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Systematics of *Juniperus* from eastern Asia based on Random Amplified Polymorphic DNAs (RAPDs)

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Abstract

DNA was examined by RAPD banding for *Junipers chinensis*, *J.c.* var. *sargentii*, *J.c.* var. *tsukusiensis*, *J. communis*, *J.c.* var. *nipponica*, *J.c.* var. *saxatilis*, *J. conferta*, *J. formosana*, *J. procumbens*, *J. rigida*, *J. taxifolia* and *J.t.* var. *lutchuensis*. The DNA data readily separated junipers of section *Sabina* from section *Juniperus*. *J.c.* var. *tsukusiensis* from Taiwan was found to be sufficiently different from *J.c.* var. *tsukusiensis* (Yakushima) to warrant the recognition of a new variety: *J. chinensis* var. *taiwanensis* R.P. Adams and C-F. Hsieh *nov. var.* *Juniperus formosana* from mainland China was found to be different from *J. formosana* from Taiwan and a new variety is recognized: *J. formosana* var. *mairei* (Lemee and Lev.) R.P. Adams and C-F. Hsieh *comb. nov.* *Juniperus communis* var. *nipponica* was found to be very distinct from *J. communis* and this supports its recognition as a variety. The recognition of *J. conferta* as a variety of *J.* rigida [*J. rigida* var. *conferta* (Parl.) Patschke] is supported by the data. The data also supports the recognition of *J. lutchuensis* Koidz. [=*J. taxifolia* var. *lutchuensis* (Koldz.) Satake] and *J. morrisonicola* Hayata [=*J. squamata* var. *morrisonicola* (Hayata) H.L. Li and H. Keng] at the specific levels.

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1. Introduction

The genus *Juniperus* consists of approximately seventy species, all of which grow in the northern hemisphere, although, *J. procera* Hochst. ex Endl. also grows southward along the rift mountains in east Africa into the southern hemisphere (Adams and Demeke, 1993). The genus is divided into three sections: *Caryocedrus* (one species, *J. drupacea* Labill.); *Juniperus* (= *Oxycedrus*, 14 species, Adams, 2000a) and *Sabina* (the remaining, approx. 55 species, Adams, 1999, 2000b,c,d, 2001).

Both sections *Juniperus* and *Sabina* are represented in Japan and Taiwan. The taxonomy of these junipers has been in flux for some time. This can be seen from Table 1. Several areas of disagreement are seen. For example, *J. procumbens* is also recognized as *J. chinensis* var. *procumbens*; *J. communis* var. *nipponica* is recognized as *J. rigida*; *J. conferta* is recognized as *J. rigida* var. *conferta*; and *J. taxifolia* var. *lutchuensis* not recognized by Farjon (1998).

In addition, the nomenclature in Taiwan has changed from the Flora of Taiwan

Table 1
Comparison of the taxonomic treatments of *Juniperus* species native to Japan and Taiwan analyzed in this study. N.A.=not analyzed

In Japan: Kitamura and Murata (1979)	Ohwi (1965)	Farjon (1998)	DNA data
J. chinensis	*J. chinensis*	*J. chinensis*	*J. chinensis*
J.c. var. *sargentii*	*J.c.* var. *sargentii*	*J.c.* var. *sargentii*	*J.c.* var. *sargentii*
J.c. var. *procumbens*	*J.c.* var. *procumbens*	*J. procumbens*	*J. procumbens*
J.c. var. *sargentii*	*J.c.* var. *sargentii*	*J.c.* var. *tsukusiensis*	*J.c.* var. *tsukusiensis*
J. communis:			
J.c. var. *hondoensis*	*J.c.* var. *hondoensis*	–	N.A.
J.c. var. *nipponica*	*J.c.* var. *nipponica*	= *J. rigida*	*J.c.* var. *nipponica*
J.c. var. *saxatilis*	*J.c.* var. *montana*	–	aff. *J.c.* var. *saxatilis*?
J. rigida	*J. rigida*	*J. rigida*	*J. rigida*
J.r. var. *conferta*	*J. conferta*	*J.r.* subsp. *conferta*	*J.r.* var. *conferta*
J. taxifolia	–	*J. taxifolia*	*J. taxifolia*
J.t. var. *lutchuensis*	–	*J. taxifolia*	*J. lutchuensis*
In Taiwan: Flora of Taiwan (1994)	Li and Keng, 1954	Farjon (1998)	DNA data
J. chinensis. var. *tsukusiensis*	*J.c.* var. *tsukusiensis*	*J.c.* var. *tsukusiensis*	*J.c.* var. *taiwanensis*
J. formosana	*J. formosana*	*J. formosana*	*J. formosana*
J. formosana var. *concolor*	*J. f. var. concolor*	*J. formosana*	Extinct?
J. squamata	*J. s. var. morrisonicola*	*J. squamata*	*J. morrisonicola*

(1975) to 1994. In 1975, four *Juniperus* taxa were recognized: *J.*. var. *tsukusiensis*; *J. formosana*; *J. formosana* var. *concolor* and *J. squamata* var. *morrisonicola*, whereas in the 1994 Flora of Taiwan, *J. squamata* var. *morrisonicola* was recognized as merely *J. squamata*.

The purpose of this paper is to examine *Juniperus* species from eastern Asia (principally Japan and Taiwan) by the use of data from Random Amplified Polymorphic DNAs (RAPDs) to better understand the systematics of these junipers. We have also added to the analysis samples of *J. formosana* Hayata (China), J. communis (Sweden), and *J. communis* var. *saxatilis* (Switzerland and Mongolia) for comparisons.

2. Materials and methods

Specimens used in this study: *J. chinensis*, Adams 8536, 8537, Osezaki Point, Shizuoka Prefecture, Japan; *J.c.* var. *sargentii* Adams 8683, 8684 Matsumae-Jyoshi Park, Hokkaido, Japan (collected by Naotoshi Yohsida), Adams 8689, cultivated, Iwate Prefecture, Japan (collected by Naotoshi Yohsida); Adams 8580, Mt. Hayachine, Iwate Prefecture, Japan and Adams 8688, Mt. Kirigishi, Hokkaido, Japan (collected by Naotoshi Yohsida); *J.c.* var. *tsukusiensis*, Adams 9061, 9062, Mt. Chingshui, Taiwan, cult., Taiwan Forestry Institute, Taiwan; *J.c.* var. *tsukusiensis*, Adams 8805, 8806, Yakushima, Japan (by Jin Murata), *J. conferta*, Adams 8585, 8586, Tottori Sand Dunes, Japan (provided by Jin Murata); *J. communis* var. *communis*, Adams 7846, 7848, Stockholm, Sweden; *J.communis* var. *nipponica*, Adams 8579, Mt. Hayachine, Iwate Prefecture, Japan and Adams 8690, cultivated, Kidaka, Hokkaido (provided by Naotoshi Yohsida); *J.c.* var. *saxatilis*, Adams 7618, 7619, Switzerland; *J.c.* var. *saxatilis*, Adams 7589, 7590, Altai Mts., Mongolia, putative *J.c.* var. *saxatilis*; Adams 8685, 8686, 8687, cultivated, Teshio Experimental Forest of Hokkaido University, Hokkaido, Japan (provided by Naotoshi Yohsida); *J. formosana*, Adams 6772, 6774, Gansu, China; *J. formosana*, Adams 8781, Fujian, 400 m, China (by Zhen-Yu Li); *J. formosana*, Adams 9059–9060, Mt. Chingshui, Taiwan; *J. formosana*, Adams 9046, seedling, 30 m, Chungte tunnel, Taiwan; *J. formosana*, Adams 9065–9066, 430 m, Lushui, Taiwan; *J. formosana*, Adams 9071–9072, 3230 m, Shihmenshan, Taiwan; *J. procumbens* (Seib. ex. Endl.) Miq., Adams 8398, Hillier Gardens, UK, Acc. #76.2779, Adams 9150, Arnold Arboretum, Acc. #1163-56; *J. rigida*, Adams 8544, 8545, Gifu Prefecture, Japan (provided by Jin Murata); *J. taxifolia*, Adams 8448, 8449, Bonin Islands, Japan (provided by Jin Murata), *J. taxifolia* var. *lutchuensis*, Adams 8541, 8542, Oshima Island, Japan. Voucher specimens are deposited at SRCG herbarium, Baylor University.

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 the hot CTAB protocol (Doyle and Doyle, 1987) with 1% (w/v) PVP added to the extraction buffer. The RAPD analyses follow that of Adams and Demeke (1993). Ten-mer primers were purchased from the University of British Colombia (5′–3′): 116: TAC GAT GAC G; 134: AAC ACA CGA

G; 153: GAG TCA CGA G; 204: TTC GGG CCG T; 212: GCT GCG TGA C; 218: CTC AGC CCA G; 239: CTG AAG CGG A; 249: GCA TCT ACC G; 250: CGA CAG TCC C; 265: CAG CTG TTC A; 327: ATA CGG CGT C; 338: CTG TGG CGG T; 346: TAG GCG AAC G; 347 TTG CTT GGC G; 375: CCG GAC ACG A; 391: GCG AAC CTC G; 413: GAG GCG GCG A; 431: CTG CGG GTC A.

PCR was performed in a volume of 15 μl containing 50 mM Tris–HCl (pH 9), 2.0 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 μM primers, 0.3 ng genomic DNA, 15 ng BSA and 0.6 unit of Taq DNA polymerase (Promega). A control PCR tube containing all components, but no genomic DNA, was run with each primer to check for contamination. DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). The thermal cycle was: 94°C (1.5 min) for initial strand separation, then 40 cycles of 38°C (2 min), 72°C (2 min), 91°C (1 min). Two additional steps were used: 38°C (2 min) and 72°C (5 min) for final extension.

Bands that occurred once or did not show fidelity within the two replicated samples of each taxon were eliminated. It should be noted that these bands contain very useful information for the study of genetic variance and individual variation, but are merely "noise" in the present taxonomic study. Bands were scored in four classes: very bright (=6); medium bright (=5), faint (=4) and absent (=0). See Adams and Demeke (1993) for details on electrophoresis and RAPD band scoring.

Similarity measures were computed using absolute character state differences (Manhattan metric), divided by the maximum observed value for that character over all taxa (=Gower metric, Gower, 1971; Adams, 1975a,b). Principal coordinate analysis (PCO) of the similarity matrix follows Gower (1966).

3. Results and discussion

3.1. Section Sabina

Preliminary analysis (Adams, in preparation) of the leaf oils revealed the plants collected as *J.c.* var. *sargentii* were of two oil types: high bornyl acetate, low sabinyl acetate (8683, 8684, 8689) and low bornyl acetate, high sabinyl acetate (8580, 8688). So all of these specimens were used for the DNA analysis to examine the RAPDs of these chemical polymorphisms.

Fig. 1 shows the minimum spanning network for the *Juniperus* in section *Sabina* analyzed in this study. The major trend is the separation of *J. chinensis* taxa from *J. procumbens*, Japan, *J. morrisonicola* Hayata, Taiwan, and *J. squamata* Buch.-Ham. ex. D. Don, Gansu, China. Kitamura and Murata (1979) and Ohwi (1965) treated *J. procumbens* as a variety of *J. chinensis*, but this study supports Farjon (1998) in maintaining *J. procumbens* as a distinct species. Adams (2000c) showed that *J. morrisonicola* and *J. squamata* are distinct at the specific level in both RAPDs and leaf essential oils, and this analysis re-affirms their distinctness.

It appears that five taxa are present within the *J. chinensis* complex (Fig. 1): *J.c.* var. *chinensis*, *J.c.* var. *tsukusiensis* (Yakushima), *J.c.* var. *tsukusiensis* (Taiwan),

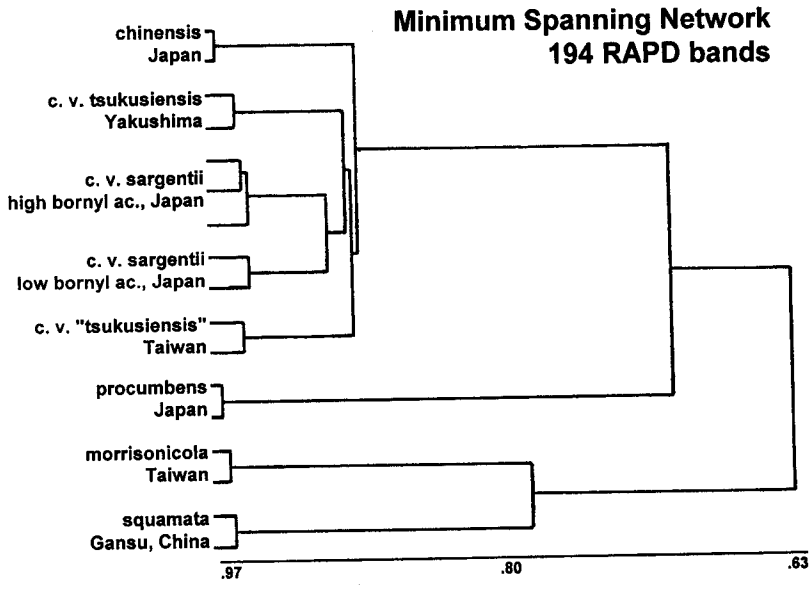

Fig. 1. Minimum spanning network based on 194 RAPD bands for *Juniperus* section *sabina*. Notice the distinct nature of *J. squamata*, *J. morrisonicola* and *J. procumbens*.

J.c. var. *sargentii* (high bornyl acetate, low sabinyl acetate), and *J.c.* var. *sargentii* (high sabinyl acetate, low bornyl acetate).

In order to facilitate the examination of the *J. chinensis* taxa, both *J. morrisonicola* and *J. squamata* were removed from the data set. The similarities were re-calculated and principal coordinates analysis was performed on the similarity matrix involving the five *J. chinensis* groups (Fig. 2). PCO revealed the groups (Fig. 2) were about equally similar. A significant point is that *J. chinensis* var. *tsukusiensis* from Yakushima did not cluster with *J.c.* var. *tsukusiensis* from Taiwan (Figs. 1 and 2). The Taiwan population of *J. chinensis* var. *tsukusiensis* occurs near (but not on) the summit of Mt. Chingshui, Taiwan. It is very isolated and consists of an estimated few hundreds of individuals. In the PCO it appears somewhat removed from the other varieties of *J. chinensis* (Fig. 2). But its linkage to other varieties of *J. chinensis* is at a comparable level of similarity. Clearly, it is not the same as *J.c.* var. *tsukusiensis* from Yakushima (the type locality and only population). It seems consistent to recognize this genetic variation expressed as the population on Mt. Chingshui as a distinct variety: *J.c.* var. *taiwanensis* R.P. Adams and C-F. Hsieh, *var. nov.*, TYPE: Mt. Chingshui, Taiwan, 2200 m, Sheng-you Lu 14498 (holotype at TAIF).

Frutices procumbentes, ramuli terminalibus ascendentibus. Foliae squamiformiae apicibus obtusis ca 1 mm longae et latae. Galbuli globosi 5–8 mm in diametro. Semina 3 erecta trigono–elliptica.

Prostrate shrubs, terminal branchlets ascending, leaves scale like, apices obtuse, about 1 mm long, 1 mm wide. Scale leaves appear as a string of beads. Female cones, about 5 mm in diameter. Seeds 3, erect, triangular–elliptical.

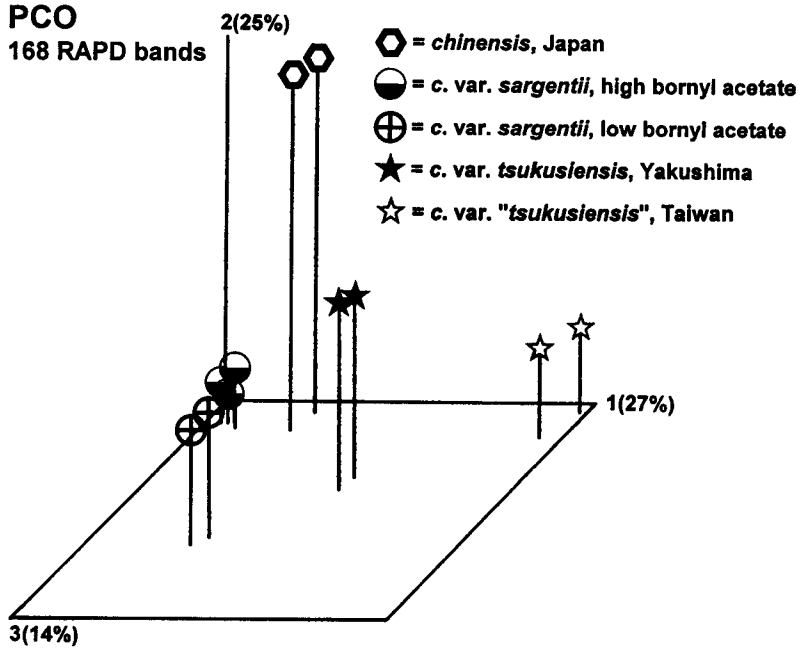

Fig. 2. PCO ordination of the *J. chinensis* taxa. Note the separation of *J.c.* var. *tsukusiensis*, Japan from *J.c.* var. "*tsukusiensis*", Taiwan. The two types of *J.c.* var. *sargentii* are: high bornyl acetate (half circles) and high sabinyl acetate (cross circles).

This variety differs from the typical variety by being a prostrate shrub and having scale leaves that are very short and wide (appearing as a string of beads) and with glands that are raised (as opposed to sunken in the typical variety). It differs form *J.c.* var. *tsukusiensis* in having scale leaves that are very short and wide and having the ultimate branchlets shorter (1–1.5 cm) than in *J.c.* var. *tsukusiensis* (1.5–2 cm).

J.c. var. *taiwanensis* is known from only one location: Mt. Chingshui. This area is a protected area, but due to the very small population, the taxon should be considered as threatened, but not endangered.

The three high bornyl acetate *J.c.* var. *sargentii* plants form a loose group that is distinct from the high sabinyl acetate *J.c.* var. *sargentii* plants (Figs. 1 and 2). Additional field work and sampling will be needed to understand these variations.

3.2. Section *Juniperus*

Analysis of *Juniperus*, sect. *Juniperus* is shown in Fig. 3. Four major groups are present: *J. communis–conferta–rigida*; *J. formosana*; *J. lutchuensis*; and *J. taxifolia*. Adams (2000a), using both leaf essential oils and RAPDs, found *J. lutchuensis* (=*J. taxifolia* var. *lutchuensis*) to be distinct from *J. taxifolia* and recognized *J. lutchuensis* as a separate species. This study reconfirms the previous work (Adams, 2000a).

Two sub-groups are apparent within the *J. communis–conferta–rigida* complex:

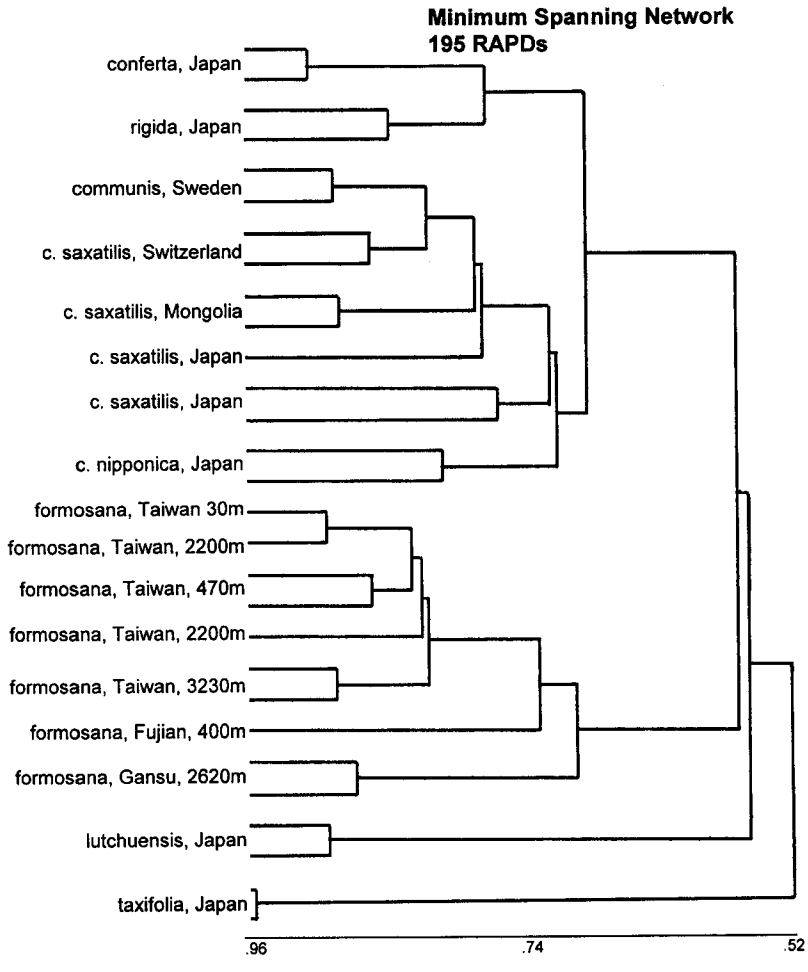

Fig. 3. Minimum spanning network based on 195 RAPD DNA bands *Juniperus* section *Juniperus*. See text for discussion.

J. communis–nipponica and *J. conferta–rigida* (Fig. 3). *J. conferta* has been treated as a species and as a subspecies of *J. rigida* (Table 1). Previously, Adams (2000a) found that the leaf essential oils and RAPDs supported the treatment of *J. conferta* as a variety, *J. rigida* var. *conferta* (Parl.) Patschke and this study reconfirms that decision.

The plants collected as *J. communis* var. *nipponica* barely cluster separately from *J. communis* (Fig. 3). The samples of putative *J. communis* var. *saxatilis* from Japan (Fig. 3) proved to be difficult to classify. The samples within the *J. communis–conferta–rigida* complex were re-analyzed and a PCO was performed. This resulted in an ordination (Fig. 4) that may more clearly show these complex relationships. Note the *J. conferta–rigida* group and that *J. c.* var. *nipponica* is quite distinct (Fig.

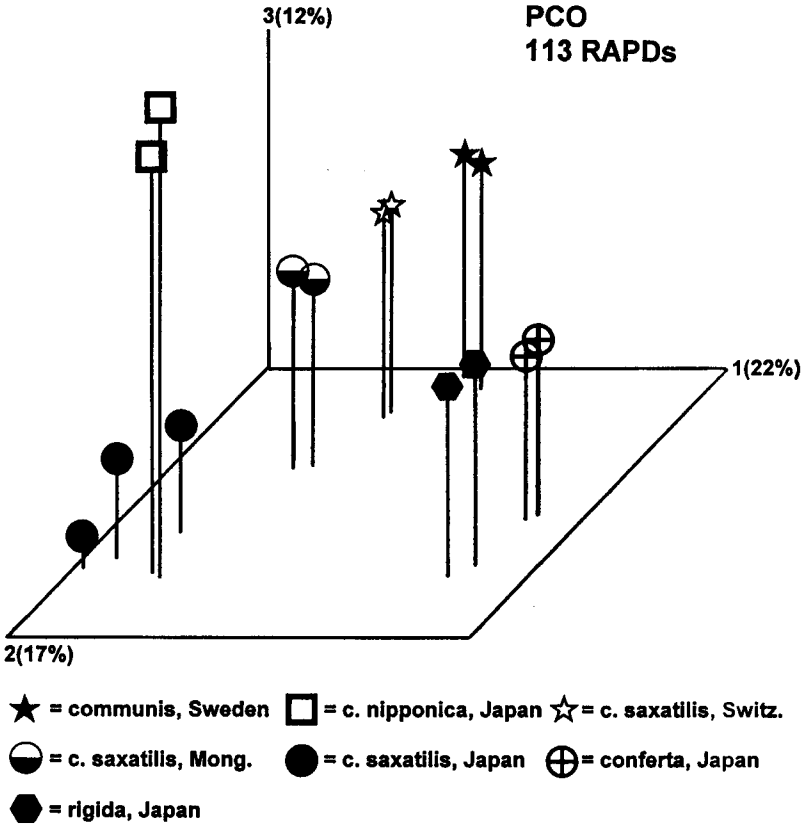

Fig. 4. Principal coordinate analysis (PCO) of the *J. communis–conferta–rigida* complex. Note the distinctness of *J.c.* var. *nipponica* and the sub-cluster of *J. conferta–J. rigida*.

4). The divergence and distinct nature of *J.c.* var. *nipponica* supports its continued varietal recognition.

It is interesting to note the gradient of *J. communis* and *J.c.* var. *saxatilis* in Fig. 4. Note that from upper right to lower left, one finds: *J. communis*, Sweden (solid stars); *J.c.* var. *saxatilis*, Switzerland (open stars); *J.c.* var. *saxatilis*, Mongolia (half circles); and *J.c.* var. *saxatilis*, Japan (solid circles). Adams (2000a) proposed that *J.c.* var. *saxatilis* from Mongolia be recognized as *J. siberica* Burgsd. From Fig. 4, the plants from Mongolia are about as distinct as *J. communis* var. *saxatilis* (Switzerland) is distinct from *J. communis* var. *communis* (Sweden). This would indicate that might be more appropriate to recognize the Mongolian plants as a distinct variety.

The loose clustering of the Japanese *J.c.* var. *saxatilis* plants (Fig. 3) indicates that additional studies are needed to resolve the status of these specimens. It may be that hybridization and introgression is responsible for the poorly defined clustering of *J.c.* var. *saxatillis* from Japan.

The second large cluster (Fig. 3) is composed of *J. formosana*. All of the specimens from Taiwan form a cluster(Fig. 3) and the plants from mainland China appear quite differentiated. The *J. formosana* specimens were utilized to calculate a new similarity matrix which was factored by PCO. These results (Fig. 5), show all the *J. formosana* from Taiwan in the foreground (Fig. 5), then the Fujian specimen and the Gansu specimens in the background. The mainland populations are quite different from those on Taiwan (in fact as different as *J. rigida* is from *J. communis* (Fig. 3). The mainland *J. formosana* has been described as *J. mairei* Lemee and Lev. from Yunnan. Based on the present data, it appears that the mainland portion of *J. formosana* should be recognized at least at the variety level: *Juniperus formosana* var. *mairei* (Lemee and Lev.) R.P. Adams and C-F. Hsieh, *stat nov.*

BASIONYM: *Juniperus mairei* Lemee and Lev., Monde Pl. 2(16): 20 (1914). E.E. Maire, Jong-tohouan, Arnold Arboretum, bar code #38339.

Finally, it should be mentioned that we searched the only known location of *J. formosana* var. *concolor* Hayata near the seashore (ca. 30 m) and found only one seedling. This seedling shows high affinity to *J. formosana* from Mt. Chingshui (2200 m) (Fig. 3, solid star in Fig. 4). It appears that Mt. Chingshui is the likely source of the seed that produced that seedling. No plants of *J. formosana* var. *con-*

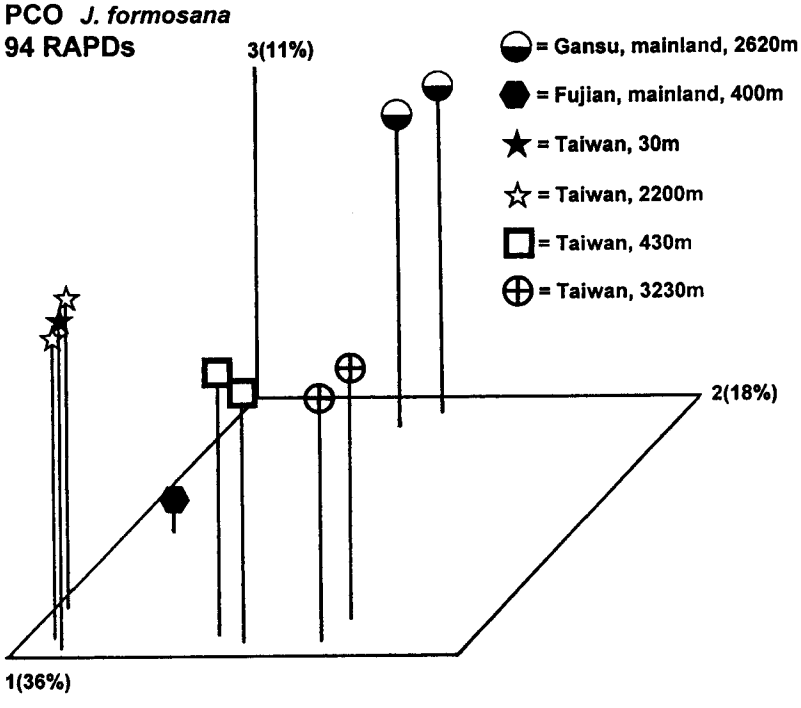

Fig. 5. PCO of the *J. formosana* samples. Notice the separation of the mainland samples (Gansu, Fujian) from the Taiwan samples.

color were found and it is presumed that the plant used as the type specimen for var. *concolor* is now extinct.

In summary, we found the RAPD markers to be in good agreement with classical morphological taxonomy (Table 1). However, there are a couple of cases where nearly morphological identical taxa exhibited large differences in their DNA. These "cryptic" species challenge our traditional taxonomic framework. Hopefully, these additional data will provide with a more stable classification as well as encourage continued research on the near-Eastern *Juniperus*.

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References

Adams, R.P., 1975a. Numerical-chemosystematic studies of infraspecific variation in *Juniperus pinchotii*. Sudw. Biochem. Syst. Ecol. 3, 71–74.

Adams, R.P., 1975b. Statistical character weighting and similarity stability. Brittonia 27, 305–316.

Adams, R.P., 1999. Systematics of multi-seeded eastern hemisphere *Juniperus* based on leaf essential oils and RAPD DNA fingerprinting. Biochem. Syst. Ecol. 27, 709–725.

Adams, R.P., 2000a. Systematics of *Juniperus* section *Juniperus* based on leaf essential oils and RAPD DNA fingerprinting. Biochem. Syst. Ecol. 28, 515–528.

Adams, R.P., 2000b. Systematics of smooth leaf margin Juniperus of the western hemisphere based on leaf essential oils and RAPD DNA fingerprinting. Biochem. Syst. Ecol. 28, 149–162.

Adams, R.P., 2000c. Systematics of the one seeded Juniperus of the eastern hemisphere based on leaf essential oils and random amplified polymorphic DNAs (RAPDs). Biochem. Syst. Ecol. 28, 529–543.

Adams, R.P., 2000d. The serrate leaf margined *Juniperus* (Section Sabina) of the western hemisphere: Systematics and evolution based on leaf essential oils and Random Amplified Polymorphic DNAs (RAPDs). Biochem. Syst. Ecol. 28, 975–989.

Adams, R.P., 2001. Geographic variation in leaf essential oils and RAPDs of *J. polycarpos* K. Koch in central Asia. Biochem. Syst. Ecol. (in press).

Adams, R.P., Demeke, T., 1993. Systematic relationships in *Juniperus* based on random amplified polymorphic DNAs (RAPDs). Taxon 42, 553–572.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bull. 19, 11–15.

Farjon, A., 1998. World Checklist and Bibliography of Conifers. Royal Botanic Gardens, Kew.

Flora of Taiwan, 1975. Li, H-L., Liu, T-S., Huang, T-C., Koyama, T., De Vol, C.E. (Eds.), Epoch, Taipei, Taiwan, pp. 538–544.

Flora of Taiwan, 1994. Huang, T.-C., Hsieh, C.-F., Keng, H., Hsieh W.-C., Tsai, J.-L. (Eds.), Editoral committee, Taipei, Taiwan, pp. 591–595.

Gower, J.C., 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53, 326–338.

Gower, J.C., 1971. A general coefficient of similarity and some of its properties. Biometrics 27, 857–874.
Kitamura, S., Murata, G., 1979. Colored Illustrations of woody plants of Japan, vol. II. Hoikusha, Osaka.
Li, H.L., Keng, H., 1954. Icones gymnospermum formosanarum, Taiwania 5, 25–83.
Ohwi, J., 1965. In: Meyer, F.G., Walker, E.H. (Eds.), Flora of Japan. Smithsonian Institute, Washington, DC.