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GENETIC VARIATION IN RUNNING BUFFALO (Trifolium stoloniferum Muhl. ex A. Eaton) USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD): YEAR TWO OF A TWO YEAR STUDY

A Final Report for Region 3, U. S. Fish and Wildlife Service
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INTRODUCTION

Trifolium stoloniferum Muhl. ex A. Eaton, commonly known as running buffalo clover, is a highly stoloniferous perennial herb belonging to the family Fabaceae. The species is known historically from nine states in the midwestern United States and was thought to be extinct from 1940 until the early 1980's, when it was rediscovered in West Virginia. Additional populations were discovered subsequently in Indiana, Kentucky, Ohio, West Virginia, and Missouri. Running buffalo clover was listed as an endangered species by the U. S. Fish and Wildlife Service in 1987. Since then a number of populations, particularly in Kentucky and West Virginia, have been found resulting in the need to revise the recovery plan, which was written in 1989. Most populations are small (10-200 plants), but several populations of more than 1,000 individuals are known.

Past genetic studies of T. stoloniferum using allozymes suggest low genetic diversity in this species (Hickey et al. 1991; Hickey and Vincent 1992). These studies also indicate that smaller populations have lower levels of diversity than larger ones, the majority of the diversity occurs among populations, and that gene flow between subpopulations is limited, even between those that are separated by short distances. Recently a technique that provides a high resolution view of the genetic differences between individuals has been developed. This technique, called RAPDs (Random Amplified Polymorphic DNA, Williams et al. 1990), involves short (10-mer) primers that are used to amplify segments of genomic DNA taken from individual plants. If two

plants are identical genetically, a given primer will amplify the same array of bands because the same priming sites (where the primers anneal) will be present in the same places. If a priming site is lost (or gained) or there has been an insertion or deletion between primer sites, then different bands will result. The array of bands for an individual are visualized by the patterns seen after electrophoresis in agarose gel. Fragments generated from one individual and not from another are called Random Amplified Polymorphic DNA markers. RAPD markers are used because they often reveal population differences not found with allozymes (Brauner et al. 1992; Crawford et al. 1994; Meyer et al. 1993; review in Crawford 1996). In addition, RAPDs only require small amounts of leaf material, which is an important consideration when studying rare plants.

The purpose of this study was to assess genetic variation within and among T. stoloniferum populations throughout its known geographic distribution using RAPD markers. The specific questions examined include:

1. **How much RAPD diversity exists in the species?** The ability of a population or species to adapt to environmental changes and to persist is determined in large measure by the genetic variation it harbors. There is often a correlation between diversity in molecular markers (e. g., allozymes, RAPDs) and diversity in morphological and physiological features.

2. **How is the diversity in the species distributed?** When recovering rare species, it is important to determine how genetic variation is apportioned within and among populations. Knowing which populations differ from each other

genetically is necessary to determine which ones should receive the most protection in order to preserve the maximum genetic diversity of the species.

3. Do larger populations contain more genetic variation than smaller ones? Ascertaining the level of genetic variation within populations of different sizes can help determine if there is a minimum population size below which genetic variation decreases.

4. Is there genetic substructuring, or a spatial distribution of different genotypes in populations or between states? Knowing if there are genetic differences between populations from different areas of the geographic range of a rare species is important information for conservation and recovery programs.

5. Do populations of T. stoloniferum consist of more than one genotype (genet)? Given the highly stoloniferous nature of T. stoloniferum, populations may not consist of more than one genotype. The amount of sexual reproduction occurring is unknown. It may be that this species reproduces primarily by vegetative means. Large populations may simply have more extensive networks of stolons than small populations rather than more genetic individuals.

METHODS

One to two leaves were collected from each of 525 plants, in 23 populations in Ohio, Kentucky, West Virginia, Missouri and Indiana in April 1994 - June 1996. In

large populations leaves were collected along a randomly placed transect while leaves were collected evenly throughout the smaller populations. Locations and sizes for each population are indicated in Table 1 and Figures 1-5. DNA was extracted from all of the 525 leaf samples. From the original sample of 525 plants in 23 populations, 390 individuals from 21 populations produced repeatable bands for each of the five primers examined and are included in this analysis (Table 2). As Table 1 indicates, most of the populations had more than one patch or subpopulation. For five of the multi-patch populations, the DNA data were evaluated by subpopulation to look for evidence of substructuring within populations.

Total DNA was extracted from 0.07-0.09 g (fresh weight) of mature leaf tissue following a miniprep extraction technique modified by Loren H. Rieseberg from Doyle and Doyle (1987). Leaf tissue was ground in 0.7 ml of 2X CTAB isolation buffer (100mM Tris-HCl, pH 8, 1.4 M NACl, 20 mM EDTA, 2% hexadecyltrimethylammonium bromide (CTAB), 1% Nabisulfite, 0.2% 2-mercaptoethanol). The grindate was incubated at 60° C for 30-60 minutes, extracted once with chloroform-isoamyl alcohol (24:1), centrifuged at high speed for 2 minutes and the supernatant collected. Nucleic acids were precipitated by adding 0.46 ml isopropanol, recovered by high speed centrifugation for 2 minutes, washed in 0.8 ml 76% EtOH/0.01 M NH_4OAc and resuspended in 0.1 ml 10 mM NH_4OAc/25mM EDTA.

The protocol for amplifications is a modification of the procedure of Williams et al. (1990). Reactions were performed in volumes of 25 ul containing 1.5 mM MgCL, 100 uM each of dATP, dCTP, dGTP and dTTP, 0.2 uM primer, 1.5 ul genomic DNA

(an amount that gave reliable amplifications) and 0.75 unit of Taq polymerase. Amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 40 cycles of 1 minute at 94° C, 2 minutes at 35° C and 2 minutes at 72 °C. Amplifications were performed with five primers, A-3, A-4, A-7, A-8 and A-9 from Operon Technologies. Amplification products were analyzed by electrophoresis on a 2% agarose gel in 1 X TBE (tris-borate-EDTA) buffer and detected by staining with ethidium bromide and photographed on a UV transilluminator. A 100 base pair ladder of DNA fragments (BRL) was included on each gel as a size reference. Amplification products from individuals of each population and each primer were initially electrophoresized on separate gels. One to three individuals, containing bands representing every band present in a population, were identified from these gels, amplified a second time and run on a second gel containing these individuals from every population. Running individuals from each population on the same gel was done to ensure the identification of reproducible similarities and differences in bands between populations.

The degree of RAPD similarity within and between populations was measured by calculating pair-wise similarity values between individuals using a computer program written by Vera Ford, University of California Davis (unpublished). The individual similarity values were then averaged for a measure of within-population similarity and again for between-population comparisons. Only bands that were scorable (1 or 0) in both individuals were used in this computation. Bands not clear or unscorable between individuals were not included. A similarity measure of 1.00

indicates that all individuals being compared are genetically identical.

The calculation for pairwise individual similarity is:

$$Sij = \frac{(2 \times Bij)}{(Bi + Bj)}$$

Where:

Bij = the number of bands scored 1 for both individuals i and j.

Bi = the number of bands scored 1 for individual i and likewise for j.

The calculation and assumptions for average within and between population or group similarity is: Assume the first population consists of individuals numbered n1 to n2 and the second population consists of individuals numbered m1 to m2.

Average pairwise similarity of individuals in the first population:

$$\frac{\Sigma \text{ Sij for all i and j with } n1 <= i < j <= n2}{\frac{(1+n2-n1) \times (n2-n1)}{2}}$$

Average similarity of individuals for the second population:

$$\frac{\Sigma \text{ Sij for all i and j with } m1 <= i < j <= m2}{\frac{(1+m2-m1) \times (m2-m1)}{2}}$$

The average between groups 1 and 2 is:

$$\frac{\Sigma Sij \text{ for all i and j with } n1<=i<=n2,\ m1<=j<=m2}{(1+n2-n1)\ X\ (1+m2-m1)}$$

The program computes the similarity of RAPD bands (for example, all the same bands = 1.0, no bands in common = 0.0) for all pairwise comparisons of individual plants within a population. That is, every plant is compared to every other plant. The within-population similarity is the mean of all these plant-by-plant comparisons. The program also computes the plant-by-plant RAPD band similarity for individuals from different populations, and averages the similarities for these comparisons. This gives the between-population similarities. The higher the similarities, the lower the diversity. For example, if all plants in a population had a similarity value of 1.0 that would mean they are all the same and thus there is no diversity. By contrast, if all plants had different bands (none in common), then the similarity would be 0.0 and the diversity would be as high as possible.

Average similarity comparisons both within and between-populations are given in Table 3. The averages of all between-population values in comparison to the within-population values are listed in Table 4. The average RAPD marker similarity comparisons by state are indicated in Table 5. In order to determine if similarity comparisons were significantly different between and within populations, a paired t-test was performed on each comparison.

RESULTS AND DISCUSSION

The five primers used in this study produced a total of 59 different RAPD bands among all the plants examined. All populations exhibited some variation, that is, no similarity values of 1.0 were obtained between individuals of any of the populations (Table 3). These results contrast markedly with those of Hickey and Vincent (1992) using allozymes; they detected no variation in 14 of the 28 populations examined.

The within similarity values range from 0.927 to 0.983 indicating that variation was found within each population and subpopulation sampled (Table 3 and 4). While populations do consist of more than one genotype, the high within-population similarity values (Table 3 and 4) indicate that each population does not consist of large numbers of genetically different individuals or individuals with very different arrays of RAPD bands. These values suggest that asexual reproduction, as would be expected because of the stoloniferous nature of this taxon, is likely to be an important if not the primary, mode of reproduction for this species.

Although RAPDs detected variation between all populations, the high average similarity values in Tables 3 and 4 indicate that T. stoloniferum contains low levels of genetic diversity which are consistently low across the range of the species. The similarities between populations range from 0.793 to 0.946 with an average of 0.884, indicating that populations of T. stoloniferum are genetically distinct (although not highly so) from one another (Table 3 and 4). The high genetic similarity within this species can be illustrated by comparing these results with those of a RAPD study on

Solidago albopilosa, a rare (only 80 populations are known) goldenrod endemic to Kentucky (E. Esselman, unpublished). Ten populations (129 individuals) have a between population average similarity of 0.413 while average within-population similarity was 0.702.

As Hickey et al. (1991) suggested, it is difficult to determine if the low allozyme diversity in this species has contributed to its rarity. Because of the small number of remaining populations and their geographical isolation, it is possible that populations have, in the past or are currently experiencing, genetic bottlenecks and that they now contain only a subset of the original genetic variance at one time present in the species. However, because of the consistently low diversity both within and between-population comparisons it is also possible that this species is characterized by low diversity and rarity may be due to other factors such as habitat loss.

Our results reveal that the highest levels of diversity in T. stoloniferum are distributed among populations. The average within-population similarity values are always higher (thus lower diversity) than any of the between-population comparisons (Table 3 and 4); the mean value is 0.884 between and 0.952 within all populations. The results of a paired t-test indicate that these values are significantly different ($T > 0.0001$). The higher within values suggest that gene flow between populations is very low or nonexistent. This is not surprising because many populations are geographically isolated and gene flow between them would be expected to be difficult; these results are concordant with those obtained by Hickey et al. (1991) using isozymes.

Further, these workers suggested that there is little gene flow between subpopulations and our RAPD data support this hypothesis for every population in which data for subpopulations were analyzed for average similarity as compared with similarity for the whole population (Table 3 and 4). The range of within similarity values between Miami B and Miami L was 0.982-0.983 (mean of 0.9825), while the average similarity between these subpopulations was 0.946. The average within-subpopulations similarities for Shaver's 1 and 2 are 0.959 and 0.958 (average of 0.9585). The mean similarity between these populations was 0.925. The range of similarity values among the Niehaus subpopulations was 0.859 - 0.920, with a mean of 0.901. The mean similarity within subpopulations of Niehaus was 0.935. The higher similarities within each subpopulation rather than between suggests that there is little gene flow even between subpopulations separated by small distances. The results of a paired t-test indicate that, when comparing all populations and subpopulations (Table 2) there are significant differences between the levels of diversity within and between-subpopulations ($t > 0.0065$) and the highest levels of diversity are found among subpopulations within a population. In each of the subpopulations examined (Table 2) there were unique bands in at least one of the subpopulations, indicating substructuring within populations. Newberry subpopulation 2 contains four bands not found in Newberry 1. Niehaus subpopulations 1, 4 and 8 have one band not present in Niehaus 6; Niehaus 1 and 8 have one band not found in 4 and 6 while Niehaus 1 has one band not found in the other subpopulations. Miami subpopulation B has two unique bands not detected in Shaver's 2, and Meagher 1 has 4 bands not found in

Meagher 2. There is also evidence of substructuring in the Congress Green and Otter Creek populations. The Congress Green population consists of six distinct patches (Table 1), and 10 RAPD markers were found that were unique to one or four of the patches. The Otter Creek population consists of three patches and had nine RAPD markers unique to either one or two of the patches. The presence of unique bands in subpopulations or patches further supports the conclusion of Hickey et al. (1991) that there is low gene flow even between patches that are within several meters of each other.

In addition to genetic substructuring at the population level there is also evidence of structuring at the state level. There were two RAPD bands restricted to two states, one in Ohio and one in Missouri. In addition to the presence of bands unique to states, there were also significant differences in the level of diversity within a state verses the level of diversity between states ($t > 0.0368$). The diversity levels between states are always higher than the within-state value, indicating that even at the state level more of the diversity occurs between populations from different states as compared to populations of the same state. The probable reason for this pattern is that within a state all populations sampled (and known) were from rather localized areas (Figures 1-5) and are disjunct from populations in other states. The only exceptions are the Ohio and Indiana populations, which are from nearby localities.

When average similarities are compared within states, the states with the lowest similarities (highest diversities) also contain the largest number of populations (Table 5). West Virginia and Ohio have the highest level of diversity, the values

being nearly identical to each other (Table 5). Kentucky populations have an intermediate level, followed by Indiana and Missouri, with only one or two populations respectively. Given that more diversity exists between rather than within populations of the species as a whole, it is not surprising that states with the most populations also have the highest diversity. When comparing similarities between states, there is not a pattern between geographical proximity and degree of RAPD marker similarity (Table 5). The highest similarity value is between Kentucky and West Virginia (0.899), which are next to one another, but Ohio and Indiana, with populations separated by fewer than 20 miles, have one of the lowest similarities. These are additional data suggesting little or no gene flow between populations.

The level of genetic diversity in populations of T. stoloniferum is not always correlated with population size (Tables 2 and 3). If the similarity values are averaged for all populations and subpopulations, the lowest values (highest diversity) are seen in Cane Forest Middle, followed by Niehaus, Milford and the Crouch Knob populations. The Niehaus and Crouch Knob populations are the two largest ones sampled and do have lower within similarities than 81% of the populations surveyed (Table 1 and 3). However, Cane Forest Middle is a smaller population and Milford was the smallest sampled. This indicates that the smallest populations may have high levels of RAPD diversity; by contrast, Hickey et al. (1991) found some smaller populations to have no allozyme diversity.

The lowest within population similarity (highest diversity) is seen at the subpopulation level in Niehaus subpopulation 1 (Table 2 and 3). This is probably the

largest continuous subpopulation of the species across its range (1500 rooted crowns). It consistently has a higher percentage of flowering individuals than other subpopulations. This may provide more opportunities for sexual reproduction and genetic recombination, thus generating higher diversity than in other populations or subpopulations. These results indicate that population size alone may not be a good indicator of levels of genetic diversity in this species. Other factors, such as the frequency of flowering, may be better indicators.

In summary, our results with RAPDs agree in certain respects with Hickey et al. (1991) who studied allozyme diversity in T. stoloniferum . Both studies show quite clearly that levels of genetic diversity are low in the species as a whole. There are however, some basic differences between the two studies and they have important implications for the recovery of T. stoloniferum:

1. In contrast to Hickey et al. (1991), we have demonstrated that no population is a single genet and even the smallest populations may contain high levels of diversity. The allozyme study indicated no diversity in half of the populations sampled. Our results suggest that even the smallest populations must be saved in order to conserve maximal genetic diversity in the species. That is, conserving only a few of the largest populations of the species, as suggested by Hickey et al. (1991), would not be effective as a means of maintaining most of its diversity.

2. Our RAPD results, in agreement with the allozyme data of Hickey et al. (1991), demonstrate that a significantly greater level of diversity resides among

as compared to within populations. The implications of these results are clear on this point: to conserve maximum levels of diversity, management efforts should focus on preserving as many populations as possible across the range of the species.

3. Our study detected genetic substructuring at the state, population and subpopulation levels. At the subpopulation level, substructuring could be correlated with higher rates of flowering at one site. These results have important implications for management. Management strategies to promote flowering (sexual reproduction) may increase genetic diversity in the species.

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Table 1. Populations of T. stoloniferum sampled from 1994-1996 for RAPD analysis. Leaf material was collected from 2 additional populations in Kentucky, Dinsmore (Boone Co.) and Stoney Point (Fayette Co.), but DNA amplification was not possible. (BAD = Bluegrass Army Depot)

STATE/County	SITE NAME (# = location on state maps - Fig. 1-5)	ESTIMATED POPULATION SIZE (# rooted crowns)	NUMBER OF SUB POPULATION SAMPLED
OHIO/Hamilton	**1.** Miami Fort	500	2
Hamilton	**2.** Congress Green	150	6
Hamilton	**3.** Niehaus	2500	4
Clermont	**4.** Milford HSC	20	1
Hamilton	**5.** Newberry WS	200	2
KENTUCKY/Madison	**1.** Otter Creek (BAD)	200	3
Madison	**2.** Upper Viny Fork (BAD)	150	4
Madison	**3.** Cane Forest Middle (BAD)	125	3
Madison	**4.** K-Ridge West (BAD)	100	2
Woodford	**5.** Wilhoit House	75	2
WEST VIRGINIA/Tucker	**1.** Fernow EF, Big Springs Gap	500-1000	8
Randolph	**2.** Rock Camp Run	1000-1500	4
Randolph	**3.** Rich Mountain West, Microwave	150	1
Randolph	**4.** Upper John's Run "A"	230-1000	1
Randolph	**5.** Shaver's Mountain	225-350	2
Randolph	**6.** Synder Run	500	1
Randolph	**7.** Laurel Mountain	125	2
Randolph	**8.** Crouch Knob	3000	1
MISSOURI/Madison	**1.** Meagher (native)	200	2
Crawford	**2.** Misty Valley Ridge (transplanted)	800	1
INDIANA/Dearborn	**1.** Hidden Valley	2500	2

Table 2. Abbreviations, number of leaves sampled and analyzed for each population and subpopulation.

Abbreviations for populations and subpopulations (in parentheses)		# of leaves sampled per population	# of plants included in analysis
Milford	(Mil)	10	10
Newberry subpopulation 1	(Ne1)	8	8
Newberry subpopulation 2	(Ne2)	20	18
Niehaus subpopulation 1	(Ni1)	30	11
Niehaus subpopulation 4	(Ni4)	12	12
Niehaus subpopulation 6	(Ni6)	7	6
Niehaus subpopulation 8	(Ni8)	16	17
Miami Fort subpoplation B	(MiB)	14	14
Miami Fort subpoplation L	(MiL)	19	16
Congress Green	(Con)	29	23
Otter Creek	(Ott)	30	27
Upper Viny Fork	(Upp)	28	21
Cane Forest Middle	(Can)	24	21
K- Ridge West	(Kri)	17	14
Wilhoit	(Wil)	20	15
Fernow	(Fer)	30	20
Rich Mountain	(Ric)	20	14
Upper John's Run "A"	(UpJ)	17	9
Shaver's subpopulation 1	(Sh1)	11	7
Shaver's subpopulation 2	(Sh2)	12	8
Laurel	(Lau)	20	9
Rock Camp	(Roc)	30	14
Synder Run	(Syn)	10	10
Crouch Knob	(Crk)	31	26
Misty Valley	(Mis)	15	9
Meagher subpopulation 1	(Me1)	15	10
Meagher subpopulation 2	(Me2)	15	6
Hidden Valley	(Hid)	15	15

Table 3. Within and between population comparisons based on average RAPD marker similarity. Population abbrevations are explained in Table 2.

	Mil	Ne1	Ne2	Ni1	Ni4	Ni6	Ni8	MmB	MmL	Con
Mil	0.939	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ne1	0.891	0.933	-----	-----	-----	-----	-----	-----	-----	-----
Ne2	0.899	0.885	0.955	-----	-----	-----	-----	-----	-----	-----
Ni1	0.892	0.885	0.888	0.917	-----	-----	-----	-----	-----	-----
Ni4	0.891	0.859	0.880	0.890	0.946	-----	-----	-----	-----	-----
Ni6	0.891	0.894	0.891	0.899	0.920	0.950	-----	-----	-----	-----
Ni8	0.885	0.865	0.880	0.902	0.901	0.895	0.927	-----	-----	-----
MiB	0.908	0.860	0.908	0.902	0.906	0.915	0.910	0.983	-----	-----
MiL	0.910	0.871	0.921	0.911	0.910	0.916	0.907	0.946	0.982	-----
Con	0.895	0.864	0.916	0.901	0.897	0.911	0.894	0.923	0.946	0.950
Ott	0.892	0.868	0.906	0.879	0.890	0.901	0.875	0.910	0.906	0.913
Upp	0.892	0.889	0.909	0.870	0.884	0.898	0.861	0.900	0.903	0.892
Can	0.881	0.831	0.894	0.859	0.865	0.859	0.861	0.889	0.891	0.893
Kri	0.888	0.803	0.887	0.879	0.886	0.902	0.879	0.902	0.933	0.918
Wil	0.901	0.874	0.876	0.878	0.899	0.913	0.880	0.907	0.899	0.891
Fer	0.874	0.886	0.866	0.880	0.884	0.892	0.874	0.902	0.894	0.884
Ric	0.890	0.873	0.900	0.873	0.877	0.906	0.870	0.901	0.899	0.897
UpJ	0.892	0.890	0.885	0.864	0.876	0.899	0.855	0.890	0.899	0.903
Sh1	0.885	0.869	0.887	0.883	0.884	0.913	0.880	0.922	0.906	0.885
Sh2	0.880	0.870	0.875	0.887	0.893	0.910	0.875	0.906	0.916	0.895
Syn	0.879	0.876	0.896	0.871	0.880	0.898	0.872	0.914	0.899	0.889
Lau	0.914	0.851	0.896	0.881	0.908	0.905	0.894	0.938	0.912	0.903
Roc	0.884	0.880	0.891	0.879	0.877	0.899	0.862	0.873	0.890	0.887
Crk	0.870	0.835	0.872	0.839	0.817	0.843	0.821	0.871	0.886	0.859
Mis	0.879	0.885	0.866	0.850	0.873	0.888	0.855	0.879	0.892	0.861
Me1	0.862	0.861	0.850	0.843	0.845	0.850	0.850	0.862	0.862	0.827
Me2	0.841	0.869	0.835	0.845	0.833	0.866	0.835	0.854	0.851	0.825
Hid	0.874	0.837	0.888	0.810	0.871	0.816	0.877	0.896	0.911	0.903

Table 3 continued.

	Ott	Upp	Can	Kri	Wil	Fer	Ric	UpJ	Sh1	Sh2
Mil	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ne1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ne2	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ni1	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ni4	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ni6	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ni8	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
MiB	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
MiL	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Con	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
Ott	0.945	-----	-----	-----	-----	-----	-----	-----	-----	-----
Upp	0.913	0.954	-----	-----	-----	-----	-----	-----	-----	-----
Can	0.908	0.880	0.920	-----	-----	-----	-----	-----	-----	-----
Kri	0.912	0.909	0.897	0.951	-----	-----	-----	-----	-----	-----
Wil	0.905	0.906	0.876	0.908	0.959	-----	-----	-----	-----	-----
Fer	0.908	0.914	0.878	0.899	0.910	0.964	-----	-----	-----	-----
Ric	0.929	0.912	0.906	0.919	0.904	0.896	0.960	-----	-----	-----
UpJ	0.914	0.905	0.877	0.911	0.899	0.883	0.908	0.951	-----	-----
Sh1	0.903	0.896	0.868	0.913	0.926	0.900	0.909	0.902	0.959	-----
Sh2	0.903	0.895	0.867	0.916	0.911	0.885	0.902	0.903	0.925	0.958
Syn	0.915	0.900	0.884	0.895	0.901	0.911	0.911	0.899	0.914	0.924
Lau	0.917	0.901	0.901	0.923	0.923	0.920	0.914	0.899	0.921	0.910
Roc	0.907	0.887	0.878	0.902	0.900	0.889	0.910	0.892	0.905	0.910
Crk	0.866	0.848	0.865	0.875	0.868	0.856	0.853	0.846	0.878	0.876
Mis	0.867	0.901	0.836	0.891	0.793	0.887	0.876	0.898	0.882	0.896
Me1	0.847	0.860	0.832	0.860	0.861	0.885	0.865	0.862	0.880	0.876
Me2	0.843	0.869	0.804	0.855	0.869	0.878	0.850	0.877	0.881	0.876
Hid	0.900	0.889	0.880	0.915	0.853	0.863	0.898	0.814	0.892	0.881

Table 3 continued.

	Syn	Lau	Roc	Crk	Mis	Me1	Me2	Hid
Mil	-----	-----	-----	-----	-----	-----	-----	-----
Ne1	-----	-----	-----	-----	-----	-----	-----	-----
Ne2	-----	-----	-----	-----	-----	-----	-----	-----
Ni1	-----	-----	-----	-----	-----	-----	-----	-----
Ni4	-----	-----	-----	-----	-----	-----	-----	-----
Ni6	-----	-----	-----	-----	-----	-----	-----	-----
Ni8	-----	-----	-----	-----	-----	-----	-----	-----
MiB	-----	-----	-----	-----	-----	-----	-----	-----
MiL	-----	-----	-----	-----	-----	-----	-----	-----
Con	-----	-----	-----	-----	-----	-----	-----	-----
Ott	-----	-----	-----	-----	-----	-----	-----	-----
Upp	-----	-----	-----	-----	-----	-----	-----	-----
Can	-----	-----	-----	-----	-----	-----	-----	-----
Kri	-----	-----	-----	-----	-----	-----	-----	-----
Wil	-----	-----	-----	-----	-----	-----	-----	-----
Fer	-----	-----	-----	-----	-----	-----	-----	-----
Ric	-----	-----	-----	-----	-----	-----	-----	-----
UpJ	-----	-----	-----	-----	-----	-----	-----	-----
Sh1	-----	-----	-----	-----	-----	-----	-----	-----
Sh2	-----	-----	-----	-----	-----	-----	-----	-----
Syn	0.950	-----	-----	-----	-----	-----	-----	-----
Lau	0.900	0.984	-----	-----	-----	-----	-----	-----
Roc	0.903	0.890	0.949	-----	-----	-----	-----	-----
Crk	0.880	0.876	0.873	0.941	-----	-----	-----	-----
Mis	0.879	0.885	0.865	0.823	0.980	-----	-----	-----
Me1	0.874	0.883	0.862	0.848	0.908	0.952	-----	-----
Me2	0.863	0.879	0.846	0.815	0.914	0.937	0.957	-----
Hid	0.812	0.840	0.884	0.864	0.794	0.848	0.842	0.952

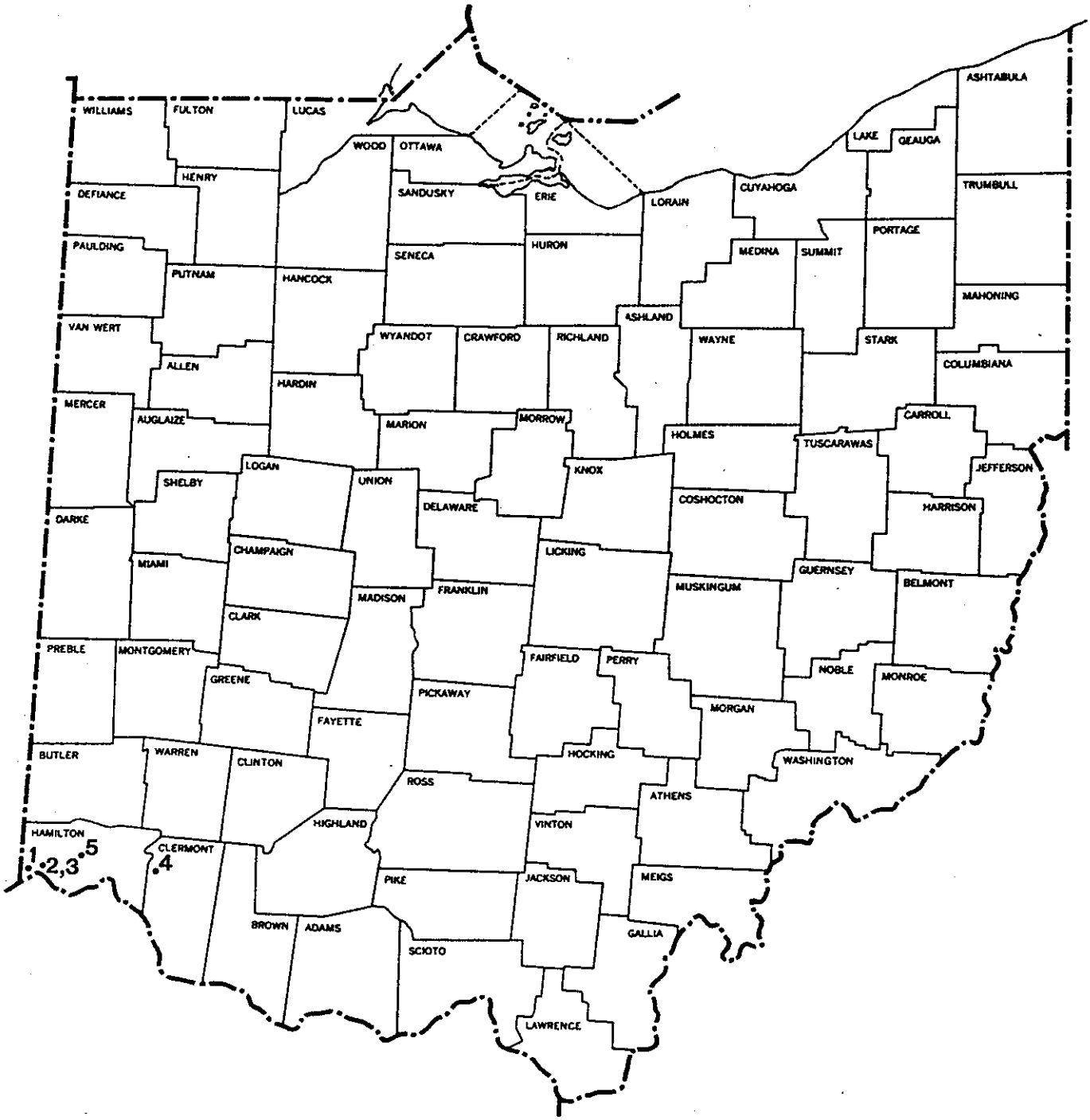
Table 4. Average RAPD marker similarity comparisons within and among populations.

Population	Range of average similarity values	Average similarity between populations	Average similarity within a population
Milford	0.914-0.874	0.886	0.939
Newberry 1	0.891-0.803	0.867	0.933
Newberry 2	0.921-0.835	0.887	0.955
Niehaus 1	0.902-0.810	0.876	0.917
Niehaus 4	0.902-0.833	0.881	0.946
Niehaus 6	0.920-0.817	0.892	0.950
Niehaus 8	0.910-0.821	0.875	0.927
Miami B	0.946-0.854	0.900	0.983
Miami L	0.946-0.851	0.903	0.982
Congress Green	0.946-0.825	0.892	0.950
Otter Creek	0.929-0.843	0.896	0.945
Upper Viney Fork	0.914-0.848	0.892	0.954
Cane Forest	0.906-0.804	0.873	0.920
K-Ridge	0.933-0.803	0.895	0.951
Wilhoit	0.926-0.793	0.890	0.959
Ferno	0.920-0.856	0.889	0.964
Rich	0.929-0.850	0.894	0.960
Upper John's Run "A"	0.914-0.814	0.887	0.951
Shaver's 1	0.926-0.880	0.897	0.959
Shaver's 2	0.924-0.875	0.895	0.958
Snyder	0.915-0.812	0.890	0.950
Laural	0.938-0.840	0.899	0.984
Rock Creek	0.910-0.846	0.887	0.949
Crouch Knob	0.886-0.815	0.856	0.941
Misty Valley	0.908-0.793	0.871	0.980
Meagher 1	0.937-0.843	0.862	0.952
Meagher 2	0.937-0.804	0.857	0.957
Hidden Valley	0.915-0.794	0.865	0.952

Table 5. Average RAPD marker similarity comparison by state.

	Missouri	West Virginia	Kentucky	Ohio	Indiana
Missouri	0.920	-----	-----	-----	-----
West Virginia	0.870	0.897	-----	-----	-----
Kentucky	0.853	0.899	0.901	-----	-----
Ohio	0.856	0.875	0.887	0.898	-----
Indiana	0.828	0.861	0.887	0.868	0.952

Figure 1. Locations of T. stoloniferum populations sampled in Ohio in 1994. (Numbers correspond to sites listed in Table 1.)



OHIO

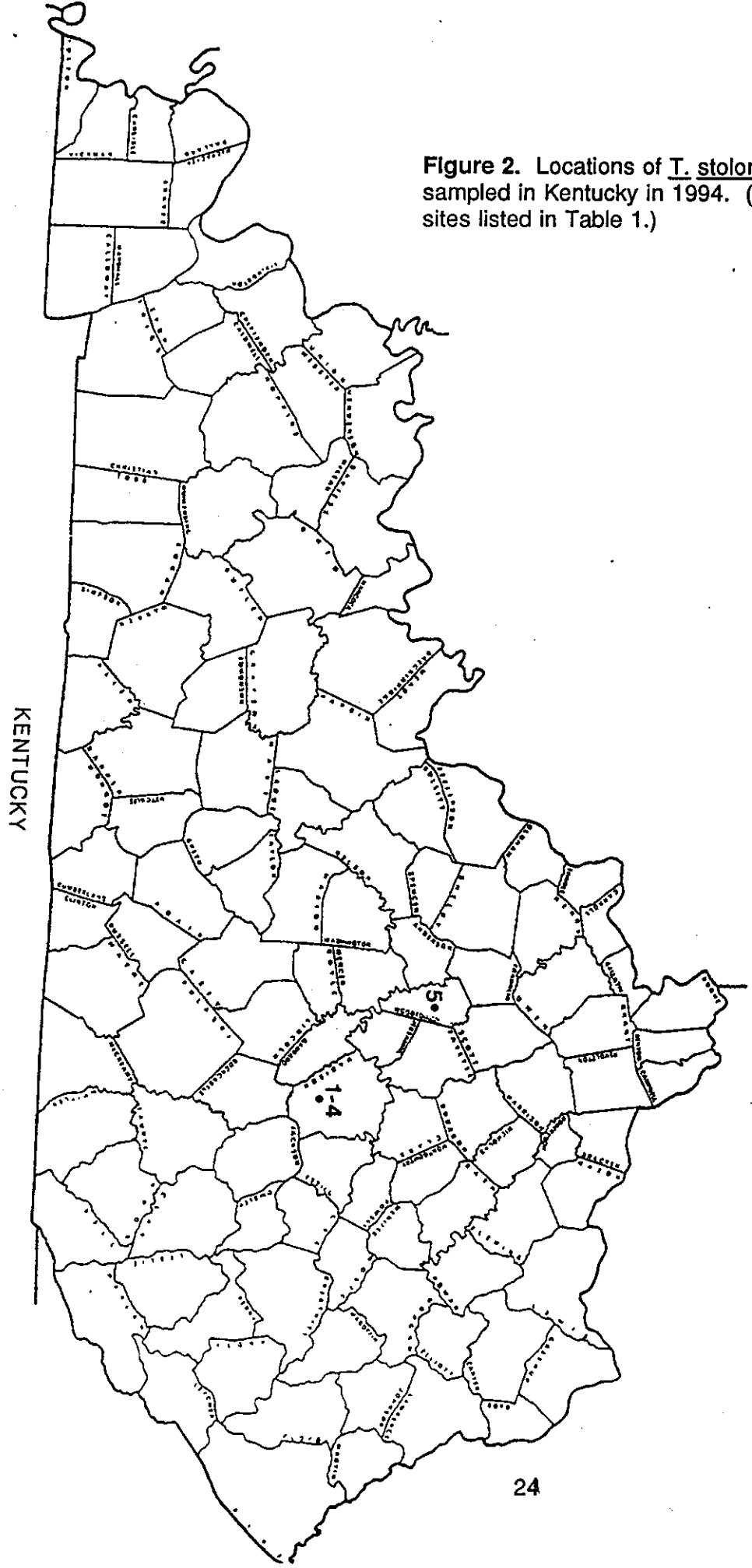


Figure 2. Locations of T. stoloniferum populations sampled in Kentucky in 1994. (Numbers correspond to sites listed in Table 1.)

Figure 3. Locations of T. stoloniferum populations sampled in West Virginia in 1994-1996. (Numbers correspond to sites listed in Table 1.)

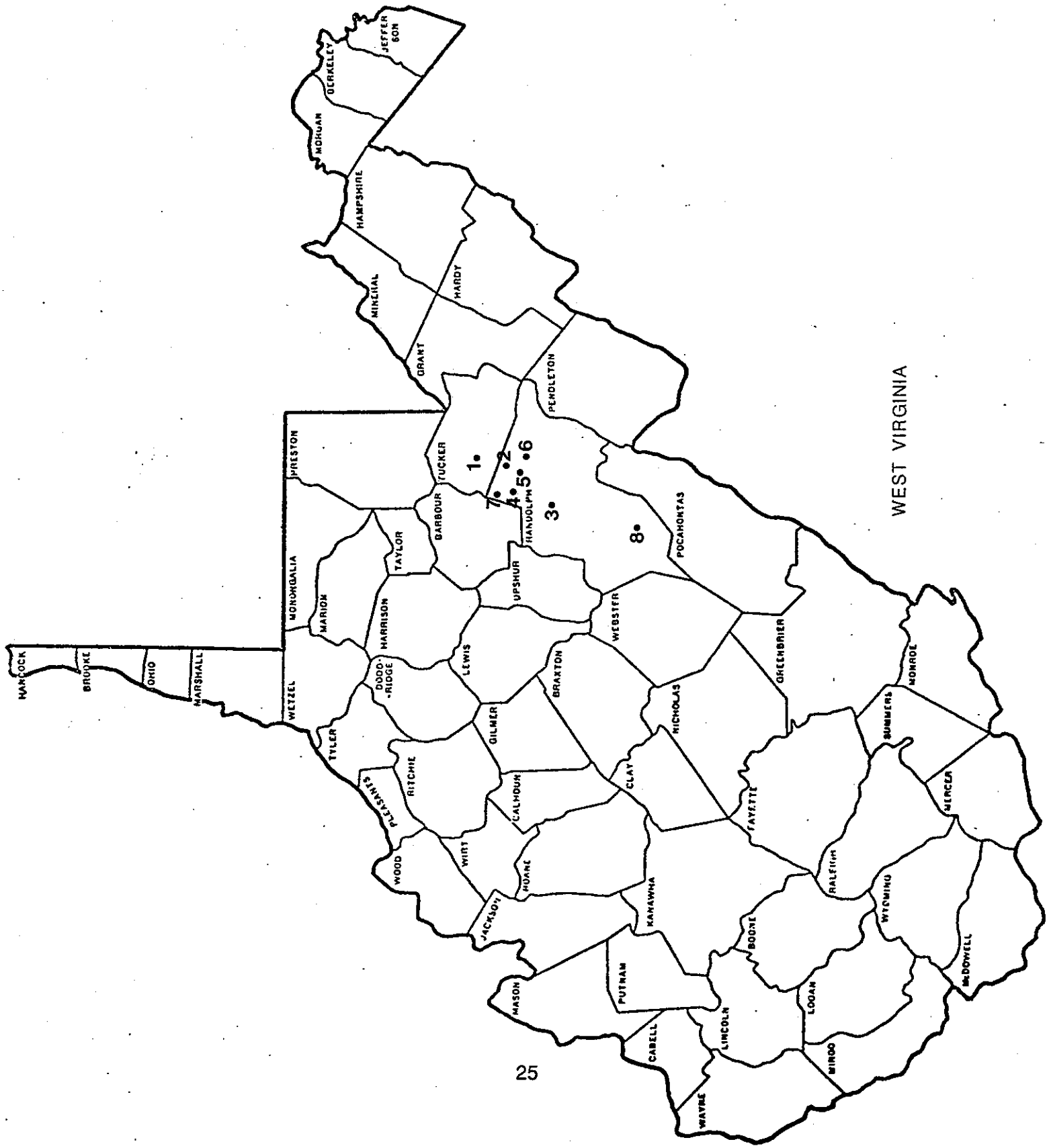
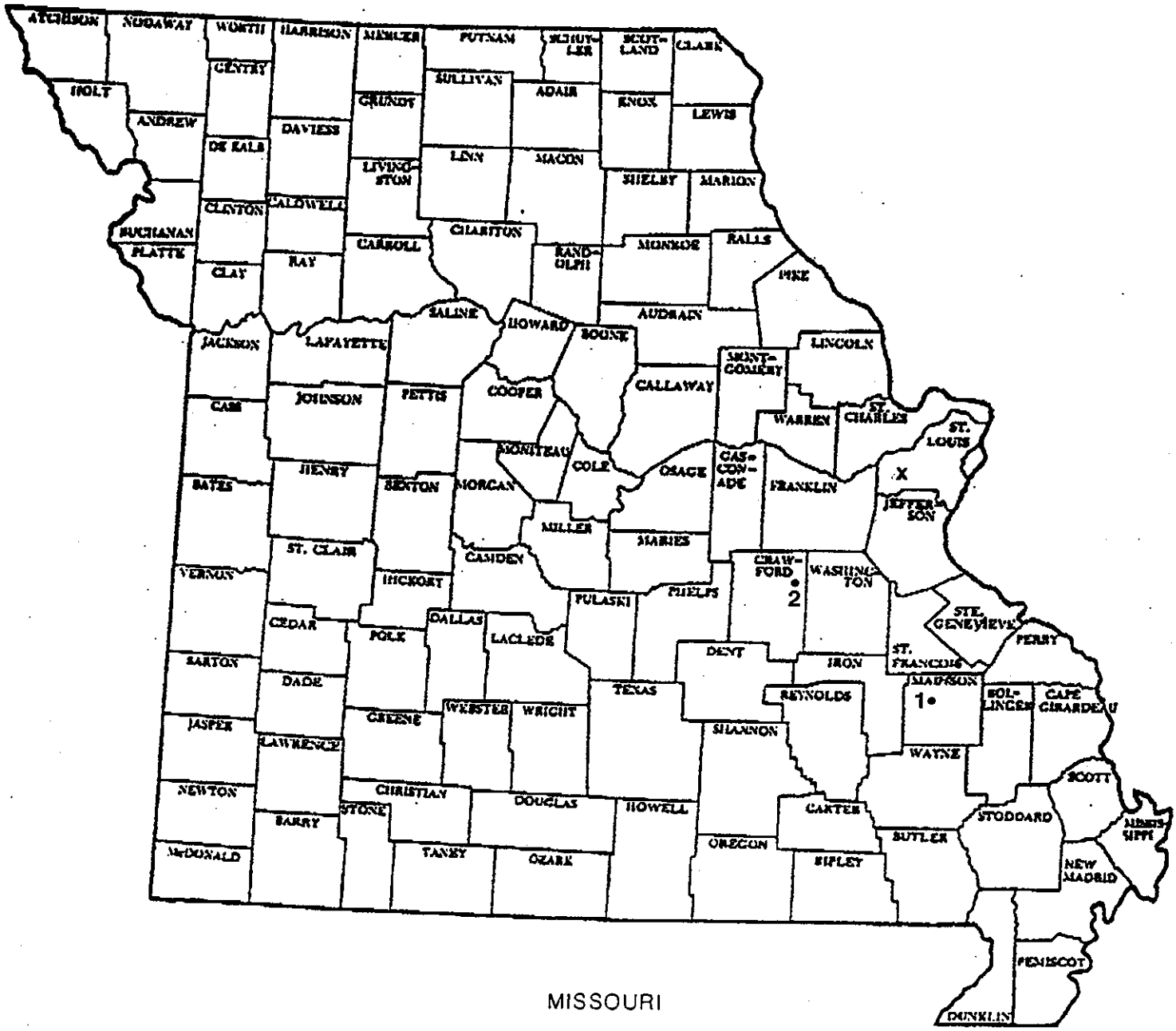
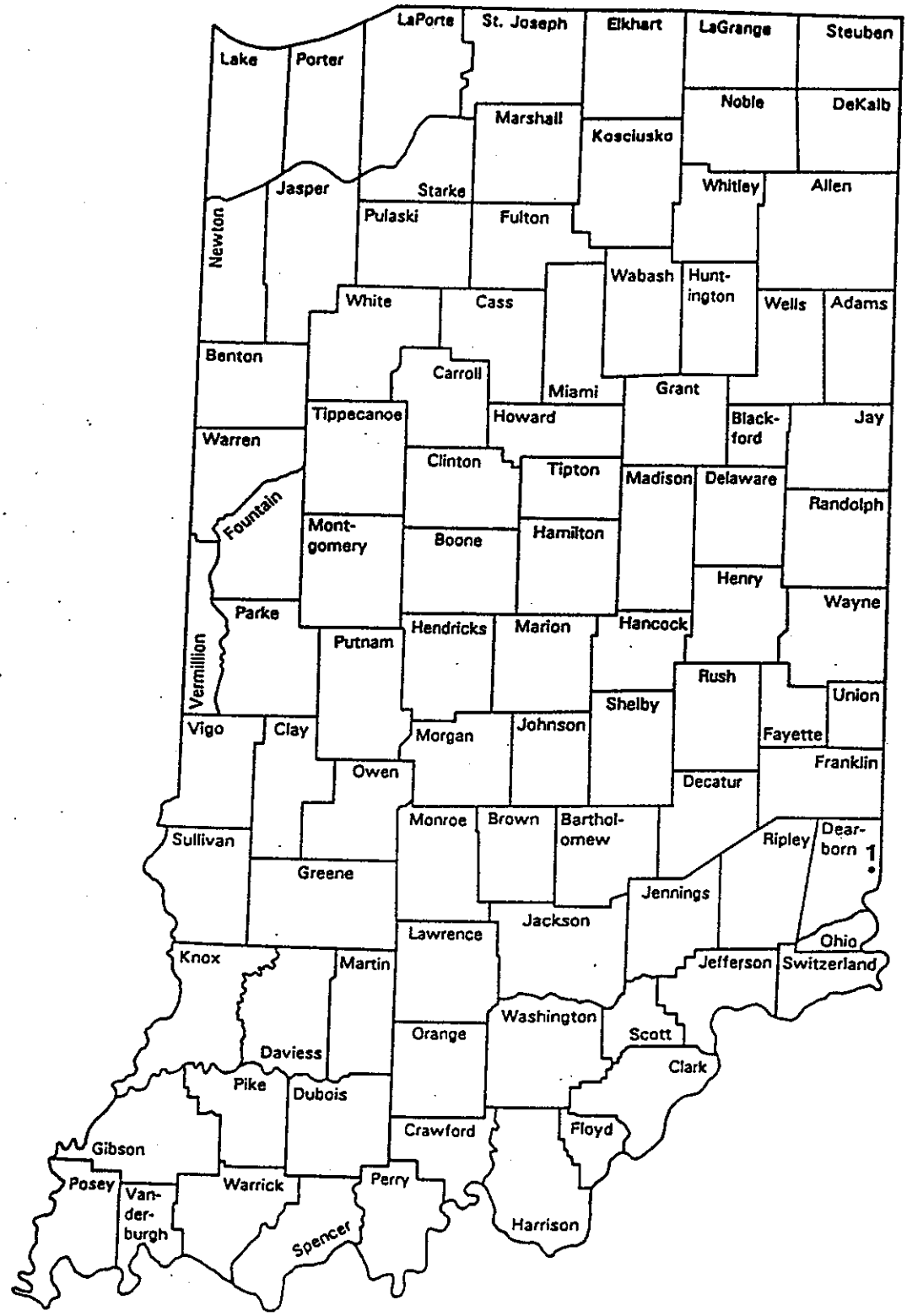


Figure 4. Locations of T. stoloniferum populations sampled in Missouri in 1995. "X" marks the origin of the dirt pile at which T. stoloniferum was discovered in Madison County. T. stoloniferum was subsequently transplanted from the Madison County "dirt pile population" via cuttings and seed to Crawford, Texas and Wayne Counties. All three populations were sampled for RAPD analysis, however, results are given only for the samples taken from the transplanted Crawford County population which was established from seed and had the most repeatable results. The native population later discovered in Madison County in 1994 represents site 1. (Numbers correspond to sites listed in Table 1.)



MISSOURI

Figure 5. Location of the T. stoloniferum population (Hidden Valley) sampled in Indiana in 1996.



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