolutionary history of the genus, and verify whether *M. bardunovii* and *M. speluncarum* are distinct from *M. pygmaea.*

Materials and methods

Plant materials

Megadenia plants from all known localities in Russia and from three localities from China were included in this study. The sampling included 10 plants from each of 3 localities in Tunka Valley (the Eastern Sayan Mountains, Buryat Republic, Russia) and 10 plants from the only known population on Chandalaz Ridge (Sikhote-Alin Mountains, Primorskii Krai, Russia). In each population, leaf material was collected from plants chosen at random approximately 5 m apart in a way that was not damaging to the plants sampled. Leaf material of *M. pygmaea* was

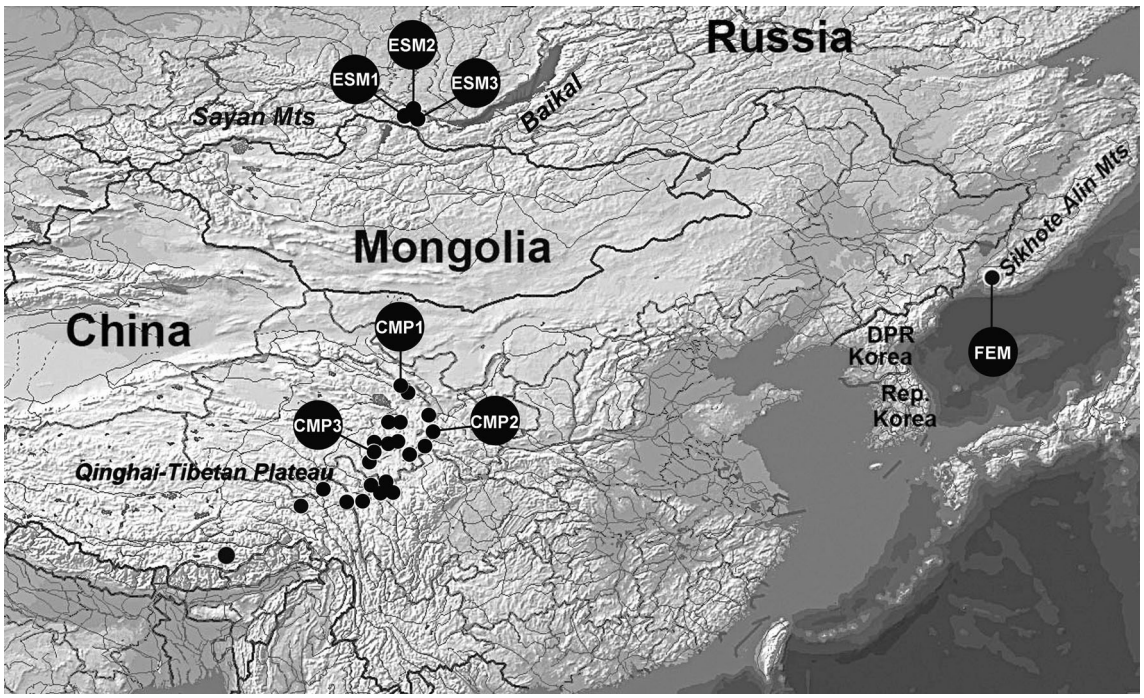

Fig. 1 Geographic distribution of *Megadenia pygmaea* Maxim. according to data from the Chinese Virtual Herbarium (http://pe.ibcas.ac.cn/sptest/syninvok.aspx) and the Global Biodiversity Information Facility Portal (http://www.gbif.org) and the sampling sites of *M. pygmaea* (CMP1, CMP2, and CMP3), *M. bardunovii* (ESM1, ESM2, and ESM3), and *M. speluncarum* (FEM). Codes and sampling location details are given in Table 1

obtained from two herbarium specimens (Table 1) and from a living plant, which was collected in the understory of a sea buckthorn forest and cultivated in laboratory conditions by one of the authors (P.G. Gorovoy). The populations from Tunka Valley and Chandalaz Ridge have previously been described as distinct species and are herein referred to as *M. bardunovii* and *M. speluncarum,* respectively; the *Megadenia* plants growing in China are referred to as *M. pygmaea*. Voucher specimens of *M. speluncarum* population are held at the Herbarium of the Institute of Biology and Soil Science, Vladivostok (VLA), and specimens representing three *M. bardunovii* populations sampled are deposited at the Herbarium of the Siberian Institute of Plant Physiology and Biochemistry, Irkutsk (IRK). Previous studies have shown that the genus *Biscutella* can be considered a relative of genus *Megadenia* (German and Al-Shehbaz 2008). To assess the level of divergence between the genera leaf material was sampled from two *Biscutella laevigata* L. plants cultivated from seeds in the Botanical Garden of Komarov Botanical Institute (St. Petersburg, Russia).

DNA extraction, amplification, and sequencing

Total DNA was extracted from herbarium specimens using a DNeasy Plant Mini kit (Qiagen, Maryland, USA) according to the manufacturer's protocol and from fresh leaves of individual plants using the modified CTAB method, as described in Artyukova et al. (2004). The DNA was then used to amplify noncoding sequences from the cytoplasmic and nuclear genomes using published conditions and the primers listed in Table 2. From nuclear DNA (nrDNA), we amplified the ITS region of the nuclear ribosomal DNA as a single fragment comprising ITS1–5.8S–ITS2. In addition, we amplified a fragment between the second and fourth exons of the nuclear *CHI* gene encoding chalcone flavanone isomerase, one of the key enzymes involved in protection against ultraviolet light and occurring as a single copy in *Arabidopsis thaliana* (Shirley et al. 1992; Kuittinen and Aguadé 2000). From the mitochondrial DNA (mtDNA), we amplified fragments located between the second and third exons of two genes (*nad1* and *nad7* subunits of NADH dehydrogenase). We used the following regions of chloroplast DNA (cpDNA): 5′end of the *trnK* intron, the $trnH^{GUG}$–*psbA*, *petG–trnP*, $trnS^{GCU}$–$trnG^{UUC}$, *rps16–trnQ*, and *rpL32–*$trnL^{UAG}$ intergenic spacers, and the *trnT–trnF* region including the *trnT–trnL* and *trnL–trnF* intergenic spacers and the *trnL* intron. Most of these regions have been shown to be useful in resolving species-level phylogenetic relationships in other species (Parisod and Besnard 2007; Dong et al. 2012).

Table 1 Details of *Megadenia* plant material investigated in this study with code, locality, and collection information

Species, population code	Latitude (N), longitude (E)	Altitude (m a.s.l.)	Locations	Habitat	Collection details[a]
Megadenia bardunovii					
ESM1	51°39′27″N, 101°34′17″E	919–924	Russia: Buryat Republic, Eastern Sayan Mts, Tunka valley, the left bank of Irkut River	At an output of brook, on moist rubble, sparsely	S.G. Kazanovsky and Y.N. Pochinchik; 28.08.2009; voucher 9398 (IRK)
ESM2	51°39′30″N, 101°34′10″E	932	Russia: Buryat Republic, Eastern Sayan Mts, Tunka valley, the left bank of Irkut River	Along a bank of brook	S.G.Kazanovsky and Y.N. Pochinchik; 28.08.2009; voucher 9404 (IRK)
ESM3	51°38′58″N, 101°29′22″E	948	Russia: Buryat Republic, Eastern Sayan Mts, Tunka valley, the left bank of Irkut River	Along a bank of brook, sparsely	S.G.Kazanovsky and Y.N. Pochinchik; 27.08.2009; voucher 9409 (IRK)
M. speluncarum					
FEM	43°01′37″N, 133°00′58″E	509	Russia: Primorskii Krai, Partizansky District, Sikhote-Alin Mountains, south slope of Chandalaz (Lozovy) Ridge	At the cave entrance, on moist limestone soil	P.G. Gorovoy et al.; 8.07.2009; voucher 10454 (VLA)
M. pygmaea					
CMP1	–	2200	China: Qinghai Province, 60 km north of Xining city	In the forest with *Hippophae rhamnoides*	P.G. Gorovoy; 15.08.2007
CMP2	–	2,200	China: Gansu Province, Yuzhong County, Xinglongshan	Wetland beside canal	Zhong Taixu; 29.07.1991; voucher 01604743[b] (PE)
CMP3	34°21′54″N, 100°11′34″E	3,950	China: Qinghai Province Maqên County, Dawuxiang, along the Deleni He	Steep slope, on moist soil in deep shade under rock	Ho Ting-nung et al.; 6.08.1993; voucher 01125088[b] (PE)

[a] Collector name, data collection, voucher (herbarium: IRK = Herbarium of Siberian Institute of Plant Physiology and Biochemistry, Irkutsk, Russia; VLA = Herbarium of Institute of Biology and Soil Science, Vladivostok, Russia; PE = Chinese National Herbarium, Institute of Botany, the Chinese Academy of Sciences, Beijing, China)

[b] All information is from the annotation on the herbarium vouchers

Sequencing of the PCR-amplified products was carried out in both directions with an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, USA) using a BigDye terminator v. 3.1 sequencing standard kit (Applied Biosystems, Foster City, USA) and the same primers that were used for amplification. In addition, internal primers were used for sequencing of the *trnS–trnG* and the *trnT–trnF* regions (Table 2). Sequence reads were assembled and edited in the Staden Package v. 1.5 (Bonfield et al. 1995). For each locus studied, the sequences were aligned manually using the programme SeaView v. 4 (Gouy et al. 2010). DNA fragments that contained substitutions, insertion/deletion (indel), and/or microsatellite variants were retested (reamplified and resequenced) to verify that our results are repeatable.

Data analysis

Analyses of polymorphisms and divergence between the *Megadenia* plants at noncoding sites and in complete sequences of each region studied were performed in DnaSP v. 5.10 (Librado and Rozas 2009). Boundaries of the noncoding regions within the studied loci were determined by comparison with the published sequences of *Arabidopsis thaliana* for the *CHI* gene and the complete mitochondrial and chloroplast genomes (GenBank accession numbers AJ287321, Y08501, and AP000423, respectively). Sites containing gaps were excluded from the estimations of nucleotide diversity and divergence.

The method of statistical parsimony (SP) was employed to determine the relationships among the cpDNA haplotypes using the TCS programme (Clement et al. 2000). We produced the networks for each cpDNA region. Variation in length in mono- and dinucleotide repeats and indels were included in the data sets because repeatability tests allowed us to exclude PCR errors. Each indel was considered as a single mutation event, and all indels were coded as substitutions (A or C). The 5-bp fragmental inversion within the *rpL32–trnL* spacer of the CMP2 individual could be generated by only a single mutation event; although it resulted in three polymorphic sites, it was coded as one character. For the subsequent analyses, we combined seven

Table 2 Primers, fragment sizes, and GenBank accession numbers for sequences of the 11 noncoding regions from the cytoplasmic and nuclear genomes used in this study

Region	Primers, nucleotide sequence (5′ → 3′)		Fragment size, bp	GenBank accession numbers
	Forward	Reverse		
trnH–psbA[a]	CGCGCATGGTGGATTCACAATCC	GTTATGCATGAACGTAATGCTC	375–386	HG792638–HG792646
trnS–trnG[a]	AGATAGGGATTCGAACCCTCGGT	GTAGCGGGAATCGAACCCGCATC	1,419–1,437	HG792665–HG792672
	GCGGGTATAGTTTAGTGGTAAAA[i]	TTTTACCACTAAACTATACCCGC[i]		
rps16–trnQ[b]	GTTGCTTTYTACCACATCGTTT	GCGTGGCCAAGYGGTAAGGC	524–530	HG792656–HG792664
rpL32– trnL[b]	CTGCTTCCTAAGAGCAGCGT	CAGTTCCAAAAAAACGTACTTC	751–1,072	HG792690–HG792698
petG–trnP[c]	GGTCTAATTCCTATAACTTTGGC	GGGATGTGGCGCAGCTTGG	531–543	HG792673–HG792680
trnT–trnF[d]	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	1,568–1,605	HG792681–HG792689
	GGTTCAAGTCCCTCTATCCC[i]	GGGGATAGAGGGACTTGAAC[i]		
trnK intron[e]	AAATTCGAATGGAAGCTCG	GTATCAAGGGAGAATTCAGATAAC	344–346	HG792647–HG792655
ITS region[f]	AGGAGAAGTCGTAACAAG	GTTTCTTTTCCTCCGCT	722–725	HG792603–HG792611
	TCCGTAGGTGAACCTGCGG[i]	TCCTCCGCTTATTGATATGC[i]		
CHI gene region[g]	GTGGAAGGGAAAAACTACGGAGGAG	AAGATGATAGTATCCCTGAAACCGG	435–709	HG792630–HG792637
nad1/2–3 [h]	GCATTACGATCTGCAGCTCA	GGAAGCCGATTAGTTTCTGC	1,087–1,088	HG792612–HG792620
nad7/2–3 [h]	GCTTTACCTTATTCTGATCG	TGTTCTTGGGCCATCATAGA	1,089	HG792621–HG792629

[a] Shaw et al. (2005); [b] Shaw et al. (2007); [c] Huang et al. (2002); [d] Taberlet et al. (1991); [e] Parisod and Besnard (2007); [f] Wen and Zimmer (1996); [g] Kuittinen et al. (2002); [h] Duminil et al. (2002); [i] additional primers for cyclic sequencing only

chloroplast data sets into a single data matrix, identified the haplotypes for *Megadenia* and *Biscutella* using DnaSp, and constructed a network from a combine data set.

To confirm the position of the *Megadenia* genus in the family Brassicaceae, we performed phylogenetic analyses of the cpDNA data using additional sequences of seven cpDNA regions for 15 taxa, which were obtained from the complete chloroplast sequences presented in GenBank (accession numbers: AP000423, AP009366, AP009367, AP009368, AP009369, AP009370, AP009371, AP009372, AP009373, AP009374, AP009375, AP009376, DQ231548, GQ861354, JX205495). Each cpDNA region was aligned separately and combined into a single data set used in the phylogenetic analyses. Both the maximum parsimony (MP) and maximum likelihood (ML) approaches were used as optimality criteria (as implemented in PAUP* v. 4b10, Swofford 2003). For the MP analysis, all of the characters were treated as unordered and equally weighted, with gaps treated as missing and coded as single additional binary characters using the "simple indel coding" method (Simmons and Ochoterena 2000) as implemented in the programme FastGap v. 1.2 (Borchsenius 2009). Full heuristic tree searches were performed with 1,000 random addition sequence replicates, starting trees obtained via stepwise addition, tree bisection and reconnection (TBR) branch swapping and the MulTrees option in effect. The ML analysis was performed using the sequence evolution model GTR + I + G, which was selected with Modeltest 3.6 (Posada and Crandall 1998) based on the AIC criterion. To assess branch support we performed bootstrap analysis with 1,000 replicates.

To determine the divergence time of *Megadenia* cpDNA haplotypes, we performed Bayesian analyses (BA) of the cpDNA data with the software package BEAST v. 1.7.4 (Drummond and Rambaut 2007), which includes the BEAUti, BEAST, Tracer, TreeAnnotator, and FigTree programmes and enables simultaneous estimations of the phylogenetic tree and divergence times. The input files for BEAST analyses were created in BEAUti. The main parameters and priors used were the uncorrelated log-normal relaxed molecular clock, Yule model of speciation, and GTR + I + G model of evolution, which received the best AIC score in Modeltest. Given the lack of known fossils and extant close relatives of two Biscutelleae genera, we resorted to secondary calibration to calibrate the clock models. Although this approach can produce unreliable dates, it may be useful when other calibrating information is absent, and the normal distribution is thought to be a useful prior to minimise uncertainty in imported dates (Ho 2007; Ho and Phillips 2009). Divergence time estimates in Brassicaceae vary with the markers, their mutation rates, and calibration points used (Franzke et al. 2011). The older divergence time estimates for Brassicaceae reported in Beilstein et al. (2010) are weakly congruent with the recent dating estimates within the angiosperms (Bell et al. 2010; Magallon et al. 2013) and are thought to be exaggerated (Franzke et al. 2011). On the contrary, time estimates published by Couvreur et al. (2010) agree with the results of most previous studies, and following the other researchers (e.g., Karl and Koch 2013) we used the age estimates from Couvreur et al. (2010) as secondary calibration points in the BEAST analyses. A prior placed at or

near the root is believed to have the greatest potential to inform node height relative to all terminals (De Bruyn et al. 2013), and we performed the divergence-time estimate calculations using age estimates for the crown and the core Brassicaceae nodes (the means of 37.6 and 32.3 million years ago (mya), respectively). The constraint was applied using normally distributed priors, with the means and standard deviations determined by the published means and highest probability distributions for corresponding nodes in Couvreur et al. (2010) tree. The chains were run for 5×10^7 generations and sampled every 5,000 generations, discarding the five first millions generations as burn-in. To ensure convergence of the chains, we conducted two independent runs, and samples from the two chains were combined into a single trace using LogCombiner, part of the BEAST package. Convergence and the effective sample size (ESS) of the estimated parameters in each single chain and in the combined trace were inspected using Tracers, and each ESS was above 1,000. The programme TreeAnnotator, also part of the BEAST package, was used to build the maximum clade credibility tree and summarise the time estimations, including the average node ages along with their 95 % highest posterior density (HPD) intervals and the posterior probabilities (PP) on each branch. The results were visualised using FigTree.

In addition, we estimated approximate divergence time (T) between Biscutelleae taxa based on sequence divergence (D) of the *CHI* gene introns and published evolution rate for introns of this nuclear gene ($\mu = 0.506–1.113 \times 10^{-8}$ substitutions per site per year, Huang et al. 2012) and using the formula $T = D/2\mu$ (Nei and Kumar 2000).

Results

Variation in nuclear, mitochondrial, and chloroplast sequences

In total, the sequences of 11 regions from three plant genomes were successfully amplified and sequenced for 43 *Megadenia* samples and two samples of *B. laevigata*. All samples within each population of *Megadenia* had identical sequences at all 11 DNA regions, as did two samples of *B. laevigata*. One sequence of each region from every population and sequences for one sample of *B. laevigata* were deposited in GenBank (Table 2) and included in the analyses.

In our study, no intra-individual polymorphisms were detected upon the direct sequencing of PCR products for both the multicopy ITS and low-copy *CHI* gene nuclear fragments. In *Megadenia* and *Biscutella*, the sequence lengths were 723 and 449 bp for the *CHI* fragments and 700 and 697 bp for the ITS, respectively. The ITS region in all of the *Megadenia* plants surveyed showed the same sequence, with only a single substitution found within the ITS1 sequence in one individual of *M. pygmaea* (CMP2). The level of nucleotide diversity in *Megadenia* at this region was very low ($\pi = 0.00041$); the sequence divergence between *Megadenia* and *Biscutella* was 10.15 %. The nucleotide diversity in the genus with regard to the *CHI* gene was 0.00184. The intron sequences of this gene in *M. bardunovii* and *M. speluncarum* were found to be identical, differing in two substitutions from *M. pygmaea* sequences, with a genetic divergence of 0.48 % between them. The average genetic divergence between *Megadenia* and *Biscutella* for introns of the *CHI* gene was 19.66 %. Based on these values and published evolution rate for these introns, the split time between *Megadenia* samples from China and Russia can be dated to 0.48–0.22 mya and the divergence time between the two Biscutelleae genera may be dated back to 22.48–10.22 mya.

For the mitochondrial genome, two DNA fragments (1,088 and 1,089 bp in length) from the *nad1* and *nad7* subunit genes of NADH dehydrogenase, respectively, were sequenced. In both *Megadenia* and *Biscutella*, the sequence of *nad1* intron 2 was 980 bp in length and differed in the presence of an 87-bp insertion from the corresponding sequences of other Brassicaceae species available in GenBank. The sequence lengths of *nad7* intron 2 in the two genera (1,067 bp) were found to be comparable to the lengths of this intron in most Brassicaceae (an average length of 1,065 bp). In all *Megadenia* plants surveyed, the intron sequences for the two genes are identical and differ from *B. laevigata* sequences in six (*nad1* intron 2) and 11 (*nad7* intron 2) substitutions. The average genetic divergence between the two genera in the introns of the two mitochondrial genes is 0.83 %. For the chloroplast loci of *Megadenia,* the sequence lengths aligned were 384 bp for *trnH–psbA*, 349 bp for the *trnK* intron, 531 bp for *rpS16–trnQ*, 1,452 bp for *trnS–trnG*, 531 bp for *petG–trnP*, 945 bp for *rpL32–trnL*, and 1,608 bp for *trnT–trnF*. All of the regions examined are rich in adenine and thymine, with AT contents between 64.09 (*petG–trnP*) and 76.64 % (*rpL32–trnL*), which is consistent with the nucleotide composition of noncoding chloroplast regions. The detailed analysis showed differences in the variability of the seven cpDNA regions within the genus. No polymorphisms were found in the *Megadenia* sequences at the *petG–trnP* spacer, and only slight differences in the length of three mononucleotide repeats were observed for the *trnK* intron, which had been found to be polymorphic in the *B. laevigata* complex (Parisod and Besnard 2007). For the five other regions, three types of polymorphisms were identified among the aligned sequences: single-nucleotide polymorphisms, 1-bp and multi-base indels, and length variation in mono- and dinucleotide repeats. In addition, within the

Table 3 Genetic divergence between *Megadenia* species and among *Biscutella and Megadenia* genera at noncoding sequences of seven chloroplast intergenic regions and at the combined sequences

cpDNA region	Length of aligned noncoding sequences, bp	Comparison (substitutions/bp)			
		M. pygmaea versus *M.speluncarum*	*M. pygmaea* versus *M. bardunovii*	*M. speluncarum* versus *M. bardunovii*	*Biscutella* versus *Megadenia*
psbA–trnH	320	0.00815	0.00474	0.01064	0.09124
rps16–trnQ	464	0.00457	0.00000	0.00457	0.07855
rpL32–trnL	994	0.00847	0.00370	0.00780	0.09762
trnS–trnG	1,394	0.00418	0.00418	0.00590	0.04711
trnT–trnF	1,498	0.00069	0.00207	0.00138	0.04001
trnK intron	348	0.00000	0.00000	0.00000	0.04720
petG–trnP	323	0.00000	0.00000	0.00000	0.07395
Combined sequence	5,353	0.00339	0.00256	0.00415	0. 05886

rpL32–trnL spacer of a single *M. pygmaea* individual (CMP2), a small inversion was found with two flanking sequences (20 nucleotides) that were reversely complemented to each other. The total length of the combined cpDNA sequences in *Megadenia* varied from 5,526 bp in the CMP2 accession of *M. pygmaea* to 5,720 bp in *M. bardunovii*. After aligning against *B. laevigata* sequence (5,822 bp), the alignment showed a consensus length of 6,060 bp, comprising 5,353 bp of noncoding sites. In the *Megadenia* plastid genome, all of the polymorphisms were found in noncoding sequences, with transversions (tv) being more prevalent than transitions (ts). Twenty substitutions, including 5 ts and 15 tv, separated the sequences of *M. bardunovii* and *M. speluncarum*. Eight substitutions (1 ts and 7 tv) were found between the sequences of *M. bardunovii* and *M. pygmaea*, whereas twelve nucleotide differences (4 ts and 8 tv) were detected between the *M. pygmaea* and *M. speluncarum* sequences. The nucleotide diversity (π) in *Megadenia* ranged from zero for the *petG–trnP* and *trnK* intron to 0.00509 for *trnH–psbA*. For the combined sequences of seven cpDNA regions, the π-value was 0.00234 at the genus level and varied from 0 in *M. bardunovii* and *M. speluncarum* to 0.00126 in *M. pygmaea*. The nucleotide divergence between the *Megadenia* species and between *Megadenia* and *B. laevigata* at each polymorphic cpDNA region and for the combine sequence data is summarised in Table 3. The levels of divergence varied from 0.26 (*M. bardunovii* vs. *M. pygmaea*) to 0.42 % (*M. bardunovii* vs. *M. speluncarum*) at noncoding sites of the seven cpDNA regions. The average sequence divergence within *M. pygmaea* (0.14 %) was less than that obtained between *M. pygmaea, M. bardunovii*, and *M. speluncarum*. *Megadenia* and *B. laevigata* showed 5.88 % sequence divergence.

In addition to nucleotide polymorphisms, *M. pygmaea, M. bardunovii*, and *M. speluncarum* were discriminated from each other by a few 1-bp indels and by length variations in mono- and di-nucleotide repeats. Moreover, a large deletion (185 bp) in *rpL32–trnL* and a 7-bp insertion in *trnH–psbA* could be considered as synapomorphies for *M. speluncarum* and *M. pygmaea*, distinguishing them from *M. bardunovii*. It should be noted that *M. speluncarum* shared no substitutions or indels with *M. bardunovii*, whereas the *M. pygmaea* sequences showed eight common substitutions with the *M. speluncarum* plants and twelve common substitutions with *M. bardunovii*. Thus, any of six polymorphic cpDNA regions can distinguish *M. bardunovii* from all other *Megadenia* plants, and any of the following spacers, *psbA–trnH*, *rps16–trnQ*, *rpL32–trnL*, or *trnS–trnG*, can be used to discriminate *M. bardunovii*, *M. speluncarum*, and *M. pygmaea* from each other. Three specimens of *M. pygmaea* representing geographically isolated populations differed from each other at any of following spacers: *psbA–trnH*, *rpL32–trnL*, and *trnS–trnG*. The pairwise genetic distances (F_{ST}) varied from 0.81 (*M. pygmaea* vs. *M. speluncarum*) to 1.00 (*M. bardunovii* vs. *M. speluncarum*).

Genealogical and phylogenetic analyses of cpDNA haplotypes and divergence time estimates

We identified six haplotypes among 43 individuals of *Megadenia* and two plants of *B. laevigata* based on all of the nucleotide and indel polymorphisms across the seven cpDNA regions. Three populations of *M. bardunovii* were found to share a single haplotype, H1. Haplotype H2 was found in all of the *M. speluncarum* plants, and the H3, H4, and H5 haplotypes were present in *M. pygmaea* from the CMP2, CMP3, and CMP1 localities in the eastern edge of the QTP, respectively. To visualise all phylogenetic relationships among the haplotypes (including extinct or non-sampled) we used SP method that are thought to be better

suited for the analysis of recently divergent genetic lineages (Posada and Crandall 2001; Koch and Matschinger 2007; Kiefer et al. 2009; Chen et al. 2012). The SP network constructed at a 95 % confidence limit highlights the significant divergence between the *Megadenia* haplotypes, which are connected in a single network with a lack of many intermediate haplotypes. The haplotype of *B. laevigata* representing a sole sister genus to *Megadenia* (German and Al-Shehbaz 2008) is highly divergent from the *Megadenia* haplotypes and remained disconnected with the *Megadenia* haplotypes at a 90 % parsimony limit (network not shown). The haplotypes of two taxa were connected in a single network only if the connection limit was greatly increased. Although an increase in the number of steps may lead to inaccuracies, the network polarized with *B. laevigata* could provide some information on the position of a putative common ancestor of all of the *Megadenia* haplotypes observed and give insight into the evolutionary history of the genus. All of the sampled *Megadenia* haplotypes held tip positions and fell into three distinct groups, each separated from the others by at least fourteen mutational steps (Fig. 2). Haplotype H1 (*M. bardunovii*) likely diverged first because it shares ancestral indels (a 185-bp insertion in *rpL32–trnL* and a 7-bp deletion in *psbA–trnH*) with *Biscutella* and other Brassicaceae. Seven mutational events separate haplotype H1 from the inferred and most likely extinct ancestral haplotype that had given rise to the extant haplotypes of the genus. The haplotypes found in *M. speluncarum* (H2) and *M. pygmaea* (H3–H5) appear to be descendants of another nonsampled or extinct haplotype, which is separated by nine mutational steps from the inferred most recent common ancestor of all of the *Megadenia* haplotypes observed (Fig. 2). These results could imply that there are three divergent maternal lineages within the genus, namely the Eastern Sayan (ES) and Far Eastern (FE) lineages, each of which is represented by a fixed private haplotype, and the Chinese lineage (CM) comprising a few allied and rather divergent haplotypes.

To specify the position of the *Megadenia* genus in Brassicaceae and estimate divergence time within the genus, cpDNA region data from the full chloroplast sequences for 15 species were used in phylogenetic analyses. The cpDNA region sequences of these species were aligned with the sequences obtained in the present study. In the *trnT–trnF* alignment of Brassicaceae taxa, the sequences of the *trnL* intron and *trnL–trnF* intergenic spacer varied in length due to the presence of a large insertion within the *trnL* intron and repeats of *trnF* pseudogenes in some species (Koch et al. 2007). Neither the insertion in the intron nor the *trnF* pseudogenes was found in the sequences of *Megadenia* or *Biscutella*; the ambiguous region with the *trnF* pseudogene repeats within the *trnL–trnF* spacer was removed from the alignment. The final length of the combined data matrix

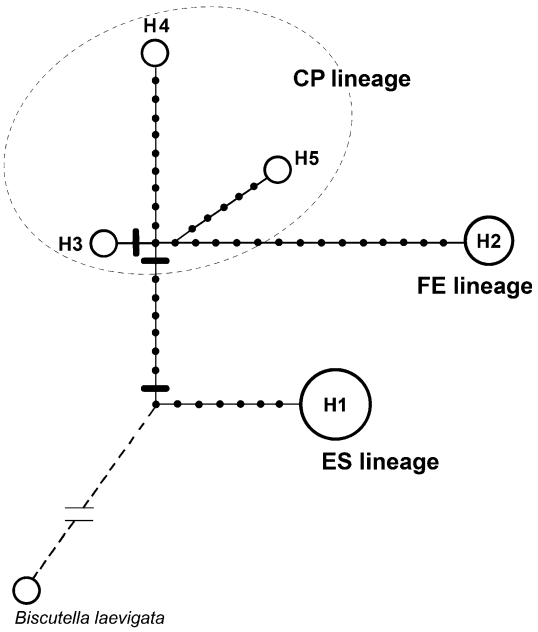

Fig. 2 Statistical parsimony network of haplotypes detected from the seven noncoding chloroplast DNA regions in *Megadenia* and *Biscutella* accessions. *Opened circles* represent sampled haplotypes of *M. bardunovii* (H1), *M. speluncarum* (H2) and *M. pygmaea* (H3, H4 and H5), and their sizes are proportional to their frequencies. The *black dots* indicate the inferred intermediate haplotypes (undetected in our study or extinct), and *short black bars* depict multi-based indels. *Each solid line* interconnecting *two haplotypes* represents one mutational step for which parsimony is supported at the 95 % level, and a *dashed line* designates nonparsimonious connection between *Megadenia* network and *Biscutella* haplotype. Three lineages recognised in *Megadenia* are shown and haplotypes of the CP lineage are encircled with *dashed line*

comprising the sequences of seven regions was 7,197 characters (analysed using ML and BA) or 8,009 when the binary-coded indel characters were added (MP analysis). In total, 5,078 sites were constant, and 1,449 sites were potentially parsimony informative (including 350 coded indels). All of the analyses revealed trees with almost consistent topologies. The MP analysis yielded one most parsimonious tree of 4,399 steps (CI 0.7706, HI = 0.2294, RI = 0.7438); the log-likelihood score of the ML tree was −27,004.5886. All of the trees were rooted using two *Aethionema* species as outgroups because the basal position of this genus has been confirmed in previously published phylogenetic reconstructions based on different genes, gene sets, and genomes (e.g., Bailey et al. 2006; Beilstein et al. 2006, 2008; Franzke et al. 2009; Couvreur et al. 2010). *Megadenia* and *B. laevigata* formed an independent monophyletic clade (Fig. 3), which was well supported by high bootstrap (BS) values (96 % BS for MP, 93 % BS for ML analyses). All of the *Megadenia* accessions were grouped into a distinct cluster (100 % BS for MP and ML) separated from *Biscutella* by a long branch. As in the SP network, *M. bardunovii*,

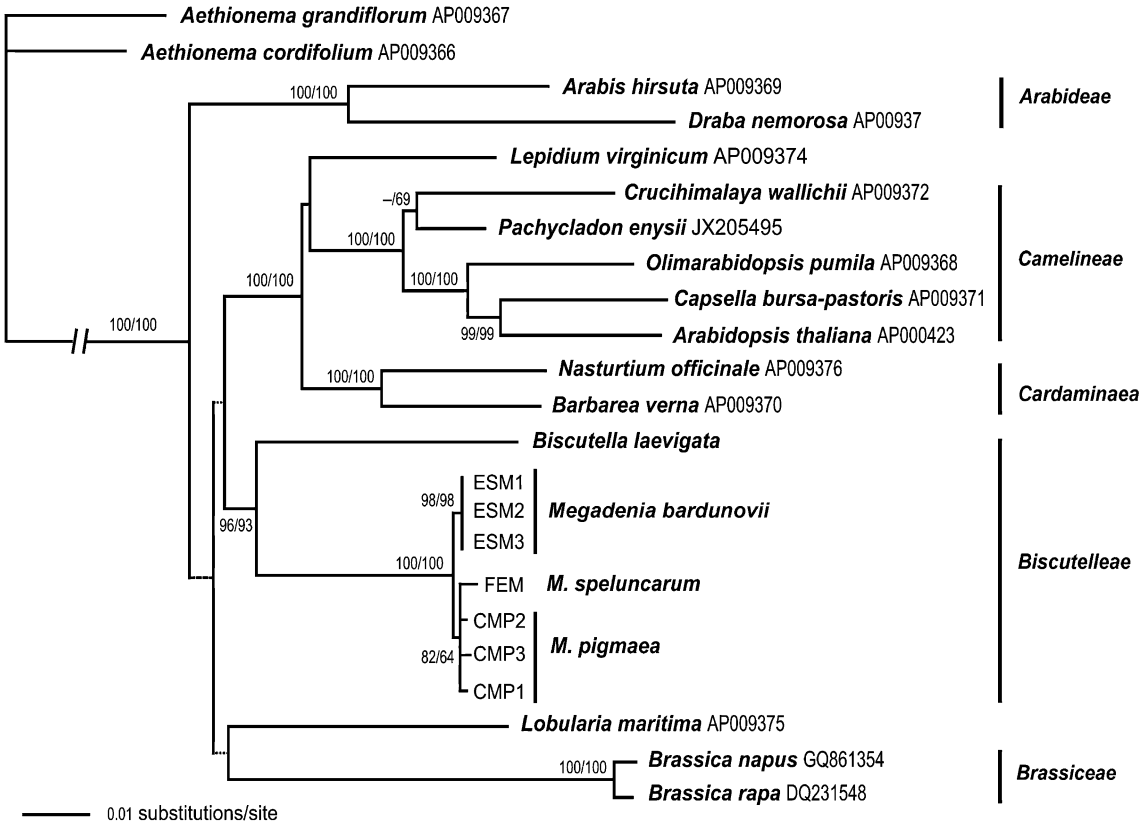

Fig. 3 Maximum likelihood tree showing the phylogenetic position of *Megadenia* within the Brassicaceae based on combined sequences of seven cpDNA regions. *Numbers* at the nodes indicate bootstrap values (>50 %) for MP (*left*) and ML (*right*) analyses. *Dashed lines* indicate the clades that are not supported by both the MP and the ML analyses. Information about tribal assignments according to Warwick et al. (2010) is given in the *right margin*

representing the ES lineage, was placed as sister to the moderately supported group (82 % BS for MP and 64 % BS for ML) containing all of the other *Megadenia* haplotypes, with unresolved relationships among them. Other species grouped with strong support in clusters according to the tribes Arabideae, Brassiceae, Cardamineae, and Camelineae. In our data set, the latter two tribes and *Lepidium virginicum* L. represented the Brassicaceae Lineage I sensu Beilstein et al. (2006) and formed a well-supported clade (100 % BS for MP and ML analyses), though the relationships between this clade and the other tribes remained uncertain.

Coalescent analyses performed in BEAST under an uncorrelated relaxed molecular clock yielded a maximum clade credibility tree comparable to that obtained with the ML and MP analyses (Fig. 4). The divergence of the ES lineage had preceded the splitting between the FE and CM lineages, with differentiation within the CM lineage likely occurring thereafter. Using the node ages derived from Couvreur et al. (2010) as calibration points, the age of the *Megadenia* stem was estimated to be 23.18 mya (95 % HPD: 26.75–16.74 mya). The divergence between the ES lineage and the common ancestor of two other lineages was estimated to have occurred in the late Early Pleistocene, at approximately 1.15 mya (95 % HPD: 1.80–0.61 mya). The next divergence event in *Megadenia* occurred between the FE and CM lineages, at approximately 0.82 mya (95 % HPD: 1.28–0.41 mya), most likely at approximately the same time when differentiation among the populations of the CM lineage in the QTP had begun (0.66 mya; 95 % HPD: 1.06–0.30 mya). The broad and overlapping ranges of divergence dates presented here do not allow precise estimates of divergence time among the cpDNA lineages of *Megadenia*. We noted that these age estimates based on calibration points derived from Couvreur et al. (2010) were one and a half times younger than the dates calculated with the secondary calibration points derived from the Beilstein et al. (2010) study. However, the 95 % HPD intervals of the estimated ages for *Megadenia* lineages calculated with different calibration points largely overlapped and fall within the same time period.

Discussion

Megadenia is one of 23 Brassicaceae genera endemic to East Asia (Manchester et al. 2009). In previous classification

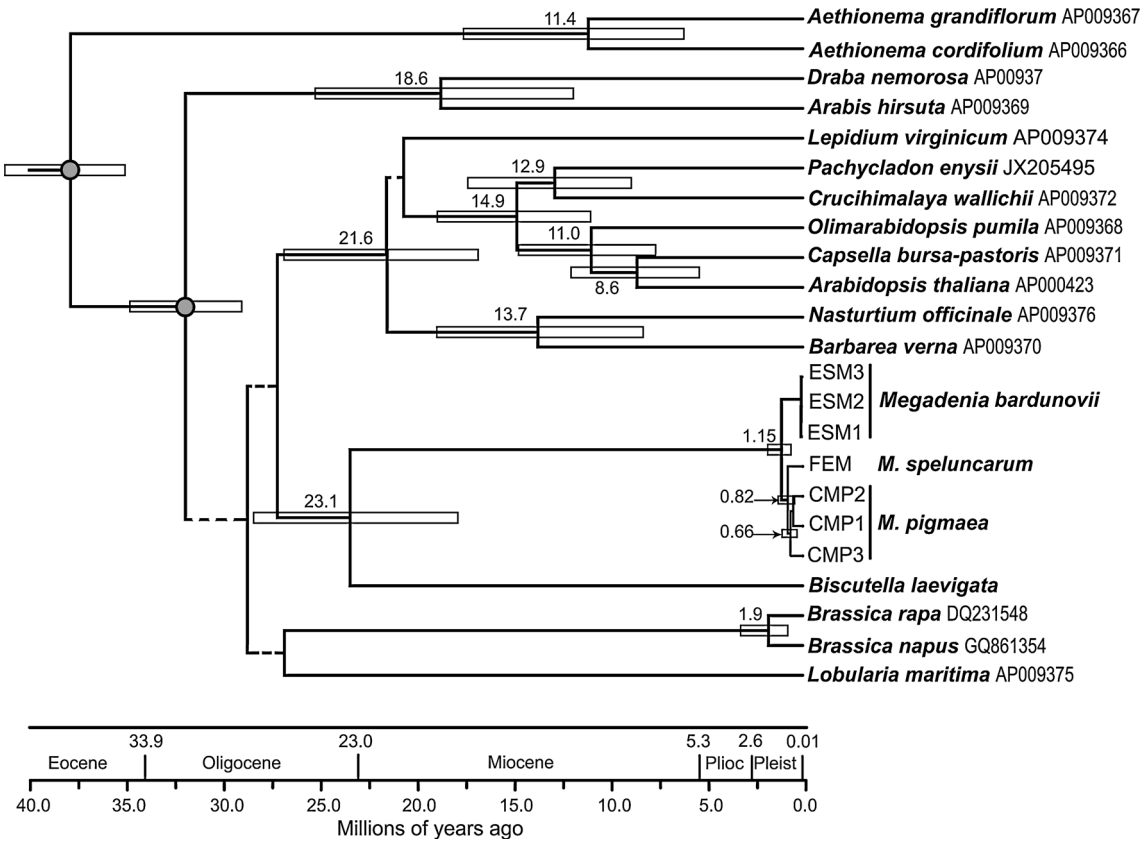

Fig. 4 Chronogram representing the maximum clade credibility tree based on seven cpDNA region sequences generated from BEAST analysis under a relaxed molecular-clock model. *Dashed lines* indicate the clades with a posterior probability value below 0.90. *Horizontal open bars* represent the 95 % highest posterior density (HPD) intervals of nodal age estimates and *node heights* (in million years) represent their mean values. Only *bars* on nodes with a posterior probability >0.97 are shown. *Grey circles* indicate nodes used for calibration of the trees based on the dates published in Couvreur et al. (2010). Geological time scale (Cohen et al. (2013) is shown at the *bottom*

systems, the genus *Megadenia* had been attributed (usually together with the genus *Biscutella*) to different tribes based mainly on the fruit type (see in Dorofeyev 2004; German et al. 2009; Warwick et al. 2010). Of the Brassicaceae, the genus *Biscutella* can be considered a sole distant relative of genus *Megadenia*. Based on ITS sequence data, *Megadenia* and *Biscutella* have been placed in the re-established tribe Biscutelleae Dumort., which was outside all other tribes and lineages known within the family, though this inclusion had only weak support (German and Al-Shehbaz 2008; German et al. 2009; Warwick et al. 2010). These genera also form a separate, but weakly supported ($\approx$50 % BS) clade in the MP and ML analyses of the mitochondrial *nad7* intron 2 data set including sequences of 40 Brassicaceae genera available in GenBank (data not shown). Using the cpDNA data, we provide strong support for the monophyly of the bigeneric tribe Biscutelleae (Figs. 3, 4). The age range of the split between the two genera (26.75–16.74 mya) calculated with calibration points derived from Couvreur et al. (2010) falls in the Late Oligocene–Early Miocene (approx. 28.1–15.97 mya), which is roughly consistent with the age range (22.48–10.22 mya) for that split calculated using the nucleotide substitution rate in noncoding sites of the low-copy nuclear *CHI* gene. The splitting of Biscutelleae genera coincided in time with the radiation within the Lineage I (Fig. 4) though the absolute timing of divergence varied with the calibration points used. Note that three major evolutionary lineages identified in the family (Beilstein et al. 2006) appear to have started radiation at around the same time, in the Oligocene–Early Miocene (28.2–21.4 mya, Couvreur et al. 2010; 35.6–30.8 mya, Beilstein et al. 2010).

Originating at least in the Early Miocene, the genus *Megadenia* is currently thought to consist of only one species, *M. pygmaea*, with three disjunct areas of distribution separated by distances of approx. 2,000 and 2,900 km. To our knowledge, plant checklists for the intervening territories contain no mention of *Megadenia*. In each area of distribution, the genus has different habitat preferences. In the southeastern edge of the QTP, *M. pygmaea* occupies diverse forest habitats at high elevations

(2,100–4,200 m), whereas the genus in the Eastern Sayan and Sikhote-Alin Mountains is confined to highly specific habitats at lower elevations (930 and 500 m, respectively). The patchy distribution of preferred habitats in the Tunka Valley led to the rare occurrence of *M. bardunovii* in the Eastern Sayan Mountains, whereas a strong connection with a specific habitat at the limestone cave of *M. speluncarum* may explain the uniqueness of its population in Primorskii Krai. *M. speluncarum*, and *M. bardunovii* are poorly known and are very similar to *M. pygmaea* in morphology. However, some clear discriminating features have recently been revealed in the anatomy of petioles, including the shape of petiole cross-sections, the number of prominences on the adaxial side, the outlines of median vascular bundles and the location of small lateral vascular bundles (Gorovoy et al. 2011).

The present molecular study based on noncoding sites in the cytoplasmic and nuclear genomes revealed that the levels of genetic divergence within the genus vary among the markers utilised. We did not detect a notable divergence within the genus at the noncoding mtDNA and nrDNA regions examined. Indeed, we found a limited divergence between *M. pygmaea* and the two *Megadenia* species from Russia only at intron sequences of the low-copy *CHI* gene. This lack of variation in nuclear and mitochondrial markers can argue in favour of the recognition of a single species in *Megadenia*, with no clear intrageneric subdivision. However, the limited applicability of ITS data for elucidating divergence has been demonstrated at low taxonomic levels (e.g., Meredâ et al. 2008; Lu et al. 2010; Artyukova and Kozyrenko 2012). Recent divergence, rapid radiations, and conservative genome evolution are believed to result in low sequence variation of multicopy nrDNA ITS regions because the fixation of new mutations among all of the units of the tandem arrays of ITS copies may require a long time (Möller and Cronk 1997). Similarly, it is suggested that gene conversion and efficient mismatch repair mechanisms might contribute to homogenise the mtDNA sequences in plants and to the low nucleotide substitution rates in this genome (Davila et al. 2011).

In contrast to nuclear and mitochondrial markers, appreciable divergence between *Megadenia* from three disjoint parts of the genus range was detected by analysing noncoding sequences from the chloroplast genome, and the nucleotide divergence revealed is comparable to the divergence between closely related species in certain other genera, e.g., *Capsella*, *Iris, Gentiana*, and *Oxytropis* (Slotte et al. 2006; Kozyrenko et al. 2009; Mishiba et al. 2009; Artyukova and Kozyrenko 2012). Our analyses clearly confirm the subdivision of the genus into three maternal lineages, ES, FE, and CM, matching three species with geographically separated distributions: *M. bardunovii, M. speluncarum*, and *M. pygmaea*. These three cpDNA lineages have no shared haplotypes (Fig. 2) and differ from each other by multiple substitutions and indels. Apomorphic sites were found for each lineage, which can be applied in the delimitation of the species. In addition, two synapomorphic indel regions in lineages CM and FE distinguish them from lineage ES and other Brassicaceae genera, including *Biscutella*. This finding might suggest that lineage ES is more closely related to the last common ancestor of the tribe Biscutelleae than the EF and CM lineages, and its sister position to the rest of the *Megadenia* lineages was well supported in all of the phylogenetic analyses. A large number of possible missing haplotypes in the CM lineage might have been lost through extinction but still might exist outside our sample. Indeed, apart from Russia, where our samplings were intensive, sampling of the CM lineage was limited to three localities, which are geographically distant from each other and harbour divergent haplotypes. Thus, a common ancestor of these haplotypes might occur in some populations scattered mainly across the southeastern edge of the QTP, which were not covered by our sampling. To our knowledge, the majority of *M. pygmaea* samples have been found in the Hengduan Mountains (Biodiversity occurrence data, accessed through GBIF Data Portal, data.gbif.org, accessed 2013 Oct 30), which are thought to be a refuge for many plant species endemic to East Asia (Qiu et al. 2011).

The current discontinuity of the genus range might have resulted from recent long-distance dispersal or might represent a relict of a former continuous distribution. If we consider all of the *Megadenia* populations from different geographical locations as one species, a long-distance dispersal would have to be assumed; however, the dispersal capability of this genus is limited due to a lack of traits facilitating long-distance dispersal. Small one-seeded mericarps of *Megadenia* might be dispersed to a new (nearby) suitable habitat by running water and/or wind, though the mericarps of *M. speluncarum* is dispersed mainly by gravity, and dispersal beyond the limits of the population is doubtful (E.V. Boltenkov, personal observations). We did not find any evidence from the plastid data for long-distance dispersal events between the lineages of *Megadenia*. Our data showed that no gene flow (via seed) has occurred between them since they began to diverge, and each of the lineages experienced an independent history. It is more likely that the three lineages of the *Megadenia* plastid genome arose by vicariance. Vicariant allopatric speciation associated with the geologic events has been proposed as the main mechanism of species diversification on the QTP and adjacent areas (Wen et al. 2014 and references therein). Under such a scenario, the formerly continuous range of the ancient progenitor of

Megadenia likely was fragmented due to historic events, such as orogeny, volcanism, and glaciations, which led to the formation of insurmountable barriers to dispersal or gene flow. The dating analysis showed that diversification within the genus that led to the extant cpDNA lineages likely occurred during a relatively short time period (1.15–0.66 mya) in the Early–Middle Pleistocene. The genus most likely began diversifying with the divergence of the ES lineage from a common ancestor of the FE and CM lineages and the split between the FE and CM lineages most likely occurred shortly thereafter (Fig. 4). Given that molecular dating within Brassicaceae is controversial (see in Franzke et al. 2011 and references therein) and strongly depends on the markers and the calibration points used (Franzke et al. 2009; Beilstein et al. 2010; Couvreur et al. 2010), our tentative results based on a single locus (cpDNA) and secondary calibration approach should be interpreted with caution. Despite these caveats, the estimated timescale for the divergence within *Megadenia* fits with the known geological and climate events for this geographical area in the Pleistocene. Recent orogenic events in the Eastern Sayan Mountains (3–0.6 mya, Arzhannikova et al. 2011), intense uplifts of the southeast margin of the QTP (1.1–0.6 mya, Wu et al. 2001; Zhou et al. 2006), and desert expansion events in the Asian interior at 1.2 and 0.7 mya (Guo et al. 2004; Ding et al. 2005; Wu et al. 2007) might have led to the formation of physical and eco-climatic impassable barriers. The adaptation to local environments, together with strong isolation, could lead to genetic differentiation and trigger an incipient stage of allopatric speciation. The absence of the *Megadenia* populations from the intervening areas, the lack of any intermediate haplotypes, and distant haplotype relationships between the extant lineages may indicate the extinction of many local populations (Dixon et al. 2009).

Thus, although *M. bardunovii*, *M. speluncarum*, and *M. pygmaea* are very similar in morphology and indiscernible in the nuclear and mitochondrial markers studied, our data for the plastid genome reveal their distinctness. Based on the lack of a direct genealogical relationship among the three cpDNA lineages, their non-overlapping distribution, the differences in habitat preferences, and in the anatomy of petioles, we are of the opinion that *Megadenia* from disjointed parts of the genus range should be treated as distinct species or at least subspecies. Further analyses with a broader sampling of *M. pygmaea* over the entire species range in China and the use of additional nuclear genome markers are necessary to uncover its evolutionary history and to determine more precisely the taxonomic ranks of *M. bardunovii* (Popov 1954), *M. speluncarum* (Vorob'ev et al. 1976), and *M. pygmaea* (Maximowicz 1889).

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