

**INFRASPECIFIC VARIATION IN *HESPEROCYPARIS*
GOVENIANA AND *H. PYGMAEA*: ISSRS AND TERPENOID
DATA**

Robert P. Adams

Biology Department, Baylor University, Box 727
Gruver, TX 79040, USA
Robert_Adams@baylor.edu

Jim A. Bartel

U.S. Fish and Wildlife Service, Carlsbad Fish and Wildlife Office
6010 Hidden Valley Road, Suite 101
Carlsbad, CA 92011-4213

ABSTRACT

Hesperocyparis (*Cupressus*) *goveniana* and putative *Cupressus goveniana* subsp. *gibsonensis* plus *H. pygmaea* were analyzed by Inter-Simple Sequence Repeats (ISSRs). The ISSRs analyses support the continued recognition *H. pygmaea* and *H. goveniana*, but the recognition of *Cupressus goveniana* subsp. *gibsonensis* Silba was not supported. *Phytologia* 91(2):277-286 (August, 2009).

KEY WORDS: *Hesperocyparis* (= *Cupressus*) *goveniana*, *Cupressus goveniana* subsp. *gibsonensis*, *H. pygmaea*, ISSR, Inter-Simple Sequence Repeats, terpenes, DNA fingerprinting, systematics.

The coastal California species of *Hesperocyparis* (= *Cupressus*) are often found in very small populations and several are endangered. The taxonomy of the western hemisphere cypresses has recently changed as DNA sequencing of the classical *Cupressus* species (Little et al., 2004; Little, 2006) demonstrated that the western hemisphere cypresses are a separate genus from the eastern hemisphere cypresses. The eastern hemisphere cypresses maintained the name *Cupressus* and *Callitropsis* was applied to the western hemisphere cypresses plus *Callitropsis nootkatensis* (D. Don) Oersted ex D. P. Little. However, Debreczy, et al. (2009) argued, on morphological

grounds, that *Callitropsis nootkatensis* is a monotypic genus. Sequencing of two additional nuclear genes and petN-psbM (Adams et al., 2009) supported the recognition of *Callitropsis nootkatensis* as a monotypic genus. Thus, *Callitropsis* could not be applied to the western hemisphere cypresses, so Adams et al. (2009) erected a new genus, *Hesperocyparis*, for these cypresses.

Silba (2003) proposed the recognition of several new subspecies of *Cupressus abramsiana* Wolf: *C. a.* subsp. *locatellii* Silba, Eagle Rock, CA; *C. a.* subsp. *opleri* Silba, Bracken Brae Forest, Santa Cruz, CA; *C. a.* subsp. *neolomondensis* Silba, Majors Creek, CA; and *C. a.* subsp. *butanoensis* Silba, Butano Ridge, CA. *Cupressus a.* subsp. *butanoensis* was recognized (Adams et al., 2009) as *Hesperocyparis abramsiana* var. *butanoensis* (Silba) Bartel & R. P. Adams. Silba (2003) also proposed *C. goveniana* Gordon subsp. *gibsonensis* Silba and *C. macrocarpa* Hartw. subsp. *lobosensis* Silba. However, the proposed new subspecies are morphologically rather indistinct. If these new subspecies are accepted, then these taxa need to be considered under the endangered species act.

Adams and Bartel (2009) examined the volatile leaf oils of *H. goveniana* (Gordon) Bartel, *C. g.* subsp. *gibsonensis* Silba and *H. pygmaea* (Lemmon) Bartel (Fig. 1). The leaf oils of these taxa appear to separate them into 2 groups (Fig. 1) composed of *H. pygmaea* and *goveniana* - *gibsonensis*. There was some variation among the three populations of *H. pygmaea* (Fig. 1) and a hint of differences between *goveniana* and *gibsonensis*. These differences appear to be in the nature of geographic variation. Adams and Bartel (2009) concluded that, based on the leaf terpenoids, there was insufficient evidence to support the recognition of Silba's *Cupressus goveniana* subsp. *gibsonensis*.

To gather additional genetic information about the validity of these subspecies, analyses using Inter-Simple Sequence Repeats (ISSRs) were conducted. It should be noted that the leaf samples utilized in the present study were taken from the same trees analyzed by Adams and Bartel (2009).

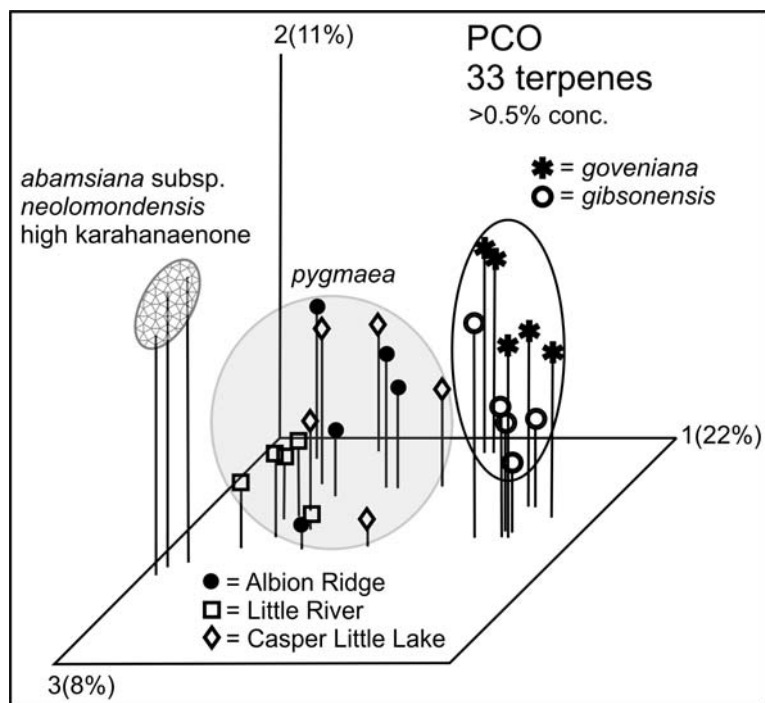


Figure 1. PCO of *Hesperocyparis goveniana*, *Cupressus* g. subsp. *gibsonensis*, *H. pygmaea* plus three plants of *C. abramsiana* subsp. *neolomondensis* (from Adams and Bartel, 2009).

MATERIALS AND METHODS

Plant material - Specimens used in this study: *H. abramsiana* (C. B. Wolf) Bartel, Bonny Doon, Santa Cruz Co., CA, *Bartel 1598a-e*; *H. abramsiana* var. *butanoensis*, Pescadero Creek County Park, Butano Ridge Fire Rd., San Mateo Co., CA, *Bartel 1605a-e*; *C. abramsiana* subsp. *locatellii* Silba, Eagle Rock, Santa Cruz Co., CA, *Bartel 1599a-e*; *C. abramsiana* subsp. *neolomondensis* Silba, Wilder Ranch State Park, Santa Cruz Co., CA, *Bartel 1604a-e*; *C. a.* subsp. *opleri* Silba, Bracken Brae, Santa Cruz Co., CA, *Bartel 1600a-e*; *H. goveniana*, SFB

Botanical Reserve, Monterey Co., CA, *Bartel 1596a-e*; *C. goveniana* subsp. *gibsonensis* Silba, Point Lobos Ranch, Monterey Co., CA, *Bartel 1595a-e*; *H. pygmaea*, Albion Ridge, Mendocino Co., CA, *Bartel 1601a-e*; Little River Airport, *Bartel 1602a-e*; Casper Little Lake Rd., CA, *Bartel 1603a-e*; *C. macrocarpa* subsp. *lobosensis* Silba, Point Lobos State Reserve, Allan Memorial Grove, Monterey Co., CA, *Bartel 1593a-e*, East Grove, *Bartel 1594a-e*; *H. macrocarpa* (Hartw.) Bartel, Crocker Grove, 100 m n of 17 Mile Drive and Madre Lane intersection, Monterey Co., CA, *Bartel 1597a-e*. Bartel specimens are held in his personal herbarium in Carlsbad, CA.

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 using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia, CA). ISSR primers were purchased from the University of British Columbia (5'-3 seq., annealing temperature used'): 807: AGA GAG AGA GAG AGA GT (50°C), 808: AGA GAG AGA GAG AGA GC (50°C), 811: GAG AGA GAG AGA GAG AC (50°C), 812: GAG AGA GAG AGA GAG AA (50°C), 836: AGA GAG AGA GAG AGA GYA (54°C), 840: GAG AGA GAG AGA GAG AYT (54°C), 841: GAG AGA GAG AGA GAG AYC (54°C), 847: CAC ACA CAC ACA CAC ARC (58°C), 861: AGC AGC AGC AGC AGC AGC, (58°C), 881: GG TGG GGT GGG GTG (50°C), 886: VDV CTC TCT CTC TCT CT (50°C), 887: DVD TCT CTC TCT CTC TC (54°C), 895: AGA GTT GGT AGC TCT TGA TC (50°C), 900: ACT TCC CCA CAG GTT AAC ACA (50°C).

PCR stock solutions (Taq, primer, and buffer) were made in bulk so that all the PCR reaction tubes for a primer were prepared using the same bulk stock. This is a critical factor for minimizing variation in band intensities from sample to sample (see Adams, Flournoy and Pandey, 1998, for protocols to minimize PCR band variation). PCR was performed in a volume of 15 μl containing: 7.5 μl Epi-Centre 2X buffer E (containing 0.4 mM of each dNTP, final conc. = 0.2 mM), 0.75 μl primer (0.6 μM final conc.), 0.75 μl Epi-Centre Fail-Safe Taq (0.75 unit/rxn.), 6 μl genomic DNA (0.3 ng/rxn.). A negative 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.). Samples were run in duplicate to insure reproducibility (Adams, Flournoy and Pandey, 1998). A temperature profile was obtained for each well of the thermocycler to be sure that no variation existed among wells in the heating/ cooling block. The thermal cycle used was: 94° C (1.5 min) for initial strand separation, then 39 cycles of 91° C (1 min), 50°C (or 54°C or 58°C, see above) (2 min), 72° C (2 min). Two additional steps were used: 91° C (1 min), 50°C (or 54°C or 58°C) (2 min) and 72° C (5 min) for final extension. The temperature inside a PCR tube containing 15 µl buffer was monitored with a temperature probe, quantitated and printed for each step for each of the 40 cycles for every PCR run (Adams, Flournoy and Pandey, 1998) to insure that each cycle met temperature specifications and that each PCR run was exactly the same. Amplification products were analyzed by electrophoresis on 1.5% agarose gels, 70V, 55 min, and detected by staining with ethidium bromide. The gels were visualized over UV light and scanned to digital images. The digital images were size normalized in reference to pGem® DNA size markers before band scoring. Bands were scored as present (4 = faint, 5 = bright, 6 = v. bright) and absent (0). Bands that were inconsistent in replicate analyses were not scored.

Associational 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, 1975). Principal coordinate analysis (PCO) was performed by factoring the associational matrix using the formulation of Gower (1966) and Veldman (1967). It should be noted that problems of homology of RAPD DNA bands on agarose gels can be significant (Rieseberg, 1996), but these errors can be accounted for using multivariate statistical methods (PCO) (see Adams and Rieseberg, 1998). A minimum spanning diagram was constructed by selecting the nearest neighbor for each taxon from the pair-wise similarity matrix, then connecting those nearest neighbors as nodes in a network (Adams, et al. 2003).

RESULTS AND DISCUSSION

The use of 14 ISSR primers resulted in 98 scoreable bands among the taxa. PCO of the association matrix removed five significant eigenroots accounting for: 15.2, 14.0, 9.1, 7.1, and 6.2% of

the variance among the OTUs. It is noteworthy that there was considerable variation among individuals and this is reflected in the relatively small amount of variance that was extracted by the first 3 eigenroots.

Ordination of the taxa shows that *H. goveniana* and *H. pygmaea* are very well resolved from *H. abramsiana* and *H. macrocarpa* (Fig. 2).

There appears to be considerable variation among the *H. pygmaea* individuals (Fig. 2). In addition, there is also variation among the *goveniana* - *gibsonensis* individuals (Fig. 2).

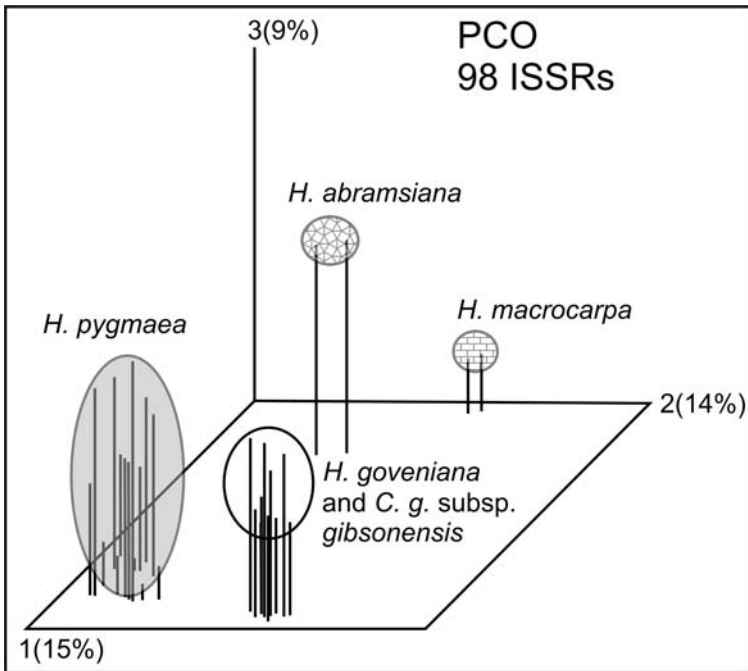


Figure 2. PCO of *H. goveniana*, *Cupressus goveniana* subsp. *gibsonensis*, and *H. pygmaea* with exemplars of *H. abramsiana* and *H. macrocarpa*.

In order to better visualize the variation among populations of *H. pygmaea*, a new PCO was performed which contained only the *H. pygmaea* individuals plus two exemplar *H. goveniana* samples. The PCO using 78 ISSRs resulted in four eigenroots accounting for 16.6, 12.8, 10.0, and 8.9% of the variance among individuals. Clearly there are considerable differences among *H. pygmaea* individuals (Fig. 3). There is a trend for the Casper Little Lake population (plus 2 individuals from the Albion Ridge population) to cluster together (Fig. 3). The balance of the Albion Ridge and Little River population plants are interspersed (Fig. 3). There was also a trend in the terpenoids to subdivide these populations (Fig. 1), but the pattern is slightly different

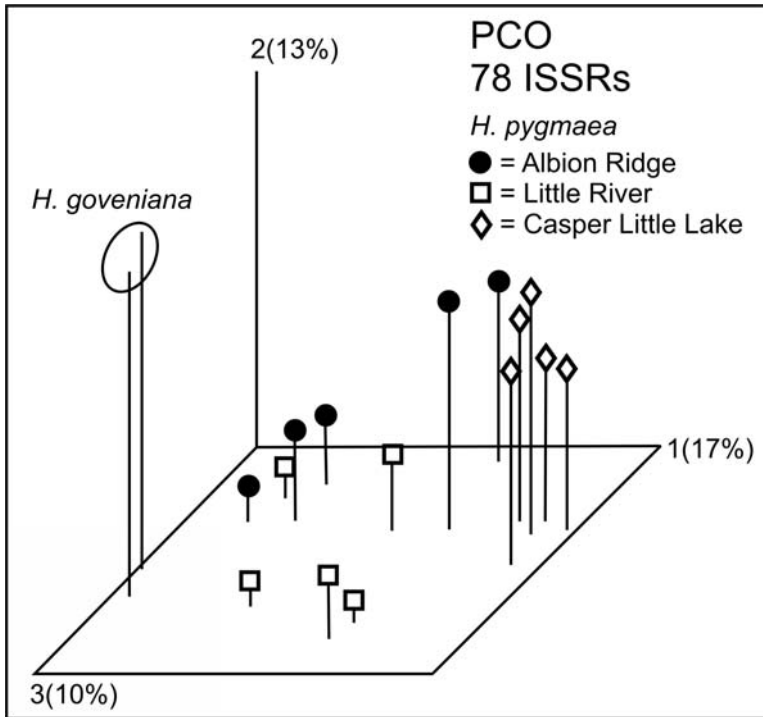


Figure 3. PCO based on 78 ISSRs for *H. pygmaea*. Two individuals of *H. goveniana* were included in the PCO.

than in the ISSR data (Fig. 3). It seems likely that these populational differences are normal geographic variation and should not to be recognized as separate taxa.

The variation between *H. goveniana* and *C. g.* subsp. *gibsonensis* was further examined using 75 ISSRs for their ten samples plus two individuals of *H. pygmaea*. PCO resulted in four eigenroots that accounted for 23.1, 19.5, 11.7, and 10.2% of the variance among these OTUs. Ordination reveals (Fig. 4) that *H. goveniana* and putative *C. g.* subsp. *gibsonensis* form a large group, with some separation between the taxa. However, as these putative subspecies are each in

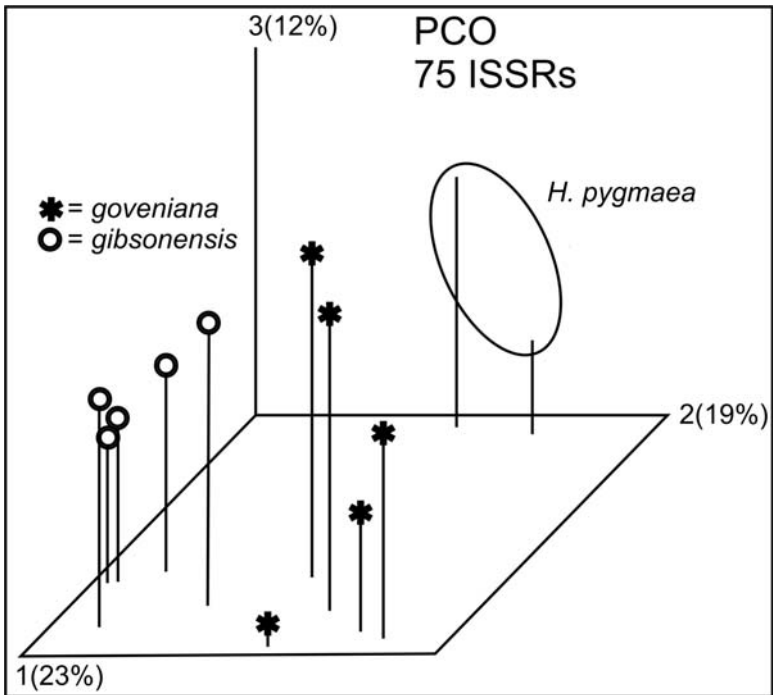


Figure 4. PCO of *H. goveniana* and *C. g.* subsp. *gibsonensis* individuals, plus two exemplars of *H. pygmaea*.

distinct (and disjunct) populations, geographic differentiation could well explain this clustering.

In summary, taking both the terpenoid and ISSR data into consideration, there appears to be sufficient genetic differentiation to support the recognition of *H. pygmaea*, but there is insufficient differentiation to support the recognition of Silba's *Cupressus goveniana* subsp. *gibsonensis*.

ACKNOWLEDGEMENTS

This research supported in part with funds from U. S. Fish and Wildlife Service, Grant 814307J011. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service. Thanks to Tonya Yanke for lab assistance.

LITERATURE CITED

- Adams, R. P. 1975. Statistical character weighting and similarity stability. *Brittonia* 27: 305-316.
- Adams, R. P. 2008. *Junipers of the world: The genus Juniperus*. 2nd Edition. Trafford Publ., Vancouver, B.C., Canada.
- Adams, R. P., J. A. Bartel and R. A. Price. 2009. A new genus, *Hesperocyparis*, for the cypresses of the western hemisphere. *Phytologia* 91(1): 160-185.
- Adams, R. P. and J. A. Bartel. 2009. Geographic in the leaf essential oils of *Hesperocyparis (Cupressus) abramsiana*, *H. goveniana* and *H. macrocarpa*: Systematic implications. *Phytologia* 91(2): 226-243.
- Adams, R. P., T. Demeke and H. A. Abulfatih 1993. RAPD DNA fingerprints and terpenoids: clues to past migrations of *Juniperus* in Arabia and east Africa. *Theoret. Appl. Genetics* 87: 22-26.
- Adams, R. P., L. E. Flourney and R. N. Pandey. 1998. Obtaining reproducible patterns from random polymorphic DNA amplification (RAPDs). *in* Conservation of Plant Genes III: conservation and Utilization of African Plants. R. P. and J. E. Adams, eds., Missouri Botanical Garden, St. Louis.

- Adams, R. P. and L. H. Rieseberg. 1998. The effects of non-homology in RAPD bands on similarity and multivariate statistical ordination in *Brassica* and *Helianthus*. Theoret. App. Gen. 97: 323-326.
- Bartel, J. A., R. P. Adams, S. A. James, L. E. Mumba and R. N. Pandey. 2003. Variation among *Cupressus* species from the western hemisphere based on random amplified polymorphic DNAs. Biochem. Syst. Ecol. 31: 693-702.
- Debreczy, Z., K. Musial, R. A. Price and I. Rácz. 2009. Relationships and nomenclatural status of the Nootka cypress (*Callitropsis nootkatensis*, Cupressaceae) Phytologia 91(1): 140-159.
- Gower, J. C. 1971. A general coefficient of similarity and some of its properties. Biometrics 27: 857-874.
- Gower, J. C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53: 326-338.
- Little, D. P., A. E. Schwarzbach, R. P. Adams and C-F. Hsieh. 2004. The circumscription and phylogenetic relationships of *Callitropsis* and the newly described genus *Xanthocyparis* (Cupressaceae). Amer. J. Bot. 91: 1872-1881.
- Little, D. P. 2006. Evolution and circumscription of the true cypresses (Cupressaceae, Cupressus). Syst. Bot. 31: 461-480.
- Rieseberg, L. H. 1996. Homology among RAPD fragments in interspecific comparisons. Mol. Ecol. 5: 99-105.
- Silba, J. 2003. Field observations of *Cupressus* in central and coastal California, July 2002 to January 2003. J. Intl. Conifer Pres. Soc. 10: 1-49.
- Veldman, D. J. 1967. Fortran programming for the behavioral sciences. Holt, Rinehart and Winston Publ., NY.