Inter-simple sequence repeat (ISSR) diversity within Monarda fistulosa var. brevis (Lamiaceae) and divergence between var. brevis and var. fistulosa in West Virginia

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Kimball, R. T. (Dept. of Zoology, University of Florida, Gainesville, FL 32611 U.S.A.; rkimball@zoo.ufl.edu), D. J. Crawford (Department of Ecology & Evolutionary Biology, University of Kansas, Lawrence, KS 66045 U.S.A.; dcrawford @ku.edu); J. R. Page (Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.; jrpage@sas.upenn.edu) & P. J. Harmon (West Virginia Nongame Wildlife & Natural Heritage Program, P.O. Box 67, Elkins, WV 26241, U.S.A.; pharmon@mail.dnr.state.wv.us). Inter-simple sequence repeat (ISSR) diversity within *Monarda fistulosa* var. *brevis* (Lamiaceae) and divergence between var. *brevis* and var. *fistulosa* in West Virginia. Brittonia 53: 511–518. 2001.—Inter-simple sequence repeat (ISSR) banding patterns were used to examine genetic diversity within and among populations of *Monarda fistulosa* var. *brevis*, a rare taxon restricted to several populations in limestone glades and barrens in eastern West Virginia and Virginia. More than 34% of the total ISSR diversity in var. *brevis* occurred among populations, which is high when compared to the few other rare species that have been examined for ISSR variation. Prior studies demonstrated that var. *brevis* is morphologically distinct from the more widespread var. *fistulosa*, and that the differences are maintained when the two varieties are grown together in a uniform environment. The present study utilizing ISSR markers indicated that the two varieties are distinct, though quite similar genetically, and this is concordant with prior investigations documenting their morphological and habitat differences. However, the ISSR results suggest that the two varieties have diverged relatively recently and/or there is a low level of gene flow between them.

Key words: Lamiaceae, *Monarda fistulosa* var. *brevis*, genetic diversity, inter-simple sequence (ISSR) variation.

Monarda fistulosa L. (wild begamot) is a morphologically variable and geographically widespread species with several infraspecific taxa recognized to accommodate this variation (Scora, 1967). One of the varieties was described by Fosberg and Artz (1953) based on specimens collected in the Smoke Hole region, Pendleton County, West Virginia. *Monarda fistulosa* var. *brevis* is distinguished by its shorter stature (<30 cm), slightly serrate, glabrous leaves, pustulated glandular calyx lobes, and comose corollas. By contrast, *Monarda fistulosa* var. *fistulosa* is typically 50–120 cm tall and has serrate leaves that are glabrous to sparsely pubescent with short hairs on the upper surface and elongate trichomes along the veins of the lower surface (Scora, 1967). *Monarda fistulosa* var. *fistulosa* is widely distributed in the central and eastern United States and Canada in a variety of habitats including woods, thickets, and prairies. By contrast, var. *brevis* is known only from limestone glades and barrens, primarily in West Vir-

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ginia, but it also has been collected in Virginia. Because of the differing habitat preferences, the two varieties are not known to occur together. *Monarda fistulosa* var. *brevis* is one of a number of taxa endemic or largely restricted to limestone outcrops in this area (Bartgis, 1993). Both Fosberg and Artz (1953) and Scora (1967) reported that the morphological features distinguishing var. *brevis* from var. *fistulosa* in nature were retained when plants were grown in the greenhouse under optimal conditions or in a garden. Thus, the diminished stature and other features of var. *brevis* appear to have a genetic basis and are not the result of environmental influence.

One purpose of the present study was to determine the level and apportionment of genetic diversity within and among populations of var. *brevis*. This information is of potential conservation value. The second purpose was to ascertain whether var. *brevis* is distinguishable at molecular marker loci from the nearest populations of *Monarda fistulosa* var. *fistulosa* that could be located (several miles removed). This study was limited in scope and did not address the issue of genetic variation and relationships in the entire *M. fistulosa* complex (Scora, 1967). Rather, the purpose was to see if var. *brevis* is divergent in discrete genetic markers that often distinguish populations of the same taxon or closely related taxa (Brunell & Whitkus, 1997). Such divergence would indicate that not only is var. *brevis* distinct morphologically but it has also diverged at other loci and that there is little effective gene flow between it and var. *fistulosa* in West Virginia.

A variety of molecular markers is now available for the study of rare plants (Crawford, 1997; Avise, 1994; Wolfe & Liston, 1998), and those based on the polymerase chain reaction (PCR) are increasingly utilized because very little plant material is required—a particularly valuable attribute when studying rare plants. Another advantage of these markers is that they are usually variable within and among populations of the same or closely related taxa. In the present study, we used a PCR-based technique in which DNA regions called inter-simple sequence repeats (ISSR) are amplified (Wolfe & Liston, 1998). This method often detects variation, even within rare species where little or no variation may be detected with other commonly employed markers (e.g., Esselman et al., 1999).

Materials and Methods

Leaves were collected along linear transects through natural populations (Table I) and the leaves were kept fresh at 4°C (on wet ice) during transportation to the laboratory. Total DNA was extracted from leaf material using a modification of the miniprep technique of Doyle and Doyle (1987), as described by Esselman et al. (1999). ISSR amplifications were likewise done using the methods described in Esselman et al. (1999). The three ISSR primers were $(GT)_6AY$, $(CA)_7YC$, and $(CAC)_4RC$. Bands were amplified by PCR and resolved by running in 1.0% agarose gels in TAE buffer until the front (tracking dye) had migrated 10 cm, stained for 30 min with ethidium bromide, and destained for 1.0 hr. ISSR profiles were recorded digitally using an Alpha Innotech imaging system (Alpha Innotech Corporation). The digital imaging files were analyzed with the BioMax 1D image analysis software (Eastman Kodak Company). Fragment sizes were estimated based on 1-kb ladder size standards (Gibco BRL) according to the algorithm provided in the BioMax 1D software. Fragment sizes were designated as loci for each primer. Bands were scored as diallelic for each locus (present = 1; absent = 0).

All individual plants and primers were amplified and run twice. The gels were scored in two ways, which will here be designated as "conservative" and "comprehensive." In the first method, only bands that amplified strongly and were well-resolved in both replicates were scored as present. In the second method, bands that were bright and robust in one of the amplifications were scored, even if they did not appear in one of the replicates. In most instances, the lack of replication of a given band was the result of poor amplification of all bands.

The similarity index of Nei and Li (1979) was employed to calculate pairwise simi-

TABLE 1
POPULATIONS OF *MONARDA FISTULOSA* EXAMINED FOR ISSR VARIATION. DESIGNATIONS SAME AS FOR FIGURE 1. VOUCHER SPECIMENS DEPOSITED AT OS.

Population designation	No. of individuals	Location
Var. *brevis*		
B1	14	South end of Cave Mtn., 0.5 mi NE of Eagle Rock, 2200 ft, Pendleton Co., WV. *Crawford 1600.* 24 Jun 1999
B2	15	Bull Hollow, 1600 ft, Grant Co., WV. *Crawford 1603.* 7 Jul 1999
B3	9	Cave Mtn., 1 mi N of Eagle Rock, 2200 ft, Pendleton Co., WV. *Crawford 1601.* 6 Jul 1999
B4	13	Hopeville Gorge, Grant Co., WV. *Crawford 1602.* 6 Jul 1999
B5	12	Above Big Bend campground, Pendleton Co., WV. *Crawford 1605.* 8 Jul 1999
Var. *Fistulosa*		
F1	1	Bull Hollow, 1400 ft, Grant Co., WV. *Crawford 1604.* 8 Jul 1999
F2	4	Roadside on steep slope along county rt. 1–13, 9 mi SW of Philippi, Upshur Co., WV. *Crawford 1606.* 14 Jul 1999
F3	2	Side of rd. at edge of pasture along CR 4–4, 1 mi S of Teater, Upshur Co., WV. *Crawford 1607.* 14 Jul 1999
F4	1	Along Rt. 374, opposite entrance to Rock House State Park, Hocking Co., OH. *Crawford 1609.* 18 Jul 1999
F5	10	Fence line, 18.5 mi S of Romney on River Rd., Hardy Co., WV. *Crawford 1610.* 21 Jul 1999
F7	12	In pasture, 2 mi SE of U-4 Motel on rt. 33–28, Pendleton Co., WV. *Crawford 1612.* 8 Aug 1999

larities of bands for all plants as described by Esselman et al. (1999). Calculations were done using RAPDPLOT 3.0 (Black, 1995). Average similarities were calculated for plants from the same and different populations. The values range from 0.0 (when no bands are shared) to 1.0 for identical banding patterns. A distance matrix (calculated as 1-similarity) was constructed and used to generate a neighbor-joining tree.

ISSR locus diversity and the percentage of variable loci were determined for all populations of var. *brevis* and for three populations of var. *fistulosa* (Table I). These statistics were not calculated for populations F1, F3, and F4 of var. *fistulosa* because of low sample size. ISSR locus diversity was calculated with the Shannon-Weaver information statistic with the Brillouin formula for eliminating the bias of finite sample size (Peet, 1974; Whitkus et al., 1998). Diversity was estimated within each population and across all populations. The apportionment of diversity within and among populations was determined according to Lewontin (1972).

TABLE 2
GENE DIVERSITY STATISTICS FOR POPULATIONS OF VAR. *BREVIS* AND VAR. *FISTULOSA* FOR COMPREHENSIVE (AND CONSERVATIVE) DATA

Population	No. of individuals	Variable loci (%)	Genetic diversity
Var. *brevis*			
B1	14 (12)	63.6 (68.2)	0.106 (0.128)
B2	15 (13)	80.3 (78.8)	0.135 (0.146)
B3	9 (9)	77.3 (78.8)	0.136 (0.143)
B4	13 (11)	65.2 (66.7)	0.105 (0.106)
B5	12 (8)	83.3 (78.8)	0.158 (0.145)
Var. *fistulosa*			
F2	4 (4)	45.5 (43.9)	0.074 (0.071)
F5	10 (8)	72.7 (74.2)	0.134 (0.130)
F7	12 (12)	65.2 (74.2)	0.124 (0.143)

Results

Results of the comprehensive and conservative data sets were comparable. Both sets of results are presented, with results of the conservative data set in parentheses below and in Table II. The results of the comprehensive data set will be used for discussion and to construct the neighbor-joining tree, because it includes more individuals than the conservative data set (63 vs. 53 for var. *brevis* and 26 vs. 24 for var. *fistulosa*). In the comprehensive data set, a total of 66 loci were scored; 65 were variable in both

varieties and one was invariant in var. *fistulosa*. The conservative data set includes 65 loci, all of which were variable in both varieties. For the 89 individuals included in the population sampling, 16.2% of the bands did not replicate.

Using the Shannon-Weaver index, the total diversity within var. *brevis* (calculated over all five populations examined) was 0.194 (0.204). Genetic diversities within populations of var. *brevis* ranged from 0.105 to 0.158 (Table II), with a mean value of 0.128 (0.134) for the five populations. The proportion of total diversity in var. *brevis* existing among populations was 0.34 (0.35); i.e., 34% (35%) of the ISSR diversity in var. *brevis* was distributed among populations and 66% (65%) was within populations. For the three populations of var. *fistulosa*, total diversity was 0.180 (0.188) and average diversity within the three populations was 0.111 (0.115). The proportion of total diversity residing among populations was 0.38 (0.39). The percentage of variable loci in the eight populations is given in Table II.

With the comprehensive data set, one locus was restricted to var. *fistulosa*, where it was detected in 50% of the individuals. Five low-frequency loci (4–19%) were detected in var. *brevis* but not in var. *fistulosa*; the same low-frequency loci were exclusive to var. *brevis* in the conservative data set. In the comprehensive data set, there were four loci present in a frequency greater than 80% in var. *brevis* that occurred in less than 50% in var. *fistulosa*. Conversely, six loci occurred in a frequency greater than 80% in var. *fistulosa* but in less than 50% in var. *brevis*.

The neighbor-joining tree, constructed from distances among all plants in all 11 populations of the two varieties (in the comprehensive data set), is shown in Figure 1. It may be seen that all individuals of each variety except one plant of var. *brevis* (from B5) formed two distinct groups. A neighbor-joining tree from the conservative data set differed only in that a different individual from one population of var. *brevis* grouped with var. *fistulosa*.

Discussion

The two aspects of genetic diversity which are important in the study of plant species, particularly those that are rare, are the level of diversity and how the diversity is partitioned within and among populations (Hamrick et al., 1991; Hamrick & Godt, 1996). The level of genetic variation within populations and species is of interest because it can affect the ability to adapt to changing environmental conditions. Whether single-locus molecular markers are indicative of variation within the genome, and thus for adaptive features, is a matter of discussion (Schaal et al., 1991; Storfer, 1996; Lande & Shannon, 1996). Despite the lack of compelling evidence that diversity at single-locus molecular markers reflects whole-genome diversity, the markers represent a simple, albeit possibly imperfect, method for assessing genetic diversity within and among populations of a species. PCR-based markers are increasingly used for measuring genetic variation in rare species and varieties of plants because they typically are more variable than allozymes (Wolfe & Liston, 1998; Crawford, 1997) and thus may be more informative.

At present, there are few studies of ISSR diversity in plants against which the results for the two varieties of *Monarda fistulosa* may be compared. In general, populations of var. *brevis* appear to have higher diversity than is found in other rare species. Four populations of the rare grass *Calamagrostis porteri* A. Gray subsp. *insperata* (Swallen) C. W. Greene in Ohio have 10–21% variable loci (Esselman et al., 1999). In addition, variable loci in populations of two rare species from the Juan Fernández Islands, Chile (*Lactoris fernandeziana* Phil. [Lactoridaceae] and *Myrceugenia fernandeziana* Johow [Myrtaceae]) can be anywhere in the range of 0–37.3% in populations of the former and 13–28% in populations of the latter species (Crawford et al., unpubl. data). The values for populations of var. *brevis* are comparable to those obtained for more widespread, common species (Wolfe et al., 1998). The Shannon-Weaver mean diversity found in populations of var. *brevis* (0.128) is higher than the values found in popula-

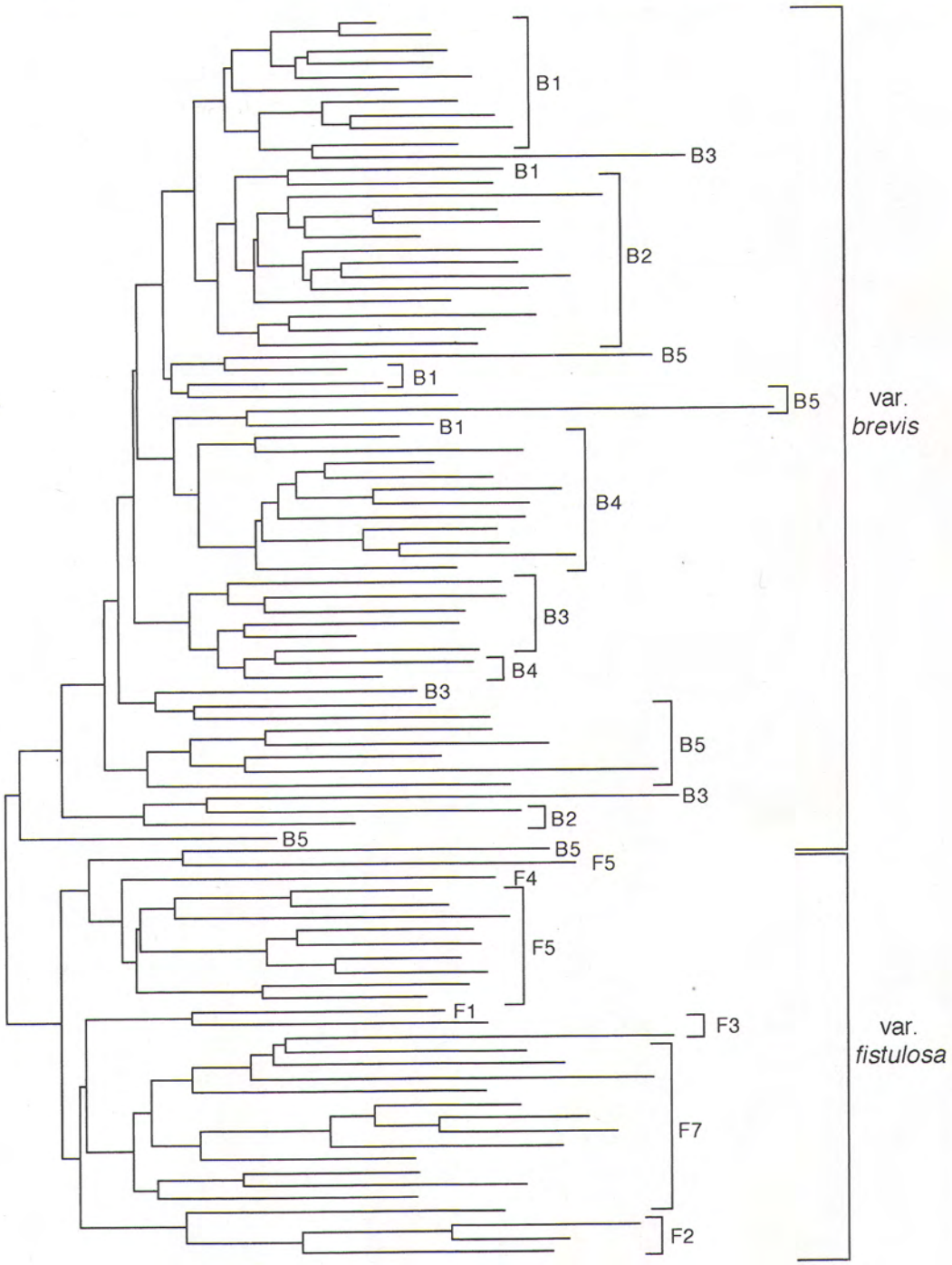

FIG. 1. Neighbor-joining tree of Nei and Li similarity coefficients (with midpoint rooting) for all individuals of *Monarda fistulosa* var. *fistulosa* and var. *brevis*. Population designations are the same as in Table I.

tions of *Lactoris* or *Myrceugenia* (<0.070; Crawford et al., unpubl. data).

In var. *brevis* as a whole, the percentage of variable loci (98.5%) is much higher than the percentages found at the species level in the two previously mentioned rare endemic species from the Juan Fernández Islands (50–55%) and *Calamagrostis porteri* subsp. *insperata* with only 24% variable loci (Esselman et al., 1999). Total ISSR Shannon-Weaver diversity in var. *brevis* (0.194) is much higher than the 0.080 value calculated for the species *Lactoris fernandeziana* and for several other endemics from the Juan Fernández Islands (Crawford et al., unpubl. data). The diversity found in var. *brevis* is comparable to the value found for var. *fistulosa*.

The ISSR data indicate that var. *brevis* does not exhibit the reduced diversity often found in rare taxa, which is attributed to factors such as founder effects, drift, and inbreeding in small populations (Barrett & Kohn, 1991; Ellstrand & Elam, 1993). The more geographically and ecologically restricted var. *brevis* has levels of diversity comparable to that found in West Virginia populations of the more widespread and variable var. *fistulosa*, but the limited number of populations sampled may provide an underestimate of ISSR diversity in var. *fistulosa*.

We can also compare the proportion of ISSR diversity found among populations of var. *brevis* to results obtained for other markers as well as the meager information available for ISSR markers in other species. The most extensive data set exists for allozymes, for which Hamrick and Godt (1989) in their survey reported a mean value of 24.8% for 52 endemic species of plants, which is slightly lower than the 34% found in var. *brevis*. In *Lactoris fernandeziana*, over 74% of the ISSR diversity resides among populations, but the high level of interpopulational diversity in this narrow endemic may result in part from its highly selfing mating system (Bernardello et al., 1999) as compared to the outcrossing var. *brevis*. Bussell (1999) discussed results of studies in which Shannon-Weaver analysis of another PCR-based marker (RAPD) was used to assess variation among populations. The 34% found for var. *brevis* is toward the high end of the range (15–38%) for outcrossing species. The values obtained for species may be influenced by the spatial scale of populational sampling, and sampling from a few widely distributed populations of a widespread species could inflate estimates of proportion of interpopulational diversity (Loveless & Hamrick, 1984; Hamrick & Godt, 1996). However, in var. *brevis*, sampling was extensive from throughout the narrow range of the taxon, and the relatively high value obtained is not likely the result of inadequate sampling.

Hypervariable molecular markers such as ISSR and RAPD, in addition to their utility for assessing genetic diversity within species, are also of value for evaluating the circumscription of infraspecific taxa and for assessing the boundaries and relationships of closely related species (e.g., Spooner et al., 1997; Brunell & Whitkus, 1997; Wolff & Morgan-Richards, 1998; Esselman et al., 2000; Huang & Sun, 2000; Wolfe & Randle, 2001). In the present study, ISSR data suggest that var. *brevis* is distinct yet closely related to var. *fistulosa*. Individuals of the two taxa, with the exception of one plant, form distinct groupings in the neighbor-joining tree (Fig. 1), indicating that they are consistently separable on the basis of ISSR loci. Despite forming two groups, the varieties are quite similar at ISSR loci as indicated by the almost total lack of unique alleles in either; rather, differentiation results from frequency differences at several loci. The high ISSR similarity between the two varieties may be the result of recent divergence and/or low levels of gene flow between them, but available data do not allow for assessing the relative importance of divergence time and gene flow. There are no documented occurrences of plants with morphological features suggesting that they are intervarietal hybrids. This does not, however, preclude the possibility of gene flow between the two varieties, although their occurrences in different habitats may limit intervarietal gene flow. As indicated above, var. *brevis* retains its distinguishing morphological characters when grown in the garden and greenhouse (Fosberg & Artz, 1953; Scora, 1967), indicating that the

characters have a genetic basis rather than being the result of environmental influences. Variety *brevis* grows well in a common garden with var. *fistulosa* (P. J. Harmon, pers. obs.), but it is not known whether var. *fistulosa* could grow on the limestone where var. *brevis* occurs. The combined molecular and morphological data support the recognition of those populations assigned to var. *brevis* as a distinct taxon, either at the varietal or specific level (the level depending to some extent on personal preference).

There are several conservation implications of the results of this study, particularly when viewed within the context of the garden and greenhouse studies of Fosberg and Artz (1953) and Scora (1967). The ISSR data, together with the morphological and ecological differences between var. *brevis* and var. *fistulosa*, indicate that the two represent separate gene pools and that var. *brevis* is not just a minor variant within the *M. fistulosa* complex. Thus, regardless of the taxonomic level at which var. *brevis* is recognized, it clearly should be conserved because it does represent a distinctive genetic entity. Secondly, the high proportion of ISSR diversity residing among populations of var. *brevis* suggests that conservation of maximal diversity within the variety requires preservation of all populations.

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