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Genetic Diversity in Native and Invasive *Rubus* (Rosaceae)

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GENETIC DIVERSITY IN NATIVE AND INVASIVE *RUBUS* (ROSACEAE)

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GENETIC DIVERSITY IN NATIVE AND INVASIVE *RUBUS* (ROSACEAE)

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Invasive species are an increasing threat to biological diversity as well as a leading cause of recent species' extinctions. Invasives spread quickly and efficiently, and the U.S spends millions of dollars annually in the control and eradication of these species. More information is necessary in order to predict which species may become invasive. *Rubus* (Rosaceae) was chosen for study because this genus includes various ploidy levels, reproductive modes, and species that are invasive as well as native. Three *Rubus* species were chosen to represent apomictic and tetraploid invasives (*Rubus armeniacus*), a sexual and diploid native species (*R. occidentalis*), and a sexual and diploid invasive species (*R. phoenicolasius*). Specimens were collected across the U.S. and two different genetic fingerprinting techniques were used; Amplified Fragment Length Polymorphism (AFLP) and Randomly Amplified Fingerprints (RAF). Using three AFLP primers and two RAF primers, genetic similarity was determined and phylograms were constructed. Through statistical analysis and phylogram data it was determined that there might be slightly more genetic diversity in native *R. occidentalis* than in invasive *R. phoenicolasius*. Genetic diversity between apomictic and tetraploid *Rubus armeniacus* and the two sexual and diploid *Rubus* species were so similar that no distinction could be made, although the mean pairwise distances and mean number of alleles were significantly different. It was

also found that geographic distance and genetic similarity do not appear to be related in these three *Rubus* species. During the course of this study it was also observed that the AFLP technique produced more alleles than the RAF technique, although this difference was not significant.

INTRODUCTION

What is an Invasive Species?

Invasive species are a very hot topic in biology because of their negative impact on species, ecosystems, and economies. Invasive species are not native to a particular ecosystem and cause, or are likely to cause, economic or environmental harm (NISC 2006). Invasive species and are often called alien, exotic, introduced, or foreign species. Many exotic plant species have been introduced deliberately by humans for timber, medicine, forage, fiber, food crops, erosion control, or as ornamentals (Baker 1974, 1986). They have also been introduced accidentally by impure crop seeds, adhesion to domesticated animals, and contaminated soil surrounding the roots of nursery plants (Baker 1986). According to the U.S. Department of Agriculture invasive species info (2008), some of the most notable invasive plants in the United States are Canada thistle (*Cirsium arvense*), garlic mustard (*Alliaria petiolata*), Japanese honeysuckle (*Lonicera japonica*), tree of heaven (*Ailanthus altissima*), purple loosestrife (*Lythrum salicaria*), and kudzu (*Pueraria lobata*). Invasive plant species have become a global ecological and economical problem, cost the U.S. ~$34,000 billion in losses and damages annually (Pimentel 2005). In other regions, numerous other species have become problematic such as Australian acacia (*Acacia mearnsii*) which has invaded North America, South America, Asia, Europe, Pacific, and Africa (GISD 2008).

Invasive plant species are aggressive plants that have been transported to an area that lacks the insects, diseases, and foraging animals that naturally keep the organism's

growth in check (Callaway and Aschehoug 2000). A reduction in detrimental selection pressures in the new habitat, allows for rapid growth; thus, the key to becoming invasive is the ability to adapt (Lee 2002). There are ~17,000 native plants in the U.S. and an estimated 5,000 introduced species (Morin 1995 and Morse et al. 1995, in Pimentel et al. 2005). In Hawai'i alone, 50% of all plants are introduced, and of the nine *Rubus* species found there, only two are native (Randell et al. 2004). Invasive species dominate areas by outcompeting native species leading to possible extinction (Gurevitch and Paddilla 2004), making these species a significant component of global change (Vitousek et al. 1996). An example of this is the zebra mussel (*Dreissena polymorpha*) in the Mississippi River basin; this mussel has put over 60 endemic mussel species at risk of extinction (Ricciardi et al. 2003).

Once invasive species were recognized as a problem, the primary goal has been to understand why such species are so successful. This information could be used to predict the likelihood of a species becoming invasive and potentially reduce the number of new problems. It has been suggested that invasive plants possess characteristics that allow them to outcompete their native counterparts. Several authors proposed that plants that produce fruits with many lightweight seeds would have more effective dispersal (Baker 1965; Werner and Platt 1976; Primack 1978; Kawano 1981; Greene and Johnson 1994). Invasiveness might also be characterized by the ability of the plant to self-fertilize. Self-fertilization gives plants the advantage of being able to reproduce when no other individuals are near, allowing them to be excellent colonists (Baker 1965). Huenneke and

Vitousek (1990) proposed that multiple reproductive strategies composed of asexual and sexual modes might be the key to invasiveness.

In the mid 1990's plant height, life form, and competitiveness were thought to indicate invasiveness (Pysek et al. 1995). Height and life form can help determine dispersal rate. Competitiveness can be associated with an invasive's ability to spread due to reaching reproductive maturity faster and having a short interval between large seed crops (Rejmanek 1996). Daehler (1998) stated that plants that reproduce vegetatively, lack pregermination seed treatment requirements, have perfect flowers, and a persistent fruit will be more likely to be invaders.

Muth and Pigliucci (2006) analyzed many traits (phonological, architectural, size and fitness) of invasive and non-invasive Asteraceae. However, Muth and Pigliucci (2006) found no significant differences. They concluded that the difference between invasive and non-invasive Asteraceae species is based more on introduction histories than the physical traits of the organism (Colautti and MacIsac 2004; Puth and Post 2005). Others suggested that phenotypic differences expressed among closely related invasives and non-invasives are due to multiple trait interactions and not necessarily fitness differences (Grotkopp et al. 2002; Muth and Pigliucci 2006). The general conclusion is that, although certain traits occur more often in alien plant species than in native species (Sakai et al. 2001), there is no simple biological predictor of invasiveness (Muth and Piglucci 2006).

Genetics of Invasives

For many years, the invasiveness of a plant was thought to depend on its overall physical tolerance. Researchers considered that the lag time, between the introduction of a species and its invasion, was needed to establish a sufficient population size; now however, it is thought that lag time is used to accumulate adequate levels of genetic variation to be able to respond to natural selection (Ellstrand and Schierenbeck 2000; Lee 2002). High levels of additive genetic variance have been found in source populations that possess traits that might facilitate invasiveness (Hard 1993; Carrol 2001). A temporary bottleneck can expose genes to selection pressures that were not normally exposed to selection; this can contribute to the rapid rate of evolution found in invasive plant species (Reznick 2001).

Hybridization can also be an important factor in a plant's invasiveness. Hybridization can cause new gene interactions, masking or deletions of deleterious recessive alleles, or the transfer of favorable genes; this can lead to faster growth, greater size, and increased aggression (Rieseberg 1999; Ellstrand and Schierenbeck 2000). These new gene interactions give hybrids the ability to have a phenotype similar to one parent or the other, be intermediate between the parents, or extreme (Rieseberg 1995). All of these factors make hybridization a powerful evolutionary stimulus for invasiveness (Ellstrand and Schierenbeck 2000). Ayres and Strong (2006) proposed that the formation of self-compatible hybrids be added to the list of mechanisms that allow hybrids to promote the evolution of invasiveness. It is recognized that hybridizations are only responsible for a fraction of invasive plants, but human dispersal and disturbance

accelerate the process and increase the opportunities for hybrid lineages to become established and invasive (Ellstrand and Schierenbeck 2000). A good example of an invasive hybrid is *Rubus* "*cuneifolius*" in South Africa. Presumably, introduced *R. cuneifolius* from the southeastern U.S. hybridized with another *Rubus* species in South Africa, increased its ploidy level, and became highly invasive (Spies and Du Plessis 1985; Sutherland et al. 2005).

Hybrids can cause new species by diluting or assimilating native species genotypes until there are no pure natives, leading to species replacement (Huxel 1999). *Rubus hawaiensis,* an endemic Hawaiian raspberry, is facing a different type of problem due to hybridization. Hybrids between *R. hawaiensis* and introduced *R. rosifolius* are sterile so genetic assimilation or dilution is not a big concern, but the negative impact of reproductive effort lost by *R. hawaiensis* is an issue (Randell et al. 2004).

Polyploids, like hybrids, can promote invasive characteristics. There are two types of polyploids; the first is a permanent hybridization that produces plants with higher ploidy levels are known as allopolyploids (Levin 1983). In these plants, homeologous chromosomes do not pair during meiosis yielding a condition known as fixed heterozygosity. Many cultivated crop species such as cotton and wheat are allopolyploids. The other type of polyploid is an autopolyploid which arises from parents belonging to the same species (Soltis 2000). They also tend to have higher levels of heterozygosity and exhibit less inbreeding depression than diploids from similar species (Soltis 2000).

Once a plant has been introduced into a new area, it typically experiences a founder effect. Founder effect occurs because colonizing individuals contain only a subset of the genetic variation present in the source population. Moreover, reduced genetic diversity and small population size may lead to further changes caused by genetic drift (Husband and Barrett 1991). An example of an invasive species that underwent founder effect is *Rubus alceifolius*. *Rubus alceifolius* genetic diversity was studied in its native range of southeast Asia as well as its introduced range of Madagascar and neighboring Indian Ocean islands; greater genetic diversity was found in its native range (Amsellem et al. 2000). However, multiple introductions of an invasive can reduce or even eliminate founder effect. For example, kudzu (*Pueraria lobata)* has relatively high genetic diversity among populations due partly to multiple introductions (Pappert et al. 2000).

Reproductive system has a major influence on the amount of genetic diversity in newly established invasive populations. Most weedy plants are selfing or apomictic; combined with founder effect, this creates a population with low genetic diversity (Husband and Barrett 1991). Individuals that reproduce by outcrossing have more genetic variation but can lack individuals with which to mate. Self-compatible individuals have less genetic variation but do not have the problem of finding a mate. Apomictic species have little to no variation, but if the genotype is successful, there is no need to change the genotype (Campbell et al. 1997). The best solution might be an apomictic species with a good genotype that reproduces to some degree by outcrossing.

Rubus

The plant genus *Rubus* (Rosaceae) is a good candidate for studying the biology of invasives because it includes many hybrids, polyploids, and apomicts and also includes several invasive species. *Rubus* comprises between 400 and 750 species traditionally divided into 12 subgenera; the three largest are *Idaeobatus, Malachobatus*, and *Rubus* (Focke, 1910, 1911, 1914; Robertson 1974). Species of *Rubus* are important as fruit crops yielding over 100,000 tons annually for jam making, canning, freezing and flavorings (Jennings 1995). *Rubus* can also be an ornamental, invasive weed, and important in early forest succession being among the first plants to colonize disturbed habitats (Thompson et al. 1995; Hummer 1996; Howarth et al.1997).

Polyploidy and hybridization are prevalent in *Rubus.* Approximately 60% of all *Rubus* species are polyploid and only subgenera *Idaeobatus*, *Dalibarda*, and *Anoplobatus* are largely diploid (Thompson 1997). Ploidy in *Rubus* ranges from 2*x* (*x* = 7) to 14*x*, to possibly 18*x* (Thompson 1997). Hybridization occurs mostly between closely rated species and in some cases between subgenera (Alice et al. 2001). Apomixis is most common in subgenus *Rubus* and is always associated with polyploidy (Gustafsson 1942; Einset 1951).

Six invasive *Rubus* are recognized by the Global Invasive Species Database (GISD 2008). *R. alceifolius* is introduced in Australia, Madagascar, Mayotte, Reunion and Mauritius (Amsellem et al. 2000). *R. armeniacus* is introduced in South Africa, Asia, eastern Europe, and North America. *R. ellipticus* is recognized on the “top 100 world worst” invasive. It is introduced in Hawai’i, and England. *R. moluccanus* is introduced in

many Pacific islands, Indonesia, Philippines, Thailand, Vietnam, Mauritius, and Reunion. *R. niveus* is introduced in southeastern Africa, Florida and Hawai'i, northern South America including the Galapagos, and Tasmania. *R. rosifolius* is introduced in New Caledonia, Papua New Guinea, Solomon Islands, Vanuatu, Indonesia, Malaysia, Reunion, Mauritius, Seychelles, Hawai'i, and French Polynesia.

Subgenus *Rubus* are blackberries and dewberries in which the ripe fruit does not detach from the receptacle (Focke 1914). Ploidy level ranges from 2*x* to 5*x,* apomixis is common (Einset 1951; Jennings 1995), and most species occur in Europe and western Asia, and from North America to central South America. From this subgenus I studied *R. armeniacus*, also known as Himalaya berry. It is a facultative pseudogamous apomictic tetraploid (Nybom 1986). This is invasive species from the Caucausus region of western Asia can also be found commonly in North America west of the Rocky Mountains and scattered in the northeastern U.S. (Fig. 1). Its current status as a noxious weed makes it illegal to bring into the U.S. without a permit (Hummer, 1996). It was introduced to the Pacific Northwest in the 1800s as a larger berry for consumption (Jennings 1988). Morphologically *R. armeniacus* is a large, mounding species with stout canes, robust prickles, and palmately 5-foliate leaves (Fig. 2).

Subgenus *Idaeobatus* are raspberries in which the ripe fruit separates from the receptacle (Focke 1914). These *Rubus* are primarily diploid (2*n*=2x=14) and sexual (Thompson 1995, 1997). Several species were domesticated and bred as fruit crops such as *R. idaeus* (red raspberry). The domestication of raspberries involved the selection of favorable traits such as fewer, stouter canes, stronger fruiting branches and larger fruit

(Jennings 1995). From this subgenus *R. occidentalis* (section *Idaeanthi*, series *Occidentales*) and *R. phoenicolasius* (section *Idaeanthi*, series *Nivei*) were studied (Focke 1914).

Rubus occidentalis is known as eastern black raspberry, and it is native throughout much of North America (Fig. 3) excluding the far western regions (Gleason and Cronquist, 1991; Alice et al. in mss.) It appeared in cultivated form in the 1830's (Jennings 1995). The stems have a waxy glaucus covering, the undersides of the leaflets are densely tomentose, and the fruit is a cluster of many purple to black drupelets with tufts of white hairs between the drupelets (Fig. 4).

Rubus phoenicolasius is known as wine raspberry. This is an invasive species from eastern Asia and can now be found scattered in eastern North America (Fig. 5). This species was introduced in the 1890s as breeding stock for new raspberry cultivars. The main distinguishing characteristic is its long reddish-purple glandular hairs on all plant parts (Alice et al. in mss.; Fig. 6).

Objectives

The main objectives of this research were to: 1) compare the genetic variation found in invasive *Rubus* (*Rubus phoenicolasius* and *R. armeniacus*) compared to native *Rubus* (*R. occidentalis*), 2) compare genetic variation in sexual and diploid *R. occidentalis* and *R. phoenicolasius* to apomictic and tetraploid *R. armeniacus,* and 3) determine if genetic similarity is associated with geographic distribution.

Objective 1:

- H_0 = No difference in genetic diversity between the introduced and invasive *Rubus phoenicolasius* and native *R. occidentalis*.
- H_1 = Introduced *Rubus* will have less genetic diversity.

Objective 2:

- H_0 = No difference in genetic diversity in between apomictic and tetraploid *Rubus armeniacus* and sexual and diploid *R. occidentalis* and *R. phoenicolasius.*
- H_1 = Sexual and diploid *Rubus* will have more genetic diversity than apomictic and tetraploid *Rubus armeniacus.*

Objective 3:

- H_0 = No relationship between geographic distance and genetic similarity.
- H_1= *Rubus* from geographically proximate locations will be more genetically similar than *Rubus* from geographically distant locations.

Techniques

To determine the best possible techniques to use in this study I evaluated various population genetics techniques. There are many available genetics techniques including allozymes, isozymes, RFLP (restriction fragment length polymorphisms), AFLP (amplified fragment length polymorphism), SSR (simple sequence repeats), and RAPDs (random amplified polymorphic DNA) that have been successfully used. Allozymes and isozymes are enzymes produced by genes. Because the products are proteins, there is often not much variation among individual samples (Hamrick and Godt 1990).

Allozymes and isozymes are a quick cheap and reliable method for studying population genetics. Most researchers have abandoned these techniques for more specialized and variable genetic markers.

VNTR (variable number tandem repeats), and RFLP, SSR are all co-dominant markers making them more informative than a molecular marker with full dominance (Jones et al. 1997). VNTR are sequences of DNA that have many repeats; each individual has a different number of repeats. These repeats are flanked by sequences which can be used to extract a section of DNA containing the repeats for analysis. This can be done with restriction enzymes.

RFLPs (restriction fragment length polymorphisms) were the most commonly used technique in genetic diversity studies in plants (Pejic 1998). RFLPs require large quantities of high-quality DNA and a sizable lab and specialized equipment due to the use of radioactivity (Pejic 1998). Depending on the level of polymorphism in a population it might be more effective than isozymes or allozymes (Jones et al. 1997). SSRs (simple sequence repeats) or microsatellites are polymorphic loci present in eukaryotic genomes containing repeats of 1 to 6 base pairs in length (Pejic 1998). SSRs have been frequently used in plant genetics and show extensive variation among individuals (Akkaya et al. 1992; Senior and Heun 1993; Wu and Tanksley 1993). SSRs are more informative then RAPDs (random amplified polymorphic DNA) because SSRs are co-dominant markers. They have the advantage of increased levels of polymorphism and allow detection of heterozygosity (Russell et al. 1997; Waldron et al. 2002). However, SSRs are very labor intensive involving the initial cloning and sequencing of

every locus (Akkaya et. al. 1995). Also there can be problems with reproducibility of data (Demeke et al. 1997; Karp et al. 1997).

RAPDs, AFLPs, and RAFs (randomly amplified fingerprints) are PCR (polymerase chain reaction) based dominant markers meaning they offer less information than co-dominant markers. Thus, one can only detect the presence or absence of the dominant allele and not heterozygosity. Their advantage is that they can produce data about population structure and genetic diversity (Kesseli et al. 1992) and the nucleotide sequence does not have to be known (Vos 1995).

RAPDs are random amplified polymorphic DNA (William et. al. 1990). RAPDs are easy to perform, quick, and can produce a lot of data (Heun et al. 1993; Russell et al 1993; Fernando and Cass 1996). In this process, short random primers are developed to anneal with the template and begin amplification. If the primers anneal too far apart or if the 3'-ends are not facing each other, the DNA will not be amplified. This allows for a unique result for each individual. Their main problem is lack of reproducibility.

AFLPs are amplified fragment length polymorphism. This method is highly reproducible and allows for the specific co-amplification of a large number of restriction fragments which can be analyzed simultaneously (Vos 1995). Information content and the average number of alleles are higher than for SSRs while the lowest level of polymorphism is obtained with AFLPs. However, AFLPs are more efficient due to their ability to reveal several bands in a single amplification. The efficiency index is 10 times higher in AFLPs than other methods (Pijic 1998); up to 100 markers can be detected per PCR (Thomas 1995; Vos 1995).

RAFs (randomly amplified fingerprints) are a new modified DNA amplification fingerprinting technique. RAFs and AFLPs have similar efficiency and reliability (Waldron 2002). There are many advantages of this process over AFLPs. RAFs are much cheaper and quicker than AFLP there is no requirement for enzymatic preparation of templates, and there is only one PCR reaction in RAFs compared to two PCR reactions in AFLPs (Waldron 2002). Although RAFs have not been used in a plant genetic diversity study that I know of, its similarities to AFLPs indicate its potential for success. RAFs have an advantage over AFLPs in fingerprinting individuals though both technologies can be used in a complementary fashion for some genetics studies (Waldron 2002).

Nearly all of these molecular techniques have been used in studies of *Rubus.* The SSR technique has been successfully used in *Rubus* to genetically map raspberry and blackberry (Strafne et al 2005).Weber (2003) used RAPD markers effectively to determine relationships among 16 *R. occidentalis* accessions as well as their relationship to other *Rubus* species. RFLP was used with *R. occidentalis* and *R. pensilvanicus* to reveal that the larger number of genotypes found in a *R. occidentalis* was related to its reproductive mode (Nybom and Schaal1990). Amsellem et al. (2000) used AFLP to assess the genetic diversity of *R. alceifolius* in its native range and its introduced range. Their research determined that *R. alceifolius* has higher levels of genetic diversity in its home range than in its introduced range. Kollmann et al. (2000) also used AFLP and allozymes to compare genetic variation in *R. bifrons* and *R. armeniacus*. Lindqvist-Kreuze et al. (2003) used three AFLP primers to determine the genetic diversity of *R.*

arcticus. It was also used to determine the genetic diversity between six populations of *R. arcticus* (Lindqvist-Kreuze et al. 2003).

In this study both AFLP and RAF were used in a complementary fashion to determine the genetic diversity of three *Rubus* species. AFLP was selected because of its reproducibility, its large number of alleles, and its previous use with similar *Rubus* species (Kollmann et al. 2000). RAF was selected because it also produces reproducible results and it is fairly simple and inexpensive, though it has not been used in *Rubus* or any other plant group for a genetic diversity study. The approach to use both AFLP and RAF will offer the opportunity to compare the utility of these two techniques in *Rubus*.

MATERIALS AND METHODS

Plant Material for Genetic Studies

During the summer of 2007, specimens of *Rubus occidentalis* and *R. phoenicolasius* were collected from the eastern U.S. and *R. armeniacus* specimens were obtained from central California to southern Oregon in 2006. The following *Rubus* samples were obtained: 16 *R. armeniacus* from two states (Fig. 1), 89 *R. occidentalis* from 19 states (Fig. 3), and 27 *R. phoenicolasius* from 13 states (Fig. 5). Several young leaves were removed from the first-year primocane of each individual and placed into a Tiger-pak zip-loc plastic bag containing silica gel to desiccate the tissue. First-year canes and/or reproductive material (flowers and/or fruits) were collected as morphological vouchers and deposited in the WKU Herbarium. Samples were commonly collected from roadsides, fence lines, and other disturbed sites. At each collection site information was gathered such as general location, GPS location, digital photos of the specimen, and characteristics such as height and habit. Specimens selected for analysis were chosen to best represent the known geographic distribution of each species.

DNA extraction

All of the young leaf tissue was frozen using liquid nitrogen and ground into a fine powder with a mortar and pestle. I used 0.02 grams of powdered leaf tissue for DNA extraction using a DNeasy plant mini kit (Qiagen, Germantown, MD) and determined DNA concentration using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE).

AFLP

The AFLP protocol follows the manufacturer's instructions (Applied Biosystems, Foster City, CA). First, the *Mse*I and *Eco*RI adaptor pairs were annealed by placing them into a 95ºC water bath for 5 minutes; then I allowed them to cool at room temperature for 10 minutes before centrifuging at 14,000 rpm for 10 seconds. Next I prepared the Enzyme Master Mix containing 0.1 µL of 10X T4 DNA ligase buffer with ATP (New England BioLabs, Ipswich, MA), 0.1 µL of 0.5 M NaCl, 0.05 µL 1 mg/mL BSA, 0.02 µL of 50,000 units/ µL *Mse*I (New England Biolabs Ipswich, MA), 0.005 µL of 100,000 units/µL *Eco*RI, 0.033 µL T4 DNA ligase, and 0.692 µL nanopure water. I gently mixed the solution, centrifuged it for 10 seconds, and placed it on ice for no longer than 1-2 hours.

Next, I performed the restriction ligation reactions with each tube containing 1.0 µL 10X T4 DNA ligase buffer with ATP, 1.0 µL 0.5M NaCl, 0.5 µL 1.0 mg/ml BSA, 1.0 µL *Mse*I adaptor, 1.0 µL *Eco*RI adaptor, 1.0 µL Enzyme Master Mix, and 5.5 µL DNA for a total of 500 ng. The solution was mixed, placed in a centrifuge for 10 seconds, and incubated at 37ºC for 2 hours in a thermocycler. I diluted the restriction ligation reaction 189 µL of AE buffer and mixed it thoroughly.

Preselective amplification allowed the sequences with adaptors annealed to both ends to amplify exponentially and become dominant in the product. The preselective amplification involved 4.0 µL of diluted DNA prepared by restriction-ligation, 1.0 µL AFLP preselective primer pairs, and 15.0 µL of AFLP Core Mix. This solution was vortexed, centrifuged, and placed in the thermocycler. The PCR parameters were 72ºC

for 2 minutes, 20 cycles of 94ºC for 20 seconds, 56ºC for 30 seconds, 72ºC for 2 minutes, followed by a final extension of 60ºC for 30 minutes. Then I prepared the template for the next step by adding 10 µL of preselective amplification reaction product to 190 µL of AE buffer.

Selective amplification targets only the specific *Mse*I and *Eco*RI fragments used. I combined 3.0 µL diluted preselective amplification reaction product, 1.0 µL fluorescently labeled *Mse*I primer- Cxx (Table 1) (three different *Mse*I primers were used), 1.0 µL fluorescently labeled *Eco*RI primer AGG, and 15.0 µL of AFLP Core Mix. The PCR parameters started with 94 ºC for 2 minutes, then cycles with 94 ºC for 20 seconds, 66ºC for 30 seconds, and 72ºC for 2 minutes. Each cycle the annealing temperature was decreased 1ºC until 56ºC was reached. The final cycle was repeated 19 times, then incubated at 60ºC for 30 minutes.

Lastly, I combined 24 µL HiDye formamide, 0.5 µL of Gene Scan (ROX-500) size standard, and 0.5 µL of selective amplification product into a 96-well plate. Before loading into an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA), I mixed these samples and placed them in a thermocycler at 95ºC for 5 minutes followed by a quick chill on ice for 5 minutes.

RAF

The RAF reaction comprises 4.0 µl of master mix (Promega, Madison, WI), 5.0 µl of fluorescently labeled primer (designed by Schlipalius et al. 2001; see Table 1) and 10 – 40 ng of DNA. I vortexed, centrifuged, and placed the solution in a thermal cycler (MJ Research, Ramsey, MN). The PCR profile consists of a denaturation at 94°C for 5

minutes, then 30 cycles of 94°C for 30 seconds and 1 minute at each of 57°C, 56°C, 55°C, 54°C, and 53°C. The final extension step is 72°C for 5 min. 1.0 µl of ROX-500 size standard (Applied Biosystems, Foster City, CA) and 10 µL HiDye formamide are added to each PCR product, mixed, and placed into a 96-well plate. After heating the plate at 95°C for 5 minutes and placing it on ice for 3-5 minutes, I loaded it into an ABI 3130 genetic analyzer. The RAF process must be performed in the dark due to the fluorescently labeled primer and light sensitive ROX size standard.

I used the Genemapper 3.7 program (Applied Biosystems, Foster City, CA) to determine the number and size of the fragments produced for both AFLP and RAF. The band width was 1.5 and peak height ranged from 12 to 15 depending on primer. I deleted fragments that appeared in the negative control and in the samples because they represent experimental artifacts. I scored non-artifactual fragments for each individual as present (1) or absent (0). Then I made a binary coded data matrix for analysis using PAUP 4.0 b10 (Swofford 2002).

All RAF and AFLP data were combined into a single matrix. I performed parsimony (MP) analyses using heuristic searches with 10,000 replicates of random stepwise-addition of taxa and TBR branch swapping on all three species together and on each species separately. To assess confidence for groupings, I conducted bootstrap analysis with 10,000 replicates and simple addition of taxa. Bootstrap values of 50% or higher were retained and placed on the resulting phylogenies. I also analyzed the combined three species data set by Neighbor Joining (NJ) using *Rubus armeniacus* as the outgroup.

Due to having only 16 *Rubus armeniacus* samples, the number of individuals of *R. occidentalis* and *R. phoenicolasius* was reduced from 25 to 16. The sample size was reduced by removing individuals that were clustered together geographically, but still retained the overall geographic distribution. Pairwise distances for the sets containing 25, 16, and 10 individuals were generated and the means and standard deviations were calculated for each (Table 2). The number of alleles and means were found for each species and each technique. For each parameter scored, normality was tested (Shapiro-Wilks normality test). Due to non-normal data, a Mann-Whitney test (SYSTAT 11 ink, Chicago, IL) was performed to assess statistical significance.

RESULTS

Genetic Diversity within Species

Tests of the pairwise distances (Table 3) showed that genetic diversity between *R. occidentalis* and *R. phoenicolasius* (P=0.714, U=44223) was not significantly different. In terms of mean number of alleles per individual scored across all five primers (total number and mean/individual; Table 4), *R. occidentalis* had 9.1 and *R. phoenicolasius* had 8.2. A significance test of allele data (Table 5) determined that there was no difference (P=0.917, U=13). Additionally, the number of pairs of individuals and their respective distances were calculated and graphed (N=16; Fig. 7); for *R. occidentalis* and *R. phoenicolasius* the distances range form 0.1 to 0.6 for *R. occidentalis* and 0.1 to 0.5 for *R. phoenicolasius. R. occidentalis* had 18 distances ≥0.4 and *R. phoenicolasius* is the least diverse with only 4 pairwise distances ≥0.4 and none ≥0.5. The Neighbor-Joining analysis produced a tree in which *Rubus occidentalis* individuals had longer branch lengths than *R. phoenicolasius.* This indicate that *R. occidentalis* is more genetically diverse than then *R. phoenicolasius.*

Tests of the pairwise distances (Table 3) showed that genetic diversity between *R. armeniacus* and *R. phoenicolasius* (P<0.01, U=32292) and *R. armeniacus* and *R. occidentalis* (P<0.01, U=30909) were significantly different. This indicates that *R. armeniacus* (mean 0.308, ± 0.086, N= 16) is more diverse than *R. occidentalis* (mean 0.193, ± 0.073, N=25) and *R. phoenicolasius* (mean 0.190, ± 0.052, N=25). The number of pairs of individuals and their respective distances were calculated and graphed (N=16; Fig. 7). *R. armeniacus* ranged from 0.2 to 0.6 similarly variable with *R. occidentalis* (0.1

to 0.5) However, *R. armeniacus* appears more diverse than *R. phoenicolasius* (0.1 to 0.6). *R. armeniacus* had 17 distances ≥0.4, *R. occidentalis* had 18 distances ≥0.4, and *R. phoenicolasius* had 4 pairwise distances ≥0.4 and none ≥0.5. A Mann-Whitney U test of allele data (Table 5) determined that *R. armeniacus* is significantly less diverse than *R. occidentalis* (*P*=0.016, U=1.00) and *R. phoenicolasius* (*P*=0.021, U=1.500). *R. armeniacus* individuals also had the shortest NJ tree branch lengths of the three species.

Phylogenetic Relationships

Rubus armeniacus

Heuristic searches produced 151 equally parsimonious trees (length 152) representing multiple islands. Excluding uninformative characters, the Consistency Index (CI) was 0.418 and the Retention Index (RI) was 0.386. The presence of numerous equally parsimonious and divergent islands largely collapsed the topology in the strict consensus tree (not shown). Because of this, one tree was selected (Fig. 9) from the island comprising the majority of the trees (60.4%). The most notable result places accessions 24 and 48 in a well-supported clade (83% bootstrap). This relationship also appears in the combined Neighbor-Joining analysis showing that these two individuals *R. armeniacus* are highly divergent from the others (Fig. 7). With this exception, no other relationships received bootstrap support ≥50% and there does not appear to be any geographic structure as specimens from Oregon are interspersed among those from California.

Rubus occidentalis

Heuristic searches yielded 10 equally parsimonious trees of length 522 (strict consensus in Fig. 10). The CI is 0.428 and the RI is 0.232. The most basal clade consists of two individuals from Maine (R16) and New York (79); these are the northeastern-most samples except for one specimen from north-central Connecticut (126). The second most basal clade comprises two individuals from Pennsylvania (84) and West Virginia (58); this clade is also supported in the combined NJ tree (Fig. 8). The remaining individuals all fall into one large clade. A subclade of interest comprises samples from Wisconsin (117), Illinois (106), Arkansas (108), and Missouri (105); these are the western-most individuals analyzed and all but Wisconsin (117) from a cluster in the combined NJ tree (Fig. 8). The only group with bootstrap support (60%) consists of one specimen from southern Ohio (54) and one from Michigan (95).

Rubus phoenicolasius

Heuristic searches produced 7 equally parsimonious trees of length 452 (strict consensus in Fig. 11). The CI is 0.451 and the RI is 0.266. The phylogeny shows a sister group relationship of a clade with individuals from North Carolina (11), Illinois (105), New Jersey (75), and Tennessee (2) and a larger clade with all remaining individuals. Two small clades received bootstrap support: 1) individuals 112 and 36 from Indiana and Kentucky (78%), respectively, and 2) individuals 28 from West Virginia and 34 from Virginia (62%). Both of these clades are supported in the combined NJ tree (Fig. 8).

AFLP and RAF Comparisons

All data are based on 66 total individuals and all three species. The AFLP primer *Mse*I-CAA produced 183 alleles with a mean of 61. Primer *Mse*I-CAG had 114 alleles with a mean of 38. Primer *Mse*I-CAT had a total of 72 alleles with a mean of 24. The RAF primer RP2 had a total of 61 alleles with a mean of 20.3. Primer RP4 had 59 alleles with a mean of 19.7 (Table 4). Overall, AFLP produced an average of 123 alleles per primer and RAF produced 60 alleles per primer. The Mann-Whitney test indicates that AFLP (three primers) did not produce significantly more alleles than RAF (two primers) (P=0.427, U=39) (Table 4).

DISCUSSION

Native vs. Introduced *Rubus*

Genetic similarity based on mean pairwise distances between individuals (25) indicates that native *Rubus occidentalis* (80.7%) and introduced and invasive *R. phoenicolasius* (81.0%) are not significantly different (Fig. 2, 3). *Rubus occidentalis* also has a higher mean number of alleles per individual (9.1) than *R. phoenicolasius* (8.2), but once again the difference is not significant. Yet, there are two additional measures which weakly suggest *R. occidentalis* is more genetically diverse than *R. phoenicolasius*. *Rubus occidentalis* has more pairwise distances ≥0.4 (18) than *R. phoenicolasius* (4) and has slightly longer branches on the NJ phylogram (Figs. 7, 8). In contrast, *R. phoenicolasius* has a mean number of 8.2 alleles per individual, 4 pairwise distances ≥40%, and somewhat shorter branches on the NJ phylogram. Therefore, the null hypothesis of no difference in genetic diversity between introduced and invasive *Rubus phoenicolasius* and native *R. occidentalis* cannot be rejected, although no statistical tests of significance were performed on the NJ phylogram.

Nybom and Schaal (1990) established that substantial genetic diversity exists within a single *Rubus occidentalis* population. Weber (2003) studied genetic diversity in *R. occidentalis* using RAPD and concluded that among 16 cultivars, the average similarity was 81% and ranged from 70% to 98% between pairs of accessions. My study revealed equivocal results using the data set of 25 individuals (80.7%). However, when the data set of 16 individuals is used, the mean genetic similarity is 73%. He suggested that additional genetic diversity might be found in the extreme portions of the *R.*

occidentalis geographic distribution (Weber 2003). However, my geographic representation included *R. occidentalis* samples from the edge of its range, (e.g., Maine and Arkansas) and similar results were found. This suggests that a broader sampling range does not necessarily affect genetic diversity of *R. occidentalis*. Consequently, *R. occidentalis* may naturally contain relatively low levels of diversity compared with other sexual diploid species. Although, other *Rubus* species have similar levels of genetic identity such as the diploid *R. arcticus* and R. coreanus, with genetic similarities ranging from 72-94 % (Hong 2003 et al.; Lindqvist-Kreuze 2003 et al.).

Invasive *Rubus phoenicolasius* was predicted to have less genetic diversity than native *R. occidentalis* due to founder effect. However, this may or may not be the case; *R. phoenicolasius* genetic diversity is not significantly different than *R. occidentalis* based on mean pairwise distances and mean number of alleles, but is lower using other metrics (NJ branch lengths). The higher than expected diversity of *R. phoenicolasius* could be due to several factors. Genetic diversity of *R. phoenicolasius* has not been studied in its home range so there is no way to determine if there is less genetic diversity in its introduced range. Another issue is that *R. phoenicolasius* may have been introduced multiple times during the past 100 years. These repeated introductions can add genetic variation to the population minimizing founder effect. Moreover, *R. phoenicolasius* has been present in the U.S. since the 1890's allowing it many years of sexual reproduction to reduce founder effect. In the end, it may be a combination of all three factors contributing to produce adequate levels of variation.

Apomictic and Tetraploid vs. Sexual and Diploid *Rubus*

Genetic similarity based on mean pairwise distances between individuals shows that *Rubus armeniacus* (69.2%, N=16) is significantly different from *R. occidentalis* (72.8%, N=25) and *R. phoenicolasius* (72.2%, N=25) (Table 2, 3) are significantly different. Therefore, apomictic and tetraploid *R. armeniacus* is more diverse genetically than either of the two sexual and diploid *Rubus* species studied. Additionally, *R. armeniacus* has a lower number of pairwise distances ≥0.4 (17) than *R. occidentalis* (18) and a greater number of pairwise distances than *R. phoenicolasius* (4; Fig 7). Other measures weakly support *R. armeniacus* as being less diverse. *Rubus armeniacus* has a significantly lower mean number of alleles per individual (3.6) than *R. occidentalis* or *R. phoenicolasius* and displays the shortest branches on the NJ phylogram (Fig. 8). In this case, our null hypothesis of no difference in genetic diversity between apomictic and tetraploid *Rubus armeniacus* and sexual and diploid *Rubus* could not be rejected or supported due to conflicting data.

Kudzu (*Pueraria lobata*), another apomictic invasive, showed higher genetic diversity than expected in its introduced range with 71% (Pappert 2000). This level of variation was attributed to the fact that this species has been repeatedly introduced over the course of decades. These introductions allowed kudzu to regain a portion of its lost genetic diversity due to founder effect (Pappert 2000).

The concept of multiple introductions increasing genetic diversity could be applied to *R. armeniacus. Rubus armeniacus* was first introduced to the northwestern U.S in the 1800's for consumption (Jennings 1995). The possibility of multiple introductions

and the many years it has been present in the U.S. may have allowed *R. armeniacus* to establish substantial variation. Polyploids like *Rubus armeniacus* (4*x*) tend to have higher levels of heterozygosity and less inbreeding depression than diploids (Soltis 2000). This would give it the ability to recover quickly from founder effect or it might not have experienced founder effect at all. Its facultatively apomictic reproductive mode with some sexuality would allow it to easily establish adequate genetic diversity.

Rubus alceifolius is a similar presumably polyploid species (all subg. *Malachobatus* species that have been studied are polyploid). The genetic diversity of *R. alceifolius* was based on AFLPs in its native range and its introduced range (Amsellem et al. 2000). They stated that because of its suggested apomictic mode of reproduction, there is little genetic diversity in its introduced range (ranging from 83% to 99% similarity). Although, the native range does contain slightly more variation (ranging from 73% to 99% similarity). Another polyploid *R. fruticosus* has a similar genetic similarity range (Salvini et al. 2006).

Kollmann et. al. (2000) examined sexuality and apomixis in *R. armeniacus* and *R. bifrons* using five AFLP primers. They found that *R. armeniacus* has low genetic diversity within seed families; the genetic variants are most likely the product of sexual recombination. However, the rate of sexual reproduction (14%) found by Kollmann et al. (2000) is typical of apomicts and within the range suggested by Nybom (1995). The increased diversity, when compared to *R. occidentalis* and *R. phoenicolasius*, might indicate that *R. armeniacus* has a higher rate of sexual reproduction than suspected.

Geographic Proximity and Genetic Similarity

The combined analysis parsimony tree (not shown) failed to separate individuals by species although the Neighbor-Joining tree did (Fig. 8). This might be due to long branch attraction, the artificial attraction of non-similar individuals due to a rapidly evolving lineage (Bergsten 2006) or it could be due to high levels of homoplasy or possibly even a lack of parsimony-informative characters.

Rubus armeniacus data produced only one clade supported by bootstrap values ≥50% (Fig. 9). This clade suggests some geographical correspondence (collection sites are ~134 miles apart) yet other individuals were geographically closer. Moreover, the habitats of these two specimens were very different; accession 24 was from a site adjacent to a redwood (*Sequoia sempervirens*) grove and accession 48 was from a roadside next to an agricultural field. The remaining relationships suggested by the parsimony analysis do not indicate any observable patterns based on geographic proximity or habitat. Individuals from Oregon are interspersed with those from California and specimens from coastal sites are also mixed with specimens from montane or central valley sites. Because birds are the most likely dispersal agent of *Rubus* seeds, it is not surprising that *R. armeniacus* individuals from divergent sites appear related.

The strict consensus phylogeny of *Rubus occidentalis* includes many clades (Fig. 10). Two of the most notable clades show a better relationship with geographic proximity. One small clade comprises samples from Maine and New York although *R. occidentalis* 126 from CT is geographically closer. The second clade contains four individuals from Wisconsin, Illinois, Arkansas, and Missouri. These are western-most

samples included in the study. Thus, it appears that there may be some correspondence between geographical distance and genetic similarity for some clades, though the general lack of well-supported relationships in the phylogeny precludes a more definitive examination.

The strict consensus phylogeny of *Rubus phoenicolasius* includes two clades with bootstrap support (Fig. 11). The grouping of two samples from Indiana (112) and Kentucky (36) with a bootstrap value of 78% might suggest a geographic association although Kentucky (41) is closer to Indiana (112) and not closely related. Moreover, the clade that is sister to the remaining individuals contains samples from North Carolina, Illinois, New Jersey, and Tennessee.

The clades found to be well supported by bootstrap values (≥ 70%) do not necessarily represent geographically close individuals. The phylogenies of all three species presented here indicate that although some groups may support an association between geographic location and genetic similarity, the majority of relationships hypothesized do not. In all three species individuals from clades with bootstrap support had other geographically closer individuals that were not included. The best case of geographical proximate individuals being related to genetically similar individuals was in *R. phoenicolasius*. Most of the clades produced, contained individuals that were within 200 miles of each other. It is possible that in my study the primers used were not selective enough to produce a geographical and genetic association. Consequently, the null hypothesis of no relationship between geographic distance and genetic similarity could not be rejected.

Other studies have produced mixed results. A study by Hampe et al. (2003) revealed a strong correlation between geographic distance and genetic diversity in buckthorn (*Frangula alnus*). Campbell et al. (1997) found a similar correlation in *A. laevis* but not in *A. bartraminana.* Another study analyzing geographic distances within 20 European forests found no correlation between geographic distance and genetic diversity in *Rubus fruticosus* (Salvini et al. 2006).

Molecular Data: AFLP vs. RAF

In a comparison of the AFLP and RAF molecular techniques, AFLP (369) produced more alleles than RAF (120). In Kollmann et al.'s (2000) study, their five AFLP primers produced between 69 and 93 fragments each. Lindqvist-Kreuze et al. (2003) produced between 106 and 109 fragments using three AFLP primers. My study using three AFLP primers yielded between 72 and 183 scored fragments. This increase in the number of fragments is higher and may reflect a difference in the peak intensity used in scoring. In conclusion, while AFLP produced more scoreable fragments than RAF, there was no significant difference in the number of alleles produced in this study (Fig. 4). Other factors such as usefulness of the alleles and cost must be assessed before a determination of usefulness is made.

In conclusion, my data suggest that all three *Rubus* species have similar levels of genetic variation and that introduced and invasive *Rubus* are not as genetically depauperate as predicted due to founder effect. It also demonstrates that the apomictic and tetraploid *R. armeniacus* does not have less diversity because of its asexual mode of reproduction.

The occurrence of more genetic diversity than expected in introduced *R. armeniacus* and *R. phoenicolasius* might be the result of a combination of multiple introductions and sufficient time since introduction. Lastly, it was determined that AFLP produced more alleles than RAF although the difference was not significant, and the relative utility of each was not determined

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Table 1. RAF and AFLP primers used and their sequences.

RAF	Primer Name	Sequence
	RP2	ATGAAGGGGTT
	RP4	TGCTGGTTCCC
AFLP		
	*Mse*I CAA	GATGAGTCCTGAGTAACAA
	*Mse*I CAG	GATGAGTCCTGAGTAACAG
	*Mse*I*CAT*	GATGAGTCCTGAGTAACAT
	*Eco*RI AGG	GACTGCGTACCAATTCAGG

Table 2. Average pairwise distances and standard deviations.

No. of samples	25	16	10
R. armeniacus	-	0.308 (±0.086)	0.290 (±0.073)
R. occidentalis	0.193 (±0.073)	0.272 (±0.103)	0.159 (±0.080)
R. phoenicolasius	0.190 (±0.052)	0.278 (±0.073)	0.196 (±0.049)

Table 3. Pairwise Mann-Whitney significance test.

Species	*R. armeniacus*	*R. occidentalis*	*R. phoenicolasius*
R. armeniacus	-	-	-
R. occidentalis	*P*>0.00, U=30909	-	-
R. phoenicolasius	*P*>0.00, U=32292	*P*=0.714, U=44223	-

Table 4. Number of alleles scored for AFLP and RAF.

AFLP	*R. armeniacus*	*R. occidentalis*	*R. phoenicolasius*	Total	Mean
CAA	4	99	80	183	
CAG	20	43	51	114	
CAT	14	44	14	72	
Total	38	186	145	369	123
Mean	12.67	62	48.33		
RAF					
RP2	9	19	33	61	
RP4	11	22	26	59	
Total	20	41	59	120	40
Mean	10	20.5	29.5		
AFLP/RAF					
Total	227	204	58		
Mean	9.08	8.16	3.625		
AFLP and RAF comparisons significant test		*P*=0.427, U=39			

Table 5. Significance test P values and Mann-Whitney U values for AFLP and RAF.

Species	*R. armeniacus*	*R. occidentalis*	*R. phoenicolasius*
R. armeniacus	-	-	-
R. occidentalis	*P*=0.16, U=1	-	-
R. phoenicolasius	*P*=0.021, U=1.5	*P*=0.917, U=13	-

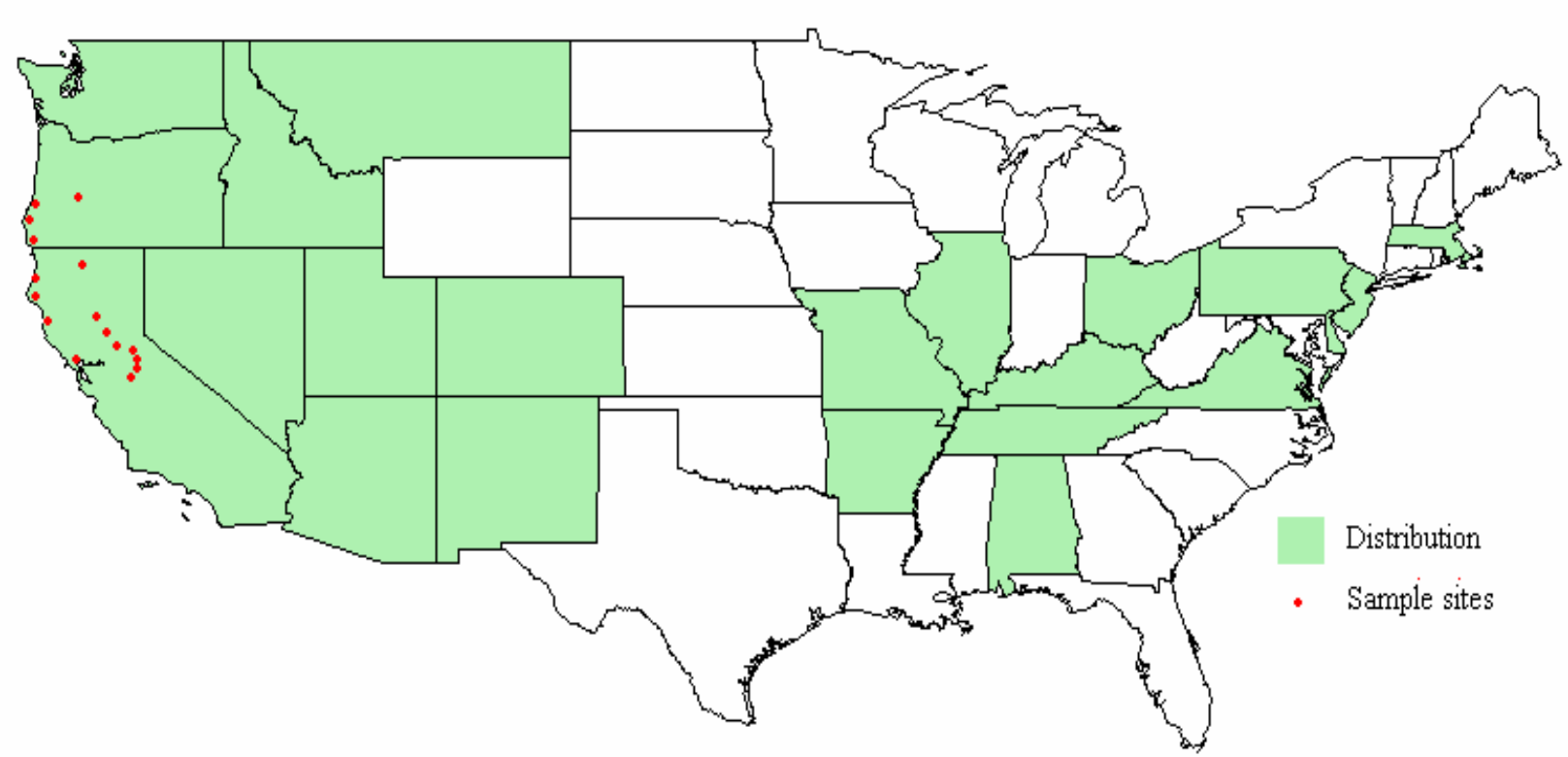

Figure 2. *Rubus armeniacus* U.S. distribution (green) map (USDA Plants Database 2008) and collection sites • .

a)

b)

c)

d)

Figure 3. *Rubus armeniacus* a) stem (photo by Ashley Wint), b) fruit (photo by http://www.ubcbotanicalgarden.org) c) flowers (photo by www.tncweeds.ucdavis.edu) d) mature plant (photo by Ashley Wint).

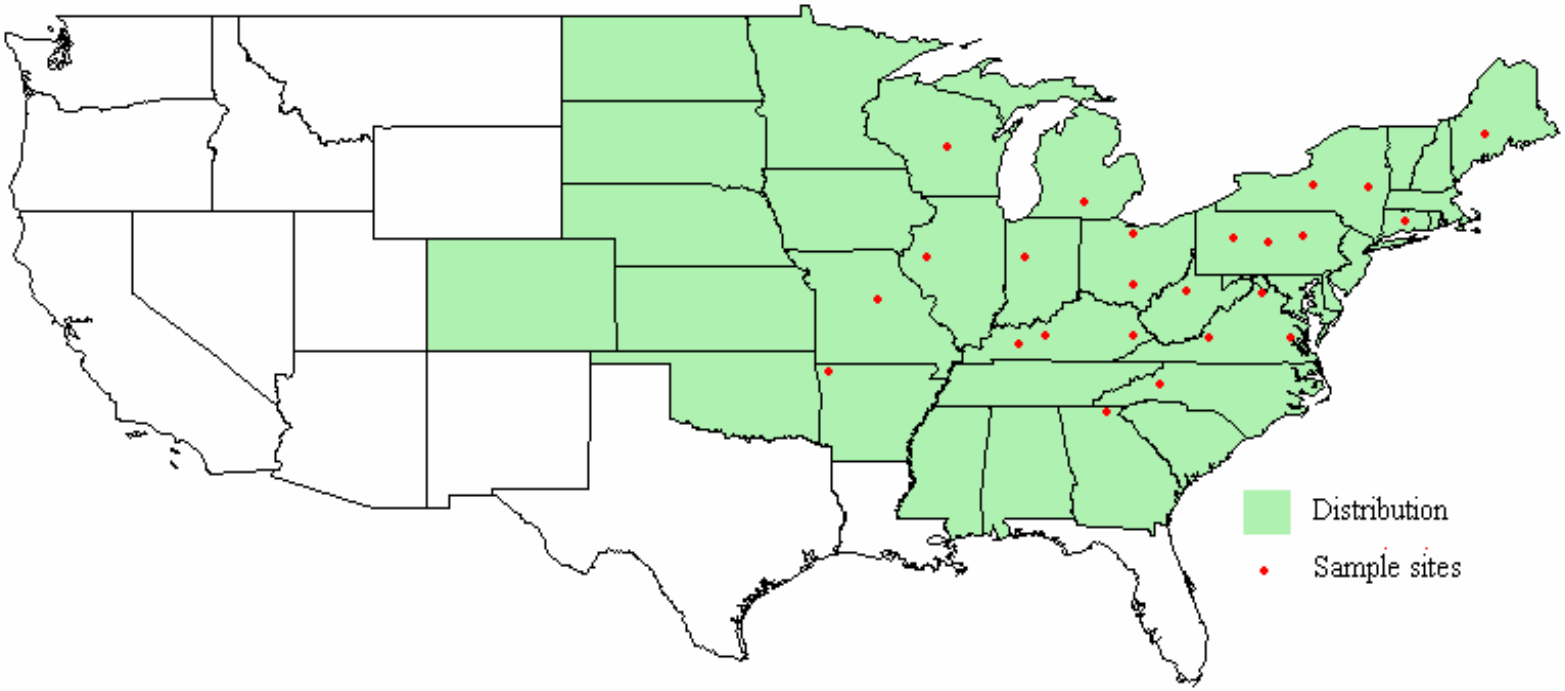

Figure 4. *Rubus occidentalis* U.S. distribution (green) map (USDA Plants Database 2008) and collection sites • .

a)

b)

c)

d)

e)

Figure 5. *Rubus occidentalis* (photos by Ashley Wint) a) fruit with tufts of hair, b) glaucus stem and leaves, c) flowers, d) mature plant, e) flower being pollinated by carpenter bee (*Xylocopa* Xylocopinae).

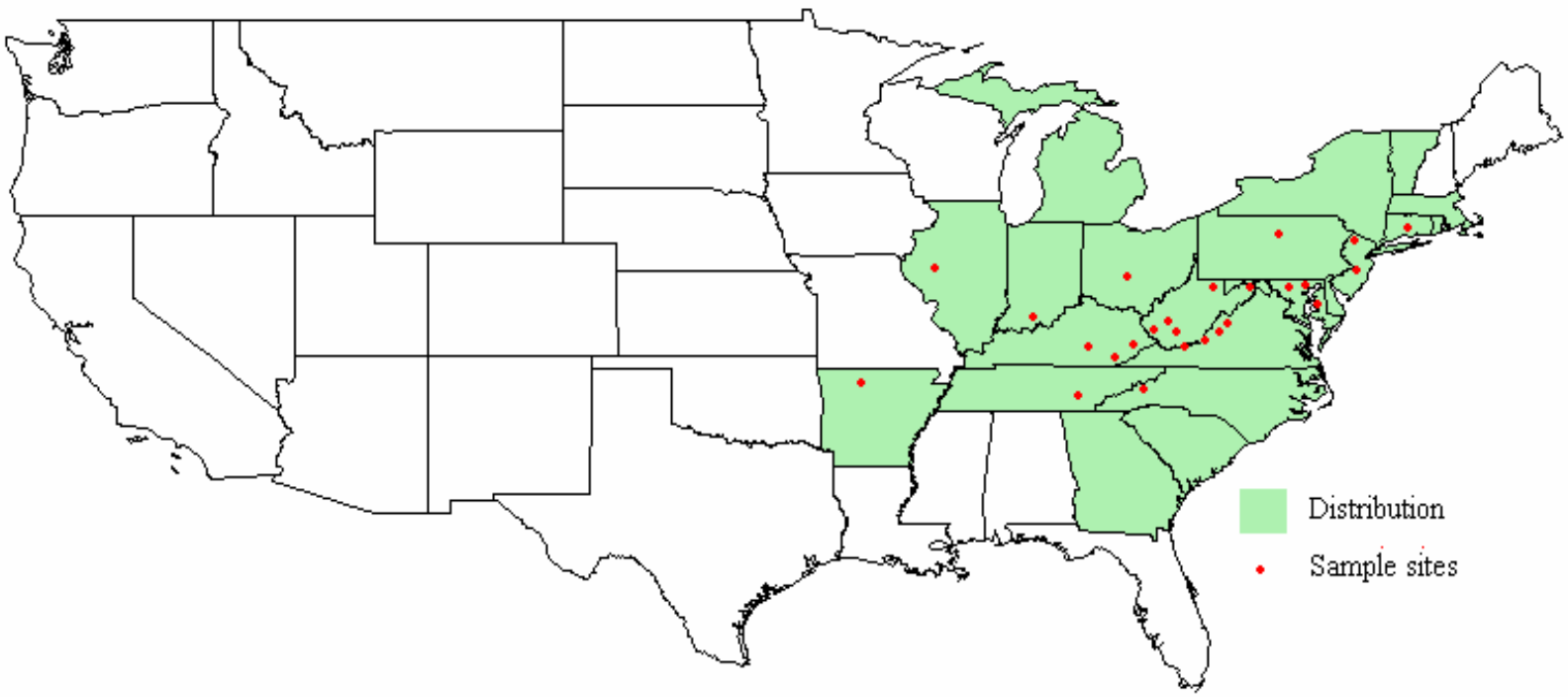

Figure 6. *Rubus phoenicolasius* U.S. distribution (green) map (USDA Plants Database 2008) and collection sites • .

a)

b)

c)

d)

Figure 7. *Rubus phoenicolasius* a) fruiting (photo by Ashley Wint), b) stem (photo by Ashley Wint), c) flowers (photo by G.A. Cooper), d) mature plant (photo by Ashley Wint).

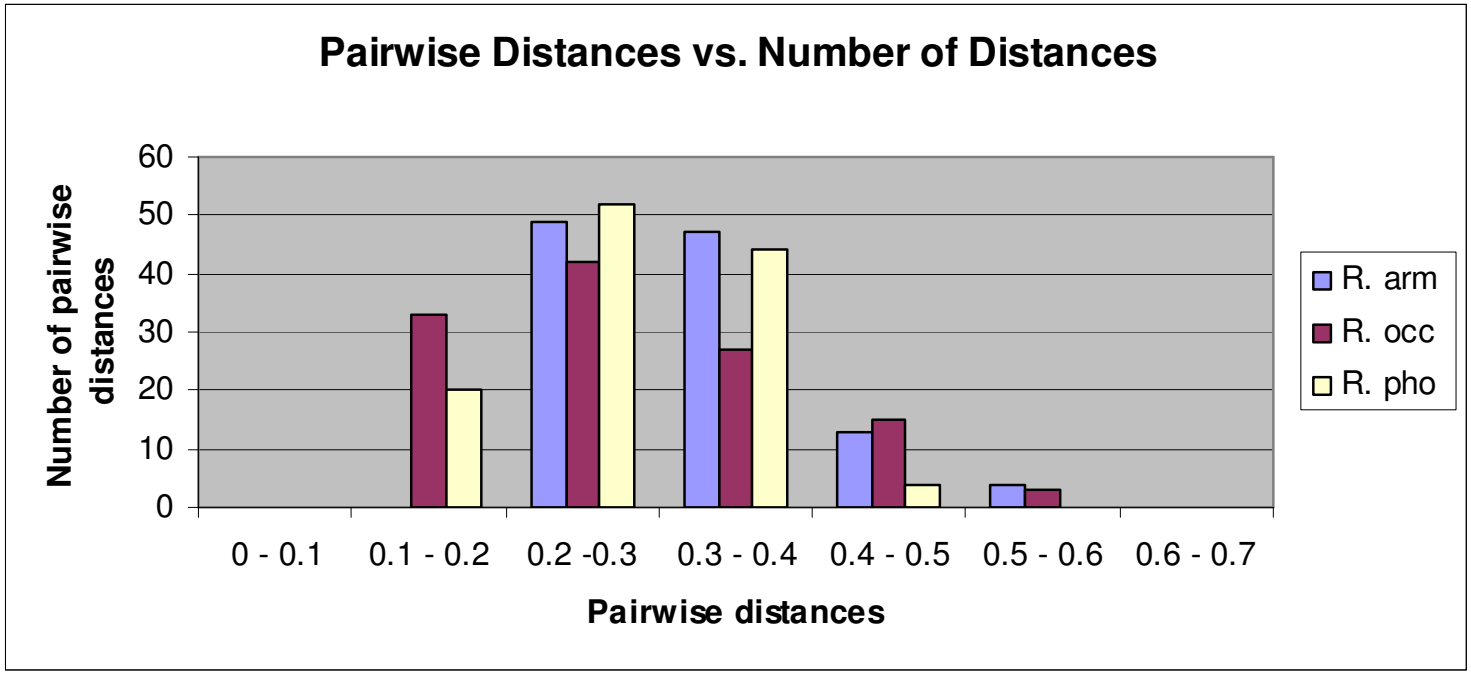

Figure 7. Graph of average pairwise distance of all individuals (*N*=16)

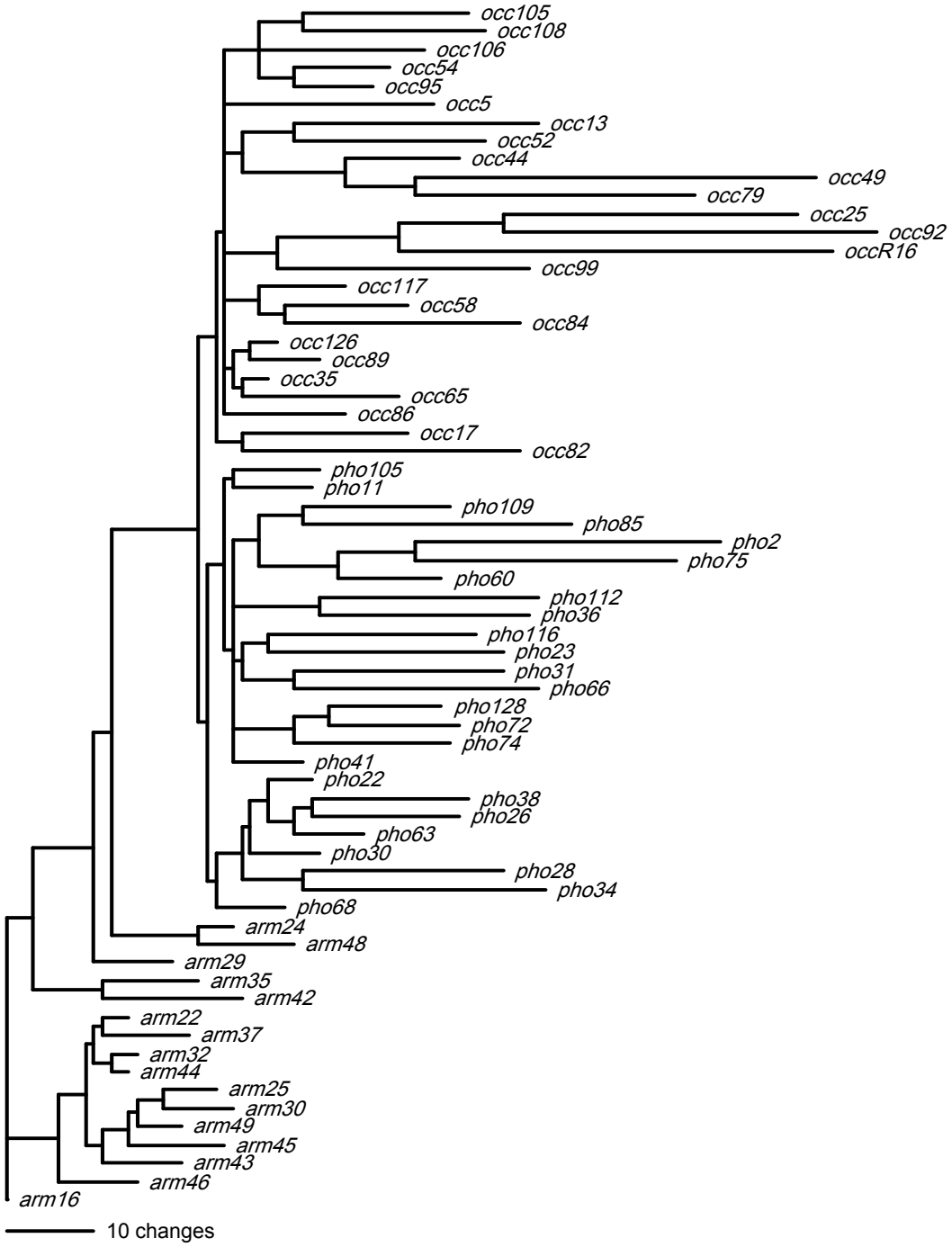

Figure 8. Combined Neighbor-Joining tree of all 66 individuals. *R. armeniacus* (arm), *R. occidentalis* (occ), and *R. phoenicolasius* (pho).

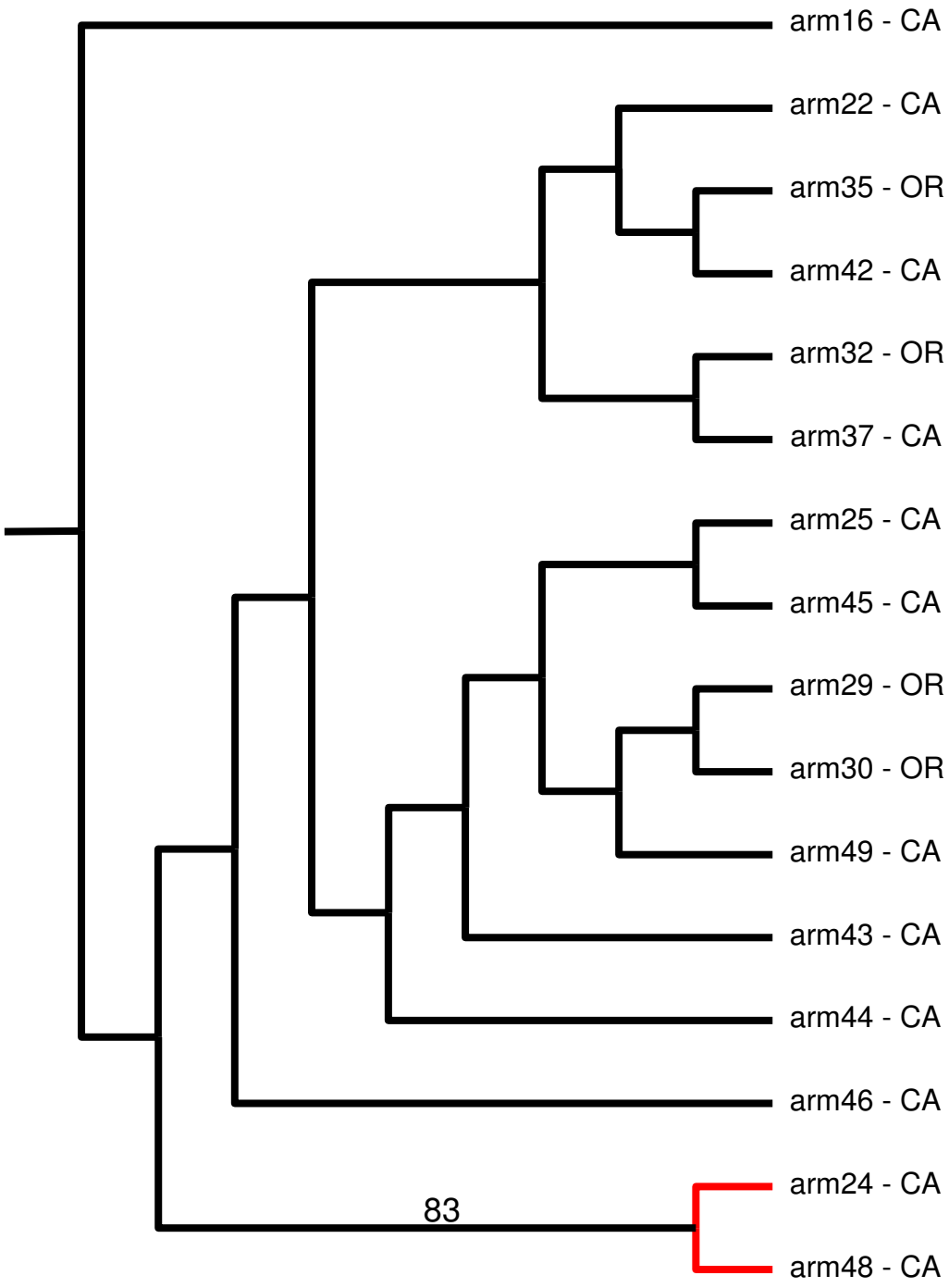

Figure 9. One of the 151 equally parsimonious trees of length 136 of *Rubus armeniacus*. CI = 0.418; RI = 0.386. Two-letter state abbreviations are used to show collection locality. Bootstrap values ≥ 50% are shown. Branches in red are highlighted for purpose of discussion.

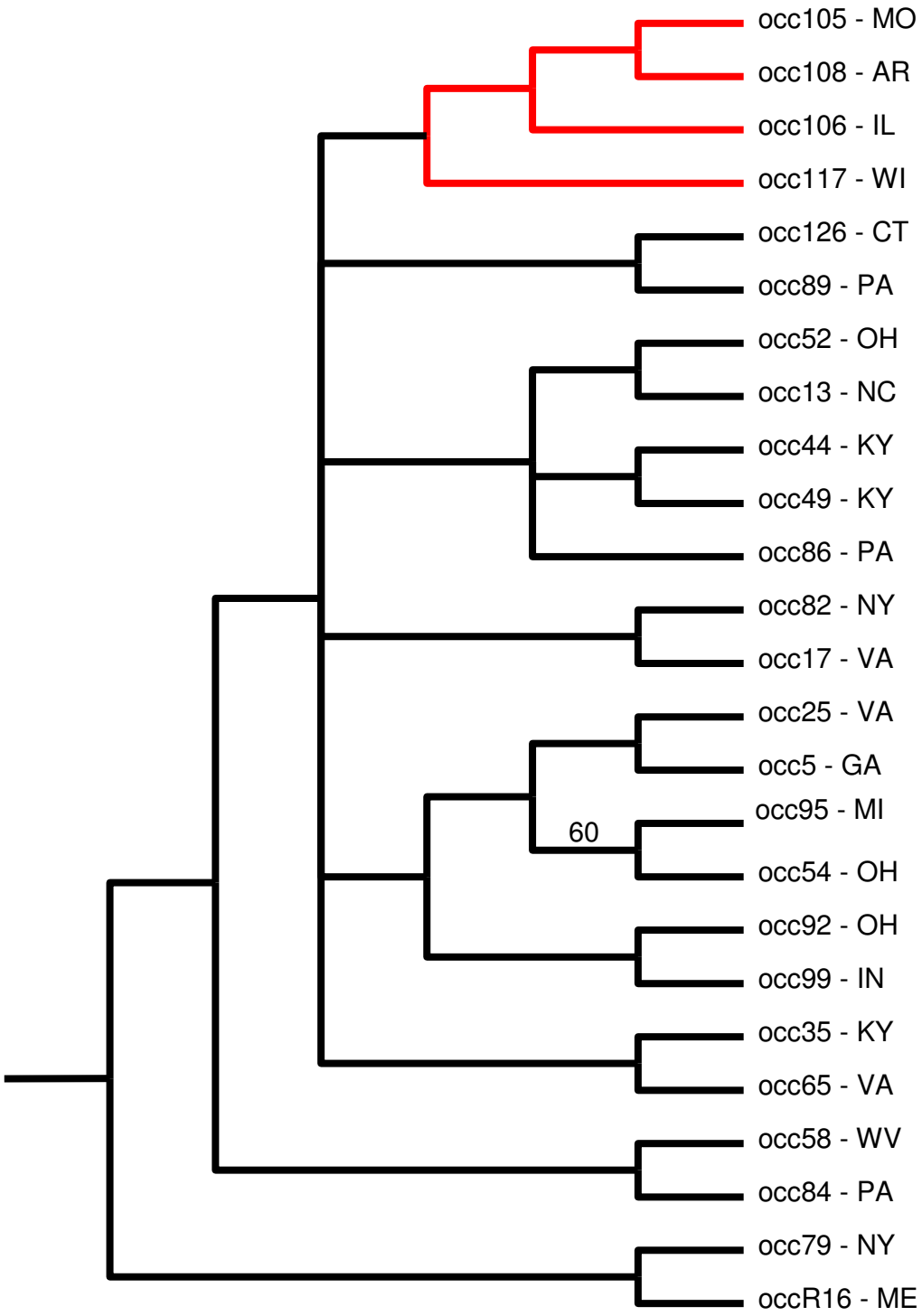

Figure 10. *Rubus occidentalis* strict consensus phylogeny of 10 equally parsimonious trees of length 522. CI = 0.428; RI = 0.232. Two-letter state abbreviations are used to show collection locality. Bootstrap values ≥ 50% are shown. Branches in red are highlighted for purpose of discussion.

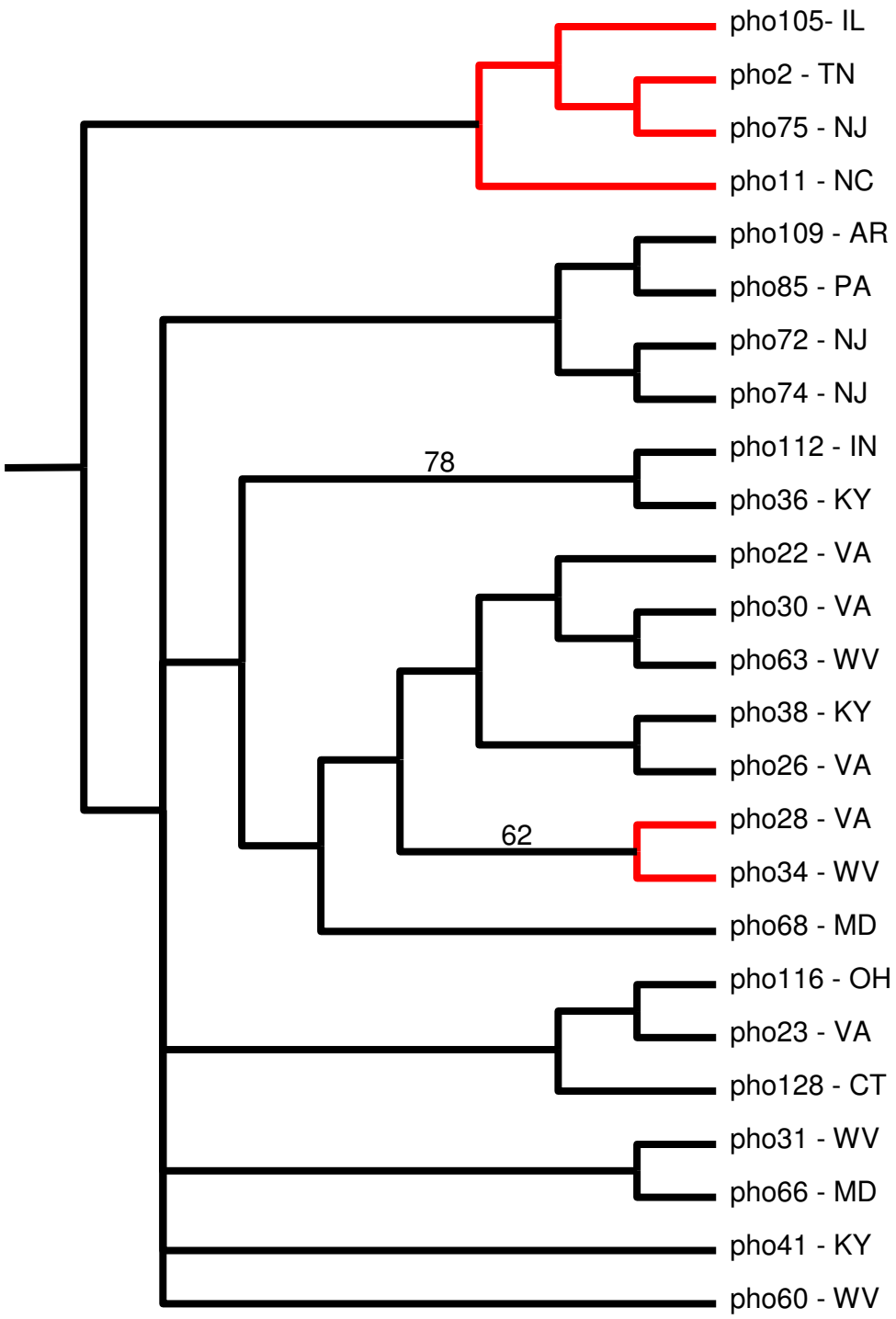

Figure 11. *Rubus phoenicolasius* strict consensus phylogeny of 7 equally parsimonious trees of length 452. CI = 0.451; RI = 0.266. Two-letter state abbreviations are used to show collection locality. Bootstrap values ≥ 50% are shown. Branches in red are highlighted for purpose of discussion.

APENDIX

List 1. People who helped in sample location gathering.

T. Osmon
E. Judziewicz
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