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Article *in* Israel Journal of Plant Sciences · August 2015

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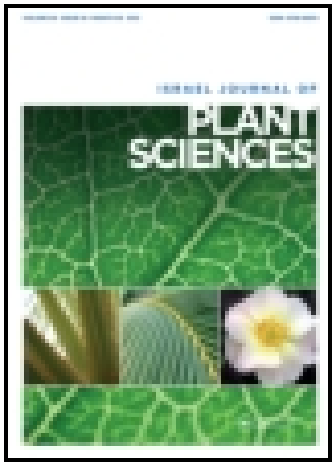

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Published online: 21 Jul 2015.

To cite this article: Sergei Volis, Tieyao Tu, Tao Deng, Marianna Zaretsky, Keren Fogel & Hang Sun (2015): Phylogeographic study of Mandragora L. reveals a case of ancient human assisted migration, Israel Journal of Plant Sciences, DOI: 10.1080/07929978.2015.1063922

To link to this article: http://dx.doi.org/10.1080/07929978.2015.1063922

Israel Journal of Plant Sciences, 2015
http://dx.doi.org/10.1080/07929978.2015.1063922

Phylogeographic study of *Mandragora* L. reveals a case of ancient human assisted migration

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(*Received 5 April 2015; accepted 11 June 2015*)

In reconstructing taxon evolution, historical biogeography is concerned with two kinds of speciation events, both resulting in a fragmented taxon distribution – vicariance and dispersal. We used PCR-RFLP of plastid DNA and a ribosomal internal transcribed spacer, sequencing of the *rps16-trnK* chloroplast region, flow cytometry (florescence-activated cell sorter; FACS), and ecological niche modeling to understand the role of these two processes in a disjunct distribution of genus *Mandragora*. The observed phylogeographic structure only partly coincided with currently recognized species. Commonly used recognition of a single species in the whole Mediterranean is not supported, given that a single haplotype observed from Morocco and Spain to Turkey is strikingly different from the haplotypes found in Israel. In the Sino-Himalayan area, the previously recognized *M. chinghaiensis* is nested within the *M. caulescens* clade indicating a very recent diversification within this lineage. And, most importantly, the obtained minimum spanning tree, observed haplotype distribution, and results of FACS call into question the existence of *M. turkomanica* as a species, and even as a lower taxonomic unit. Rather, the mandrake from Central Asia is nested within those from Israel, suggesting their closely related evolutionary history and ancient human assisted migration from Israel to Persia in historic times. Our study suggests that human assisted migration can explain the cases of disjunct species distribution for which vicariance was previously considered as the only plausible explanation.

Keywords: disjunct distribution; long distance dispersal; *Mandragora*; PCR-RFLP; phylogeny; phylogeography vicariance; ecological niche modeling

Introduction

Reconstruction of the history of a particular taxon or a group of taxa based on phylogenetic hypotheses is the major aim of modern historical biogeography (Cox & Moore 2005; Lomolino et al. 2006; Riddle et al. 2008). In reconstructing taxon evolution, historical biogeography is concerned with two kinds of speciation events, both resulting in a fragmented taxon distribution – vicariance and dispersal. Vicariance results from an emerging barrier splitting a continuous range into two or more separate parts, while (long-distance) dispersal is an active colonization across a preexisting barrier (Platnick & Nelson 1978). Because disjunct distribution patterns are common in many organism groups, understanding the relative importance of these processes is crucial for historical biogeography and has been intensively debated in the last decades. The advent of techniques to date divergences between lineages greatly increased an ability to distinguish between these two possibilities (Crisci 2001; Posadas et al. 2006). However, for relatively recent evolutionary events, dating can be problematic because of low amounts of DNA sequence divergence. In this case, inferences about vicariance vs. dispersal are based on branching orders in a phylogenetic tree. For vicariance, the lineages from the disjunct areas are expected to be reciprocally monophyletic, while for long-distance dispersal, the lineages from one disjunct area should be nested within those from the other disjunct area (e.g. Brunsfeld et al. 2001; Carstens et al. 2005).

The genus *Mandragora* L. (Solanaceae) is a taxon with a very disjunct distribution embracing Sino-Himalaya, Central Asia and the Mediterranean basin. Traditionally, speciation and distributional pattern in this taxon were explained as solely due to vicariance, viz. by the uplift of the Himalayas in the Eastern part of the range, and by receding of the Tethys Sea and aridization in the remaining range (Proskuryakova & Belyanina 1985; Ungricht et al. 1998). However, recent findings (Tu et al. 2010) suggest the possible involvement of long-distance dispersal in the history of the genus.

Here, we analyze the genus phylogeography using a combination of molecular, morphological, and caryological approaches, as well as ecological niche modeling to

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This paper has been contributed in honor of Professor Daniel Zohary.

elucidate the role of vicariance and dispersal in the genus evolutionary history.

Materials and methods

Study group taxonomy

Mandragora L. (Solanaceae) is a small genus of outcrossing perennial herbs with an exclusive Eurasian distribution. Taxonomic classification within the *Mandragora* genus is an unresolved longstanding debate. During the last three centuries, dozens of names defining species, subspecies, and varieties were proposed, creating confusion in identification and comparison of taxa from distant geographic regions. A modern revision of the genus by Ungricht et al. (1998) was a first serious attempt to resolve this confusion. Ungricht and colleagues recognized three species, *M. officinarum* L., *M. turcomanica* Mizg. and *M. caulescens* C.B. Clarke using a morphometric analysis of herbarium specimens. However, delimitation of these three species within *Mandragora* may require further revision, as for the first time, samples of all currently recognized species (and some of them live samples) became available to the authors allowing (1) a direct analysis of morphological, phenological, and caryological intrageneric variability; and (2) usage of molecular markers for species delimitation and phylogeny reconstruction. Our putative species delimitation used existing species names in population genetic and phylogenetic analyses. Samples from Israel were putatively classified as *M. officinarum* to distinguish them from other samples of Mediterranean origin named *M. autumnalis*. This distinction followed Tercinet (1950) but was based not on phenology, as all Mediterranean samples known to us flower in autumn and winter, but on size of fruits and seeds. In *M. officinarum*, Tercinet states "The fruits are much larger than those of the previous species, being as large as a small apple" (Tercinet 1950). All samples from Turkmenistan were assigned to *M. turcomanica* Mizg. Plants from Sino-Himalaya were assigned to either *M. caulescens* C.B. Clark or *M. chinghaiensis* Kuang & A.M. Lu depending on plant habit (elongated stem vs. rosette).

Plant material

We collected 133 specimens in Israel during 1999−2008 and 92 specimens in China during 2002−2008 as mature fruits, root cuttings, or fresh leaves. Other samples were either collected as seeds/root cuttings by colleagues (University of Sevilla − six accessions from Spain and 10 from Morocco; Hacettepe University − two accessions from Turkey; private − nine accessions from Turkmenistan) or obtained as seeds/root cuttings from living collections (Kew Botanical Garden − one accession from Cyprus, Radboud University − one accession from Italy and one from Morocco). Samples, collected in Israel and China, represent different sub-regions that differ in climatic conditions, soil, and vegetation. In Israel, the sub-regions included Mount Hermon, Golan Heights, Upper Galilee, Sharon plain, Mount Gilboa, Judean Mountains, and Northern Negev (see Zohary & Feinbrun-Dothan 1981 for details). The samples collected in China represented the sub-regions eastern Himalayan Mountains, eastern Tibetan Plateau, western Hengduan Mountains, and northeastern Hengduan Mountains (Zhang et al. 1997, 2002) (Table 1).

PCR-RFLP

The DNA was extracted from leaf tissues using a modified CTAB protocol (Hillis et al. 1996) or a Qiagen Dneasy Plant extraction kit (Qiagen, Hilden, Germany). A preliminary screening of a collection subset was performed for the nuclear ribosome ITS (ITS4 and ITS5) and nine universal cpDNA primer pairs: HK (*trnH*/*trnK*), K1K2 (*trnK* exon1/*trnK* exon2), DT (*trnD*/*trnT*), CS (*psbC*/*trnS*), SfM (*trnS*/*trnf*$_M$), ML (*trnM*/*rbcL*) (Demesure et al. 1995; TC (*trnT*/*psbC*) (Dumolin-Lapeque et al. 1997); B2B3 (*psbB*/*petB*), FV (*trnF*/*trnV*) (Grivet et al. 2001). The subset included 17 samples representing different Mediterranean, Central Asian, and Sino-Himalayan sub-regions/populations. The PCR thermal profile followed Demesure et al. (1995). The PCR was performed in 25 μl containing 1.0 μl of diluted DNA (1:30 for the CTAB extracted samples and 1:5 for the kit extracted samples), 2.5 μl 10× *Taq* DNA Polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.04 mM dNTP, 0.4 pmol of each of the primers, and 1 unit of *Taq* DNA Polymerase. The PCR products were checked on 1% agarose gels to verify the amplification. Five microliters of PCR products were digested with either one or two restriction enzymes (cpDNA: *HinfI*, *TaqI*, *Cfr421*, *MseI*, *SalI*, *BcuI*+*RsaI*, *Fnu4HI*+*ApaLI*, *SphI*+*DdeI*; ITS: *Hha*, *AluI*, *Rsa*, *BcuI* and *Fnu4HI*). The reaction was carried out in a total volume of 25 μl using 2 U of restriction enzyme for at least 2 h at 65°C for the *TaqI* and at 37°C for the rest of the enzymes. The digested fragments were loaded into 8−12% polyacrylamide gels, run for 2 h at 300 V and stained with ethidium bromide.

Regions that proved polymorphic in the initial screening (*trnH*/*trnK*, *trnD*/*trnT*, *trnF*/*trnV*, *trnM*/*rbcL*, *psbB*/*petB*) were further amplified and digested for the whole collection using the same protocols as above.

Sequencing

A representative subset of samples was used for sequencing the *rps16-trnK* region. PCR was performed using either Eppendorf or MJ Research (Waltham, MA) thermal cyclers in 50 μl volumes. For each sample reaction, 5.0 μl of 10× TAQ DNA polymerase buffer, 5.0 μl of 20 mM $MgSO_4$, 4 μl of 2.5mM dNTPs, 2.5 μl of 10mM of each, forward (AAA GTG GGT TTT TAT GAT CC) and reverse (TTA AAA GCC GAG TAC TCT ACC) primer, 0.5 μl of 5 u/

Table 1. Sample information including country of origin, geographic region and sub-region, and current taxonomy according to Tercinet (1950) (*M. officinarum* and *M. autumnalis*), Ungricht *et al.* (1998) (*M. turcomanica*), Kuang and Lu (1978) (*M. caulescens* and *M. chinghaiensis*).

Country of origin	Region	Sub-region	Number of samples	Current taxonomy
Israel	Near East	Mount Hermon	2	*M. officinarum* L.
Israel	Near East	Golan Heights	6	*M. officinarum* L.
Israel	Near East	Upper Galilee	43	*M. officinarum* L.
Israel	Near East	Sharon plain	2	*M. officinarum* L.
Israel	Near East	Mount Gilboa	6	*M. officinarum* L.
Israel	Near East	Judean Mountains	25	*M. officinarum* L.
Israel	Near East	Shefela	18	*M. officinarum* L.
Israel	Near East	Northern Negev	31	*M. officinarum* L.
Turkmenistan	Central Asia	–	9	*M. turcomanica* Mizg.
Turkey	Mediterranean	–	2	*M. autumnalis* Bertol.
Cyprus	Mediterranean	–	1	*M. autumnalis* Bertol.
Italy	Mediterranean	–	1	*M. autumnalis* Bertol.
Spain	Mediterranean	–	6	*M. autumnalis* Bertol.
Morocco	Mediterranean	–	11	*M. autumnalis* Bertol.
China	Sino-Himalaya	Eastern Tibetan Plateau	3	*M. chinghaiensis* Kuang & A.M. Lu
China	Sino-Himalaya	Himalaya	15	*M. caulescens* C.B. Clark
China	Sino-Himalaya	Western Hengduan Mountains	7	*M. caulescens* C.B. Clark
China	Sino-Himalaya	Northeastern Hengduan Mountains	67	*M. caulescens* C.B. Clark

μl TAQ DNA polymerase, 28 μl of sterilized distilled water, and 2.5 μl of DNA (~50 ng/μl) were mixed (Tu et al. 2008). The amplification profiles were template DNA denaturation at 95°C for 3 min, then 35 cycles of 30 s at 94°C, primer annealing at 50°C for 1 min, and primer extension at 72°C for 2 min, ending with 72°C for 10 min. The PCR products were checked on 1% agarose gel with 100 bp ladder before being purified with either the MSB® Spin PCRapace kit (Invitek, Berlin, Germany) or Illustra™ ExoStar kit (USB, Cleveland, OH). The cycle sequencing reactions were conducted in 10 μl volumes that contained 0.25 μl of BigDye 3.1, 0.5 μl of primers, 2.0 μl of purified PCR products, and 1.75 μl of sequencing buffer. The reaction conditions were 95°C for 2 min, 30 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min, ending with a 4°C hold. The sequencing reactions were run on an ABI 3730 automated sequencer (Applied Biosystems, Ben-Gurion University of the Negev).

FACS and seed size measurements

The genome sizes (C-values *sensu* Dolezel et al. 1992) of the analyzed plants were estimated by flow cytometry (florescence-activated cell sorter, FACS). Measurements were taken on a Becton Dickinson (San Jose, CA) FACS Vantage SE equipped with an air-cooled argon-ion laser as an excitation source. The 100 mg of fresh intact leaf tissues from the analyzed sample and internal reference standard were chopped together in 4 ml of nuclei isolation ice-cold buffer (0.2 M sucrose, 10 mM MES, 2.5 mM EDTA, 10 mM NaCl, 10 mM KCl, 0.15% Triton X-100, 0.1 mM spermine, and 2.5 mM DTT) (Galbraith et al. 1983 with small modifications). The crude suspension was shaken in ice for 30−50 min, filtered through a 150 μm nylon mesh and centrifuged for 10 min at 550 × g at 40°C. The pellet was dissolved in 0.5 ml of buffer and kept in ice (until the next day). Before taking measurements, another filtration through a nylon mesh of 50 μm was performed. Nuclei were stained with 10 μl of propidium iodide (PI, 2 mg/ml). Flow histograms were evaluated using the BD CellQuest™ pro V. 5.1.1 software (BD Bioscience, San Diego, CA). At least two independent measurements were performed for each sample. *Pisum sativum* cv. Citrad (2C = 9.09 pg; Dolezel et al. 1992) and *Nicotiana tabacum* Samson (2C = 10.4 pg) were selected as the primary and secondary reference standards. The sample 2C DNA content was calculated as described in Dolezel and Bartos (2005).

Fruits collected in the field and from the reproducing plants in the living collection were used to analyze the effect of geographic origin on seed weight. The datum in this analysis was average seed weight per fruit. Plants (FACS) and fruits (seed weight) of different geographic origin were compared by the Tukey−Kramer test.

Population genetic and phylogenetic analyses

PCR-RFLP

The cpDNA variation was analyzed by a hierarchical analysis of molecular variance (AMOVA; Excoffier et al.

1992) as implemented in Arlequin (Schneider et al. 2001). The total cpDNA variation was partitioned to estimate variance components for different hierarchical levels. Several criteria were used to assign the populations to groups: (1) species, which was based on current taxonomy according to Tercinet (1950) (*M. officinarum* L. and *M. autumnalis* Bertol.), Ungricht et al. (1998) (*M. turcomanica* Mizg.), and Kuang and Lu (1978) (*M. caulescens* C.B. Clark and *M. chinghaiensis* Kuang & A.M. Lu); and (2) geographic distribution only (Mediterranean, Near East-Central Asia, Sino-Himalaya) (Table 1).

Statistical significance of variance components associated with genetic differentiation of samples between populations within groups and between groups was estimated using 1000 permutations. A Euclidean square distance matrix of haplotype pairwise differences was used to construct a minimum spanning-tree and neighbor-joining clustering, the latter with NTSYSpc 2.0 (Rohlf 1998).

Diversity and differentiation parameters (within-population diversity, h_S; total diversity, h_T; differentiation for unordered and ordered alleles, G_{ST} and N_{ST}, respectively) were calculated according to Pons and Petit (1996)) using PERMUT (http://www.pierroton.intra.fr/genetics/labo/Software). This program was also used to test whether N_{ST} is larger than G_{ST}, indicative of a phylogeographic structure (a situation when closely related haplotypes are more often found in the same area than less closely related haplotypes) (Pons & Petit 1996). Significance of the difference between N_{ST} and G_{ST} was assessed with 1000 random permutations following Burban et al. (1999).

Sequencing

Sequencher 4.10.1 (Gene Codes Corporation, 2005) was used to evaluate chromatograms for base confirmation and to edit contiguous sequences. Sequences were aligned with Muscle (Edgar 2004) and analyzed by MEGA version 6 (Tamura et al. 2013). The phylogeny reconstruction was performed using maximum likelihood (ML), maximum parsimony (MP), and Bayesian analyses. The best available model of molecular evolution was selected using Bayesian Information Criterion as implemented in MEGA 6. The best-fitting model was the Hasegawa−Kishino−Yano model. Both ML and MP analyses were conducted in MEGA with partial deletion of gaps (site coverage cutoff set at 95%). The heuristic search used the nearest-neighbor-interchange (ML) and tree-bisection-reconnection (MP). In MP analysis, a strict consensus tree was constructed from the most parsimonious trees. Both analyses used a Bootstrap test of phylogeny with 1000 replications. Bayesian inference was conducted using MrBayes, version 3.2.1 (Ronquist et al. 2012). The Markov chain Monte Carlo algorithm was run for 1,000,000 generations with four incrementally heated chains, starting from random trees and sampling every 100 generations. The first 10,000 trees were discarded as burn in, and the remaining trees were used to construct a 50% majority rule consensus tree. Internodes with posterior probabilities >95% were considered statistically significant.

Niche modeling and projections

We used ecological niche modeling to analyze the geographic distribution of climatically suitable habitats of *Mandragora*. For environmental layers, we used the 19 "Bioclim" variables developed by Hijmans et al. (2005) that summarize temperature and precipitation dimensions (often monthly) of the environment into biologically relevant layers and have been intensively used in evolutionary studies using niche models (e.g. Knouft et al. 2006; Evans et al. 2009; Smith & Donoghue 2010). Data were obtained from WorldClim 1.4 Hijmans et al. (2005) with a resolution of 1 km^2. Species-occurrence data were based on presence records obtained from databases [Chinese Virtual Herbarium (http://www.cvh.org.cn/cms/cn) and Global Biodiversity Information Facility (http://data.gbif.org)], as well as labels on herbarium specimens (on loan from PE, KUN and JIU) and the authors' personal observation database. In total, there were 152 and 86 occurrence records in the Mediterranean and Sino-Himalayan regions, respectively. We did not include in the occurrence dataset locations from Turkmenistan, as we wanted to test whether the area currently occupied by mandrake in this region would fall within the predicted range.

Ecological niche modeling was performed to identify: (1) the potential current distribution, (2) the potential past distribution during the Last Interglacial period (140,000−120,000 BP), and (3) the potential past distribution during the extreme conditions during the Last Glacial Maximum (LGM) (21,000−18,000 BP). In the LGM analysis, general circulation model simulations utilized the Community Climate System Model (Collins et al. 2006).

To construct the species climatic niches, we used MAXENT v3.3.3 (Phillips et al. 2006; Phillips & Dudik 2008). The output of MAXENT consists of a grid map with each cell having an index of suitability between 0 and 1. Model predictions were projected onto the world map visualized in ARCMAP 10.2 (ESRI, Redlands, CA).

Results

PCR-RFLP of cpDNA

Thirteen haplotypes were identified in the whole collection ($n = 255$) by PCR-RFLP of cpDNA (Table 2). The haplotypes displayed a very distinct pattern of geographic distribution being clustered in three groups by the minimum spanning tree (Figure 1). The first cluster has a sole representative, a haplotype A that is found throughout the Mediterranean region (Spain, Morocco, Italy, Cyprus, and Turkey) except for Israel or elsewhere. No other haplotypes

Table 2. Description of the 13 haplotypes detected by PCR-RFLP of cpDNA.

Haplotype	FV *Taq*	FV *Hinf*	FV *Ecor*	FV *Hap*	B2B3 *Hha*	HK *Apa1/ Fnu4*	HK *Bcu1/ Rsa1*	HK SphI/ DdeI	HK *Mse*	DT *Mse*	DT *Bcu/ Rsa*	DT *Fnu4HI/ ApaLI*	DT *Cfr421*	DT *SalI*	ML *Sph/Dde*	ML *Cfr421*
A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
B	2	2	2	2	2	2	1	2	2	1	1	2	1	1	2	1
C	3	2	2	2	2	2	1	2	2	1	1	2	1	1	2	1
D	3	3	2	2	2	2	1	2	2	1	1	2	1	1	2	1
E	3	2	2	2	2	2	2	2	2	1	1	2	1	1	2	1
F	3	3	2	2	2	2	2	2	2	1	1	2	1	1	2	1
G	4	4	3	3	3	2	1	3	3	2	2	3	2	2	1	2
H	4	5	3	4	3	2	1	3	3	2	2	3	2	2	1	2
I	5	5	3	4	3	2	1	3	3	2	2	3	2	2	1	2
J	4	6	3	4	3	2	1	3	3	2	2	3	2	2	1	2
K	6	4	3	3	3	2	1	3	3	2	2	3	2	2	1	2
L	5	5	4	1	3	2	1	3	3	2	2	3	2	2	1	2
M	5	6	4	1	3	2	1	3	3	2	2	3	2	2	1	2

Columns describe the electrophoretic profiles for each PCR product and restriction-enzyme combination.

were detected for this region. The second cluster (haplotypes B−F) comprises four haplotypes found only in Israel (C−F) and the B haplotype present in both Israel and Central Asia (Turkmenistan). In Israel, the B haplotype was found at a single location on Mount Hermon. The haplotype C is the most common haplotype in Israel, but it decreases in frequency from north to south (Figure 1). In Turkmenistan, all plants possessed the haplotype B. The third cluster constitutes a group of haplotypes of Sino-Himalayan origin (G-N), with haplotype L being the most common and widespread, and the other five haplotypes being either locally common or rare. The haplotype G was found only in three plants identified as *M. chinghaiensis* and collected on the eastern Tibetan Plateau.

Analysis of genetic diversity revealed similar partitioning of among and within group variation when groups

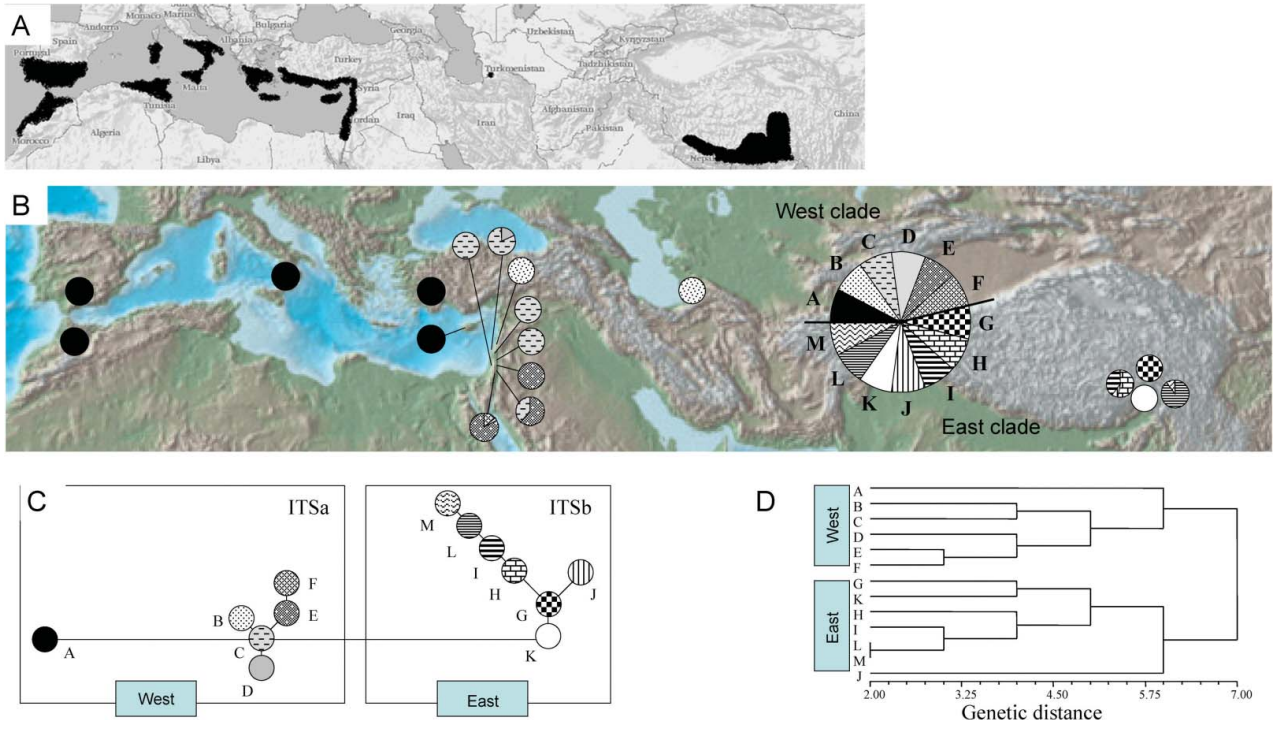

Figure 1. (A) Map showing the geographic range of *Mandragora*. (B) Distribution of 13 cpDNA haplotypes. (C) Minimum-length spanning tree of the cpDNA haplotypes. The two ITS types are superimposed. Distances between the haplotypes correspond to the number of restriction pattern differences. (D) CpDNA haplotype tree as assessed by the neighbor-joining method.

Table 3. Analysis of genetic variation in *Mandragora* with groups defined as either recognized species or geographic regions: (a) comparison of differentiation for unordered and ordered alleles; (b) AMOVA for two groupings; (c) species-specific population differentiation estimates

(a)					
	h_S	h_T	G_{ST}	N_{ST}	P ($N_{ST} > G_{ST}$)
Species	0.200 (0.199)	0.995 (0.130)	0.799 (0.203)	0.968 (0.036)	< 0.001
Regions	0.406 (0.204)	1.000 (0.135)	0.594 (0.211)	0.934 (0.024)	< 0.001
(b)					
Source of variation	d.f.	Sum of squares	Variance components	F_{ST}	
Among species	4	2804	96.6	0.966	
Within species	247	161	3.4		
Among regions	2	2741	96.5	0.965	
Within regions	249	215	3.5		
(c)					
M. officinarum				0.801	
M. autumnalis				0	
M. turcomanica				0	
M. chinghaiensis				0	
M. caulescens				0.873	

were delimited as formerly recognized species or broadly defined geographic regions (Table 3). The within-group component of diversity was very small (less than 4%). A test comparing structuring of genetic diversity for ordered vs. unordered alleles (i.e. N_{ST} vs. G_{ST}) showed that N_{ST} is significantly higher than G_{ST} ($p < 0.001$) for both groupings.

Neighbor-joining clustering carried out on a Euclidean square distance matrix of haplotype pairwise differences revealed two distinct clusters, one comprising haplotypes of Mediterranean and Central Asian origin, and one comprising Sino-Himalayan haplotypes (Figure 1).

The structure of haplotype genetic diversity within each species estimated by F_{ST} revealed either no subdivision owing to a lack of variation (in *M. autumnalis* and *M. chinghaiensis*) or a similarly high (> 0.80) subdivision in *M. officinarum* and *M. caulescens*.

PCR-RFLP of ITS

Two ribosomal types were identified in the whole collection ($n = 255$) by analysis of the ITS region using five restriction enzymes. The individuals characterized by the first type (ITSa) are from the Mediterranean and Central Asia, while all the individuals from China possess the second type (ITSb). The observed ribosomal types coincide with two major clades of cpDNA variation (haplotypes A−F and G−M) (Figure 1).

Sequencing

The *rps16-trnK* gene had 873 basepairs, of which 18 were variable, and 17 were parsimony informative. A generated majority-rule consensus tree was rooted with *Atropa*. Three methods (ML, MP, and Bayesian) produced identical topology (Figure 2). There were two major clades, Mediterranean−Central Asia and Sino-Himalaya, and the first clade had two distinct sub-clades, Mediterranean and Near East-Central Asia (all the nodes with 100% posterior probability and bootstrap support values). The Turkmenian haplotype (currently recognized *M. turcomanica*) differed by only one nucleotide from haplotypes sampled in Israel, while a single haplotype detected in Turkey, Cyprus, Italy, Spain, and Morocco, was distinctly different from the Turkmenian and Israeli ones.

In the Sino-Himalayan clade, three samples identified as *M. chinghaiensis* possessed the same haplotype. This haplotype was different from the samples identified as *M. caulescens*, but was nested within the *M. caulescens* clade (100% posterior probability and bootstrap support value).

DNA amount and seed size

The DNA content of plants collected in different regions of Israel was 6.51 ± 0.06 pg (range 5.1−7.8 pg) and did not differ from the content of plants from Turkmenistan (average 6.43 ± 0.10 pg, range 5.7−7.1 pg). The DNA content of plants from Morocco, Spain, Cyprus, Turkey, and Italy was significantly lower (4.67 ± 0.27, 4.61 ± 0.10, 4.79 ± 0.16, 4.60 ± 0.30, and 4.77 ± 0.07, respectively) and ranged from 4.3 to 5.4 pg (Figure 3).

Israeli origin seeds from 119 field collected plants and 51 cross-pollinated plants in the living collection weighed 28.9 ± 0.4 and 24.8 ± 0.7 mg, respectively. Seeds from two field-collected plants of Turkmenian origin weighed

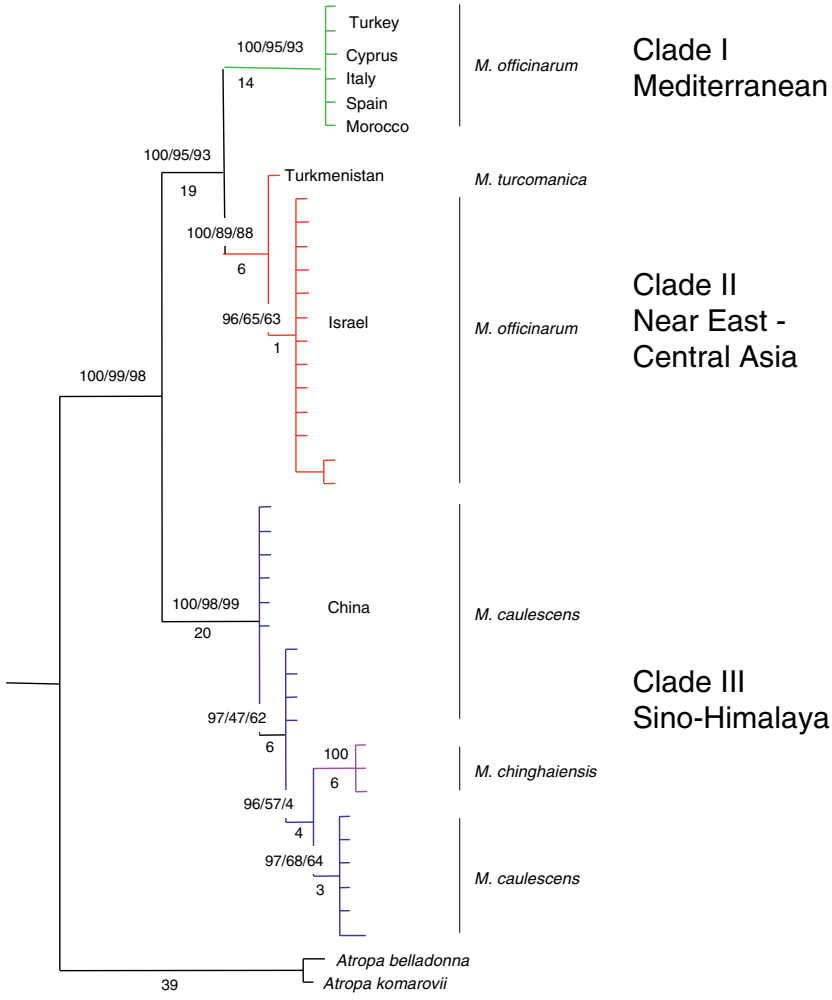

Figure 2. Phylogenetic tree of chloroplast DNA sequence of 873 bp of the *rps16-trnK* gene from 39 mandrake plants of different geographic origin. The tree was rooted with *Atropa*. Trees, created from Bayesian, ML and MP methods, had identical clade topologies. Posterior probability/bootstrap values (from left to right: Bayes/MP/ML) are adjacent to nodes, and the number of expected single-nucleotide differences is below the corresponding branch. Countries of origin and names of currently recognized species are next to the branch tips.

37.7 ± 1.0 mg. Seeds of both Israeli and Turkmenian origins were much larger than seeds of any other origin (Figure 3).

Niche modeling and projections

Niche models showed a good performance in capturing the remaining records in native prediction (AUC = 0.99, omission = 0), when calibrating a niche model using the trimmed records. Niche projection results are presented in Figure 4. The predicted distribution of *Mandragora* under the current climate corresponded well to the known distribution of the genus in both regions, the Mediterranean and Sino-Himalaya. The predicted genus distribution included the actual area of its distribution in Turkmenistan, although the occurrence data used did not include these locations as potentially of non-natural (human introduced) origin. The modeling results show that the climate of the area around the southern part of the Caspian Sea was suitable for mandrake at least for the last 20,000 years. The global distribution of *Mandragora* in both regions, the Mediterranean and Sino-Himalaya, appears to change little during the Holocene with almost no effect of glaciation on the latter.

Discussion

The results of molecular analyses clearly show geographic structuring of *Mandragora* genetic variability. This structuring coincides only partly with currently recognized taxonomy, i.e. delineation of the three species, *M. officinarum*, *M. turkomanica*, and *M. caulescens* (Ungricht et al. 1998). The obtained minimum spanning tree and observed haplotype distribution (PCR-RFLP) and phylogenetic tree (sequencing of *rps16-trnK*) call into question the existence of *M. turkomanica* as a species, and even as a lower taxonomic unit. These results are concordant with the observed

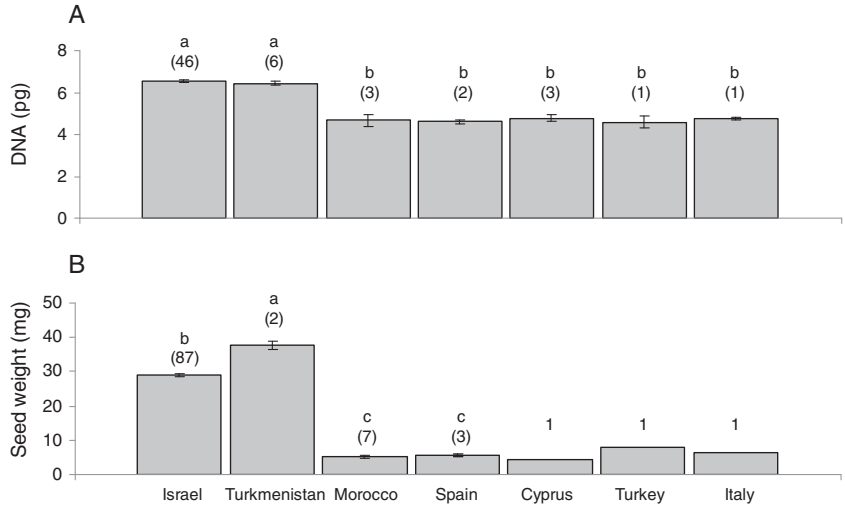

Figure 3. FACS analysis (in pg of DNA) (A) and seed weight (in mg) (B) of mandrake plants of different geographic origins. The letters above the bars denote the results from the Tukey–Kramer test, and the numbers denote the number of tested genetically different plants (FACS) and fruits (seed weight). There was one test per plant of Israeli origin, and two to three tests per plant of other origins in the FACS analysis. In determining the seed weight, the average seed weight per fruit was used as a datum.

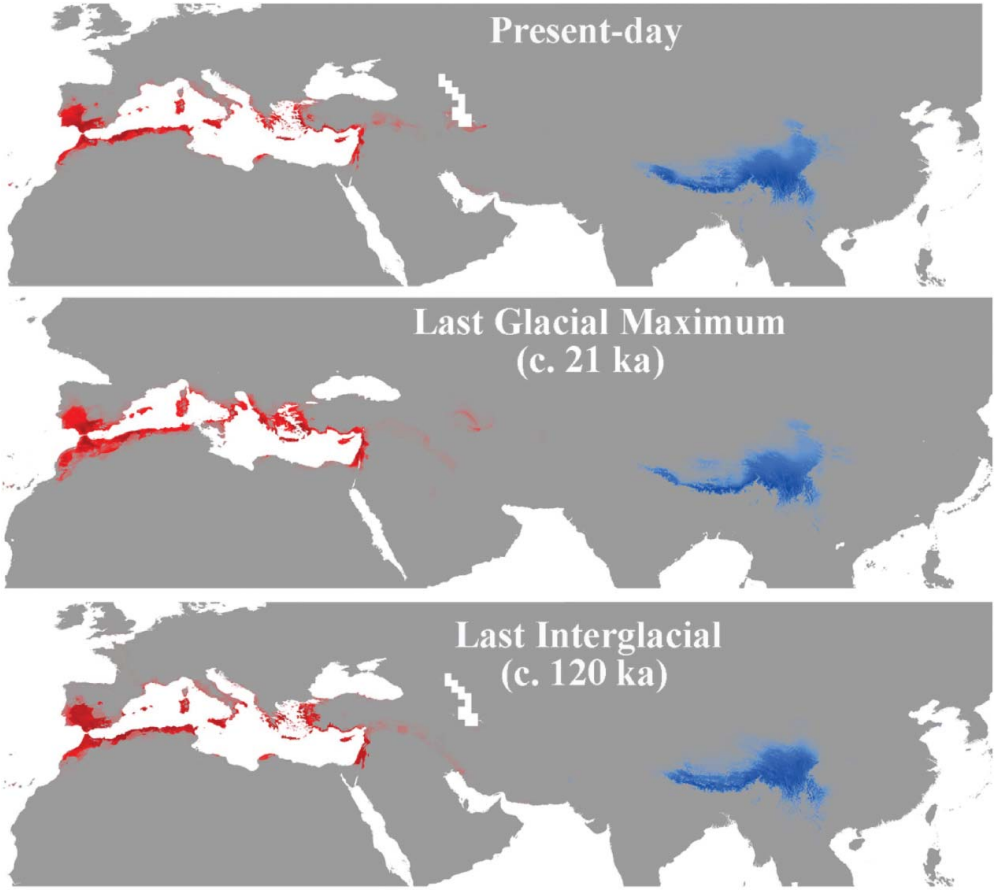

Figure 4. Climate-based predicted distribution of *Mandragora* in two regions, the Mediterranean and Sino-Himalaya, for three geological time periods.

morphological congruence (shape and size of leaf rosettes, leaves, and flowers) of *M. turkomanica* and plants from Israel (S. Volis, unpublished data) as was hypothesized earlier by Akhani and Ghorbani (2003). On the other hand, the currently recognized sole Mediterranean species (*M. officinarum* in Ungricht et al. 1998) does not appear to be a single species. A single haplotype detected by PCR-RFLP observed in most of the Mediterranean region is strikingly different from the haplotypes found in Israel and Central Asia. Similarly, sequencing of *rps16-trnK* revealed two different clades with 100% support, one including plants from Israel and Turkmenistan, and another including plants from all other Mediterranean countries.

The observed differences in DNA sequences are matched by the differences in DNA content per nucleus, which is indicative of ploidy level. Reported chromosome counts in *Mandragora* of Mediterranean origin are controversial, ranging from 24 (Lentini et al. 1988) to 96 (Murin 1978). The exact caryotype formula of Mediterranean *Mandragora* remains unknown owing to small chromosome sizes (Tu et al. 2005), but the plants of Turkmenian and Israeli origin appear to have a higher ploidy level than other Mediterranean plants. The latter is supported by a difference in seed size between plants from these two regions, with Mediterranean seeds being much smaller in size than Turkmenian and Israeli ones.

Although it would be premature to reconstruct the genus phylogenetic tree and to identify the ancestor haplotype with the current data, the dichotomy branching of mandrake into Mediterranean-Near East-Central Asian and Sino-Himalayan clades agrees with repeatedly suggested common evolutionary history of the genus, namely receding of the Tethys Sea and aridization of the Central Asia and Mediterranean in the Tertiary (Proskuryakova & Belyanina 1985; Kurbanov 1994; Ungricht et al. 1998; Sun 2002; Tu et al. 2005). The geographically isolated position of the Central Asian mandrake, nested within rather than sister to the populations from Israel, indicates a recent eastward dispersal event. However, although mandrake berry is sweet and potentially attractive to birds, a distance between Central Asian and Near East populations is too far for any bird considering the time for berry digestion and seed defecation. In fact, birds have never been observed eating mandrake fruits (in contrast to animals such as porcupines) (I. Sinev, personal observations). In addition, the vast Syrian Desert separates the two regions, making a scenario of range expansion with partial extinction highly improbable. A much more probable scenario of long-distance dispersal is by humans, who were interested in the magical, medicinal, or culinary properties of mandrake thousands of years ago (Thompson 1934; Moldenke & Moldenke 1952; Fleisher & Fleisher 1994). Climate-based modeling of the genus distribution confirmed that *Mandragora* plants can grow in the area around southern part of the Caspian sea, which is the area from which *M. turkomanica* was reported, and this area was suitable for mandrake at least the last 20,000 years. Interestingly, the distribution of mandrake locations in Turkmenistan coincides with abandoned ancient settlements (Kuznetsov 1998), and the only known location of this plant in Iran is in a vegetable garden in a village (Akhani & Ghorbani 2003). Fruits of Turkmenian and Israeli origin are sweet, tasty (sometimes even delicious) and large, while fruits from the other Mediterranean countries are not sweet, not tasty at all and small (K. Fogel and S. Volis, unpublished data).

In the Sino-Himalayan area, the evolution of mandrake appears to have a different route, in which adaptation to the opposite, as compared with the Mediterranean, climate change (cold and long winter as a result of glaciation) took place in the Quaternary (Proskuryakova & Belyanina 1985; Ungricht et al. 1998). The similar extent (number of observed haplotypes) and structure (F_{ST}) of haplotype diversity in *M. officinarum* and *M. caulescens* suggest that these two species are at similar stages of speciation. In the Sino-Himalayan area, *Mandragora caulescens* from the Himalaya-Hengduan Mountains has large, purple flowers and flower stalks elongated up to 40−60 cm long, whereas in the two studied populations of *M. chinghaiensis* from the Tibetan Plateau, the plants had small, yellow flowers and were cushioned in habit. We did not observe any intermediate individuals between these two taxa. *M. caulescens* grows in forests dominated by *Abies* and *Salix* species, or among shrubs at the forest edges. *M. chinghaiensis* habitats are plateau steppes, overgrazed meadows, and pika warrens dominated by herbs and small shrubs from such genera as *Potentilla*, *Aconitum*, *Pedicularis*, *Gentiana*, *Polygonum*, *Kobresia*, and *Przewalskia tangutica*. The fruits of *M. chinghaiensis* are hidden under leaves or sometimes underground. Like the Central Asian mandrake, *M. chinghaiensis* is nested within the *M. caulescens* clade indicating a very recent origin. However, in contrast to the latter, *M. chinghaiensis* is morphologically distinct and occupies habitats different from *M. caulescens* habitats. The habitats of the two Sino-Himalayan species are separated by only a short geographic distance (the minimum linear distance between known populations of *M. chinghaiensis* and *M. caulescens* is about 120 km, the former from Mangkang county and the latter from Chayu county of Xizang Province of Tibet). However, the two species occur at different altitudes. *M. chinghaiensis* in Mangkang county is located at 4300 m, the southern margin of the Tibetan Plateau or the northwestern margin of Hengduan Mountains. In contrast, *M. caulsecens* in Chayu county is never found at altitudes above 3900 m, and this location is closer to the center of the Hengduan Mountains. Thus, the origin of *M. chinghaiensis* cannot be attributed to long-distance dispersal or vicariance, and appears to be due to adaptive diversification within this lineage.

In summary, the results suggest that three lineages within the genus *Mandragora* have a very old history as separate taxonomic units. Two of these lineages appear to be species: (1) putative name *M. autumnalis* – distribution area covers the Mediterranean except for the eastern part (Israel and probably Lebanon and Syria); (2) putative name *M. officinarum* – the range includes Near East–Iran–Central Asia. The third lineage considered as a single species by Ungricht et al. (1998) should be split into two species following Kuang and Lu (1978): *M. caulescens* and *M. chinghaiensis*, both in Himalaya–Tibet.

In addition, the results suggest direct involvement of humans in *Mandragora* migration in ancient time. This involvement is known or presumed for many crop species or their ancestors (e.g. Zohary et al. 2012). Our study suggests that humans were a vector of dispersal for more species than we previously thought.

Acknowledgments

This project was supported by the CAS/SAFEA International Partnership Program for Creative Research Teams. We are grateful to Innokentiy Sinev for fruitful discussion of the results and Linda Olsvig-Whittaker for editing the manuscript English. This work would be impossible without the kind help of those people and organizations who provided samples of *Mandragora*. In particular, we would like to express our gratitude to Nicolay Orlovsky, Vitaliy Kuznetzov, Grigoriy Levin, Burcu Elçi, Gerard van der Weerden, and Juan Arroyo. Any new samples of known geographic origin will be greatly appreciated by the authors.

Disclosure statement

No potential conflict of interest was reported by the authors.

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