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Discovery of chloroplast capture in *Juniperus excelsa* complex by multi- locus phylogeny

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Abstract

Previous studies of nrDNA (nuclear DNA) of *Juniperus seravschanica* indicated its nuclear DNA (ITS) was from an ancestor of *J. polycarpos*. However, analysis of cpDNA (chloroplast DNA) suggested the taxon had derived its chloroplast from an ancestor of *J. foetidissima.* That study has been viewed as putative, because the ITS region is sometimes unreliable for the detection of ancestral hybrids due to concerted evolution and lineage sorting. The recent availability of several single copy nuclear genes (SCNGs) with primers specifically designed for *Juniperus* presented an opportunity to fully investigate this case of putative chloroplast capture. Three phylogenetic analyses using five SCNGs (*LHCA*4, *maldehy*, *myb*, *CnAIP*3 and 4*CL*), ITS region, and four cpDNAs (*pet*N- *psb*M, *trn*D-*trn*T, *trn*L-*trn*F and *trn*S-*trn*G) were performed on *J. seravschanica*, as well as other members of the *J. excelsa* complex: *J. excelsa*, *J. polycarpos*, and *J. p*. var. *turcomanica.* Analyses revealed incongruence between SCNGs, ITS region and cpDNA showing that *J. seravschanica* contains an ancestral *J. foetidissima*/ *J. thruifera* cp genome. In addition, the phylogenies indicate that the *J. excelsa* complex is composed of three distinct clades at the species level: *J. excelsa*, *J. polycarpos* and *J. seravschanica* and two varieties of *J. polycarpos*: *J. p.* var. *polycarpos* and *J. p.* var. *turcomanica.*

Keywords: *Juniperus,* ITS region, chloroplast DNA, single copy nuclear genes (SCNG), climate change, Pleistocene, distributions

Introduction

Climate change presents a new challenge to humankind (Scheffers *et al.*, 2016) , but in times past, climate change provided potential new habitats for *Juniperus* species. *Juniperus* is one of the most diverse genera of conifers with approximately 75 species (Adams and Schwarzbach, 2013; Adams, 2014). *Juniperus* grows on sites ranging from sand dunes near sea level (*J. maritima*, *J. communis* var. *megistocarpa*) to timberline (*J. communis* var. *depressa*, *J. zanonii*, etc. see Adams, 2014). *Juniperus* habitats include swamps, sand dunes, volcanic rock, ultra-mafic rock as well as limestone that is preferred by most juniper species (Adams, 2014). Junipers are often invasive species on disturbed sites such as those produced by glaciation and the subsequent creation of boulder strewn landscapes and terminal moraine. The glacial advances and retreats in the Pleistocene provided numerous new habitats for *Juniperus* (Adams, 1983; 2015; Wells, 1970). Thus, the cooler temperatures and often increased rainfall, converted deserts to semi-arid lands, which are prime habitat for many juniper species. In addition, *Juniperus* has evolved small, fleshy seed cones that are extensively utilized by birds that can disseminate the seeds to distant areas (cf. *J. bermudiana*, Bermuda Island - North Carolina, 1060 km; *J. brevifolia*, Azores - Portugal, 1,500 km). The spread of *Juniperus* by birds (especially section *Sabina*, with many species with soft, fleshy seed cones), and *J. communis* (sect. *Juniperus*), often assures that disturbed lands are colonized by juniper (see chpt. 8, p. 336, Adams, 2014 for a review).

The wide-spread nature of *Juniperus* often leads to sympatry and hybridization (Adams 2014). Hybridization and subsequent backcrossing to one of the parental species can result in the virtual elimination of the second's parent nuclear DNA, resulting in a taxon with nuclear DNA of one parental species, and the chloroplast of the other parental species (i.e., chloroplast capture). The idea of chloroplast capture is not new. Rieseberg and Soltis (1991) warned

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about chloroplast capture (both recent or ancient via hybridization) that could produce incongruent topologies in phylogenetic trees between nuclear and cp data. They presented evidence of chloroplast capture in 37 cases and, of those, 28 were well supported (see Table 1, in Rieseberg and Soltis, 1991). With the explosion of the use of nrDNA and cp markers, there are hundreds of examples of chloroplast capture today. A few examples of putative chloroplast capture include *Heuchera* (Soltis and Kuzoff, 1995), *Brassica napus - B. rapa* (Haider *et al.* 2009), and *Osmorhiza* (Yi *et al.* 2015). Tsitrone *et al.* (2003) proposed a model of chloroplast capture that provides some basis for the concept.

Recently, Munoz-Rodriguez *et al.*(2018) published a paper on the origin of sweet potato (*Ipomoea batatas*) and reconciling conflicting phylogenies between nuclear and chloroplast based phylogenies. They discuss two methods of possible capture in *Ipomoea batatas*, from *I. trifida.* One of the methods was hybridization of a male *I. batatas* (eg. pollen with no chloroplast) crossed with a female *I. trifida* (eg. egg with chloroplast). These hybrids could then backcross to *I. batatas* by pollen (lacking chloroplast organells) of *I. batatas.* The second method was asymmetrical hybridization, resulting in a hybrid with the nucleus of *I. batatas* and the chloroplast of *I. batatas* (see Fig, 4A, B, Munoz-Rodriguez *et al.* 2018). It should be noted in hybridization in *Juniperus* (and Cupressaceae in general), the chloroplast is transmitted via pollen (see Adams, Miller and Low, 2016).

There are only a few examples of chloroplast capture in conifers. In *Pinus* and other conifers, Hipkins *et al.* (1994) concluded that "past hybridization" and associated "chloroplast capture" can confuse the phylogenies of conifers. Bouille *et al.* (2011) found significant topological differences in phylogenetic trees based on cpDNA (vs. mtDNA sequences) in *Picea* that suggested organelle capture.

Adams, Schwarzbach and Tashev (2016) reported a case of putative chloroplast capture by plants of *J. sabina* in Bulgaria and northern Greece. The Balkan plants had nrDNA exactly the same as other *J. sabina* plants in other regions, but their cpDNA differed by only 6 MEs (SNPs + indels) from that of *J. thurifera*, but 36 MEs from typical *J. sabina* cpDNA. Adams (2016), examined four junipers in the *J. excelsa* complex (Farjon, 1992) using ITS regionand cpDNA (*pet*N-*psb*M, *trn*S-*trn*G, *trn*D-*trn*T and *trn*L-*trn*F) and found incongruent topologies between ITS region and cpDNA data sets that suggested two instances of chloroplast capture in the *J. excelsa* complex: *J. polycarpos* var. *turcomanica* seemed to have recently captured its chloroplast from *J. polycarpos* or an ancestor; and *J. seravschanica* appeared to possess an anciently captured chloroplast from an ancestor of *J. foetidissima*/*J. thurifera*.

However, ITS region may not always be reliable for the analysis of hybridization and introgression. Adams, Miller and Low (2016) examined the inheritance of nrDNA patterns in artificial hybrids between *Hesperocyparis arizonica* and *H. macrocarpa*, (closely related to *Juniperus*, Zhu *et al.*, 2018). The hybrids, based on quantative peak sizes in the sequencing chromatograms and PCO analysis, ordinated between *H. arizonica*, the paternal, chloroplast donor parent and the theoretic hybrid (with exactly equal peak heights at the 8 heterozygous sites) (Fig. 1). So, it is clear that there is some bias towards classifying hybrids, because they ordinate closer to one parent (*H. arizonica*) in this case, as they might be construed to be backcrosses or introgressants. In addition, the artificial hybrids could be placed into four groups (Fig. 2), suggesting chromosome linkage groups. Adams, Miller and Low (2016) concluded that classifying backcrosses, and introgressed individuals may underestimate the degree of introgression due to skewed inheritance towards one parent. They warned against over-reliance on nrDNA patterns when examining putative hybridization and introgression. He recent availability of several single copy nuclear genes with primers specific to *Juniperus* (Adams *et al.* 2009, Letelier *et al.* 2014) has presented an opportunity to fully investigate chloroplast capture by an ancestor of *J. seravschanica*.

Juniperus seravschanica is a part of the *J. excelsa* complex, one of the most difficult taxonomic groups of *Juniperus* (Farjon 1992). This complex consists of four morphologically cryptic taxa, and when recognized at the specific level are: *J. excelsa* M. Bieb., *J. polycarpos* K. Koch, *J. seravschanica* Kom and *J. turcomanica* B. Fedtsch. The ranks of these taxa have been changed several times (Marschall von Bieberstein 1800, 1808, Koch 1849, Boissier 1884, Fedtschenko *et al.* 1932, Komarov 1932, Riedl 1968, Zohary 1973, Browicz and Zielinsky 1982, Silba 1986, 1990, Farjon 1992, Assadi 1998, Schulz *et al.* 2005). Several studies have been performed on this complex using a variety of characters, including leaf essential oils, morphology, RAPDs (Randomly Amplified Polymorphic DNAs), isoenzymes and DNA sequences (Adams 1999, 2001, 2004, Adams *et al.* 2008, Hojjati et al 2009, Adams and Shanjani 2011, Douaihy *et al.* 2011, Adams and Hojjati 2012, 2013, Adams *et al.* 2014a, 2014b, 2014c, 2014d, 2016a, 2016b).

The primary purpose of this study was to investigate the case of putative chloroplast capture by an ancestor of *J. seravschanica* using sequences from 6 nuclear DNA regions (ITS 1 and 2; *LHCA*4, *maldehy*, *myb*, *CnAIP*3 and 4*CL*) and four chloroplast intergenic spacer regions (*pet*N- *psb*M, *trn*D-*trn*T, *trn*L-*trn*F and *trn*S-*trn*G).

TABLE 1. Location, Herbarium number and Accession Number of Accessions.

Taxon	Location	Herbarium number	Accession Number	
J. excelsa	Greece	8785 (BAYLU)	4CL	LC420805
			CnAIP3	LC420804
			ITS	LC420800
			LHCA4	LC420801
			maldehy	LC420802
			myb	LC420803
			petN-psbM	LC420796
			trnD-trnT	LC420797
			trnL-trnF	LC420798
			trnS-trnG	LC420799
J. excelsa	Greece	8786 (BAYLU)	4CL	LC420815
			CnAIP3	LC420814
			ITS	LC420810
			LHCA4	LC420811
			maldehy	LC420812
			myb	LC420813
			petN-psbM	LC420806
			trnD-trnT	LC420807
			trnL-trnF	LC420808
			trnS-trnG	LC420809
J. excelsa	Greece	14742 (BAYLU)	4CL	LC420825
			CnAIP3	LC420824
			ITS	LC420820
			LHCA4	LC420821
			maldehy	LC420822
			myb	LC420823
			petN-psbM	LC420816
			trnD-trnT	LC420817
			trnL-trnF	LC420818
			trnS-trnG	LC420819
J. excelsa	Bulgaria	13720 (BAYLU)	4CL	LC420835
			CnAIP3	LC420834
			ITS	LC420830
			LHCA4	LC420831
			maldehy	LC420832
			myb	LC420833
			petN-psbM	LC420826
			trnD-trnT	LC420827
			trnL-trnF	LC420828
			trnS-trnG	LC420829
J. excelsa	Bulgaria	13721 (BAYLU)	4CL	LC420845
			CnAIP3	LC420844
			ITS	LC420840
			LHCA4	LC420841
			maldehy	LC420842

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TABLE 1. (Continued)

Taxon	Location	Herbarium number	Accession Number	
			myb	LC420843
			petN-psbM	LC420836
			trnD-trnT	LC420837
			trnL-trnF	LC420838
			trnS-trnG	LC420839
J. polycarpos var. *polycarpos*	Azerbaijan	14162 (BAYLU)	4CL	LC420745
			CnAIP3	LC420744
			ITS	LC420740
			LHCA4	LC420741
			maldehy	LC420742
			myb	LC420743
			petN-psbM	LC420736
			trnD-trnT	LC420737
			trnL-trnF	LC420738
			trnS-trnG	LC420739
J. polycarpos var. *polycarpos*	Azerbaijan	14164 (BAYLU)	4CL	LC420755
			CnAIP3	LC420754
			ITS	LC420750
			LHCA4	LC420751
			maldehy	LC420752
			myb	LC420753
			petN-psbM	LC420746
			trnD-trnT	LC420747
			trnL-trnF	LC420748
			trnS-trnG	LC420749
J. polycarpos var. *polycarpos*	Azerbaijan	14166 (BAYLU)	4CL	LC420765
			CnAIP3	LC420764
			ITS	LC420760
			LHCA4	LC420761
			maldehy	LC420762
			myb	LC420763
			petN-psbM	LC420756
			trnD-trnT	LC420757
			trnL-trnF	LC420758
			trnS-trnG	LC420759
J. polycarpos var. *polycarpos*	Azerbaijan	14167 (BAYLU)	4CL	LC420775
			CnAIP3	LC420774
			ITS	LC420770
			LHCA4	LC420771
			maldehy	LC420772
			myb	LC420773
			petN-psbM	LC420766
			trnD-trnT	LC420767
			trnL-trnF	LC420768
			trnS-trnG	LC420769

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TABLE 1. (Continued)

Taxon	Location	Herbarium number	Accession Number	
J. polycarpos var. *polycarpos*	Azerbaijan	14168 (BAYLU)	4CL	LC420785
			CnAIP3	LC420784
			ITS	LC420780
			LHCA4	LC420781
			maldehy	LC420782
			myb	LC420783
			petN-psbM	LC420776
			trnD-trnT	LC420777
			trnL-trnF	LC420778
			trnS-trnG	LC420779
J. polycarpos var. *turcomanica*, Bajgiran	Bajgiran, Khorassan, Iran	12802 (BAYLU)	4CL	LC420735
			CnAIP3	LC420734
			ITS	LC420730
			LHCA4	LC420731
			maldehy	LC420732
			myb	LC420733
			petN-psbM	LC420726
			trnD-trnT	LC420727
			trnL-trnF	LC420728
			trnS-trnG	LC420729
J. polycarpos var. *turcomanica*	Turkmenistan	8757 (BAYLU)	4CL	LC420695
			CnAIP3	LC420694
			ITS	LC420690
			LHCA4	LC420691
			maldehy	LC420692
			myb	LC420693
			petN-psbM	LC420686
			trnD-trnT	LC420687
			trnL-trnF	LC420688
			trnS-trnG	LC420689
J. polycarpos var. *turcomanica*	Turkmenistan	8758 (BAYLU)	4CL	LC420705
			CnAIP3	LC420704
			ITS	LC420700
			LHCA4	LC420701
			maldehy	LC420702
			myb	LC420703
			petN-psbM	LC420696
			trnD-trnT	LC420697
			trnL-trnF	LC420698
			trnS-trnG	LC420699
J. polycarpos var. *turcomanica*	Turkmenistan	8759 (BAYLU)	4CL	LC420715
			CnAIP3	LC420714
			ITS	LC420710
			LHCA4	LC420711
			maldehy	LC420712
			myb	LC420713

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TABLE 1. (Continued)

Taxon	Location	Herbarium number	Accession Number	
			petN-psbM	LC420706
			trnD-trnT	LC420707
			trnL-trnF	LC420708
			trnS-trnG	LC420709
J. polycarpos var. turcomanica	Turkmenistan	8760 (BAYLU)	4CL	LC420725
			CnAIP3	LC420724
			ITS	LC420720
			LHCA4	LC420721
			maldehy	LC420722
			myb	LC420723
			petN-psbM	LC420716
			trnD-trnT	LC420717
			trnL-trnF	LC420718
			trnS-trnG	LC420719
J. sabina	Azerbaijan	14316 (BAYLU)	4CL	LC420915
			CnAIP3	LC420914
			ITS	LC420910
			LHCA4	LC420911
			maldehy	LC420912
			myb	LC420913
			petN-psbM	LC420906
			trnD-trnT	LC420907
			trnL-trnF	LC420908
			trnS-trnG	LC420909
J. sabina	Azerbaijan	14317 (BAYLU)	4CL	LC420925
			CnAIP3	LC420924
			ITS	LC420920
			LHCA4	LC420921
			maldehy	LC420922
			myb	LC420923
			petN-psbM	LC420916
			trnD-trnT	LC420917
			trnL-trnF	LC420918
			trnS-trnG	LC420919
J. seravschanica	Pakistan	8483 (BAYLU)	4CL	LC420675
			CnAIP3	LC420674
			ITS	LC420670
			LHCA4	LC420671
			maldehy	LC420672
			myb	LC420673
			petN-psbM	LC420666
			trnD-trnT	LC420667
			trnL-trnF	LC420668
			trnS-trnG	LC420669

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TABLE 1. (Continued)

Taxon	Location	Herbarium number	Accession Number	
J. seravschanica	Pakistan	8484 (BAYLU)	4CL	LC420685
			CnAIP3	LC420684
			ITS	LC420680
			LHCA4	LC420681
			maldehy	LC420682
			myb	LC420683
			petN-psbM	LC420676
			trnD-trnT	LC420677
			trnL-trnF	LC420678
			trnS-trnG	LC420679
Juniperus seravschanica	Kazakhstan	8224 (BAYLU)	4CL	LC420645
			CnAIP3	LC420644
			ITS	LC420640
			LHCA4	LC420641
			maldehy	LC420642
			myb	LC420643
			petN-psbM	LC420636
			trnD-trnT	LC420637
			trnL-trnF	LC420638
			trnS-trnG	LC420639
Juniperus seravschanica	Kazakhstan	8225 (BAYLU)	4CL	LC420655
			CnAIP3	LC420654
			ITS	LC420650
			LHCA4	LC420651
			maldehy	LC420652
			myb	LC420653
			petN-psbM	LC420646
			trnD-trnT	LC420647
			trnL-trnF	LC420648
			trnS-trnG	LC420649
Juniperus seravschanica	Kazakhstan	8226 (BAYLU)	4CL	LC420665
			CnAIP3	LC420664
			ITS	LC420660
			LHCA4	LC420661
			maldehy	LC420662
			myb	LC420663
			petN-psbM	LC420656
			trnD-trnT	LC420657
			trnL-trnF	LC420658
			trnS-trnG	LC420659

TARI: Herbarium of Research institute of Forests & Rangelands, HWANRR: Herbarium of West Azerbaijan Natural Resource Research Center, BAYLU: Baylor University Herbarium, Robert P. Adams, collector, TMUH: Tarbiat Modares University Herbarium.

A secondary purpose was to produce a well-supported phylogeny of the *J. excelsa* complex, with emphasis on resolving the specific or infraspecific levels of the four taxa in the complex.

Materials and Methods

Plant materials

Fresh leaves (~1g) were field collected (R. P. Adams), placed in 20 g activated silica gel, and desiccated for 72 h. Desiccated leaves were removed from silica and stored, frozen (-20°C) until DNA extraction. Voucher specimens (see Table 1 sample sizes) are deposited at BAYLU, Baylor University Herbarium.

DNA isolation, PCR amplification, and sequencing

Total genomic DNA was extracted from dried or fresh leaf tissues using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). The purity and quantity of genomic DNA were determined by using 0.8 % agarose gel electrophoresis. After isolation, DNAs were stored at -20°C prior to amplification. Ten single copy nuclear genes (SCNGs): type IV chlorophyll binding protein (*LHCA*4), malate dehydrogenase (*maldehy*), Myb transcription factor (*myb*), ABI3-interacting protein gene, 4-coumarate CoA ligase (*4CL*), abscisic acid-insensitive 3 (*CnABI3*), GTP binding protein gene (*cc13333*), chalcone synthase (*chs*) and heat shock protein (*hsp*) (Adams *et al.* 2009, Letelier *et al.* 2014) were tested to determine if they were informative in distinguishing *J. excelsa*, *J. polycarpos*, *J. p.* var. *turcomanica* and *J. seravschanica*. Five (5) SCNGs (*LHCA4*, *maldehy*, *myb*, *CnAIP3* and *4CL*) were found to be informative and were subsequently used for phylogenetic study.

In addition, the ITS and four chloroplast intergenic spacers: *pet*N-*psb*M, *trn*D-*trn*T, *trn*L- *trn*F and *trn*S-*trn*G (Adams *et al.* 2009, Adams and Kauffmann 2010) were amplified and sequenced.

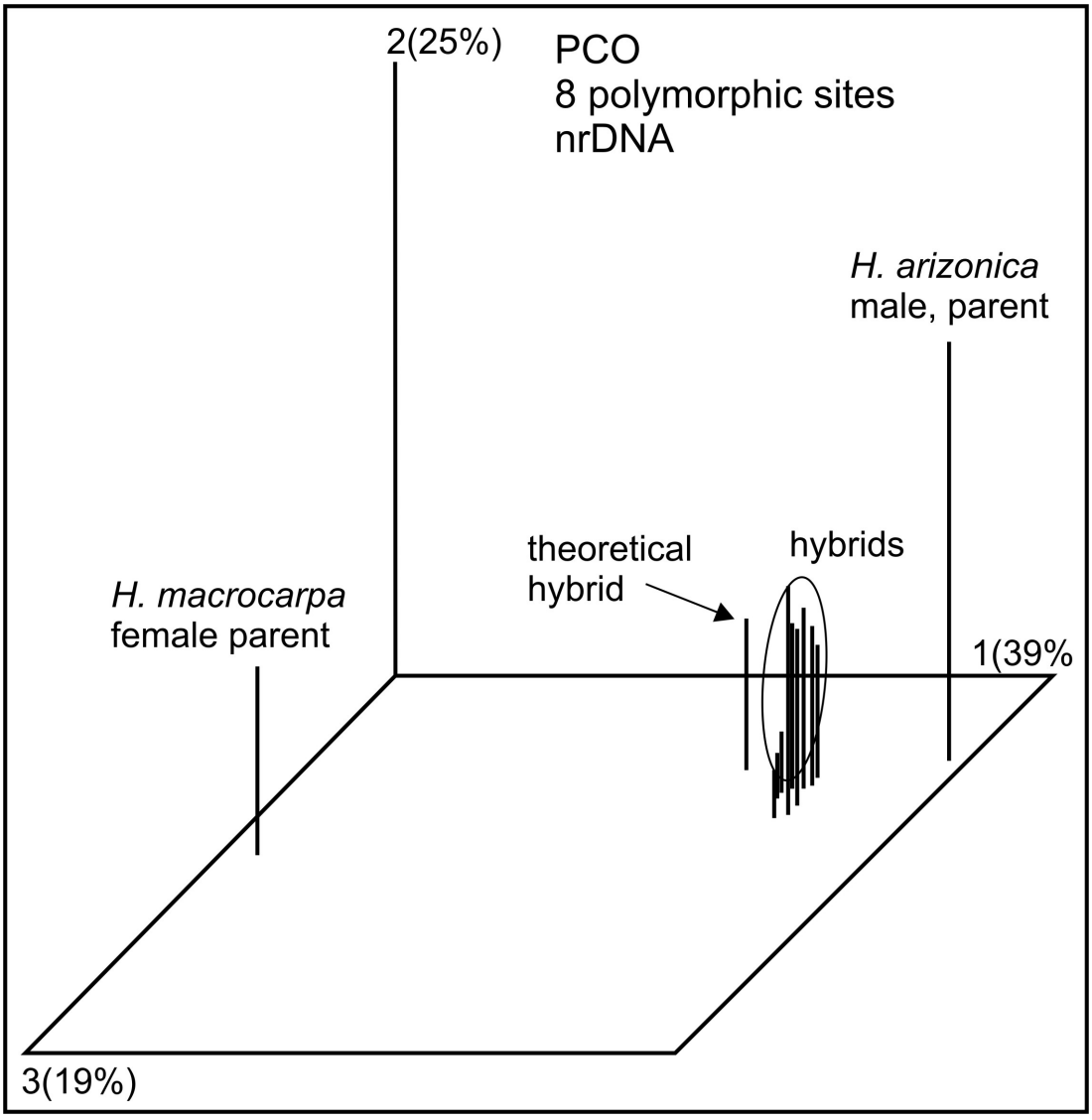

FIGURE 1. Principal coordinates ordination (PCO) of *H. arizonica* (pollen parent), *H. macrocarpa* (female parent), 18 artificial hybrids and a theoretical hybrid, based on 8 polymorphic sites using quantitative sequencing peak heights data. Adapted from Adams *et al.* 2016.

DNA amplifications were performed in 30 µl volumes containing 9 µl genomic DNA (4 ng/µl), 21 µl master mix containing 15 µl 2x buffer (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used), 1.8 µM each primer and 1.0 unit Epi-Centre Fail- Safe Taq polymerase. The reaction mixtures were amplified in a PTC-100, MJ Research Thermal Cycler. The PCR was subjected to purification by agarose gel electrophoresis (1.5% agarose, 82 v., 40 min.). In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit. The gel purified DNA band with the appropriate primers was sent to McLab Inc. (South San Francisco, CA) for sequencing in both forward and reverse directions. Sibling samples were sequenced as controls on sequencing quality.

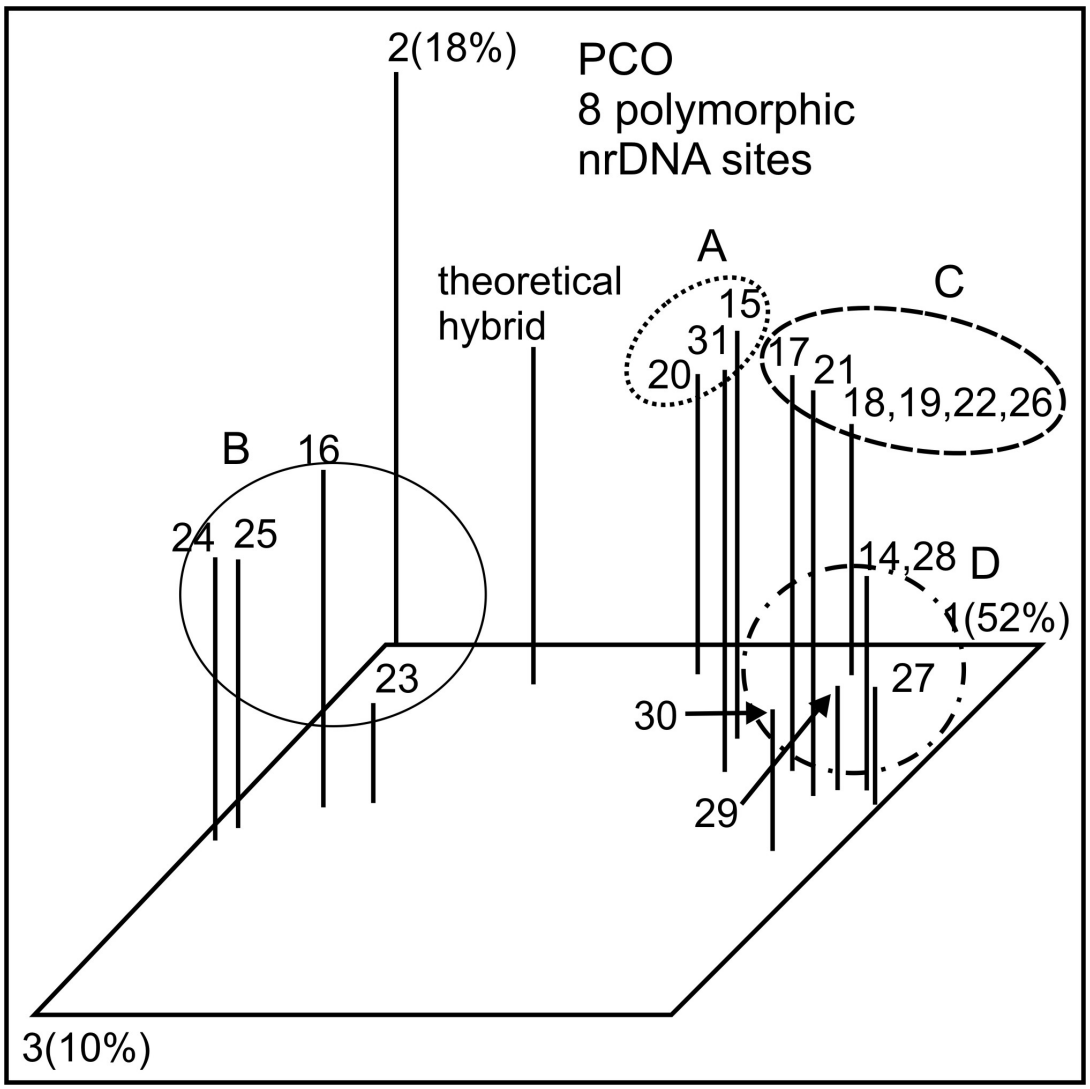

FIGURE 2. Principal coordinates ordination (PCO) of 18 artificial hybrids, and a theoretical hybrid, based on 8 polymorphic sites using quantitative sequencing peak heights data, showing 4 groups (A,B,C,D). Adapted from Adams *et al.* 2016.

Alignment and phylogenetic analyses

Each of the single datasets was aligned using the web-based version of MUSCLE (Edgar 2004; http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters followed by manual adjustment. Aligned Sequences of all data sets contained indels that were treated as missing data in phylogenetic analyses. Phylogenetic relationships were inferred using maximum parsimony (MP) and maximum likelihood (ML) methods, as well as Bayesian inference (BI). Parsimony analyses were conducted using PAUP* version 4.0b10 (Swofford 2002). The heuristic search option was employed for each dataset, using tree bisection-reconnection (TBR) branch swapping, with 100 replications of random addition sequence and an automatic increase in the maximum number of trees saved. Branch support values were estimated using a full heuristic search with 1,000 bootstrap replicates (Felsenstein 1985) each with simple addition sequence. In Bayesian analyses, models of sequence evolution were selected using the program MrModeltest version 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) (Posada and Buckley 2004). This program indicated HKY for ITS region and GTR+I for single copy nuclear genes and chloroplast DNA as the best model

for nucleotide substitution. The program MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003) was used for the Bayesian phylogenetic analyses. Posteriors on the model parameters were estimated from the data, using the default priors. The analysis was carried out with 10 million generations, using the Markov chain Monte Carlo (MCMC) search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns = 2) each with four Markov chains and trees sampled every 1000 generations. The first 25% trees were discarded as burn-in, and the remaining trees were used to build a 50% majority rule consensus tree accompanied with posterior probability (PP) values. Maximum likelihood (ML) analyses were performed using raxmlGUI (Silvestro and Michalak 2012) and the same models of sequence evolution used in Bayesian analyses. Parametric bootstrap values for ML were calculated in raxmlGUI based on 1000 replicates with one search replicate per bootstrap replicate. Tree visualization was carried out using TreeView version 1.6.6 (Page 2001).

Results

Phylogenetic analysis of 3882 nucleotide sites from five single copy nuclear genes (*LHCA*4, *maldehy*, *myb*, *CnAIP*3 and 4*CL*) yielded 61 potentially parsimony-informative nucleotide sites. The Bayesian tree shows *J. excelsa*, *J. seravschanica*, and *J. p.* var. *polycarpos* in distinct clades with very strong support (Fig. 3). The *J. excelsa* clade is quite distinct from a strongly supported clade (posterior possibility=1) that contains *J. seravschanica*, *J. p*. var. *polycarpos* and *J. p*. var. *turcomanica*. *Juniperus p*. var. *polycarpos* and *J. p*. var. *turcomanica* form a weakly supported clade (PP=0.53) that is linked with the *J. seravschanica* clade (Fig. 3).

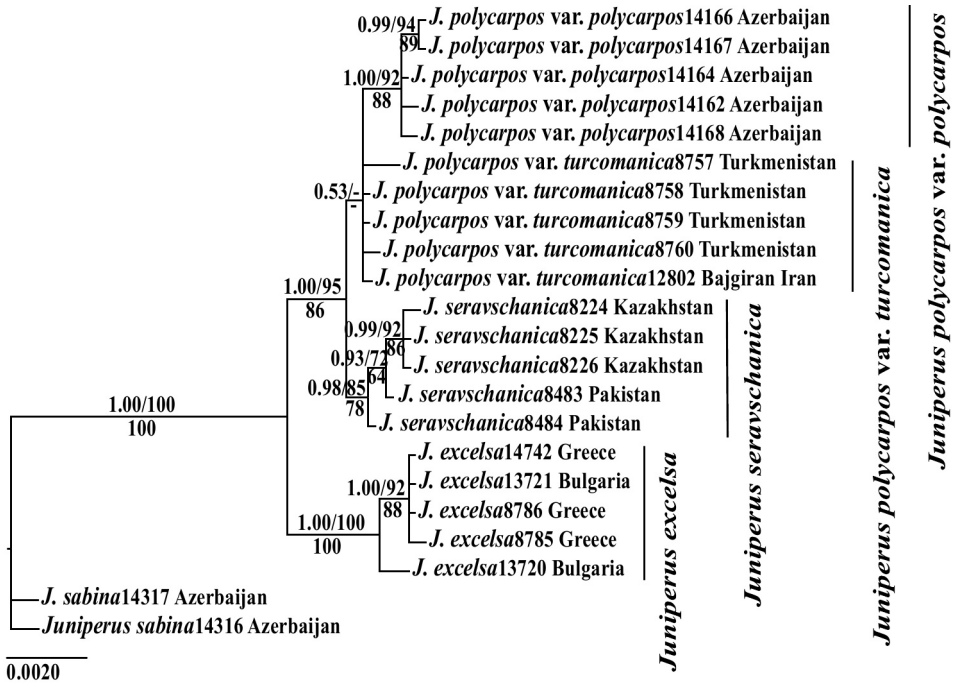

FIGURE 3. Fifty percent majority rule consensus Bayesian tree based on single copy nuclear genes (*LHCA4*, *maldehy*, *myb*, *CnAIP3* and *4CL*) combined data. Numbers above branches are Bayesian posterior possibilities (before slashes) and maximum likelihood bootstrap Support values (after slashes), while numbers under branches are maximum parsimony bootstrap support values. Values<50 % are not shown. CI= 0.886 RI= 0.951.

Sequencing the ITS region of 1272 nucleotide sites revealed 18 potentially parsimony- informative nucleotide sites, which is only 29% of the information found in the SCNGs (61 informative SNPs). The tree, using nrDNA ITS, produced two major clades (Fig. 4): a clade with very strong support, that contains *J. excelsa* and *J. p.* var. *turcomanica,* and a clade with a moderate support composed of *J. p.* var. *polycarpos* and *J. seravschanica*. However, there was strong support for the *J. p*. var. *polycarpos* clade (Fig. 4).

Examination of the 3171 nucleotide sites of *pet*N-*psb*M, *trn*D-*trn*T, *trn*L-*trn*F and *trn*S-*trn*G data resulted in the discovery of 35 potentially parsimony-informative sites. The major trend in the cpDNA tree is the distinction of *J.*

seravschanica in a clade with strong support (Fig. 5). In addition, *J. excelsa* is in a well-supported clade, sister to a clade that includes *J. p.* var. *turcomanica* and *J. p.* var. *polycarpos*. The Azerbaijan samples of *J. p.* var. *polycarpos* are in a well-supported clade. However, this tree (Fig. 5, cpDNA), was incongruent with both ~~the~~ previous trees (Fig. 3, SCNG; Fig. 4. nrDNA).

Discussion

The three trees [SCNG (Fig. 3), nrDNA (Fig 4) and cpDNA (Fig. 5)], are incompatible with each other; mainly due to the position of *J. seravschanica*. Comparing SCNGs (Fig. 3) and cpDNA (Fig. 5) trees, it appears that a "chloroplast capture", evolutionary event occurred in ancestral *J. seravschanica*. That event caused the taxon's to occupy different positions in the SCNG and chloroplast trees. Chloroplast capture, the introgression of a chloroplast from one species into another, has been frequently suggested as the explanation for inconsistencies between gene trees based on nuclear and cytoplasmic markers in plants (Tsitrone *et al.* 2003). Our finding that supports a hybrid origin for *J. seravschanica* is in agreement with Adams (2016), who concluded that *J. seravschanica* appears to possess an anciently captured chloroplast from an ancestor of *J. foetidissima*.

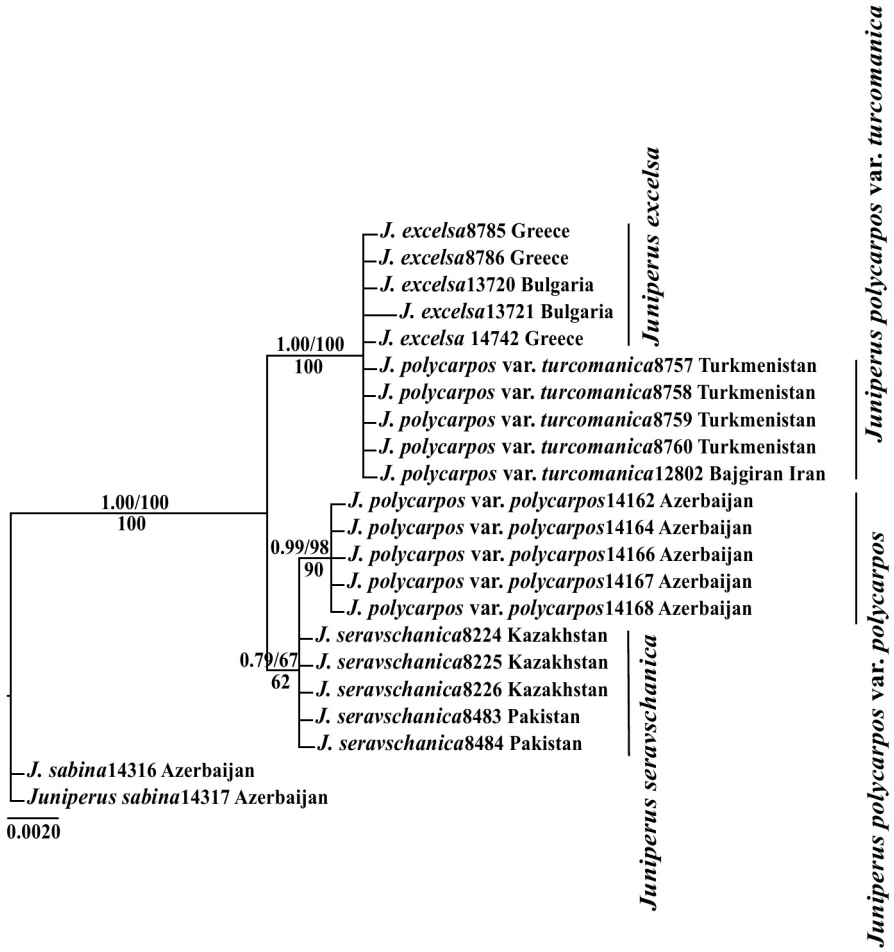

FIGURE 4. Fifty percent majority rule consensus Bayesian tree based on ITS region data. Numbers above branches are Bayesian posterior possibilities (before slashes) and maximum likelihood bootstrap support values (after slashes), while numbers under branches are maximum parsimony bootstrap support values. Values<50 % are not shown. CI= 1.000 RI= 1.000.

If one compares the position of *J. p.* var. *polycarpos* and *J. p.* var. *turcomanica* in all three trees, both SCNG (Fig. 3) and cpDNA (Fig. 3) trees have these varieties in a common clade. However, nrDNA ITS differs by having *J. p*. var. *turcomanica* in a strongly supported clade with *J. excelsa* (Fig. 4). Because the SCN genes data set contains many more informative SNPs (61) than ITS region (18 SNPs), it is prudent to favor the acceptance of the SCNG phylogeny. Adams (2016), using ITS region and cpDNA, noted the incongruence between ITS region and cpDNA and concluded that *J.*

p. var. *turcomanica* was of ~~the~~ hybrid origin involving *J. excelsa*. It seems likely, in that instance, incomplete lineage sorting of nrDNA masked the true relationships that are supported by SCNGs (Fig. 3).

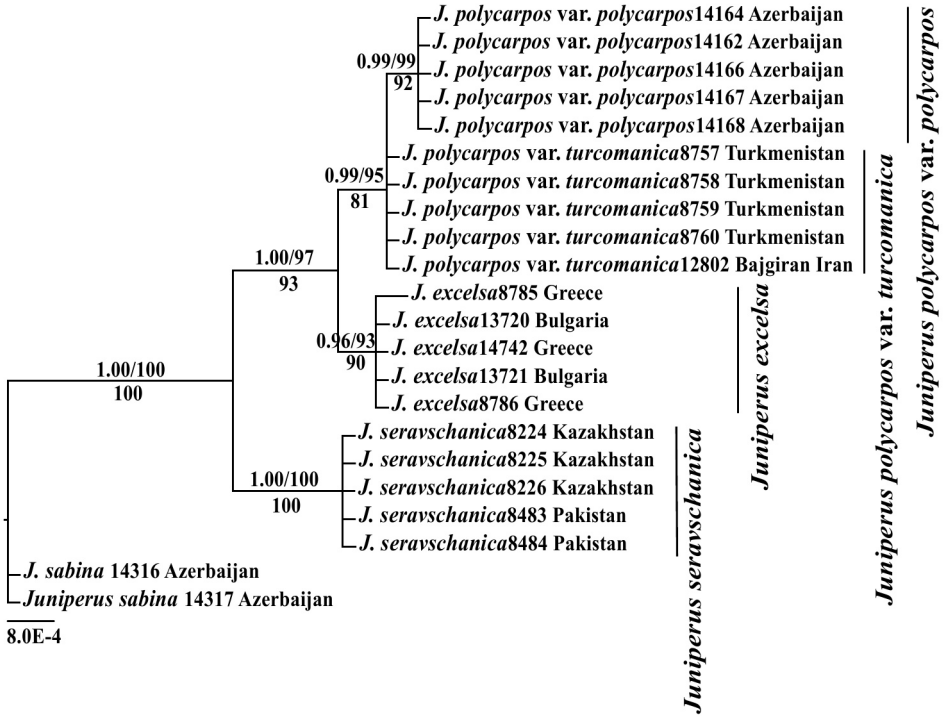

FIGURE 5. Fifty percent majority rule consensus Bayesian tree based on plastid *pet*N-*psb*M, *trn*D-*trn*T, *trn*L-*trn*F, *trn*S-*trn*G intergenic spacers combined data. Numbers above branches are Bayesian posterior possibilities (before slashes) and maximum likelihood bootstrap support values (after slashes), while numbers under branches are maximum parsimony bootstrap support values. Values<50 % are not shown. CI= 0.973 RI= 0.992.

Pleistocene climate and range expansion of ancestral *J. seravschanica*, *J. polycarpos,* and *J. foetidissima*

Information concerning the impact of climate changes on the vegetation of the middle east, especially Iran, is very limited (see Kehl, 2009 for a "State of the Knowledge" review). In contrast the paleoecology data from North America, based on pack-rat middens, tree rings, and pollen cores from sediments, is voluminous, compared to the paucity of paleo climate data in the middle east. Kehl (2009) found a few generalizations about Iran climate: the climate in northern and western Iran, was dry and cold in the stadials and moist and warm during the interstadials. However, Ebrahimi and Seif (2016) recently published a very relevant paper on Equilibrium-Line Altitudes (ELAs) in the late quaternary glaciers in Iran. They reported that ELAs were lowered by 1433m during the last glacial maximum (LGM). Because of the increasing mass of glaciers in LGM, the ELAs are disproportionally extended as the cool air from the glacier cools the area down-slope, so that ELAs may be extended into quite warmer areas. Thus, one can not assume that the vegetation zones were shifted downward by 1433m. Adams (1983) reviewed the literature on the effects of the last glacial (Wisconsin) in North America and reported vegetation zones were lowered by 300 to 1100 m throughout the southwest and Great Basin, USA in the period of 13,500 to 11,000 ybp. Wells (1970) suggested a downward displacement of *J. scopulorum* (in Colorado, Utah, Wyoming) of about 600m. It seems likely that ELAs in Iran do indicate some displacement of vegetation (and *Juniperus*) to lower and more mesic environments during glacial times, but exactly how much will have to await additional data. However, even lowering the ranges *Juniperus* species by a few hundred meters could make large differences in their ranges and provide "bridges" between mountain ranges for species to expand their distribution.

The distributions of *J. foetidissima, J. polycarpos* and *J. seravschanica* are shown in figure 6. The ranges of *J. foetidissima* and *J. polycarpos* overlap in Turkey, Armenia, Georgia and Azerbaijan (Fig. 6). Notice the presence of putative hybrids between *J. polycarpos* and *J. seravschanica* (P x S, Fig. 6) in Turkey and NW Iran, suggestive of a trail of germplasm towards the main populations of *J. seravschanica* in southern Iran and from Pakistan to Kazakhstan (Fig. 6). It would seem likely that ancestral *J. foetidissima* and *J. polycarpos* could have been sympatric on many occasions in the Turkey-Azerbaijan regions during the past.

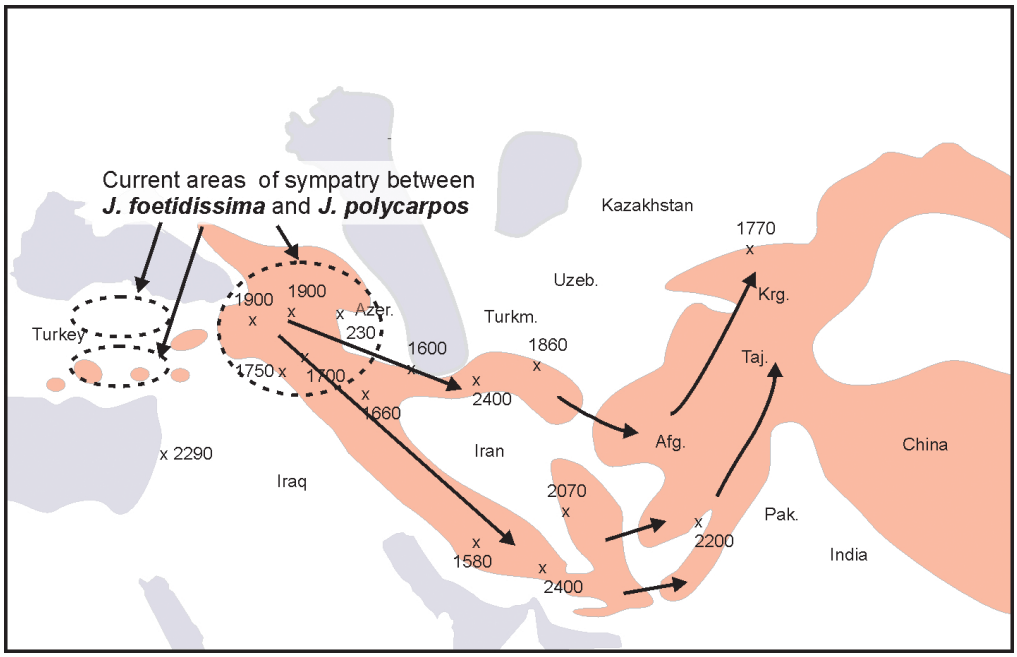

FIGURE 7. A hypothetical range expansion corridor (>2000m elevation) for ancestral *J. seravschanica* into the present range of *J. seravschanica* (depicted by arrows). Elevations (in meters) are shown at several *J. polycarpos* and *J. seravschanica* populations sampled in this study. See text for discussion.

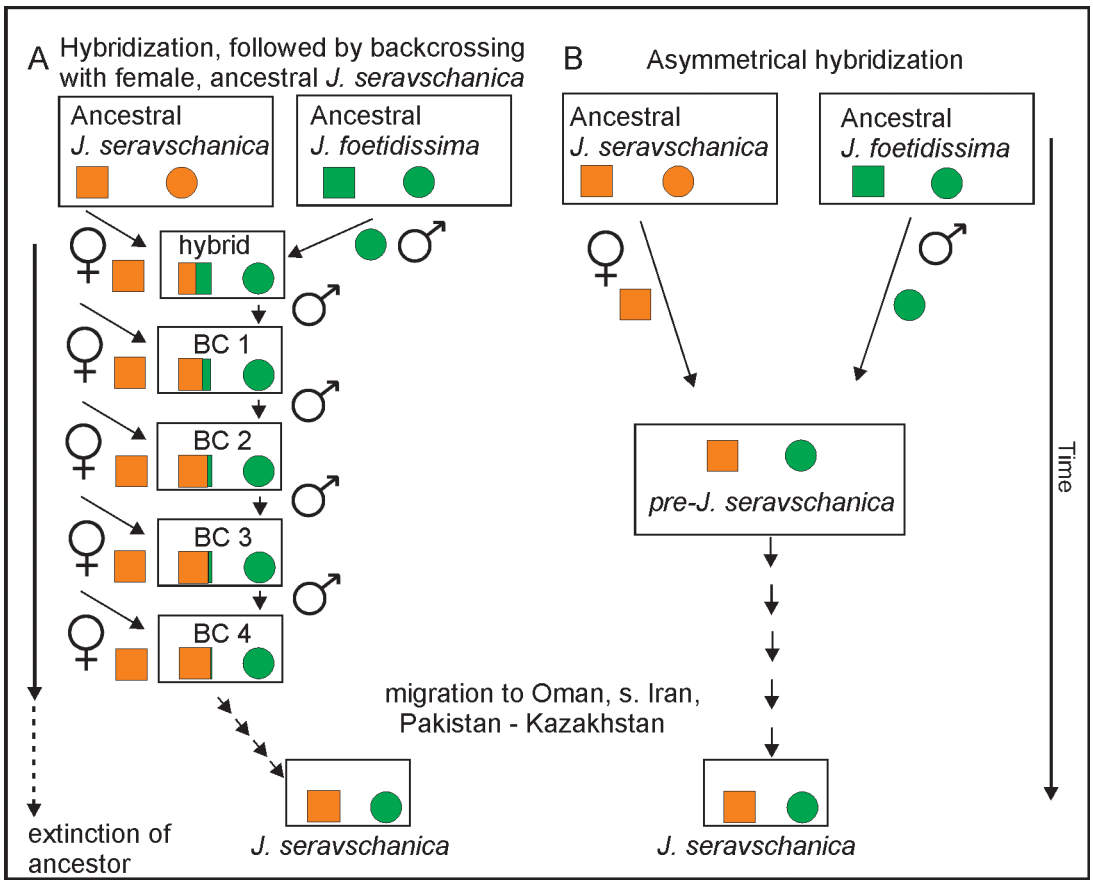

FIGURE 8. A. Two hypothetic methods of chloroplast capture. A. Hybridization, followed by backcrossing with female, ancestral *J. seravschanica*. B. Asymmetrical hybridization.

This new hybrid could have readily invaded suitable, newly formed environments during any number of the Pleistocene glacial cycles. A hypothetical migration of ancestral- *J. seravschanica* into its present range is shown

in Fig. 7. Gaps between high plateaus and mountain ranges are small (Fig. 7), and would be easily bridged by bird-disseminated ancestral—*J. seravschanica*.

Two theories seem possible for the chloroplast capture in the present example. The first is that hybridization and chloroplast capture between ancestral *J. foetidissima* and *J. polycarpos* occurred by hybridization, followed by backcrossing (introgression) with female, ancestral *J. seravschanica*. (Fig. 8A) until nearly all traces of the nuclear genes from *J. foetidissima* were eliminated. The second is asymmetrical hybridization (see Munoz-Rodriguez *et al.* 2018) in which only the parental nucleus of ancestral *J. seravschanica* and the chloroplast of ancestral *J.foetidissima* are placed into the asymmetrically produced hybrid (Fig. 8B). Additional research will be needed to determine if one of the theories (or another) is correct.

Perspective on the use of SCNGs

This study has shown that the use of single copy nuclear genes (SCNGs) provide excellent data for the determination of chloroplast capture, as well as their use in the analysis of a complex taxonomic problem. In many studies, only a few molecular markers are employed in producing phylogenies of plants. In contrast to the predominantly utilized cpDNA and ITS region data, the nuclear genome contains a vast reservoir of genes that potentially harbor abundant phylogenetic signal. Due to the limitations inherent in cpDNA and ITS markers and because of the phylogenetic potential of single-copy nuclear genes, they are increasingly being used in systematic studies. Some of the principal advantages of single- copy nuclear genes are (1) bi-parental inheritance; (2) co-occurrence of introns and exons within the same gene, yielding characters that evolve at different rates, and thus can provide phylogenetic data at different levels; and (3) a large number of independent markers (Alvarez *et al.* 2008, Li *et al.* 2017). Furthermore, SCNGs are ideal for detecting hybridization, introgression, and ancient allo-polyploidization events, whereas ITS region markers, which undergo concerted evolution, appear to be less reliable for detecting ancient hybridization and, especially, introgression events (Adams, Miller and Low, 2016; Duarte *et al.* 2010). NexGen sequencing, with the capability of finding and analyzing numerous SCNGs, will surely produce more robust taxonomies and evolutionary studies.

On a taxonomic note, the expanded data set used in this study supports the treatment of the *J. excelsa* complex as composed of three species: *J. excelsa*, *J. seravschanica, and J. polycarpos* with two varieties, *J. p.* var *polycarpos* and *J. p.* var. *turcomanica. Juniperus excelsa* is distributed in areas of Europe with a milder, Mediterranean climate (Fig. 6). *Juniperus seravschanica* is grows in semi-arid environments in central Asia and Pakistan to SE Iran (Fig. 6). *J. p.* var. *polycarpos* is found in temperate to semi-arid sites in Turkey, Caucasus, Lebanon and NW Iran (Fig. 6). In contrast, *J. p.* var. *turcomanica* grows in more- mesic areas of the Elburz and Kopet Dag mountains of Iran and Turkmenistan (Fig. 6).

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