Hybridization and introgression between *Juniperus communis* var. *saxatilis* and var. *hemisphaerica* in southeastern Spain

Robert P. Adams and Sam T. Johnson
Biology Department, Baylor University, Waco, TX, 76798, USA, robert_adams@baylor.edu

Carlos Salazar-Mendias
Departamento de Biología Animal, Biología vegetal y Ecología, Universidad de Jaén, 23071 Jaén, Spain.

and

Joaquín Altarejos
Departamento de Química Inorgánica y Orgánica, Universidad de Jaén, 23071 Jaén, Spain.

ABSTRACT

An investigation of variation between *J. communis* var. *hemisphaerica* and var. *saxatilis* in Sierra de Baza, Cazorla, Mágina, Huéscar, S. Nevada (SE Spain) and Sicily revealed 5 SNPs and 1 indel (in nrDNA) that distinguished the varieties. Chloroplast (cp) petN-psbM did not clearly separate var. *hemisphaerica* and var. *saxatilis*, however trnL-trnF contained 4 SNPs and 2 indels that distinguished *hemisphaerica* and *saxatilis.* All plants from Sierra de Baza, Cazorla, Mágina, Huéscar and S. Nevada were var. *hemisphaerica* based on ITS data. Surprisingly, these var. *hemisphaerica* plants each contained var. *saxatilis* chloroplasts, suggesting chloroplast capture by an ancient evolutionary event. Hybrids (*hemisphaerica* x *saxatilis*) were found in four of the ten S. de Baza samples. Two plants from S. Nevada contained a SNP base characteristic of var. *saxatilis* indicating introgression. Published on-line www.phytologia.org *Phytologia 102(2): 83-87 (June 24, 2020).* ISSN 030319430.

KEY WORDS: *Juniperus communis* var. *saxatilis*, *J. c.* var. *hemisphaerica*, Sierra de Baza, Sierra Nevada, southeastern Spain, Baetic mountains, hybridization, introgression, nrDNA, cpDNA, trnL-trnF.

Juniperus communis var. *hemisphaerica* (J. & C. Presl.) Parl. was described from a shrub growing on the flanks of Mt. Etna, Sicily (Adams 2014). Analyses of nrDNA (ITS) revealed that its ITS differs from other *J. communis* varieties by 5 SNPs and 1 indel (Adams and Schwarzbach, 2013), making its DNA very distinctive among *J. communis* varieties. Yet, the discovery of additional var. *hemisphaerica* populations in Europe has been futile (Adams 2014). Prostrate juniper plants in Sierra Nevada, Spain, are often treated as var. *hemisphaerica*, but they are not small, globose shrubs as in Sicily. However, their ITS DNA is identical to that of var. *hemisphaerica* from Sicily (Adams and Espeut 2020).

Recently, DNA *J. communis* 'var. *hemisphaerica'* plants from the Pyrenees were analyzed (Adams and Espeut 2020). Nearly all the plants were prostrate and all but one had identical var. *saxatilis* nrDNA for the informative 5 SNPs and indel, except plant *15401*, that had 3 SNPs from *hemisphaerica* and 2 SNPs and the indel from var. *saxatilis* (Adams and Espeut 2020). Plant 15401 was judged to be a hybrid: *J. c.* var. *hemisphaerica* x *J. c.* var. *saxatilis*, despite the fact that no *J. c.* var. *hemisphaerica* were found from that population.

To investigate the more southern range of putative *J. c.* var. *hemisphaerica*, we initiated this study using nrDNA (ITS) and more chloroplast markers: petN-psbM trnL-trnF, trnS-trnG, and trnD-trnT.

MATERIAL AND METHODS

Specimens used in this study:
Juniperus communis var. *hemisphaerica*:
France:
Pyrenees: Lab Acc. *Robert P. Adams 15401,15402,15403,15404*, horizontal plants, on limestone. Opoul, Pyrénées-orientales, France, Mount Montoulier de Périllos, 42.907° N 02.943° E, 600 m, Feb. 2018, coll. *Marc Espeut, ns 1,2,3,4*, 23
Pyrenees: Lab. Acc. *Robert P. Adams 15581-15600*(20),horizontal plants, on limestone. Opoul, Pyrénées-orientales, France, Mount Montoulier de Périllos, 42° 54' 42.82" N, 2° 50' 37.47" W. 630 m, 8 March 2019, Coll. Marc Espeut ns 1-20, Lab Acc. all horizontal, except 15598 was a sub-shrub.
Spain:
Sierra Nevada: Coll. *Robert P. Adams 7194-7195.* prostrate to 0.5m tall, with *J. sabina*. Sierra Nevada, Granada province, Spain. 37˚ 06' 17" N 3˚ 24' 51" W, 2100m, 20 Oct. 1993.
Sierra Nevada: Coll. *Robert P. Adams 15702-15703* prostrate to 0.2m tall x 3-5 m wide, abundant at Ski area, Sierra Nevada, Granada province, Spain. 37° 06' 02.54" N, 03° 24' 00.55" W 2024 m, 6 June 2019,
Sierra de Baza: Coll. *Robert P. Adams 15692-15701,* with Carlos Salazar Mendias, Joaquin Altarejos, prostrate shrubs (0.2m high x 3 to 5 m wide) to very decumbent shrubs (0.5m x 3 to 5 m wide), common on limestone with *J. sabina.* Sierra de Baza, medium elev. site 2. 37° 22' 28" N, 02° 51' 03" W. 2024 m, 4 June 2019, Granada province, Spain
Sierras de Cazorla, Segura y Las Villas: Lab Acc. *Robert P. Adams 13052*, Coll. Joaquin Altarejos JA-089, shrub, 0.5m x 1 m wide. 37º 04' 34.16" N, 2º 55' 26.54" E, elev. 1,350m, 1 Oct 2011, Las Sierras de Cazorla, Segura y Las Villas, Jaén province, Spain and Lab Acc. *Robert P. Adams 13053*, Coll. Joaquin Altarejos shrub, 0.25m x 1 m wide. JA-090, 37º 55' 31.60" N, 2º 50' 25.13" E, elev. 1,350m, 1 Oct 2011, Sierra de Cazorla, Jaén province, Spain.
Sierra de Huéscar: Lab Acc. *Robert P. Adams 13054*, Coll. Joaquin Altarejos JA-091, prostate shrub, 0.1m x 1 m wide. 38º 01' 21.57" N, 2º 34' 48.05" E, elev. 1,350m, 1 Oct 2011, Sierra de Huéscar, Granada province, Spain.
Sierra Mágina: Lab Acc. *Robert P. Adams 13055*, Coll. Joaquin Altarejos JA-092, shrub, 0.25m x 1 m wide. 37º 44' 34.85" N, 3º 27' 10.82" E, elev. 1,350m, 1 Oct 2011, Sierra Mágina, Jaén province, Spain.
Voucher specimens are deposited in BAYLU and JAEN herbaria.

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20º C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (cp markers) or K (nrDNA) (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. See Adams, Bartel and Price (2009) for the ITS primers utilized. The primers for petN-psbM, trn: SG, LF and DT regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Chromatograms were processed by use of Chromas 2.31 (Technelysium Pty Ltd.).

RESULTS

Analyses of nrDNA (1270 bp) revealed an informative 3 bp indel at site 205 and 9 SNPs but only 5 SNPs were informative (SNP 3, site 400; SNP 4, site 404; SNP 5, site 414; SNP 8, site 642; SNP 9, site 1149). Samples from the Pyrenees (Adams and Espeut 2020) are included in Table 1, along with *J.*

Table 1. DNA variation in *J. communis* from southeastern Spain compared with other areas.

			ITS Indel[1]	ITS S3[1]	ITS S4[1]	ITS S5[1]	ITS S8[1]	ITS S9[1]	LF S1	LF S2	LF Ind	LF S3	LF S4	LF Ind	
coll. #	Location	ITS class	205	400	404	414	642	1149	160	208	473	503	521	622	cp class
11206	Norway	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
11207	Norway	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
7846	Sweden	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
7847	Sweden	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15402	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15404	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15590	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15593	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15583	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15586	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15587	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15595	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15598	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15592	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15589	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15581	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15582	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15584	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15585	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15588	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15403	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15591	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15594	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15596	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15597	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15599	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15600	Pyrenees	sax	---	G	T	T	C	A	C	G	-	T	G	-	sax
15401	Pyrenees	s x h	TTT	A	C	C	C/T	A/G	C	G	-	T	G	-	sax
15695	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15697	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15699	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15701	S. de Baza	s x h	TTT/-	A/G	C/T	C/T	C/T	A/G	C	G	-	T	G	-	sax
15692	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15693	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15694	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15696	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15698	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15700	S. de Baza	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15702	S. Nevada	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
15703	S. Nevada	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
7194	S. Nevada	hemi	TTT	A	C	C	T	A	C	G	-	T	G	-	sax
7195	S. Nevada	hemi	TTT	A	C	C	T	A	C	G	-	T	G	-	sax
13052	S. Cazorla	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
13053	S. Cazorla	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
13054	S. Huéscar	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
13055	S. Mágina	hemi	TTT	A	C	C	T	G	C	G	-	T	G	-	sax
9045	Sicily	hemi	TTT	A	C	C	T	G	A	T	A	A	A	G	hemi
9046	Sicily	hemi	TTT	A	C	C	T	G	A	T	A	A	A	G	hemi

[1]ITS: ndel 205: xxxTGCTGGACGG; S400: GGACGTCCGx; S404: GGACGTCCGNGGCCx; S414: xTGAGATTT;S642: XGGGGCGGGG; S1149: xTCTTTGGTG. [2]cp trnLF S160: xAACATAA; S208: xAATTGTAC; Indel 473: ACACAATATx. [3]petN, S305: xGAACCATAC.

communis var. *communis* (Sweden), and *J. c*. var. *saxatilis* (Norway). These samples were uniform in their nrDNA that clearly separated them from *J. c*. var. *hemisphaerica* from S. de Baza, Cazorla, Las Villas, Mágina and Huéscar, S. Nevada and Sicily. An exception from the Pyrenees was sample 15401, that had 3 SNPs from *hemisphaerica* and the indel and 2 SNPs from *saxatilis* (Table 1). The S. de Baza plants are divided into 2 groups: those which have typical ITS DNA, and four (15695, 15697, 15699, 15701) that are heterozygous at all six sites with nucleotides from both *hemisphaerica* and *saxatilis*, and TTT or - for the indel site 205(Table 1). These are hybrids. Two S. Nevada plants (7194,7195) have an A (ex *saxatilis*) at SNP 9 (site 1149), suggesting they may be introgressants with gene flow from *saxatilis* into their *hemisphaerica* nrDNA in the past.

Examination of the cp data (LF region) reveals every plant analyzed (Pyrenees, S. de Baza, Cazorla, Las Villas, Mágina and Huéscar, and S. Nevada) has the *saxatilis* chloroplast! The only *hemisphaerica* cp is found in the plants from Sicily (Table 1). This appears to be a chloroplast capture evolutionary event as we have previously reported in *Juniperus* (Adams et al. 2017a,b, Adams et al. 2018, Adams et al. 2020, Farhat et al. 2019a,b, Hojjati et al. 2019).

A summary of plants in southeastern Spain and the Pyrenees shows (Table 2) that hybridization and introgression is common at S. de Baza (4 of 10 plants), and rare in the Pyrenees (1 of 29 plants). Yet, all plants analyzed have the *saxatilis* chloroplast, except the two *J. communis* var. *hemisphaerica* plants from the type locality in Sicily.

Table 2. Summary of hybridization and introgression in S. de Baza, Cazorla, Las Villas, Mágina and Huéscar, S. Nevada and Sicily, plus two kinds of plants found in the Pyrenees. IG = introgressed.

coll. #	Location	nrDNA (ITS) classif.	cp class	Overall classification and status
28, all but one	Pyrenees	v. *saxatilis*	sax	*J. communis* var. *saxatilis*, no hybridization with *J. c*. var. *hemisphaerica*, except for 15401, below.
15401	Pyrenees	sax x hemi	sax	Hybrid x *hemisphaerica*, with *saxatilis* cp
15695	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15697	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15699	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15701	S. de Baza	sax x hemi	sax	Hybrid (var. *saxatilis* x *hemisphaerica*), with *saxatilis* cp
15692	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15693	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15694	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15696	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15698	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15700	S. de Baza	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13052	S. Cazorla	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13053	S. Cazorla	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13054	S. Huéscar	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
13055	S. Mágina	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15702	S. Nevada	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
15703	S. Nevada	*hemisphaerica*	sax	var. *hemisphaerica* (ITS), with *saxatilis* chloroplast
7194	S. Nevada	*hemisphaerica*, IG *saxatilis*	sax	var. *hemisphaerica* introgressed by *saxatilis* (ITS), with *saxatilis* cp
7195	S. Nevada	*hemisphaerica*, IG *saxatilis*	sax	var. *hemisphaerica* introgressed by *saxatilis* (ITS), with *saxatilis* cp
9045	Sicily	*hemisphaerica*	hemi	var. *hemisphaerica* (ITS), with *hemisphaerica* cp
9046	Sicily	*hemisphaerica*	hemi	var. *hemisphaerica* (ITS), with *hemisphaerica* cp

It is unclear how *J. c*. var. *hemisphaerica* in Spain acquired the *saxatilis* chloroplast. Additional screening in Spain (and Europe) is needed to determine if all *J. c*. var. *hemisphaerica* have the *hemisphaerica* chloroplast, or if the phenomenon is local to southeastern Spain.

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LITERATURE CITED

Adams, R. P. 2014. The Junipers of the World: The genus *Juniperus*. 4th ed. Trafford Publ., Victoria, BC.

Adams, R. P., J. A. Bartel and R. A. Price. 2009. A new genus, *Hesperocyparis*, for the cypresses of the new world. Phytologia 91: 160-185.

Adams, R. P. and M. E. Kauffmann. 2010. Geographic variation in nrDNA and cp DNA of *Juniperus californica, J. grandis, J. occidentalis* and *J. osteosperma* (Cupressaceae). Phytologia 92: 266-276.

Adams, R. P. and A. E. Schwarzbach. 2012. Taxonomy of *Juniperus* section *Juniperus*: Sequence analysis of nrDNA and five cpDNA regions. Phytologia 94: 280-297.

Adams, R. P. and A. E. Schwarzbach. 2013. Phylogeny of *Juniperus* using nrDNA and four cpDNA regions. Phytologia 95: p. 179-187.

Adams, R. P., A. Boratynski, T. Mataraci, A. N. Tashev and A. E. Schwarzbach. 2017a. Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev in southwestern Turkey. Phytologia 99: 22-31. 396.

Adams, R. P., M. S. Gonzalez-Elizondo, M. Gonzalez-Elizondo, D. Ramirez Noy and A. E. Schwarzbach. 2017b. DNA sequencing and taxonomy of unusual serrate *Juniperus* from Mexico: Chloroplast capture and incomplete lineage sorting in *J. coahuilensis* and allied taxa. Phytologia 99: 62-73.

Adams, R. P., A. Boratynski, K. Marcysiak, F. Roma-Marzio, L. Peruzzi, F. Bartolucci, F. Conti, T. Mataraci, A. N. Tashev and S. Siljak-Yakovlev. 2018. Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams & Tashev in Macedonia, Bosnia-Herzegovina, Croatia and southern Italy and relictual polymorphisms found in nrDNA. Phytologia 100: 117-127.

Adams, R. P. and M. Espeut. 2020. Hybridization and introgression between *Juniperus communis* var. *saxatilis* and var. *hemisphaerica* in the Pyrenees Mountains, France. Phytologia 102: 9-13.

Adams, R. P. M. Socorro González-Elizondo and George M. Ferguson. 2020. Allopatric hybridization and introgression between *Juniperus scopulorum* Sarg. and *Juniperus blancoi* Mart. in northern Mexico: Unidirectional gene flow. Phytologia 102: 14-26.

Farhat, P., S. Siljak-Yakovlev, R. P. Adams, T. Robert and M. B. Dagher-Kharrat. 2019a. Genome size variation and polyploidy in the geographical range of *Juniperus sabina* L. (Cupressaceae). Botany Letters DOI: 10.1080/23818107.2-19.1613262

Farhat, P., O. Hidalgo, T. Robert, S. Siljak-Yakovlev, I. J. Leitch, R. P. Adams, and M. B. Dagher-Kharrat. 2019b. Polyploidy in the conifer genus *Juniperus*: an unexpectedly high rate. Frontiers in Plant Science 10:676. doi: 10.3389/pls.2019.00676.

Hojjati, F., R.P. Adams and R. G. Terry. 2019. Discovery of chloroplast capture in *Juniperus excelsa* complex by multi-locus phylogeny. Phytotaxa doi.org/10.11646/phytotaxa.413.1.2.