Variation in *Juniperus communis* trees and shrubs from Bulgaria: analyses of nrDNA and cpDNA regions plus leaf essential oil.

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ABSTRACT

DNA sequence data from nrDNA plus four cp DNA regions (4315 bp) was congruent with leaf essential oils data. Based on leaf essential oils, DNA sequences and morphology, *J. sibirica*, Bulgaria, should be treated as *J. communis* var. *saxatilis*. Combined data on leaf essential oils, DNA sequences and morphology indicates that *J. pygmaea*, Bulgaria, should be treated as a shrubby form of *J. communis*: ***Juniperus communis* forma *pygmaea*** (K. Koch) R. P. Adams & A. N. Tashev, **comb. nov.** Published on-line **www.phytologia.org** *Phytologia 96(2): 124-129 (April 1, 2014)*. ISSN 030319430.
**KEY WORDS:** *Juniperus communis* **forma *pygmaea*,** *Juniperus communis, J. sibirica, J. pygmaea, Bulgaria*, nrDNA, cpDNA sequences, leaf terpenes, taxonomy.

Assyov and Petrova (2012) recognized 6 native *Juniperus* species in the flora of Bulgaria: *J. communis* L., *J. excelsa* M.-Bieb., *J. oxycedrus* L*., J. pygmaea* K. Koch*., J. sabina* L. *and J. sibirica* Burgsd*.* Adams and Tashev (2012) reported that *J. oxycedrus* from Bulgaria was actually *J. deltoides* R. P. Adams, which grows from Italy eastward through Turkey. Of interest to the present work are the resolution and taxonomy of *J. communis, J. pygmaea* and *J. sibirica* (the latter two taxa treated as *J. c.* var. *saxatilis* by Adams, 2014 and Farjon, 2005, 2010). Of these 3 taxa, *J. communis* var. *communis*, grows as a small tree, whereas *J. pygmaea* and *J. sibirica* are small to spreading shrubs. They differ in their leaf morphology (Fig. 1) with *J. pygmaea* leaves being very similar to *J. communis;* those of *J. sibirica* differ by being shorter, curved and more appressed to the stem (Fig. 1). However, the leaves of the Bulgarian *J. sibirica* are quite similar to those of *J. communis* var. *saxatilis* Pall., as they are shorter and appressed as in the specimens from Mongolia and Norway (Fig. 2). Based on leaf morphology, *J. pygmaea* appears to be a shrub form of *J. communis* and *J. sibirica* appears to be *J. c*. var. *saxatilis*.

Recently, Adams and Tashev (2013) compared the leaf essential oils of *J. communis, J. pygmaea* and *J. sibirica* from Bulgaria with the oils of *J. communis*, Sweden and *J. saxatilis,* Switzerland (Fig. 3). From their analysis, the oils do not ordinate *J. communis, J. pygmaea* and *J. sibirica* from Bulgaria into separate groups, but they are generally interspersed (Fig. 3). There is a suggestion of a grouping of *J. sibirica* (oval, Fig. 3), but no clear clustering.
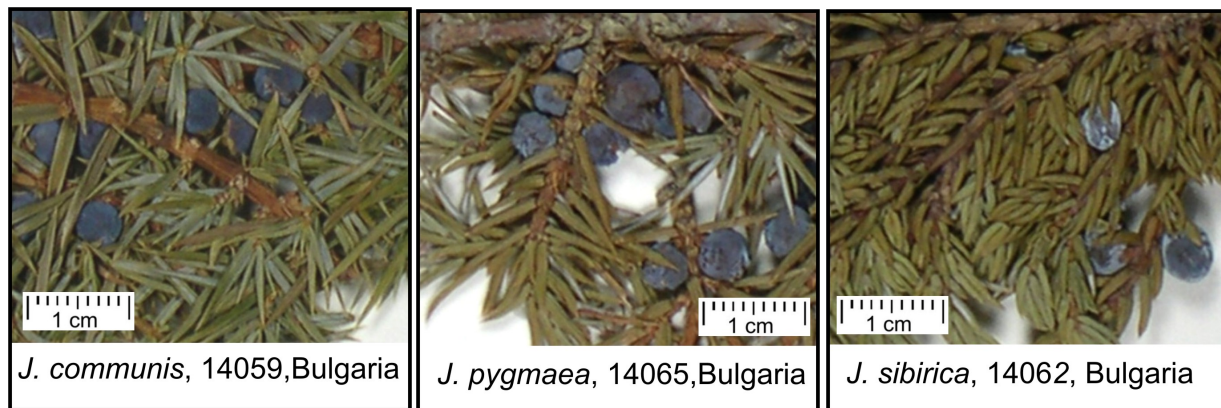
Figure 1. *J. communis, J. pygmaea* and *J. sibirica* specimens from Bulgaria.
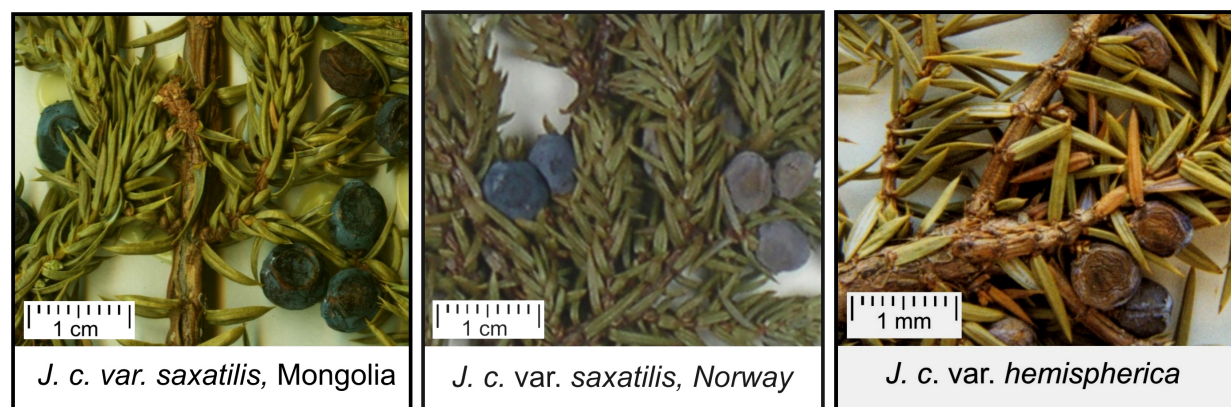
Fig. 2. Specimens of *J. c.* var. *saxatilis* from Mongolia and Norway and var. *hemispherica* from Mt. Etna.

Figure 3. PCO ordination based on 26 leaf terpenes of *J. communis, J. pygmaea* and *J. sibirica* from Bulgaria with exemplars of *J. communis*, Sweden and *J. c.* var. *saxatilis*, Switzerland.

The purpose of this study was to compare data from nrDNA and four cpDNA regions of *J. communis, J. pygmaea* and *J. sibirica* from Bulgaria with other members of *Juniperus* sect. *Juniperus* from the eastern hemisphere to determine if these taxa differ in their DNA. Note that both *J. pygmaea* and *J. sibirica* were treated as synonyms of *J. saxatilis* by Adams (2014) and Farjon (2005), but to avoid confusion, the usage as per the Flora of Bulgaria (Assyov and Petrova (2012) is used in the present paper .
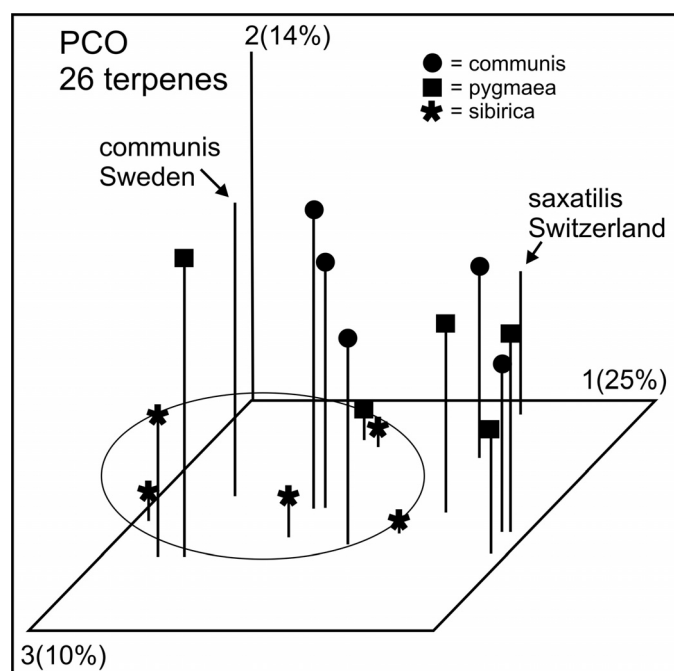
MATERIALS AND METHODS

Plant material - Bulgaria, *J. communis* var. *communis*, *Adams 13730-31, 14058-60, Alex Tashev, 2012-JC1-5*, Eastern Rhodopes, in protected site "Gumurdjinsky Shezhnik", locality "Madzharsky Kidik". On limestone rocks above the upper border of a forest of *Fagus sylvatica* ssp. *moesiaca,* 41° 14' 44.7" N; 25° 15' 31.9" E. elev. 1270 m.

J. pygmaea K. Koch, *Adams 13734-35, 14064-66, Alex Tashev, 2012-JP1-5*, Central Rhodopes. Mursalitza part, locality "Piramidata". On high-mountain meadow, on a limestone rock near a forest of *Pinus sylvestris* together with *Picea abies,* 41° 40' 22.8" N; 24° 26' 36.6" E. elev. 1756 m.

Juniperus sibirica Burgsd. (cf. *J. communis* var. *saxatilis*), *Adams 13732-33, 14061-63, Alex Tashev, 2012-JSI1-5,* Vitosha Region. Nature Park "Vitosha". Above the hut "Aleco" near the alpine timber line formed by a forest of *Picea abies*. On silicate rock together with *Vaccinium myrtillus, V. uliginosum, Ribes petraeum, Rubus idaeus, Calamagrostis arundinacea, Festuca valida (*Bulgarian endemic*),* 42° 34' 52.1" N; 23° 17' 28.0" E. elev. 1848 m.

Exemplar specimens: *J. communis* var. *communis,* Stockholm, Sweden, *Adams 8167 (7846-7848); J. communis* var. *saxatilis,* Switzerland, *Adams 11164 (7618-7621)*. Voucher specimens deposited in the Herbarium, Baylor University (BAYLU).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° 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 (petN, trnD-T, trnL-F, trnS-G) 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 and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG 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. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R6-1 (Biomatters. Available from http://www.geneious.com/) and the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v. 3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall, 1998) and Akaike's information criterion. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams et al. 2009; Adams, 1975; Veldman, 1967).

RESULTS AND DISCUSSION

Sequencing nrDNA (ITS) and four cp-regions petN-psbM, trnS-tenG, trnD-trnT and trnL-trnF yielded 4315 bp of data. The Bayesian consensus tree (Fig. 4) revealed that all the *sibirica* accessions are in a clade with *J. c.* var. *communis*, Sweden and *J. c.* var. *saxatilis*, Norway. Three of the *J. c.* var. *communis*, Bulgaria are in a well-supported clade, whereas the other two are in a clade with three *pygmaea* (Fig. 4). It is interesting that *J. c.* var. *saxatilis* is polyphyletic. Adams (2014) noted that *J. c* var. *saxatilis*, from the Japan region, should probably be treated as *J. c*. var. *nipponica*. Two of the

pygmaea plants had mixed bases in their nrDNA suggesting they may be of hybrid origin. Notice that they group with *J. c.* var. *communis* and that the other three *pygmaea* are in a separate clade.
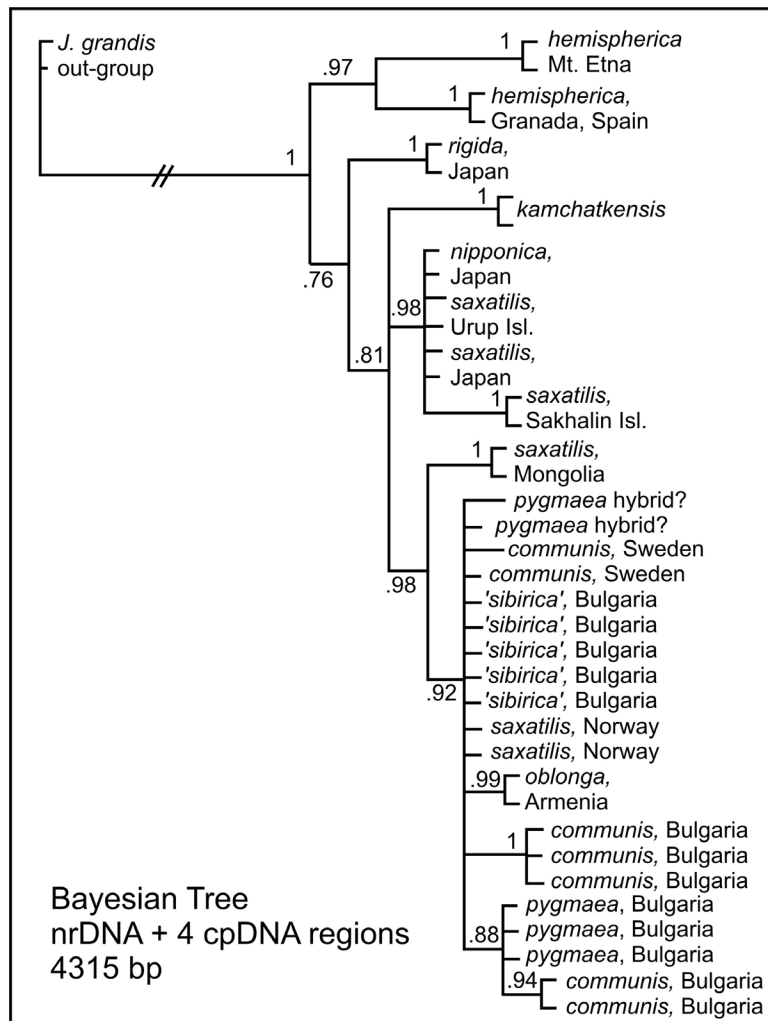

Figure 4. Bayesian tree of *Juniperus* sect. *Juniperus* taxa of the eastern hemisphere. Numbers at branch points are posterior probabilities. Two of the *pygmaea* plants had mixed bases in their nrDNA suggesting they may be of hybrid origin. Notice that they group with *J. c.* var. *communis* and the other three *pygmaea* are in a separate clade.

To examine the magnitude of the differences, a minimum spanning network was constructed (Fig. 5). *Juniperus communis*, eastern hemisphere, is divided into three groups: *J. communis*, Europe, *J. communis*, Japan and far east, and *J. c.* var. *hemispherica*, the latter divided among Mt. Etna, Sicily (type locality) and Sierra Nevada, Granada, Spain. All the samples from Bulgaria are tightly grouped with *J. communis* from Europe (Fig. 5). No variation was found among the five samples of *J. sibirica* and they have no differences from *J. communis* var. *communis* (type locality, Sweden), nor from *J. c.* var. *saxatilis* (Norway), although the *saxatilis* from Norway appears to only be a shrubby form of *J. communis* var. *communis*. Thus, based on leaf essential oils, DNA sequence data and morphology, *J. sibirica* of Bulgaria, should be treated as *J. communis* var. *saxatilis*.

The five samples of the Bulgarian *J. c.* var. *communis* (trees to shrubby trees) displayed two kinds of DNA. Two trees were like three shrubs of *J. pygmae*a, and the other three *communis* trees had DNA that was differed by 3 MEs from *J. communis* (Sweden), etc. (Fig. 5). The *J. pygmaea* samples were in two groups separated by 2 MEs (Fig. 5). This minor variation to be expected in natural populations.
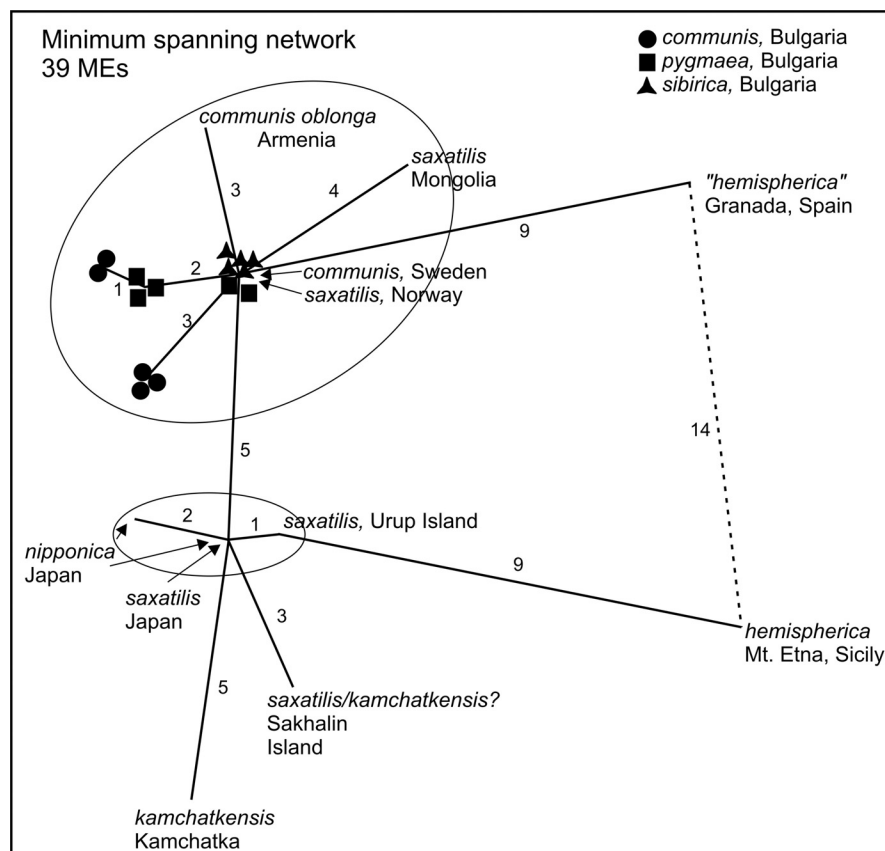
Figure 5. Minimum spanning network of *J. communis* and its varieties based on 39 MEs (mutational events = SNPs + indels). Numbers next to the links are the number of MEs. The dashed line is the second shortest link between the *hemispherica* taxa.

In short, based on leaf essential oils, DNA sequence data and morphology, *J. pygmaea* of Bulgaria, appears to be a shrubby form of *J. communis* var. *communis,* and not *J. c.* var. *saxatilis, sensu stricto* and should be recognized as but a form:

Juniperus communis* forma *pygmaea (K. Koch) R. P. Adams & A.N. Tashev, **comb. nov.**, Fig. 1 (center).
        Basionym: *Juniperus pygmaea* K. Koch, Linnaea 22: 302 (1849). Type: unknown (coll. K. Koch 1843-44, B lost in fire) "Auf dem pontischen Hochgebirge der Gaue Hemschin und Pertakrek" (Pontic Mtns., southern side of the Black Sea).
        J. communis subsp. *pygmaea* (K. Koch) Imkhan., Novosti Sist. Vyssh. Rast. 27: 10. 1990.

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