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Microsatellite analysis of relationships among North American plums (*Prunus* sect. *Prunocerasus*, Rosaceae)

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Abstract. Fifteen microsatellite primer pairs developed in sweet cherry and peach were used to explore genetic relationships among North American plums (*Prunus* section *Prunocerasus*). In all, 186 putative alleles were detected with a mean value of 12.4 per locus. The Jaccard coefficient of similarity was calculated between all pairs of accessions and their genetic similarities represented by a UPGMA dendrogram. Despite the apparent closeness among native American plums as evidenced by their ability to hybridize freely and their very similar ITS and *trn*L-*trn*F nucleotide sequences, all pairs of accessions among the North American plums shared fewer than half of their alleles. Some of the relationships suggested by the UPGMA dendrogram are congruent with current taxonomic hypotheses, but others are difficult to interpret. Further resolution of relationships among American plums will require molecular markers more variable than ITS yet less variable than microsatellites.

Key words: Rosaceae, *Prunus*, *Prunocerasus*, North American plums, microsatellites, simple sequence repeats (SSRs), genetic similarity.

Approximately 15 species of North American plums constitute *Prunus* section *Prunocerasus* (Krüssmann 1986, Rehder 1940). Recent nucleotide sequence studies have clarified relationships among subgenera and sections within *Prunus*, however, relationships among the species of section *Prunocerasus* remain unresolved. A bootstrap tree based on ITS sequences published by Bortiri et al. (2001) shows all six North American plums in their study diverging along with nine other *Prunus* species and clades pitchfork style from a polytomy. A strict consensus tree of 76 maximum parsimony trees from chloroplast *trn*L-*trn*F sequences has only slightly more structure (Bortiri et al. 2001). *P. mexicana* is sister to an unresolved group of four plum species from eastern North America with a bootstrap value of 64 supporting the monophyly of the group. *P. subcordata*, the only plum native west of the Rocky Mountains, is not a member of the clade. Similarly, another analysis of ITS sequences by Lee and Wen (2001) revealed the four North American plum species included in their study to be an unresolved monophyletic group (but which also included *P. armeniaca*, apricot). Bortiri et al.'s (2002) study of the phylogenetic utility of *s6pdh* nucleotide sequences in *Prunus* included only three species of section *Prunocerasus*. *P. maritima* and *P. mexicana* are sister groups on the maximum likelihood and parsimony trees.

P. subcordata is sister to the clade including these species along with *P. andersonii* and *P. fremontii*, two dry-fruited species of section *Penarmeniaca* from the western United States.

Microsatellite or simple sequence repeat (SSR) analysis has been used to characterize relationships among genotypes of economically valuable species of *Prunus*, including peaches (Cipriani et al. 1999, Sosinski et al. 2000), apricots (Hormaza 2002), and cherries (Struss et al. 2003). The apparent closeness of relationships among North American plums, as evidenced by their ability to hybridize freely and their very similar nucleotide sequences for ITS and *trn*L-*trn*F, suggests that microsatellite analysis might prove useful at resolving genetic relationships among species of section *Prunocerasus*. The proven cross-transportability of microsatellites among species of *Prunus* (Cipriani et al. 1999, Downey and Iezzoni 2000, Sosinski et al. 2000, Hormaza 2002, Serrano et al. 2002) eliminates the high cost of isolating SSRs and developing primers to amplify them. The purpose of this study, therefore, is to evaluate the utility of microsatellite markers originally isolated in sweet cherry and peach for revealing relationships among the North American plums.

Materials and methods

Plant material. We were able to obtain at least one accession from each of 13 species of North American plums. Also examined were several undetermined wild plums collected in Texas that we believe may be of hybrid origin. Apricot (*P. armeniaca*), classified in section *Armeniaca*, was included because it had clustered with members of section *Prunocerasus* on the phylogenetic tree of Lee and Wen (2001), and myrobalan or cherry plum (*P. cerasifera*), from the Eurasian section *Prunus*, was used for outgroup comparison. In all, we analyzed 21 accessions of North American plums and two outgroup species (Table 1). Some specimens were collected from wild plant populations; others were collected from cultivated plants on the University of California, Davis campus or from the collection of the USDA National Clonal Germplasm Repository in Davis. All sources of DNA are documented by voucher specimens deposited in the University of Wisconsin-Eau Claire herbarium (UWEC). We verified identifications against published descriptions and authentic herbarium material.

DNA extraction. Genomic DNA was isolated by grinding approximately 100 mg (wet weight) of leaves in liquid nitrogen and using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Specimens were given the optional 5-min centrifugation prior to centrifugation through the shredder column. DNA was eluted from the mini-column filter by two elutions of 50 μl each. The resulting DNA solutions were further cleaned by precipitation with 1/10 vol sodium acetate (3M, Ph 5.2) and 2 vols 100% cold ethanol. After centrifugation, washing with 70% ethanol, a second centrifugation, and air drying, the DNA was resuspended in TE buffer. DNA quality and concentration were estimated by comparing 4 ul of genomic DNA solution against a DNA standard on a 1.5% agarose gel run in 1x TBE buffer and stained with ethidium bromide. Samples were diluted to a uniform 10 ng/μl with TE buffer prior to PCR amplification.

PCR amplification. We initially chose eighteen pairs of primers for use in this study (Table 2): 15 primers were previously developed in sweet cherry (Struss et al. 2003) and three in peach (Cipriani et al. 1999, Sosinski et al. 2000). PCR reactions were performed in 11 µl volumes containing 20 ng of template DNA, 0.5 units of *Taq* DNA polymerase (Qiagen), 1x PCR buffer, 200 µM dNTPs, 250 nmol of each primer, and sterile water. We amplified DNA in a Peltier Thermal Cycler (PTC-225 DNA Engine Tetrad, MJ Research, Watertown, MA) using the following program: 1) an initial denaturation for 2 min at 94 °C; 2) 41 cycles consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and 2-min extension at 72 °C; and 3) a final extension at 72 °C for 7 min.

Microsatellite analysis. One µl of PCR reaction products was mixed with 6 µl of formamide dye, denatured at 94° for 3 min, and rapidly cooled on ice for 10 min. Amplified fragments were separated by electrophoresis on 0.25 mm thick 5.5% polyacrylamide gels and visualized using a LI-COR DNA Analyzer Gene Readir 4200. We scored bands as present (1) or absent (0). Allele size was measured with Gene Profiler

Table 1. Plant material used in the study (DPRU = USDA National Clonal Germplasm Repository accession number; voucher numbers are collections of JRR deposited at UWEC)

Reference #	Accession	Source	Voucher #
1	*P. alleghaniensis* T. C. Porter	Michigan	10467
2	*P. americana* Marshall	DPRU 1250.8	10586
3	*P. americana* Marshall	Wisconsin	10505
4	*P. americana* Marshall	DPRU 0544	10587
5	*P. angustifolia* Marshall	DPRU 1924	10588
6	*P. angustifolia* Marshall	Texas	10519
7	*P. armeniaca* L.	DPRU 1134	15089
8	*P. cerasifera* Ehrh.	UCD campus	10543
9	*P. gracilis* Engelm. & Gray	Texas	10516
10	*P. hortulana* Bailey	UCD campus	10598
11	*P. maritima* Marshall	DPRU 1737	10590
12	*P. mexicana* S. Watson	DPRU 1368.1	10591
13	*P. mexicana* S. Watson	Texas	10511
14	*P. munsoniana* Wight & Hedrick	DPRU 0546	10592
15	*P. murrayana* E. J. Palmer	Texas	10526
16	*P. orthosepala* Koehne	DPRU 0551	10594
17	*P. rivularis* Scheele	Texas	10527
18	*P. rivularis* Scheele	Texas	10528
19	*P. subcordata* Benth.	DPRU 2216	10595
20	*P. umbellata* Ell.	Texas	10520
21	*P.* sp. (*americana* × *mexicana*?)	Texas	10517
22	*P.* sp. (*americana* × *mexicana*?)	Texas	10518
23	*P.* sp. (*angustifolia* × *mexicana*?)	Texas	10512

Table 2. Microsatellite markers used in the study

Marker	Repeat Type	Size Range (bp)	No. of Putative Alleles
pchgms1	$(AC)_n$ $(AT)_n$	95–145	19
pchgms2	$(CT)_n$	115–154	16
UCD-CH10	$(CT)_n$	45–65	13
UCD-CH11	$(CA)_n$	50–156	14
UCD-CH12	$(CT)_n$	75–102	22
UCD-CH13	$(CA)_n$	107–118	4
UCD-CH14	$(CT)_n$	65–157	16
UCD-CH15	$(CA)_n$	45–55	5
UCD-CH16	$(CT)_n$ $(CA)_n$	Omitted from analysis because 7 of the 23 accessions gave no bands	
UCD-CH17	$(CT)_n$	45–65	19
UCD-CH19	$(CT)_n$	54–85	16
UCD-CH21	$(CA)_n$	Numerous bands per accession, not scored	
UCD-CH23	$(CT)_n$ $(CA)_n$	Numerous bands per accession, not scored	
UCD-CH24	$(CA)_n$	45–95	7
UCD-CH26	$(CA)_n$	62–95	19
UCD-CH27	$(CT)_n$ $(CA)_n$	50–145	3
UCD-CH31	$(CA)_n$	145–155	6
UDP96-001	$(CA)_n$	50–57	7

analysis software (Scanalytics, Fairfax, VA) and also by manual editing to increase accuracy. We used the NTSYS-pc software package (Rohlf 2000) to generate a similarity matrix by calculating the proportion of bands shared by each pair of accessions (Jaccard coefficient) and to produce a dendrogram using the unweighted pair group method based on arithmetic averages (UPGMA).

Results

PCR amplifications were produced in native American plums using all of the microsatellite primers tested and each marker was polymorphic. Although developed in species other than plums, the congeneric relationship of plums to peach and cherry allowed the successful use of these primers in section *Prunocerasus*. Occasionally no band was seen for a particular accession-primer combination, and these samples were rerun through PCR amplification and electrophoresis along with positive controls to confirm the absence of bands. One primer pair (UCD-CH16) yielded no bands for seven of the 23 accessions and was not used in the analysis. Two other pairs of primers (UCD-CH21, UCD-CH23) produced numerous bands per accession and were dropped from further analysis. Each of the other 15 markers (retained for analysis of relationships among the accessions) typically produced only one or two bands per sample; rarely three bands per accession-primer pair were seen. The number of putative alleles per locus varied from three for UCD-CH27 to 22 for UCD-CH12 with a mean value of 12.4 per locus. The total number of putative alleles expressed over the 15 loci ranged from 18 in *P. subcordata* and *P. umbellata* to 26 in *P. orthosepala*, with a mean of 21.4 per accession.

The similarity among the 23 genotypes included in this study is graphically represented by a UPGMA dendrogram (Fig. 1). *P. armeniaca* is the last genotype to join the cluster and has the most unique banding pattern. It shares only six alleles with the accession to which it was most similar, *P. mexicana* (13) and only

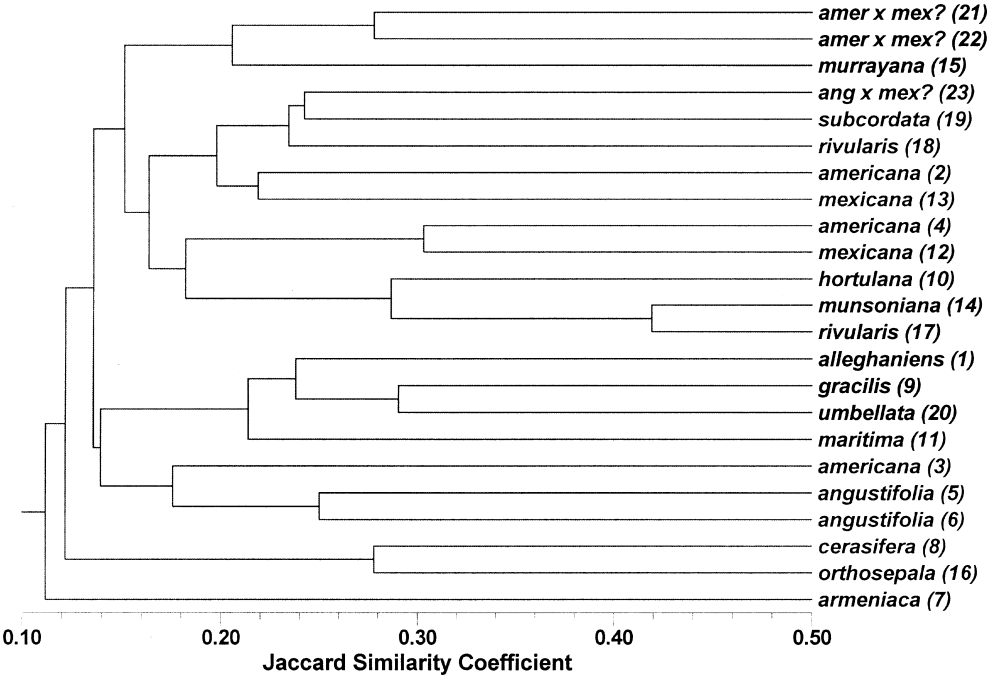

Fig. 1. UPGMA dendrogram of relationships among North American plums, based on Jaccard coefficient of similarity obtained from 186 microsatellite bands. The reference number from Table 1 is indicated in parentheses

two alleles with *P. rivularis* (18), the fewest shared between any pair of accessions. All samples from the North American section *Prunocerasus* form one large cluster, with the exception of *P. orthosepala* (later found to be misidentified, see discussion), which is most similar to *P. cerasifera*.

All pairs of accessions share fewer than half of their alleles, indicating considerable genetic divergence among North American plums, more than expected given their nearly identical ITS and *trn* sequences and ease of hybridization. Most similar to each other are *P. munsoniana* and *P. rivularis* (17), which share 13 (42%) of the 31 alleles expressed in these two species.

Although the two accessions of *P. angustifolia* cluster together on the UPGMA dendrogram, the three accessions of *P. americana* as well as the two accessions each of *P. mexicana* and *P. rivularis* do not form conspecific groups.

Discussion

Microsatellites isolated in sweet cherry and peach are of some value for evaluating relationships among species of North American plums, but they are probably not the best molecular markers available. Many of the relationships suggested by the UPGMA dendrogram are congruent with current taxonomic hypotheses and make sense given our understanding of *Prunus*. On the other hand, in three out of the four species represented by multiple accessions in our study, the accessions did not group together on the dendrogram. To us this suggests that microsatellites are evolving too rapidly in North American plums to be truly useful at resolving species relationships, even among closely related species.

Given the considerable morphological similarity of their leaves and flowers, it is not surprising that *P. alleghaniensis, P. maritima*, and *P. umbellata* cluster together. Wight (1915) placed all three in his *Maritima* group. Clustered with them is *P. gracilis,* which shares with the others an ecological preference for deep sandy soils.

Also the genetic similarity of *P. hortulana*, *P. munsoniana*, and *P. rivularis* is expected. All three species are difficult to distinguish morphologically, especially in the herbarium, where the distinction between thicket-forming shrubs and individual trees is rarely recorded on collection labels. *P. munsoniana* can only be distinguished from *P. rivularis* by its larger stature and larger leaves, and flowering herbarium specimens are mostly indistinguishable. Diggs et al. (1999) concluded that they are doubtfully separable and that *P. munsoniana* is possibly only a larger phase of *P. rivularis*. *P. munsoniana* was one of the last American plums to be named as a species; previously it had been confused with *P. angustifolia* and *P. hortulana* (Hedrick 1911).

The native American plums form a cluster distinct from the Eurasian *P. armeniaca* and *P. cerasifera*. The isolation of *P. armeniaca* supports the traditional view that apricot is not as closely related to the North American plums as one ITS study had indicated (Lee and Wen 2001).

The disjoined placement of the three samples of *P. americana* and the two samples of *P. mexicana* on the dendrogram is more difficult to interpret. All identifications are accurate given current taxonomic concepts, and leaves of the conspecific accessions appear more similar to each other than to accessions of the other species. Recent floras agree that they are separate species, but differ as to the characters used to differentiate *P. americana* from *P. mexicana,* and also as to the range of each species. Some taxonomists (e.g. Smith 1994, Steyermark 1963, following Shinners 1956) treat all individuals with pubescence on the undersides of leaves, on twigs, and on flower pedicels as *P. mexicana*, whereas other taxonomists accept a hairy variety of *P. americana* (e.g. Radford et al. 1968, Vines 1960). Clearly more populations of both species should be sampled and analyzed.

Two unidentified accessions (21 & 22), collected in east Texas where *P. americana* and *P. mexicana* come in contact, were included in the study because they are morphologically

intermediate between the two species. Based on our microsatellite analysis, there is no evidence that they belong to either *P. americana* or *P. mexicana*, nor is there evidence that they are hybrids between these two species. At the time of collection, accession 23 was believed to be a hybrid between *P. angustifolia* and *P. mexicana*. Microsatellite analysis provides no evidence to support this hypothesis. Accession 23 shares the greatest number of alleles with *P. subcordata*, a species whose native range lies over 2000 km from the site where #23 was collected.

One important consideration in interpreting the results is that despite verifying the identity of accessions, some might be misidentified. After microsatellite analysis, one identification was revised. Our accession of *P. orthosepala* shows close genetic similarity to *P. cerasifera*, which along with its Marianna hybrid is widely used as a rootstock for plums (Hartmann et al. 1990). We suspect that the scion on this particular plant died and the top has been replaced by growth from the rootstock. In fact, this was the case for our collection of *P. nigra*, but our greater familiarity with that species allowed us to detect the error in identification prior to running the experiment. Unfortunately, it was our only collection of that species available at the time so *P. nigra* could not be included in the study.

We are continuing to investigate relationships among North American plums using other molecular markers. Because ITS and *trn*L-*trn*F sequences do not provide enough variability (and microsatellites too much), we have turned our attention to a *LEAFY* intron, which in *Isoëtes* is more than four times more variable than ITS (Hoot and Taylor 2001). Preliminary sequence data appear to be supportive of our results from microsatellite analysis including a close relationship among *P. alleghaniensis, P. gracilis, P. maritima*, and *P. umbellata* and of the closer similarity of some accessions of *P. americana* to certain *P. mexicana* accessions than to other accessions of the same species.

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