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THE GENETIC STATUS OF NORTHERN CRICKET FROGS IN MINNESOTA

FINAL REPORT TO THE MINNESOTA DEPARTMENT OF NATURAL RESOURCES, NONGAME RESEARCH PROGRAM

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ABSTRACT

The northern cricket frog has a small range in Minnesota, most records are from the southeast and southwestern regions of the state. Populations of these frogs exhibited a precipitous decline in the 1980's and by the mid 1990's the northern cricket frog was considered extirpated from Minnesota. In 1998, a chorus of northern cricket frogs was heard in Bloomington, Hennepin County, Minnesota. This population is far outside the historic range of northern cricket frogs and its proximity to a shipping depot and a large urban center raised concerns that these frogs may have been purposefully or accidentally introduced. To determine the origin of this population, we sequenced 725 nucleotides of the mitochondrial cytochrome *b* gene in 45 specimens of the northern cricket frog. These samples included five specimens from the Bloomington population as well as specimens from across the range of the species. We used phylogenetic and phylogeographic techniques to analyze these data to determine evolutionary relationships among populations and to examine the correlation of these relationships with geographic distribution. The Bloomington population has DNA sequences that are unique compared to other northern cricket frog populations. This population is genetically most similar to populations from Iowa, Wisconsin, and northern Illinois. Thus it is unlikely that the Bloomington frogs were introduced from a distant population. They may have been introduced from a population in the upper midwest but given their unique DNA sequences, we think it just as likely that these frogs are native to Minnesota. We recommend that these frogs be managed as a native endangered species. We further recommend that additional surveys be undertaken to discover other populations that may be present in the state.

INTRODUCTION

The northern cricket frog (*Acris crepitans*) is a widespread species in eastern North America (Fig. 1) that has exhibited dramatic population declines in the northern portion of its range (Baker 1997; Hammerson and Livo 1999; Hay 1998; Lannoo 1998). This phenomenon first came to light in the 1970s and continues to the present (Hay 1998; Lehtinen 2002; Vogt 1981). These declines are characterized by the disappearance of cricket frogs from apparently intact habitat with no concurrent decline in populations of other amphibian species (Lanoo 1998). Possible causes include: climate (J. Irwin, pers comm.; Hay 1998); habitat alteration (Lanoo 1998); and habitat fragmentation (Hay 1998).

The historic range of northern cricket frogs in Minnesota included the southeastern and far southwestern portions of the state (Fig. 2; Oldfield and Moriarty 1994). There is an isolated record from Chisago County (JFBM 4394) collected in 1960, about 100 miles north of other records from Minnesota. Concerns about declining populations of northern cricket frogs in Minnesota were raised throughout the 1980's (Lang and Karns 1988; Pfannmuller and Wells 1981). A single specimen was collected in Houston County in 1981 (Moriarty et al. 1998) and a northern cricket frog call was reported from Houston County in 1991 (Oldfield and Moriarty 1994). By the mid 1990's the northern cricket frog was considered extirpated from Minnesota (Baker 1997; Moriarty 1998).

In 1998, a chorus of northern cricket frogs was heard near the Minnesota River within the Minnesota Valley National Wildlife Refuge in Bloomington, Hennepin County, Minnesota, and one specimen was collected (JFBM 13727; Moriarty et al. 1998). This population is far outside the historic range of northern cricket frogs (Fig. 2) and its proximity to a shipping depot and a large urban center raised concerns that these frogs may have been purposefully or accidentally introduced. This population has persisted although the number of calling males fluctuates dramatically from year to year (Fig. 3;J. Gerholdt, pers. comm.).

It is unlikely that cricket frog population in Bloomington existed prior to 1998 and was

merely overlooked. Suitable habitat did not exist prior to the mid-1970's when the wetland was created (J. Moriarty, pers. comm.). Northern cricket frogs are not thought of as long-distance dispersers probably due to their small size; however, there exist several extra-limital records for this species. These include the previously mentioned record from Chisago County and a report of northern cricket frogs as "probably common" in Franklin County, New York, near the Canadian border (Evermann 1917). Other unverified extralimital records exist in northern Michigan (L. Sargent, pers. comm.); central Wisconsin (B. Hay, pers. comm.); and western Pennsylvania (Harding 1997). There also exists a record of an introduction of northern cricket frogs in Boulder, Colorado (Livo et al. 1998). This population persisted until the lake they inhabited was drained for maintenance (Livo et al. 1998). These observations suggest the following: northern cricket frogs are capable of living out of their known range; they may be better dispersers than previously thought; and small extralimital populations may be able to persist for many years undetected.

We used phylogeographic methods to investigate the origin and status of the Bloomington population in Minnesota. Phylogeography incorporates phylogenetic information with population biology and biogeography and thus provides a good approach to this problem. In phylogeographic analyses genetic data, such as DNA sequences, are used to construct phylogenetic trees or networks that describe how genetic variants are related to one another. Genes sequenced from the mitochondrial genome are particularly suited to these types of analyses because they evolve rapidly, are inherited maternally, and do not exhibit recombination (Avise 2000). This makes them particularly useful for examining relationships among populations or closely related species. The mitochondrial genome is haploid, each mitochondria contains a single version of the mitochondrial genome, in comparison to nuclear genes where two copies are present in each cell nucleus (diploid). Unique mitochondrial sequences are referred to as haplotypes. Haplotypes are used to construct phylogenetic trees or networks that are compared with geographic or distributional data. Statistical tests are used to determine whether there is a correlation between monophyletic groups of haplotypes (clades) and geography.

Our null hypothesis was that the Bloomington northern cricket frog population are

descended from, or related to, cricket frogs native to Minnesota. The alternate hypothesis was that the Bloomington frogs are not native and therefore the result of intentional or accidental introduction. A strong test of our hypothesis requires samples from other Minnesota northern cricket frog populations. Testing this hypothesis was complicated by the fact that there were no other extant populations of the northern cricket frog in Minnesota and difficulties extracting useable DNA from preserved tissue limits the utility of museum specimens. Instead we obtained tissues of northern cricket frogs from across their range including populations from neighboring states as well as more distant localities. If the Bloomington population was more closely related to northern cricket frogs from distant populations than more proximate populations such as Iowa or Wisconsin, we could reject the null hypothesis and conclude that the frogs had been recently introduced to the state. If the Bloomington frogs were more closely related to populations from proximate populations in Iowa or Wisconsin than to southern populations, the null hypothesis would be corroborated. Given the lack of available tissues from Minnesota, we would not be able to rule out the possibility of human mediated introduction or natural dispersal.

MATERIALS AND METHODS

We obtained tissues from 45 specimens of northern cricket frogs including the population from Bloomington, Minnesota and 11 other localities across the range of this species (Fig. 1, Table 1). Sample sizes per population ranged from one to five individuals. Spring peepers (*Pseudacris crucifer),* western chorus frogs (*P. triseriata),* and gray treefrogs (*Hyla versicolor),* were included as outgroups in phylogenetic analyses (da Silva 1997). Outgroups are used to root phylogenetic trees.

Genomic DNA was extracted using QIAamp^{TM} tissue extraction kits (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations. A fragment of the mitochondrial cytochrome *b* gene was amplified using polymerase chain reaction (PCR). PCR reactions were performed on a MJ Research, Inc. PTC-100 Programmable Thermal Controller (Watertown, MA) in a total volume of 25 μl containing 1.0 μg DNA, 1.2 μM of each primer, 10X *Taq* salts (Promega, Madison, WI), 10 μM dNTPs, and 1.25 units of *Taq* DNA polymerase (Promega). The following thermal profile was used: initial denaturation at 94°C (3 min); 35 cycles of 94°C (10 sec), 52°C (20 sec), 72°C (20 sec); and a final extension at 72°C (10 min) before termination of the reaction at 4°C. Amplification products were purified using the QIA quick PCR Purification kit (Qiagen) following manufacturer's recommendations. Automated sequencing was performed using Big Dye (Perkin Elmer) terminator cycle sequencing on an ABI 3700 at the Advanced Genetic Analysis Center, University of Minnesota. Primers MVZ16-H [5' AAA TAG GAA RTA TCA YTC TGG TTT RAT 3'] and MVZ15-L [5' GAA CTA ATG GCC CAC ACW WTA CGN AA 3'] (Moritz et al. 1992) were used for amplification and sequencing and both the heavy and light strands were sequenced. Sequences were checked for accuracy of base determination and assembled using the computer program Sequencher 4.0 (Gene Codes). All sequences are listed in Appendix A.

Sequences were aligned by eye and entered into a data matrix. The numbers of haplotypes and polymorphic sites were calculated using Arlequin, version 2.0 (Schneider et al. 2000). Identical sequences were combined into single haplotypes for phylogenetic analyses. This increased the speed of the analyses but maintained all genealogical information.

Phylogenetic trees were constructed using two techniques, parsimony and maximum likelihood. Parsimony analyses search for phylogenetic trees that have the smallest number of changes in character states or, for genetic data, nucleotides. These analyses have the advantage of being fast, but can be misleading in some circumstances (Felsenstein 1978). Maximum likelihood analyses require specification of a particular model of nucleotide evolution. These analyses require more computing time than parsimony analyses but provide branch length information, a proxy for evolutionary time, and can take into account peculiarities in nucleotide evolution that can mislead parsimony (Felsenstein 1981).

Parsimony analyses were performed to determine relationships among haplotypes of cricket frogs using the computer program PAUP*, version 4.0b10 (Swofford 2001). Tree search options included: heuristic search, 100 random addition sequence replicates, and tree-bisection-reconnection. All nucleotide positions were equally weighted. Parsimony trees were evaluated using summary values reported by PAUP*. Support for branches was evaluated by calculating bootstrap values (Felsenstein 1985) using 100 pseudoreplicate bootstraps with the following options: heuristic search, simple step-wise addition option, and tree-bisection-reconnection.

The model of nucleotide substitution for the maximum likelihood analysis was chosen using the computer program Modeltest, version 3.06 (Posada and Crandall 1998). This program determines the parameters for a hierarchy of likelihood models and chooses among these models using a likelihood ratio test (Posada and Crandall 2001). This model is presumed to provide the simplest explanation of the data, i.e., it provides a reasonable model for the data but minimizes the number of assumptions in the analysis. The model and model parameters obtained were used for the maximum likelihood analyses in PAUP* and for calculation of genetic distances (see below).

Maximum likelihood analyses (Felsenstein 1981) were performed to estimate phylogeny and branch lengths under the likelihood model of sequence evolution as chosen by Modeltest. Support for internodes was evaluated by calculating bootstrap values (Felsenstein 1985) using 100 pseudoreplicate bootstraps with the following options: full

heuristic search, simple step-wise addition option, and tree-bisection-reconnection.

Pairwise genetic distances, using uncorrected percent ("p") distances and corrected distances based on the likelihood model and parameters identified using Modeltest, were calculated using PAUP*. Net between group distances were calculated using MEGA2 (Kumar et al. 2001).

A nested clade analysis was used to identify genetic relationships among populations of northern cricket frogs. This analysis was restricted to the western clade (see results) because of the vast genetic differences between the western clade and the specimens from Georgia. This analysis tested the null hypothesis of no association between haplotype variation and geography (Templeton 1998). A minimum spanning phylogenetic network was constructed using statistical parsimony with the computer program TCS, version 1.13 (Clement et al. 2000). This approach differs from parsimony and maximum likelihood analyses as it allows for the persistence of an ancestral form or haplotype that may give rise to several contemporaneous descendent haplotypes (see haplotype c, Fig. 2). Networks, therefore, are not strictly dichotomous and extant haplotypes may appear on internal nodes. Ambiguities in the network, represented by loops, were resolved using the criteria outlined by Pfenninger and Posada (2002). The network was organized into a set of nested clades following the rules of Templeton et al. (1987) and Templeton and Sing (1993) as implemented in TCS. Nested clades were used to test for significant associations between haplotypes and geographic sampling. The average clade distance (D_C) measures the geographic spread of each clade. The nested clade distance (D_N) measures how widespread a clade is relative to the distribution of its nesting clade. The interior tip distances ($I\text{-}T_C$ and $I\text{-}T_N$) measure how widespread tip clades are compared to interior clades in the nested clade network. A null distribution was created by randomly permuting the observations within a nested clade across geographical locations 1000 times while preserving clade frequencies and sample sizes (Templeton and Sing 1993). Clade distances, nested clade distances, and interior-tip distances were calculated during each iteration. The null hypothesis of no geographical associations was tested using the computer program GeoDis, version 2.0 (Posada et al. 2000). This program calculated the nested clade distance measures and their statistical significance.

RESULTS

A 725 nucleotide fragment of the cytochrome *b* gene was sequenced for 45 individuals of the northern cricket frog (Table 1), two individuals of the western chorus frog (JFBM 14325, 14310) and two individuals of the gray treefrog (JFBM 14311, 14312). Cytochrome *b* sequences (291 bases) for two individuals of the northern spring peeper were downloaded from GenBank (#'s AF488308, AF488337). Twenty-four unique haplotypes were observed among the 45 northern cricket frog specimens. The number of haplotypes observed per population ranged from one to four. The Bloomington population was characterized by a single haplotype (haplotype X) that was not observed in any of the other included populations (Table 2).

The pairwise uncorrected "p" distances and likelihood corrected distances among all unique northern cricket frog haplotypes are listed in Table 3. The observed number of haplotypes, number of polymorphic sites and average within group sequence divergence for each group are listed in Table 4.

The parsimony analysis resulted in 598 equally most parsimonious trees (Tree Length = 351, Consistency Index excluding uninformative characters = 0.8163, Retention Index = 0.8920). A strict consensus of these trees is presented in Figure 4. Two divergent clades were identified; an eastern clade containing Georgia haplotypes and a western clade containing all remaining haplotypes. Within the western clade there was very little resolution among haplotypes.

The evolutionary model of nucleotide substitution that provided the simplest explanation of the data was F81 + Γ. This model allows a single substitution type, variable base frequencies, and rate heterogeneity across all nucleotide positions (Felsenstein 1981). The estimated model parameters were: nucleotide frequencies, A = 0.4043, C = 0.1639, G = 0.1654, T = 0.2664; and the shape parameter α = 1.6296. The shape parameter α describes the Γ distribution that models rate heterogeneity. A value of 1.63 suggests that most nucleotide positions evolve relatively slowly but some positions have moderate to high rates. The maximum likelihood analysis produced a tree with a likelihood of -ln 2799.234 (Fig. 5). The results of the maximum likelihood analysis were largely consistent with the parsimony analysis. As in the parsimony analyses, two divergent

eastern and western clades were identified.

The minimum spanning haplotype network was not fully resolved; two loops were observed. The loops were resolved by breaking the connections between haplotypes D and R (via two inferred haplotypes) and between C and M (via two inferred haplotypes; see Fig. 6). The connections were broken according to geographic criteria; haplotypes are more likely to be connected to haplotypes from the same population or region than to haplotypes from distant populations (Pfenninger and Posada 2002). Haplotypes fully nested within the network resulted in a three-level hierarchy that included 17 one-step clades, seven two-step clades and three three-step clades (Fig. 7). Of these clades 1-1, 1-2, 1-3, 1-4, 1-6, 1-7, 1-8, 1-9, 1-10, 1-11, 1-12, 1-13, 1-14, 1-16, and 2-6 were not tested for geographical associations because haplotypes in these clades showed no geographical variation within the nested clade. The nested clade analysis revealed significant geographic structuring among haplotypes at two geographic scales; clade 2-7 ($P<0.000$) and clade 3-3 ($P<0.010$). Clade 2-7 contained haplotypes from Iowa, northern Illinois, Wisconsin, and Minnesota (Fig. 8). Clade 3-3 contained the haplotypes of clade 2-7 plus haplotypes from Kansas, Arkansas, southern Illinois, and Texas (Fig. 8).

DISCUSSION

The origin of the Bloomington population could not be determined unequivocally. We were surprised to observe that the Bloomington population contained a single unique haplotype not observed in any other sampled population of northern cricket frogs. The relationships of this haplotype to other northern cricket frog haplotypes could not be determined using parsimony or maximum likelihood because of the low sequence divergence among these haplotypes and assumptions of these analyses regarding persistent ancestral haplotypes. This low divergence was evident in the short terminal branches of the phylogenetic trees produced in maximum parsimony and maximum likelihood analyses.

The nested clade analysis indicated that the Bloomington population is a member of a northern group or clade whose range includes Minnesota, Wisconsin, Iowa, and northern Illinois (Fig. 8). The Bloomington haplotype (haplotype X) differs from its presumed ancestral haplotype (haplotype C) by three nucleotide differences. Because there are no other extant populations from Minnesota, and given that the Bloomington population is more closely related to northern cricket frog populations from neighboring states, we could not falsify the hypothesis that the Bloomington population is native. There is no evidence that these frogs were introduced from populations south of Iowa, and given their unique haplotype, there is no evidence they were introduced from neighboring states. Thus the available data corroborated the hypothesis that the Bloomington populations are native.

A more rigorous test of this hypothesis would require additional data. These data should be derived from additional sampling of northern cricket frog populations in neighboring states and Minnesota. Samples from these populations should include at least ten individuals per population to better survey haplotype diversity. This higher sampling would increase the possibility of finding haplotypes more closely related to the Bloomington population haplotype.

Historic tissue samples of northern cricket frogs from Minnesota should be obtained and efforts made to extract and sequence DNA from preserved museum specimens. We

have initiated applications for loans of Minnesota northern cricket frog tissues collected in the 1960s by Herbert Dessauer and held at Louisiana State University. Greater efforts should also be made to locate other populations of northern cricket frogs in Minnesota; in particular, the Chisago County locality should be visited in Spring 2004 (M. Davis, pers. comm.).

We recommend that the Bloomington population continue to be managed as an endangered native species. There is no evidence that this population is not descended from northern cricket frogs native to the state although it could have been recently established. Northern cricket frog populations may behave as metapopulations characterized by frequent local extinctions and recolonizations (Lanoo 1998). Management decisions should take the complexity and stochasticity of metapopulation dynamics into account. We recommend additional surveys for northern cricket frogs be made in the Minnesota Valley National Wildlife Refuge and surrounding areas with suitable habitat. If additional populations are discovered we advise genetic work be undertaken to determine the relationship to the existing Bloomington population, effective population size, and population limits. These data will provide guidance in subsequent management decisions.

Although outside the scope of this study, these results indicated that current subspecific and specific delineations of northern cricket frogs are inadequate. The large genetic distance between the eastern and western clades does not correspond to current subspecific designations. In particular, the populations from southeast Arkansas are currently considered to be *Acris crepitans crepitans* but the phylogeny indicates they are more closely related to *A. crepitans blanchardi*. The marked divergence between the western clade and the Georgia specimens suggests that the northern cricket frog as currently defined may consist of two separate species. Dessauer and Nevo (1969) found similar results in cricket frogs using allozymes (Fig. 9). The presence of an eastern and western clade is seen in the biogeographic patterns of other sister species in the eastern United States such as ratsnakes (Burbrink 2002); eastern fence lizards (Leache and Reeder 2002); and numerous freshwater fishes (Berendzen et al. 2003; Mayden 1988). More specimens representing additional localities across the species range are needed to test this hypothesis.

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Table 1. Cricket frog specimens examined. Fogs used in this study are listed with the number of specimens, locality, and locality ID. Latitude and longitude are approximations for some specimens. Voucher numbers or field collection numbers are given where available.

ID	Locality	*n*	Latitude	Longitude	Voucher number(s)
1	Madison Co., Iowa	5	41°17'57"	94°02'29"	JRP 1374
2	Warren Co., Iowa	5	41°20'00"	93°33'00"	JRP 1379
3	Travis Co., Texas	3	30°26'39"	97°48'09"	N/A
4	Monroe Co., Georgia	5	33°02'07"	83°54'48"	N/A
5	Fayette Co., Illinois	1	39°07'54"	88°58'18"	INHS #73
6	Rock Island Co., Illinois	3	41°26'24"	90°48'42"	INHS #199, 200, 201
7	Perry Co., Arkansas	4	34°55'46.5"	92°48'2.6"	N/A
8	White Co., Arkansas	5	35°13'22.4"	91°35'56.7"	N/A
9	Ashley Co., Arkansas	4	33°06'06"	91°38'19.9"	N/A
10	Jefferson Co. Kansas	2	38°59'19"	95°13'25"	JES 2631, 2630
11	Iowa Co., Wisconsin	3	43°01'54"	90°05'29"	N/A
12	**Hennepin Co. Minnesota**	**5**	XXXXXXX	XXXXXXX	**JFBM13727, N/A**

Table 2. Unique haplotypes and localities within northern cricket frogs. Haplotype letter assignments are arbitrary.

Haplotype	Locality	*n*	Haplotype	Locality	#
A	Jefferson Co. Kansas	1	L	Perry Co., Arkansas	1
B	Jefferson Co. Kansas	1	M	Perry Co., Arkansas	1
C	Madison Co., Iowa	3	N	Perry Co., Arkansas	1
	Warren Co., Iowa	5	O	White Co., Arkansas	1
	Rock Island Co., Illinois	1	P	White Co., Arkansas	2
D	Madison Co., Iowa	1	Q	White Co., Arkansas	1
E	Madison Co., Iowa	1	R	White Co., Arkansas	1
F	Travis Co., Texas	1		Ashley Co., Arkansas	1
G	Travis Co., Texas	1	S	Ashley Co., Arkansas	2
H	Travis Co., Texas	1	T	Fayette Co., Illinois	1
I	Iowa Co., Wisconsin	1	U	Rock Island Co., Illinois	1
J	Iowa Co., Wisconsin	2	V	Monroe Co., Georgia	3
	Rock Island Co., Illinois	1	W	Monroe Co., Georgia	2
K	Perry Co., Arkansas	1	**X**	**Hennepin Co. Minnesota**	**5**
	Ashley Co., Arkansas	1			

Table 3. Distance matrix for pairwise comparison of northern cricket frog haplotypes. Uncorrected "p" distances are on the top portion of the table. Corrected likelihood distance values are on the bottom portion of the table. Haplotype letter assignments are labeled according to Table 2. Note: Space limitations resulted in the table being split between 2 pages.

	A	B	C	D	E	F	G	H	I	J	K	L
A	***	0.0082	0.0020	0.0039	0.0039	0.0057	0.0094	0.0094	0.0058	0.0039	0.0076	0.0057
B	0.0083	***	0.0129	0.0108	0.0148	0.0148	0.0106	0.0188	0.0169	0.0148	0.0148	0.0106
C	0.0021	0.0131	***	0.0014	0.0014	0.0071	0.0070	0.0110	0.0029	0.0014	0.0055	0.0041
D	0.0039	0.0109	0.0014	***	0.0029	0.0084	0.0056	0.0125	0.0044	0.0028	0.0069	0.0055
E	0.0039	0.0151	0.0014	0.0029	***	0.0087	0.0087	0.0129	0.0045	0.0029	0.0072	0.0057
F	0.0057	0.0150	0.0071	0.0085	0.0088	***	0.0084	0.0154	0.0102	0.0084	0.0099	0.0084
G	0.0095	0.0107	0.0071	0.0057	0.0088	0.0085	***	0.0154	0.0102	0.0084	0.0099	0.0056
H	0.0095	0.0192	0.0112	0.0126	0.0131	0.0157	0.0157	***	0.0131	0.0124	0.0138	0.0110
I	0.0059	0.0172	0.0029	0.0044	0.0045	0.0103	0.0103	0.0133	***	0.0014	0.0087	0.0058
J	0.0039	0.0151	0.0014	0.0028	0.0029	0.0085	0.0085	0.0126	0.0014	***	0.0069	0.0055
K	0.0076	0.0150	0.0056	0.0070	0.0072	0.0100	0.0100	0.0140	0.0088	0.0070	***	0.0069
L	0.0058	0.0108	0.0042	0.0056	0.0058	0.0085	0.0056	0.0112	0.0058	0.0056	0.0070	***
M	0.0057	0.0150	0.0042	0.0056	0.0058	0.0085	0.0085	0.0126	0.0073	0.0056	0.0042	0.0056
N	0.0097	0.0110	0.0070	0.0056	0.0087	0.0114	0.0057	0.0140	0.0088	0.0084	0.0098	0.0028
O	0.0097	0.0110	0.0070	0.0056	0.0087	0.0114	0.0057	0.0140	0.0088	0.0084	0.0098	0.0028
P	0.0059	0.0152	0.0084	0.0084	0.0087	0.0057	0.0085	0.0155	0.0103	0.0112	0.0127	0.0112
Q	0.0095	0.0065	0.0056	0.0042	0.0072	0.0099	0.0042	0.0140	0.0088	0.0070	0.0084	0.0042
R	0.0076	0.0088	0.0056	0.0042	0.0073	0.0100	0.0042	0.0140	0.0088	0.0070	0.0084	0.0042
S	0.0078	0.0173	0.0057	0.0071	0.0073	0.0100	0.0100	0.0142	0.0088	0.0071	0.0057	0.0071
T	0.0039	0.0151	0.0028	0.0042	0.0043	0.0100	0.0100	0.0140	0.0059	0.0042	0.0084	0.0070
U	0.0039	0.0151	0.0014	0.0028	0.0029	0.0085	0.0085	0.0126	0.0044	0.0028	0.0070	0.0056
V	0.1433	0.1455	0.1354	0.1346	0.1396	0.1381	0.1376	0.1422	0.1460	0.1367	0.1315	0.1348
W	0.1458	0.1482	0.1376	0.1368	0.1418	0.1400	0.1398	0.1444	0.1484	0.1388	0.1336	0.1369
X	**0.0097**	**0.0214**	**0.0060**	**0.0076**	**0.0076**	**0.0138**	**0.0106**	**0.0168**	**0.0093**	**0.0076**	**0.0106**	**0.0091**

Table 3 (cont.).

	M	N	O	P	Q	R	S	T	U	V	W	**X**
A	0.0057	0.0096	0.0096	0.0059	0.0094	0.0075	0.0077	0.0039	0.0039	0.1236	0.1255	**0.0096**
B	0.0148	0.0109	0.0109	0.0150	0.0065	0.0087	0.0170	0.0148	0.0149	0.1252	0.1272	**0.0209**
C	0.0042	0.0069	0.0069	0.0083	0.0055	0.0055	0.0056	0.0028	0.0014	0.1177	0.1193	**0.0060**
D	0.0055	0.0056	0.0056	0.0084	0.0041	0.0042	0.0070	0.0042	0.0028	0.1171	0.1187	**0.0075**
E	0.0057	0.0086	0.0086	0.0086	0.0072	0.0072	0.0072	0.0043	0.0029	0.1208	0.1225	**0.0075**
F	0.0084	0.0112	0.0112	0.0056	0.0098	0.0098	0.0099	0.0099	0.0084	0.1197	0.1211	**0.0135**
G	0.0084	0.0056	0.0056	0.0084	0.0042	0.0042	0.0099	0.0099	0.0084	0.1193	0.1210	**0.0105**
H	0.0124	0.0138	0.0138	0.0152	0.0138	0.0138	0.0140	0.0138	0.0124	0.1228	0.1244	**0.0165**
I	0.0072	0.0087	0.0087	0.0102	0.0087	0.0087	0.0087	0.0058	0.0043	0.1256	0.1273	**0.0092**
J	0.0055	0.0083	0.0083	0.0110	0.0069	0.0069	0.0070	0.0042	0.0028	0.1186	0.1202	**0.0075**
K	0.0042	0.0097	0.0097	0.0125	0.0083	0.0083	0.0057	0.0083	0.0069	0.1147	0.1163	**0.0105**
L	0.0055	0.0028	0.0028	0.0111	0.0041	0.0041	0.0070	0.0069	0.0055	0.1172	0.1188	**0.0090**
M	***	0.0083	0.0083	0.0110	0.0069	0.0069	0.0042	0.0069	0.0055	0.1186	0.1202	**0.0090**
N	0.0084	***	0.0028	0.0138	0.0041	0.0041	0.0098	0.0097	0.0083	0.1145	0.1161	**0.0120**
O	0.0084	0.0028	***	0.0138	0.0041	0.0041	0.0098	0.0097	0.0083	0.1172	0.1188	**0.0120**
P	0.0112	0.0140	0.0140	***	0.0124	0.0124	0.0112	0.0125	0.0110	0.1243	0.1259	**0.0135**
Q	0.0070	0.0042	0.0042	0.0126	***	0.0028	0.0084	0.0083	0.0069	0.1131	0.1147	**0.0120**
R	0.0070	0.0042	0.0042	0.0126	0.0028	***	0.0084	0.0083	0.0069	0.1133	0.1149	**0.0105**
S	0.0042	0.0099	0.0099	0.0113	0.0085	0.0085	***	0.0085	0.0070	0.1208	0.1224	**0.0105**
T	0.0070	0.0098	0.0098	0.0127	0.0084	0.0084	0.0085	***	0.0042	0.1204	0.1220	**0.0090**
U	0.0056	0.0084	0.0084	0.0112	0.0070	0.0070	0.0071	0.0042	***	0.1186	0.1202	**0.0075**
V	0.1367	0.1312	0.1348	0.1443	0.1294	0.1296	0.1396	0.1390	0.1367	***	0.0028	**0.1187**
W	0.1388	0.1333	0.1369	0.1464	0.1315	0.1317	0.1417	0.1411	0.1388	0.0028	***	**0.1202**
X	**0.0091**	**0.0122**	**0.0122**	**0.0137**	**0.0121**	**0.0106**	**0.0107**	**0.0091**	**0.0076**	**0.1367**	**0.1387**	***

Table 4. Comparison of number of haplotypes and sequence variation.

	n	# of haplotypes	Number of polymorphic sites	Average within-group sequence divergence	
				uncorrected	corrected
Pseudacris crucifer	2	2	10	0.0345	0.0344
Pseudacris triseriata	2	2	3	0.0044	0.0042
Hyla versicolor	2	2	3	0.0050	0.0048
Acris crepitans	45	24	89	0.0261	0.0277
western clade	40	22	31	0.0083	0.0080
eastern clade	5	2	3	0.0028	0.0026

Figure 1. Range of northern cricket frog. Approximate historical distribution of the subspecies following Conant and Collins (1998). Black dots identify approximate sampling localities and numbers within dots correspond to the locality ID from Table 1.

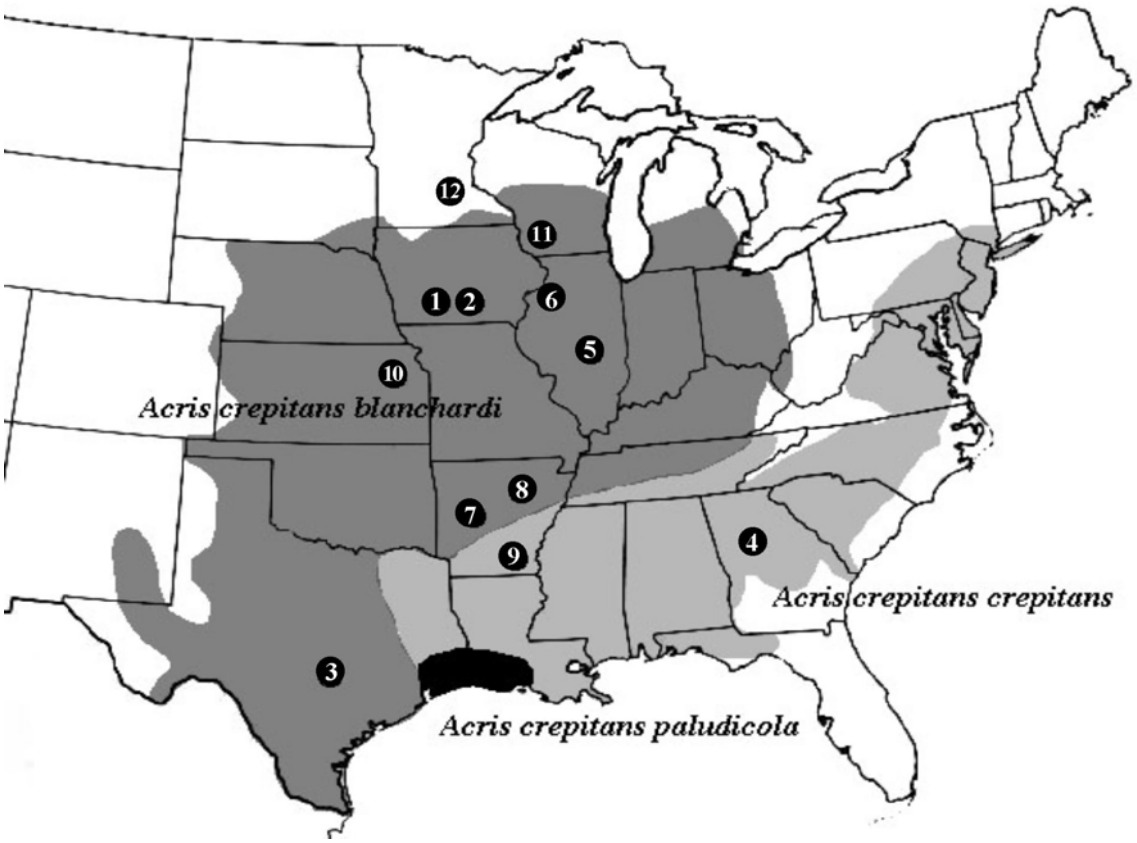


Figure 2. Historic range of cricket frogs in Minnesota. Modified from Oldfield and Moriarty 1994.

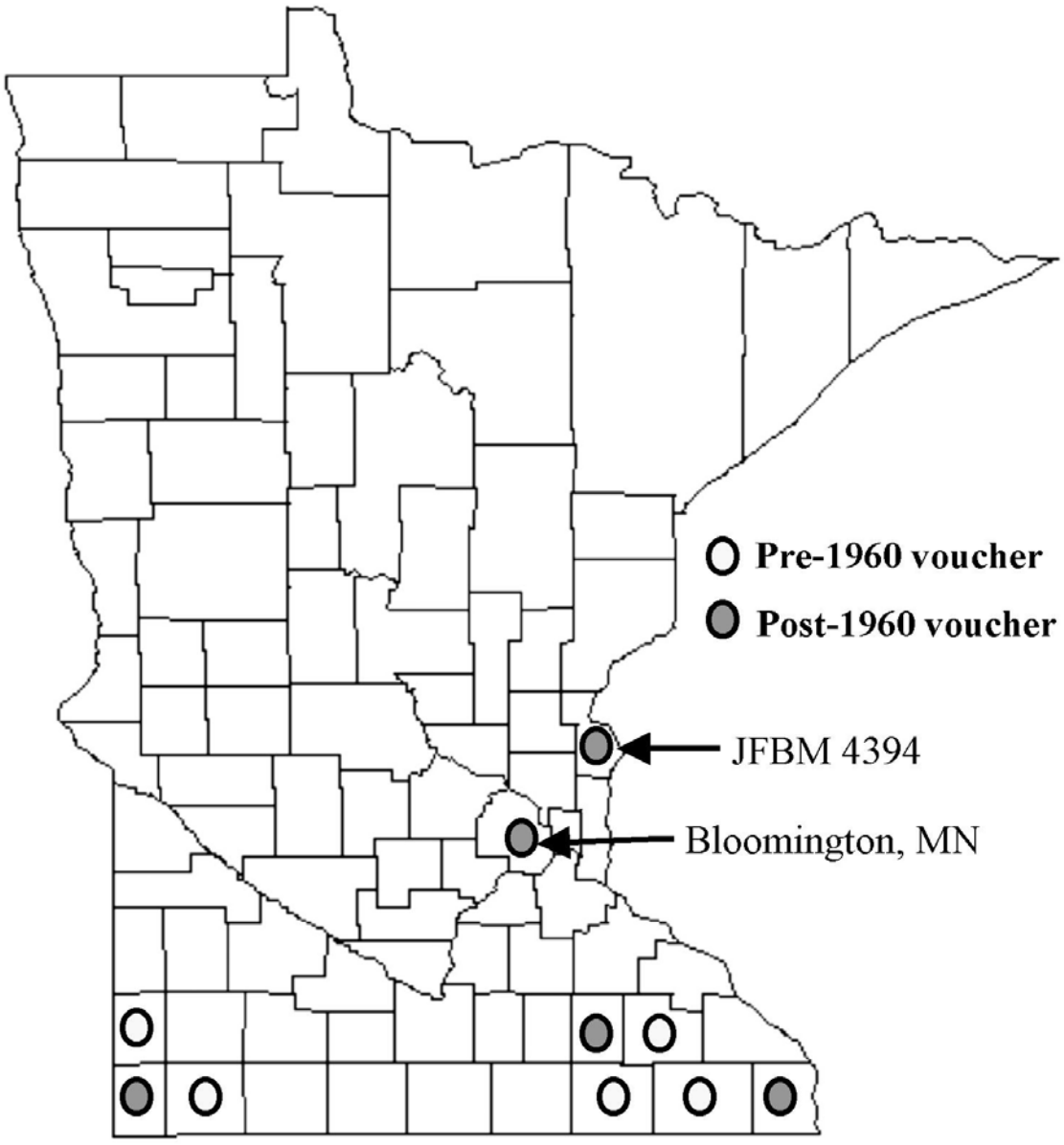


Figure 3. Yearly call survey results from Bloomington cricket frog population. From J. Gerholdt (pers. comm.).

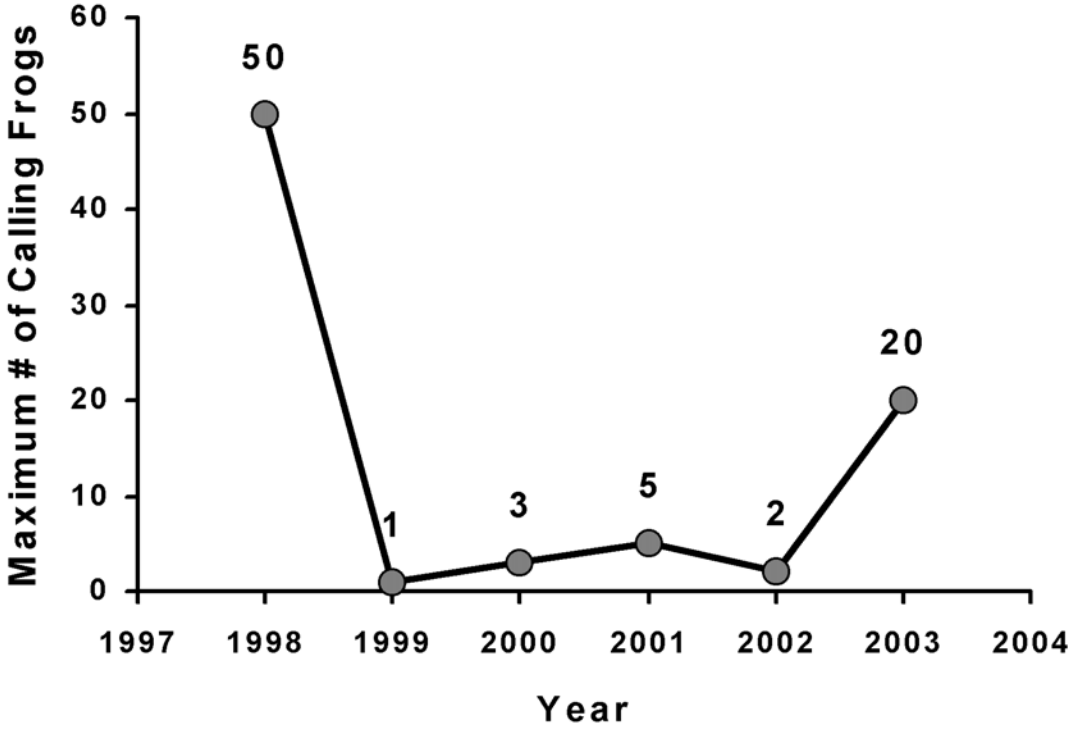


Figure 4. Strict consensus of 598 equally parsimonious trees. Numbers above nodes indicate bootstrap values (100 pseudoreplicates). TL = 351, CI excluding uninformative characters = 0.8163, RI = 0.8920. Haplotype letter assignments are labeled following Table 2.

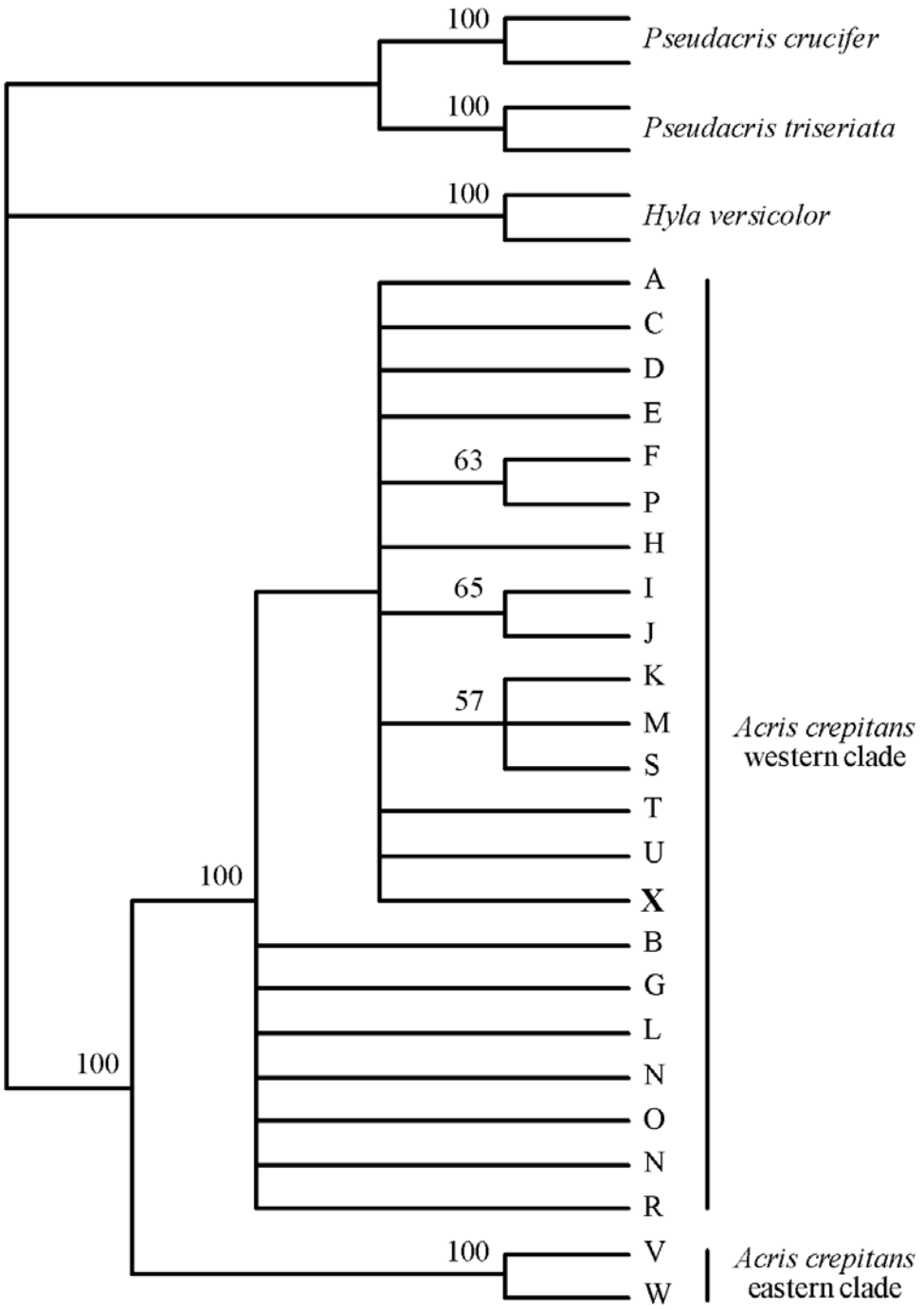


Figure 5. Topology produced in the maximum likelihood analysis (-ln 2799.23410). Numbers above nodes indicate bootstrap values (100 pseudoreplicates). Haplotype letter assignments are labeled following Table 2.

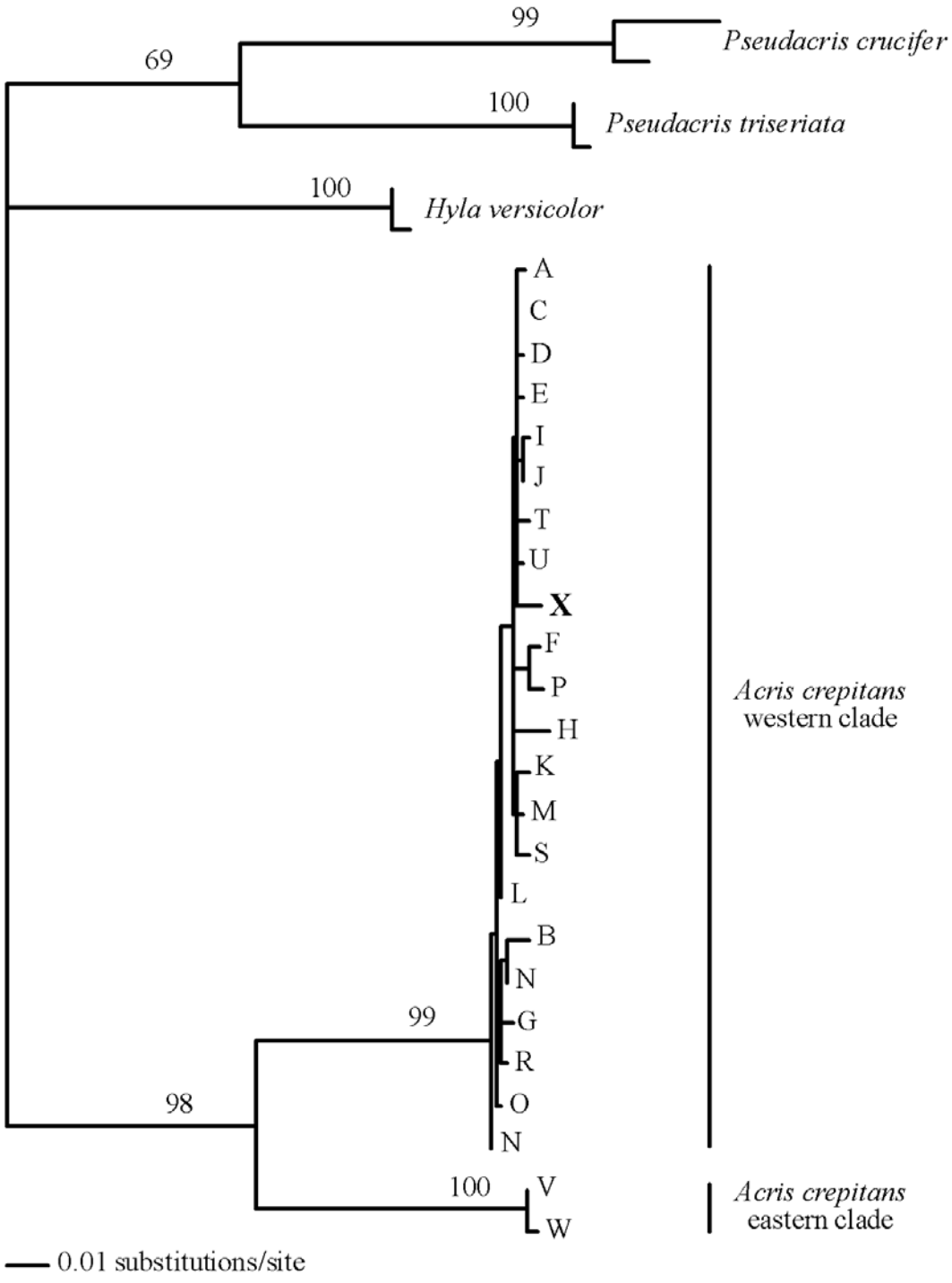


Figure 6. Statistical parsimony haplotype network. Haplotype letter assignments are labeled following Table 2. Filled circles indicate inferred haplotypes that were not present in the sample. Size of the circle is relative to the number of individuals for each haplotype. Each line represents a single mutational step connecting two haplotypes.

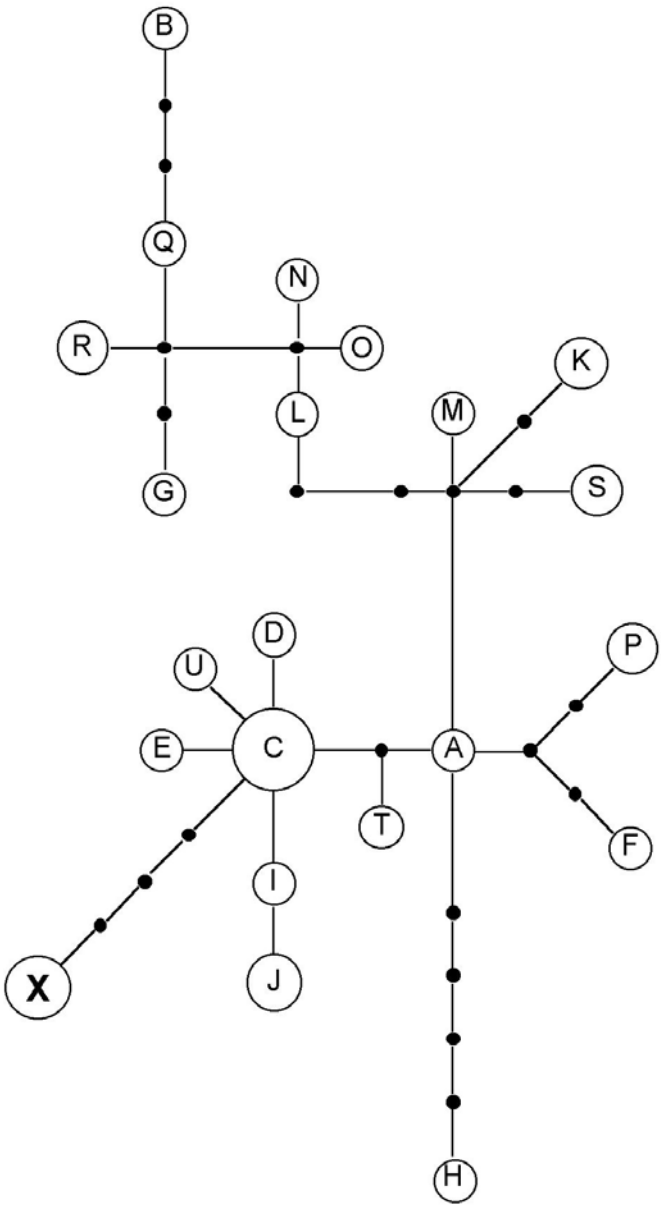


Figure 7. Statistical parsimony haplotype network with complete nesting design. Clade level designations are given within each box. Haplotype letter assignments are labeled following Table 2.

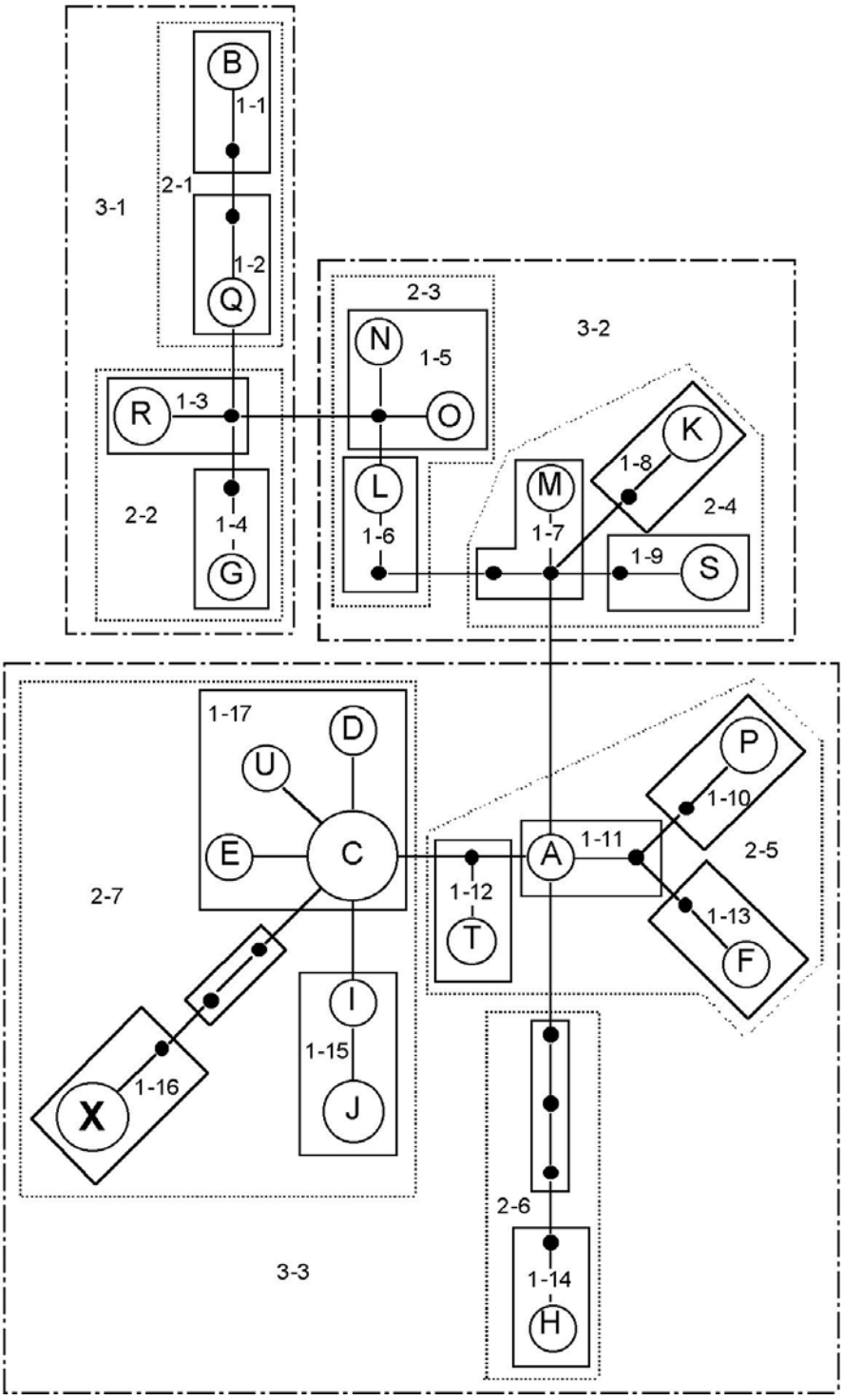


Figure 8. Statistical parsimony haplotype network, with nesting, overlaid onto map of cricket frog collection localities. For simplicity, only the most inclusive clade level (clades 3-1, 3-2, 3-3) and the statistically significant clade 2-7, surrounded by a dashed line, were included. Black dots identify approximate sampling localities and numbers within dots correspond to the locality ID from Table 1.

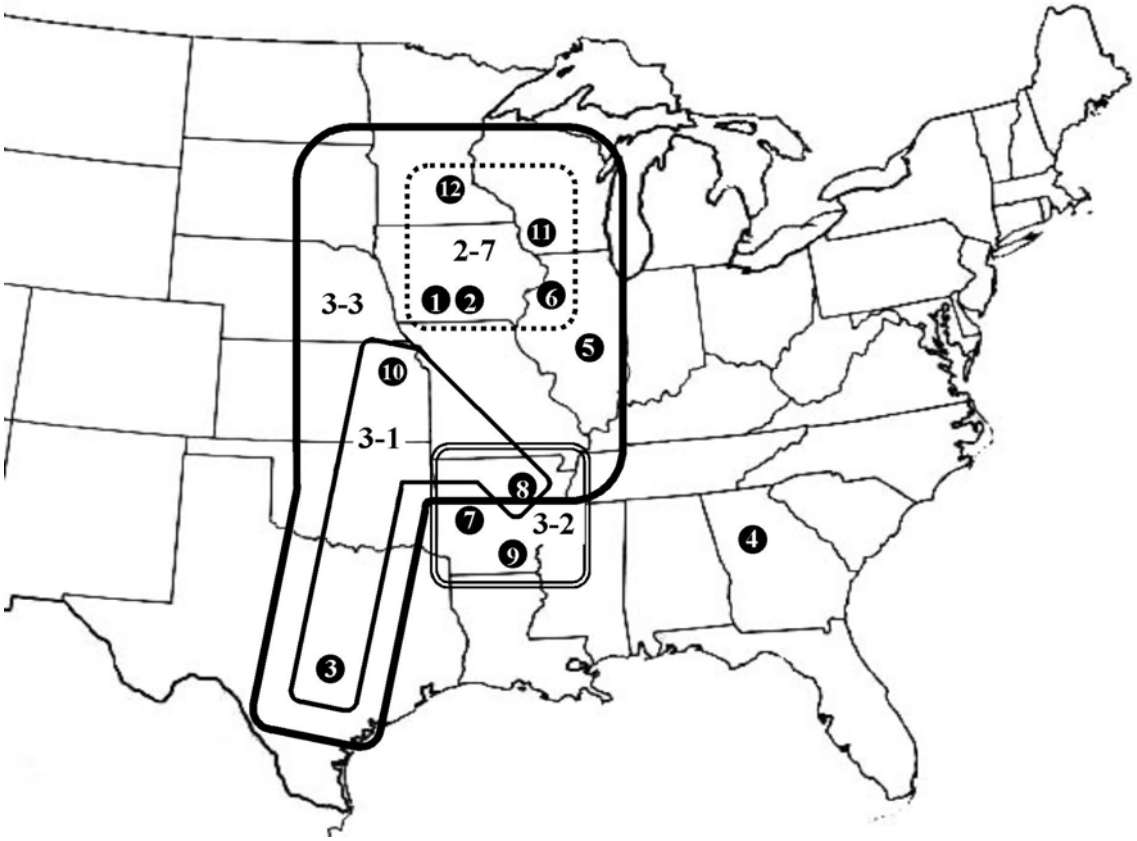
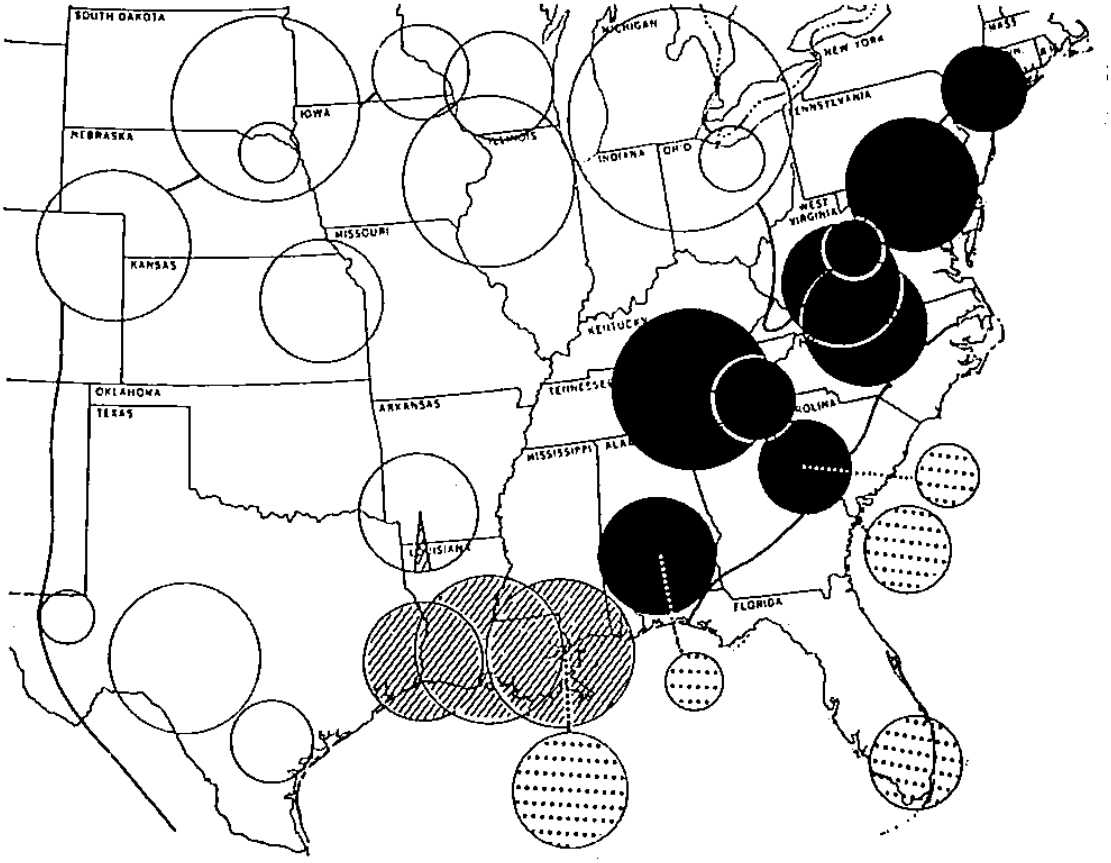
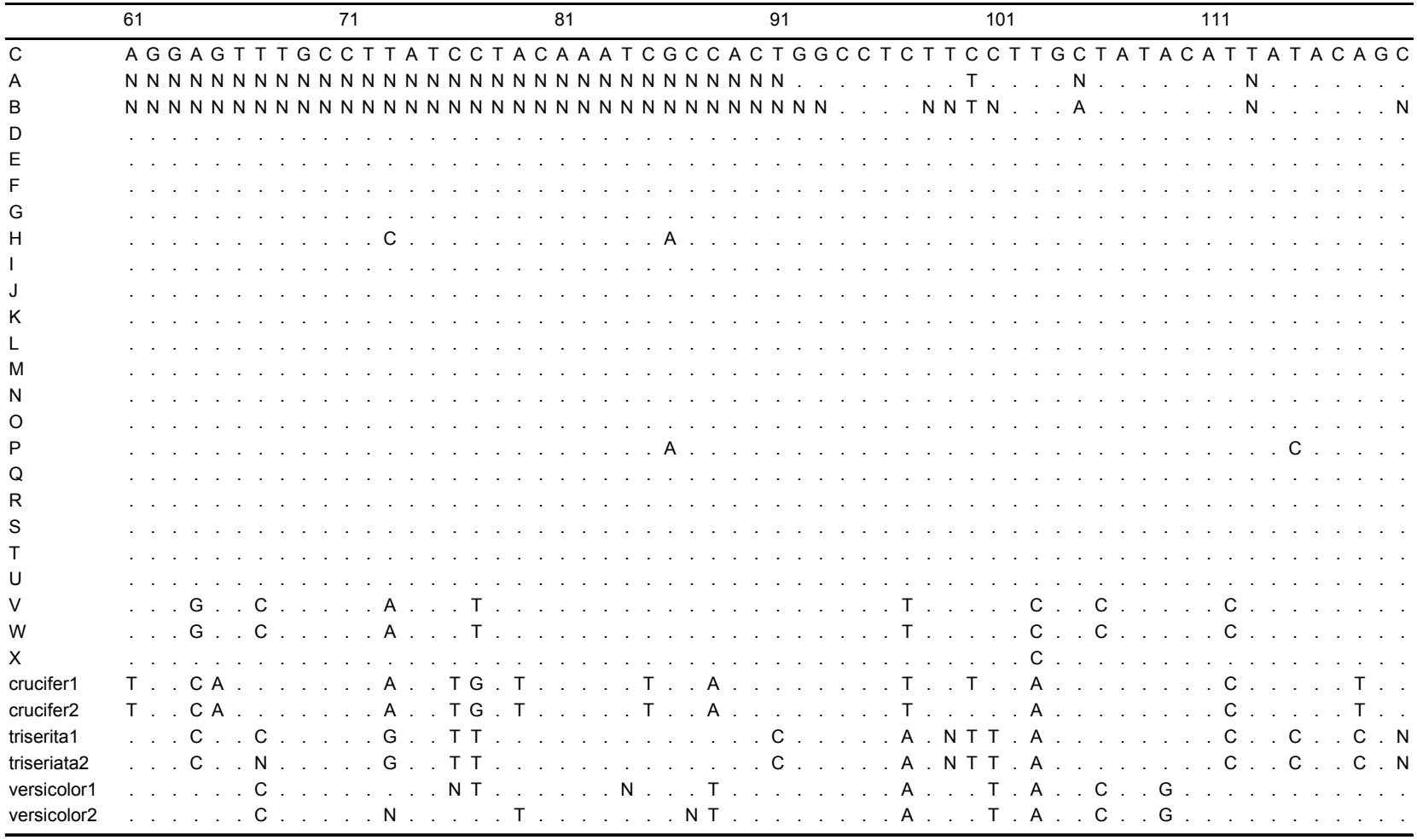


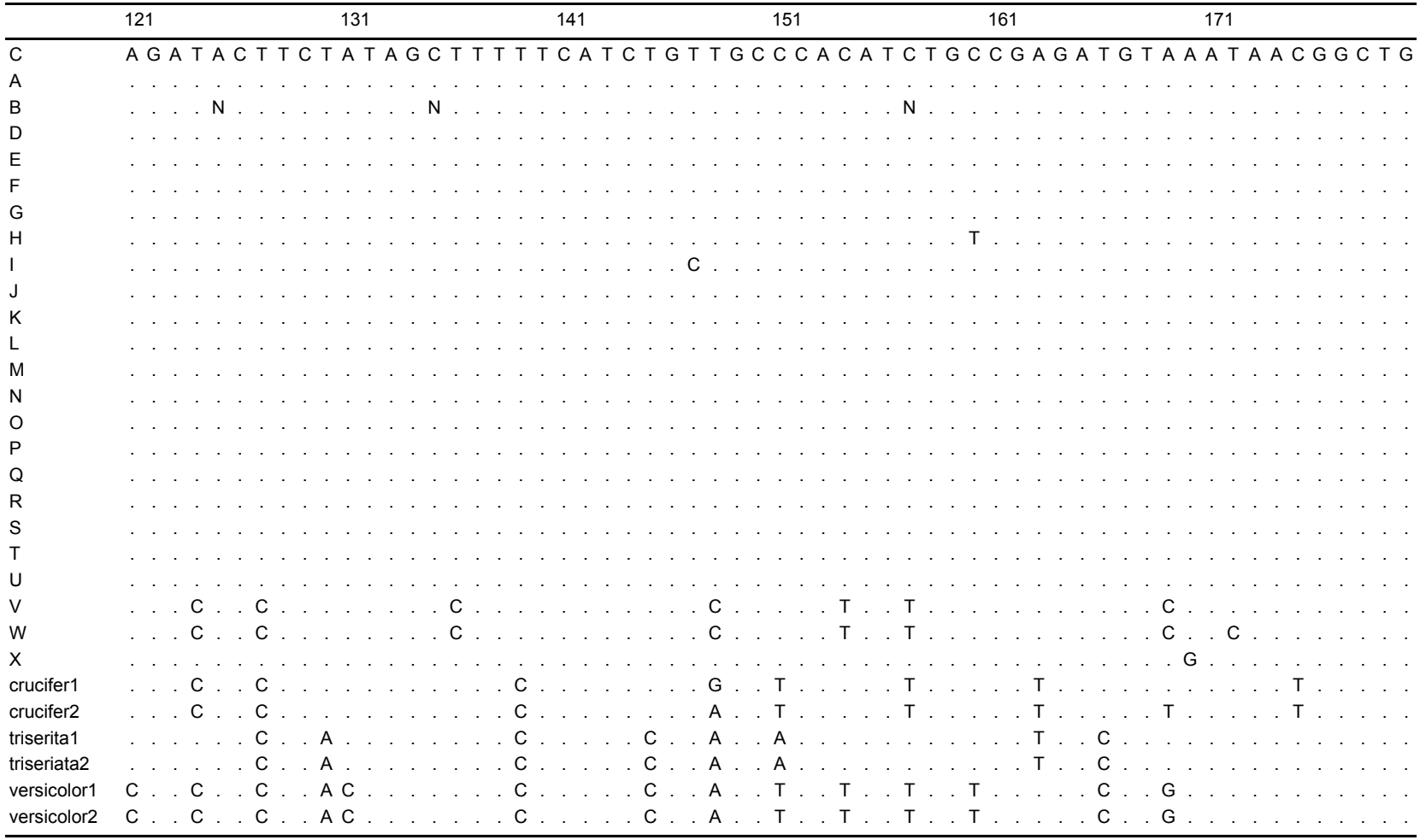
Figure 9. Geographic distribution of globin polypeptides among populations of *Acris*, from Dessauer and Nevo (1969). White circles indicate the presence of polypeptides 1, 2, 3, 4, and 5; black indicates the presence of 1s, 2s, and 3s variants; lines indicate the occurrence of variant 5s; dots indicate *A. gryllus*. Area of the circle is proportional to the sample size.

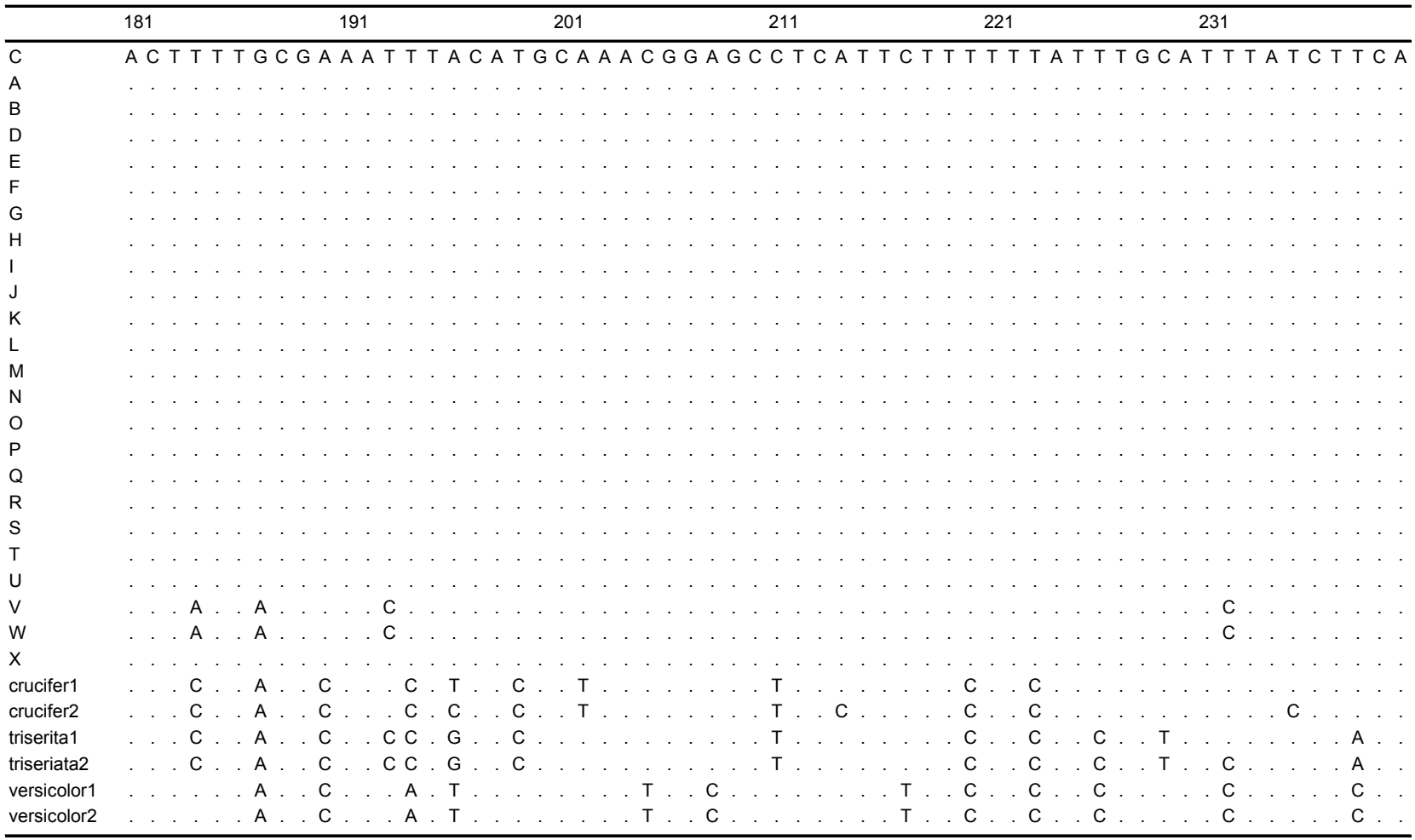


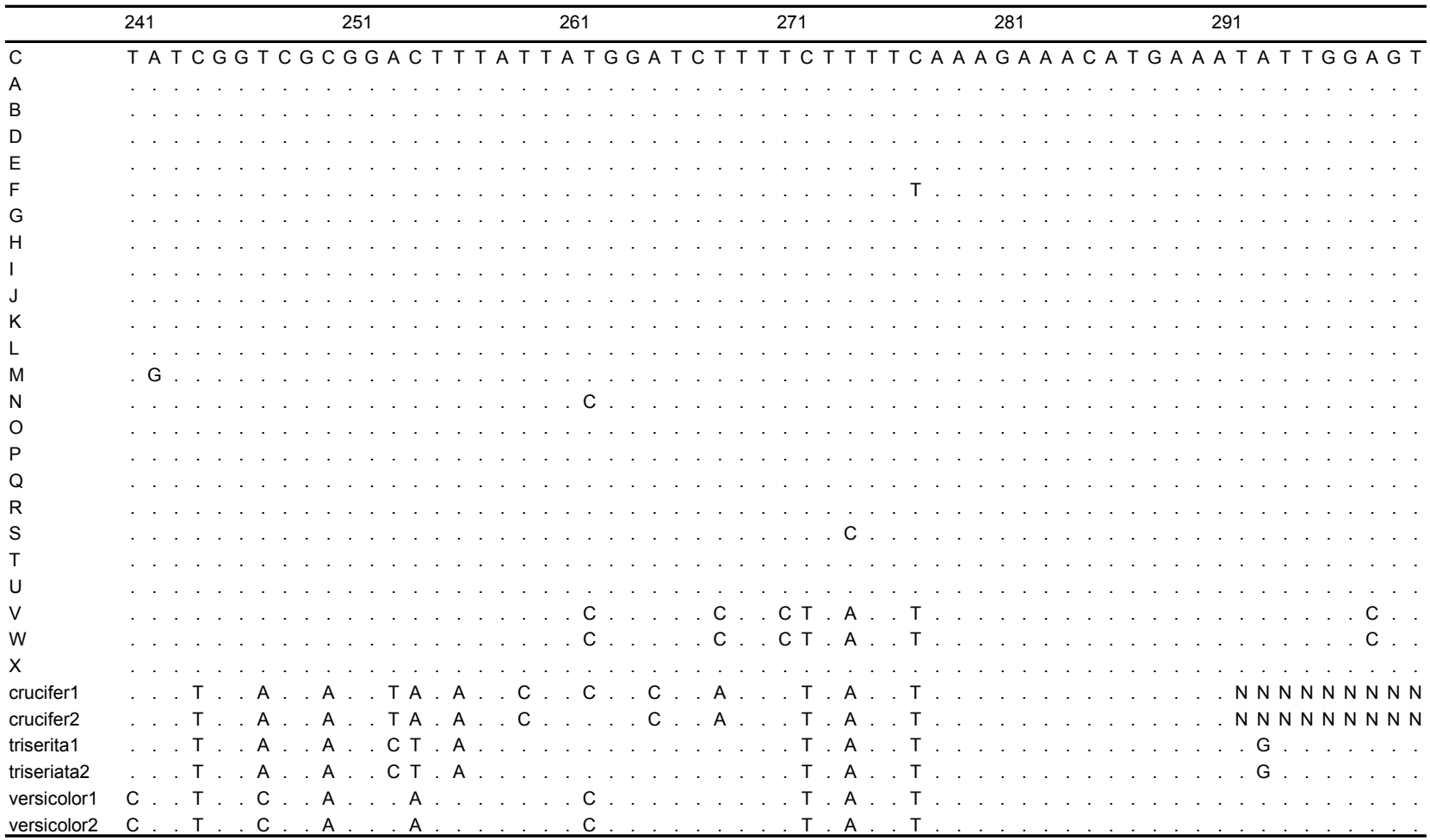
Appendix A. Complete data matrix of the 725 nucleotide fragment of the cytochrome *b* gene for all unique haplotypes. Haplotype letter assignments are labeled according to Table 2. A = adenine; G = guanine; T = thymine; C = cytosine; N = missing data.

	1	11	21	31	41	51
C	A T T T G T G G A C C T C C C A G C A C C A T C A A A C T T A T C C T C A T G A T G A A A C T A C G G C T C C C T T C T
A	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
B	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
D	. . . .
E	N N N N . . . . N . . . .
F	N N N N . . . . G . . . .
G	N N N N . . . .
H	. . . . . A . . . . T .
I	N N N N N N N N N N N N N N N N N N N N N . . . . . N . . . .
J	. . . .
K	. . . .
L	. . . . . T . . . .
M	. . . .
N	. . . . . T . . . .
O	. . . . . T . . . .
P	. . . . . N . . . .
Q	. . . .
R	. . . .
S	N N N N . N . . . .
T	. . . .
U	. . . .
V	. . . .
W	. . . .
X	N N N N N N N N N . . N . . . .
crucifer1	N . . . A . T . . . . . A . C . . . . . C . G . . A . . . . . G . . . . . T . T T . . . .
crucifer2	N . . . A . T . . T . . . . . C . . . . . G . . . C . G . . A . . . . . G . . . . . T . T T . . . .
triserita1	N N N N N N N N N N N N N N N N N . . . . . C . . . C . C . . A . . . . T . . . . C T .
triseriata2	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N . . A . . . . N . . . . T . . . . C T .
versicolor1	N N N C A . T . . . . G . . . . N T . . T . . . . N G . . G . . . . G . . A . . . T .
versicolor2	N . . C A . T . C . . . G . . . . T . . T . N . . . G . N G . . . . G . . A . . . T .

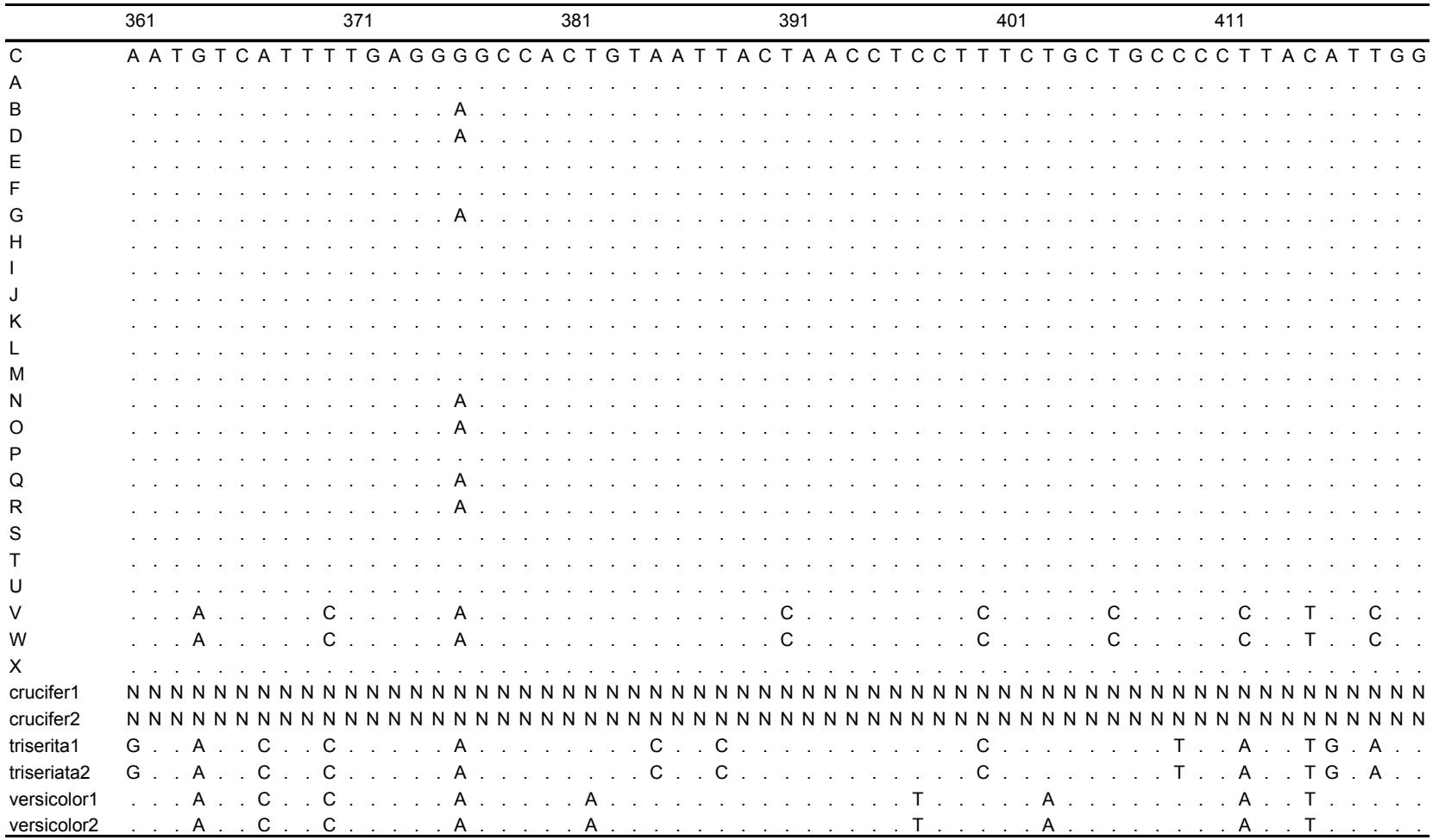

61
71
81
91
101
111
C A G G A G T T T G C C T T A T C C T A C A A A T C G C C A C T G G C C T C T T C C T T G C T A T A C A T T A T A C A G C
A N N N N N N N N N N N N N N N N N N N N N N N N N N N N . . . . . T . . . N . . . . . N . . . . . .
B N N N N N N N N N N N N N N N N N N N N N N N N N N N N N . . . N N T N . . A . . . . . N . . . . . N
D . . . . . .
E . . . . . .
F . . . . . .
G . . . . . .
H . . . . . C . . . . . A . . . . . .
I . . . . . .
J . . . . . .
K . . . . . .
L . . . . . .
M . . . . . .
N . . . . . .
O . . . . . .
P . . . . . A . . . . . C . . . . .
Q . . . . . .
R . . . . . .
S . . . . . .
T . . . . . .
U . . . . . .
V . . . G . . C . . . . A . . . T . . . . . T . . . . . C . . C . . . . . C . . . . . .
W . . . G . . C . . . . A . . . T . . . . . T . . . . . C . . C . . . . . C . . . . . .
X . . . . . C . . . . . .
crucifer1 T . . C A . . . . . A . . T G . T . . . . . T . . A . . . . . T . . T . . A . . . . . C . . . . . T . .
crucifer2 T . . C A . . . . . A . . T G . T . . . . . T . . A . . . . . T . . . . . A . . . . . C . . . . . T . .
triserita1 . . . C . . C . . . . . G . . T T . . . . . C . . . . . A . N T T . A . . . . . C . . C . . C . N
triseriata2 . . . C . . N . . . . . G . . T T . . . . . C . . . . . A . N T T . A . . . . . C . . C . . C . N
versicolor1 . . . . . C . . . . . N T . . . . . N . . . T . . . . . A . . . T . A . . C . . G . . . . . .
versicolor2 . . . . . C . . . . . N . . . . . T . . . . . N T . . . . . A . . . T . A . . C . . G . . . . . .


121 131 141 151 161 171
C A G A T A C T T C T A T A G C T T T T T T C A T C T G T T G C C C A C A T C T G C C G A G A T G T A A A T A A C G G C T G
A
B N N N
D
E
F
G
H T
I C
J
K
L
M
N
O
P
Q
R
S
T
U
V C C C T T C C
W C C C T T C C C
X G
crucifer1 C C C G T T T T
crucifer2 C C C A T T T T T
triserita1 C A C C A A T C
triseriata2 C A C C A A T C
versicolor1 C C C A C C C A T T T T C G
versicolor2 C C C A C C C A T T T T C G


181
191
201
211
221
231
C A C T T T T G C G A A A T T T A C A T G C A A A C G G A G C C T C A T T C T T T T T T A T T T G C A T T T A T C T T C A
A
B
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V A A C C
W A A C C
X
crucifer1 C A C C T C T C T C
crucifer2 C A C C C C T C T C C C C
triserita1 C A C C C G C T C C C T
triseriata2 C A C C C G C T C C C T C A
versicolor1 A C A T T C T C C C C A
versicolor2 A C A T T C T C C C C A


241 251 261 271 281 291
C T A T C G G T C G C G G A C T T T A T T A T G G A T C T T T T C T T T T C A A A G A A A C A T G A A A T A T T G G A G T
A
B
D
E
F T
G
H
I
J
K
L
M G
N C
O
P
Q
R
S C
T
U
V C C C T A T C
W C C C T A T C
X
crucifer1 T A A T A A C C C A T A T N N N N N N N N N
crucifer2 T A A T A A C C A T A T N N N N N N N N N
triserita1 T A A C T A T A T G
triseriata2 T A A C T A T A T G
versicolor1 C T C A A C T A T
versicolor2 C T C A A C T A T

	301	311	321	331	341	351
C	C A T T C T T C T C	T T C C T G G T G A	T A G C C A C C G C	A T T T G T T G G C	T A C G T C C T C C	C T T G A G G C C A
A	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
B	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
D	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
E	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
F	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
G	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
H	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
I	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
J	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
K	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
L	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
M	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
N	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
O	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
P	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
Q	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
R	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
S	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
T	. . . .	. . . .	. . . . T . .	. . . .	. . . .	. . . .
U	. . . .	. . . .	. . . .	. . . .	. . . .	. . . .
V	T G . C T . G . . .	. . T . . A . . A .	. . . .	. . . . T	. . T . . . . . A .	. . . . T . .
W	T G . C T . G . . .	. . T . . A . . A .	. . . .	. . . . T	. . T . . . . . A .	. . . . T . .
X	. . . .	. . . . . A	. . . .	. . . .	. . . .	. . . .
crucifer1	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N
crucifer2	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N	N N N N N N N N N N
triserita1	A . . C . . A T . A	. . T . . T . . T .	. . . . A . . A . .	C . . C . . C . . .	. . . . . . T . A .	. A . . . . . A . .
triseriata2	G . . C . . A T . A	. . T . . T . . T .	. . . . A . . A . .	C . . C . . C . . .	. . . . . . T . A .	. A . . . . . A . .
versicolor1	T . . . . T	. . . . . T . . T .	. . . . A . .	C . . . . T	. . T . . T	. A . . . . . T . .
versicolor2	T . . . . T	. . . . . T . . T .	. . . . A . .	C . . . . T	. . T . . T	. A . . . . . T . .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A
B
D
E
F
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H
I
J
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L
M
N
O
P
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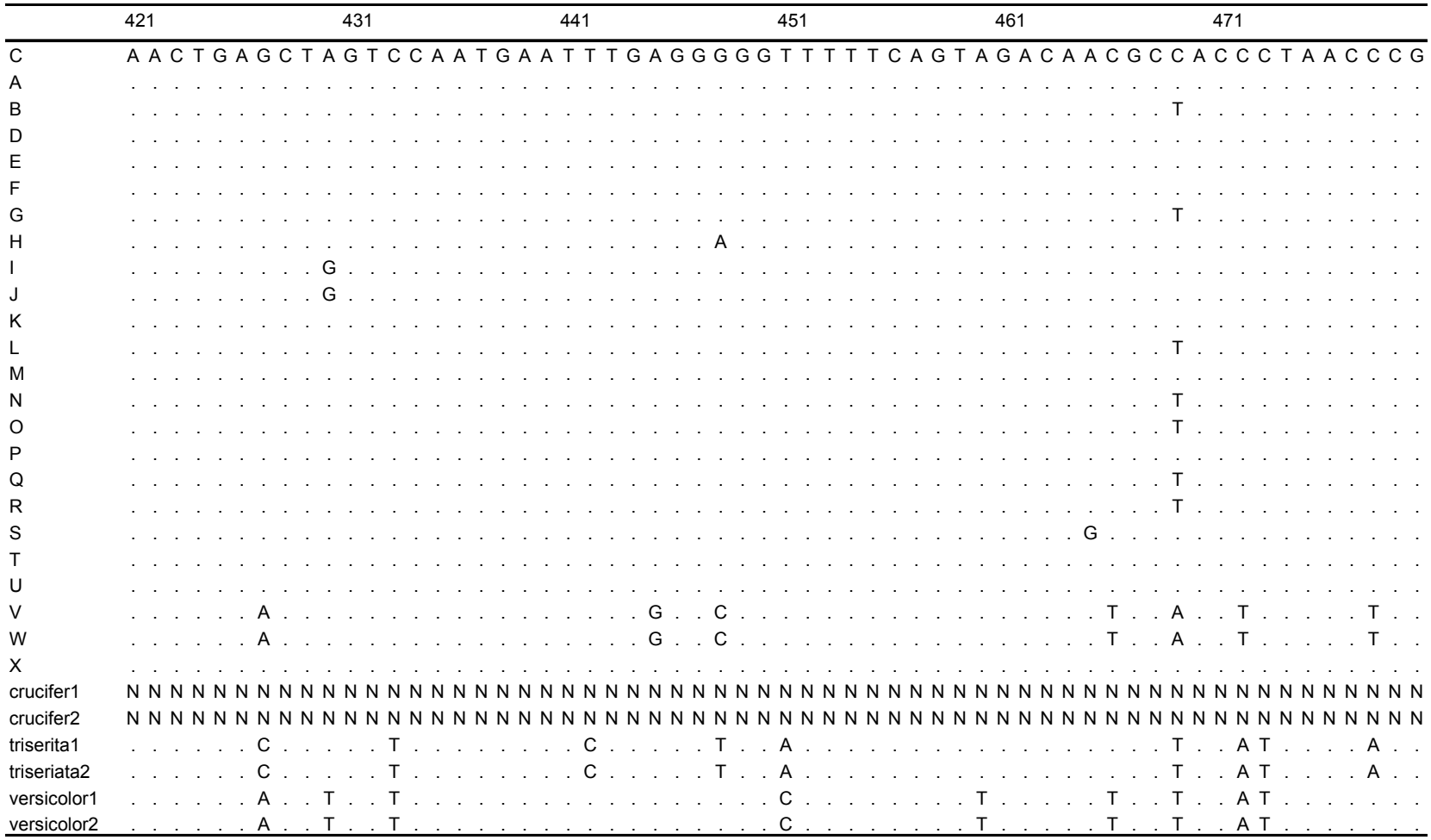
U
V
W
X
crucifer1
crucifer2
triserita1
triseriata2
versicolor1
versicolor2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C A A C T G A G C T A G T C C A A T G A A T T T G A G G G G G T T T T T C A G T A G A C A A C G C C A C C C T A A C C C G
crucifer1 N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
crucifer2 N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
triserita1
triseriata2
versicolor1
versicolor2

	481										491										501										511										521										531									
C	G	T	T	T	T	T	C	A	C	A	T	T	T	C	A	C	T	T	C	A	T	C	T	T	A	C	C	T	T	T	T	A	T	T	A	T	C	G	C	A	G	G	A	G	C	A	T	C	A	A	T	A	A	T	T	C	A	T	C	T
A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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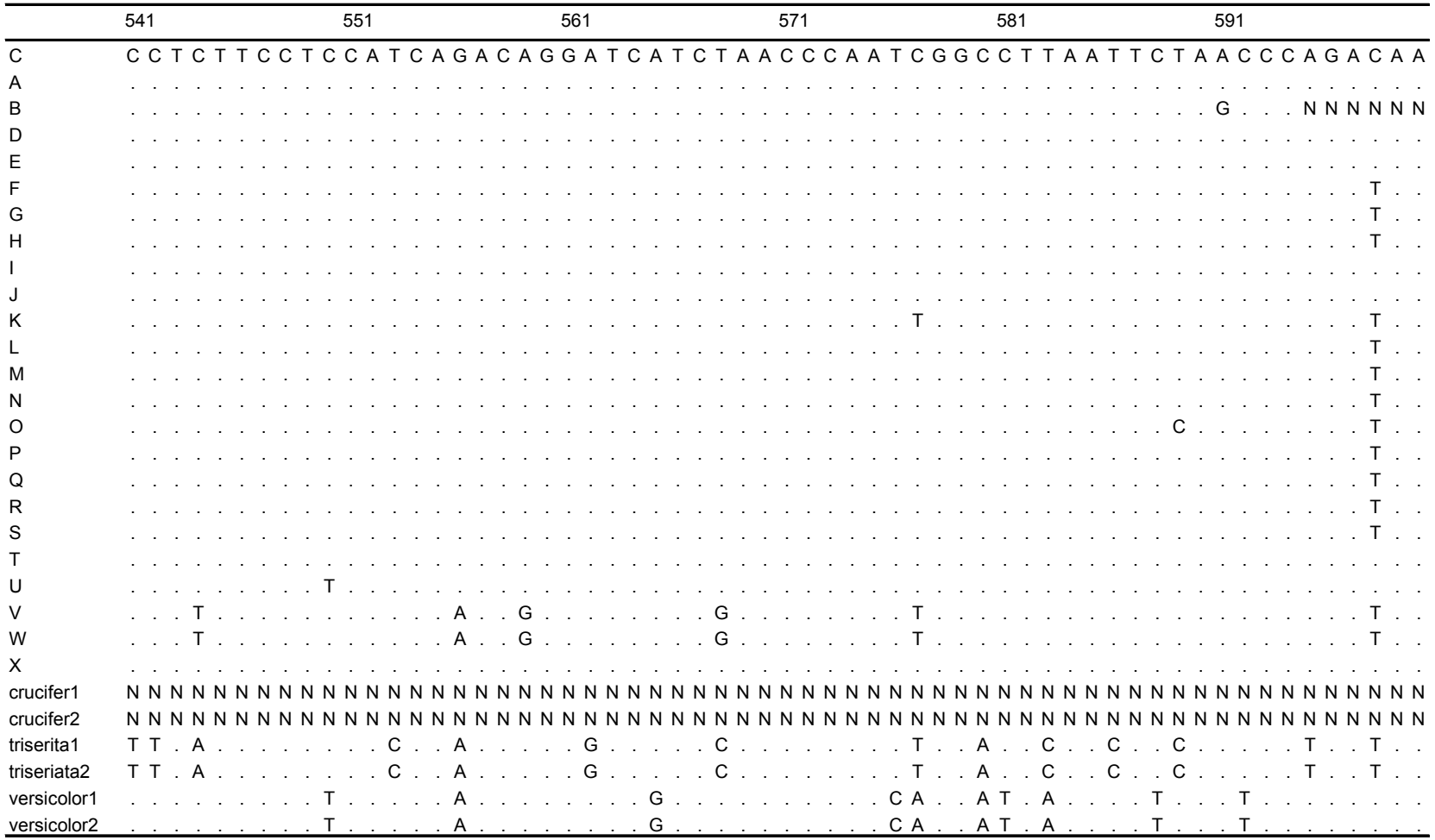
V	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
W	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.
X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
crucifer1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
crucifer2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
triserita1	A	.	.	C	.	.	T	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	C	.	C	.	.	A	.	.	.	.	.	.	.	.	T	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.
triseriata2	A	.	.	C	.	.	T	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	C	.	C	.	.	A	.	.	.	.	.	.	.	.	T	.	.	C	.	.	G	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.
versicolor1	A	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.
versicolor2	A	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.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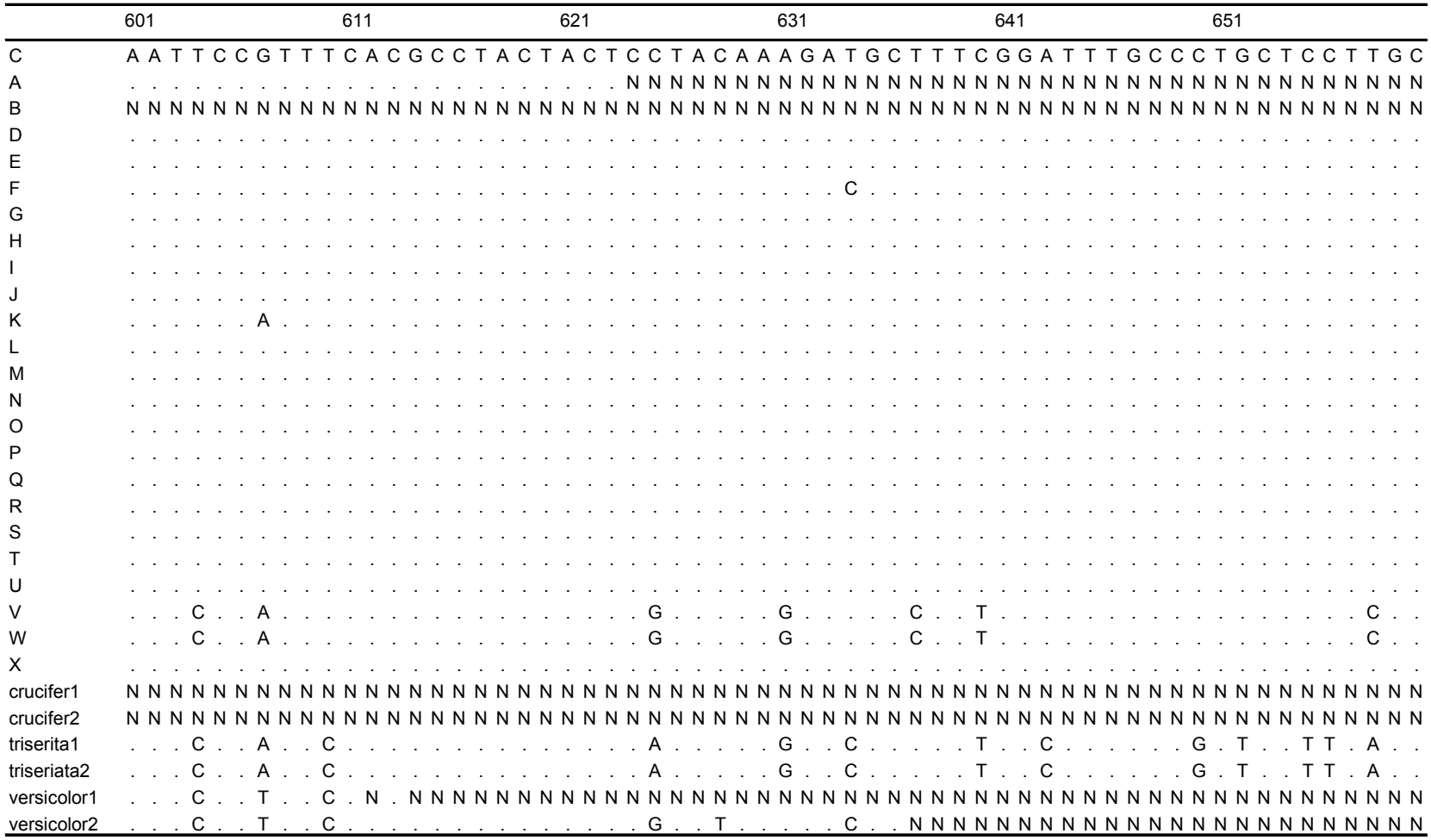
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A . . . . . N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
B N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
D . . . . . .
E . . . . . .
F . . . . . C . . . . . .
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H . . . . . .
I . . . . . .
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U . . . . . .
V . . . C . . A . . . . . G . . . . G . . . . . C . . T . . . . . C . .
W . . . C . . A . . . . . G . . . . G . . . . . C . . T . . . . . C . .
X . . . . . .
crucifer1 N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
crucifer2 N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
triserita1 . . . C . . A . . C . . . . . A . . . . . G . . C . . . . . T . . C . . . . . G . T . . T T . A . .
triseriata2 . . . C . . A . . C . . . . . A . . . . . G . . C . . . . . T . . C . . . . . G . T . . T T . A . .
versicolor1 . . . C . . T . . C . N . N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
versicolor2 . . . C . . T . . C . . . . . G . . T . . . . . C . . N N N N N N N N N N N N N N N N N N N N N N N N N N N N

	661 671 681 691 701 711 721
C	C C T T T T A G C T G C C C T C T C T A C A T T T G C C C C T A A C A T T C T A G G A N A C C C T G A C A A T T T T A C C C C N N
A	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
B	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
D	. . . . G . . . . N N N N N N
E	. . . . N N N N N N N N N N N N N N N N N N N N N N N N N
F	. . . . . . G . . . . N N . . . . N . . . . . G . . . . N N N N N N
G	. . . . . . G . . . . G . . . . . . N . N . . . . . . N N N N N N
H	. . . . G . . . . C G
I	. . . . G . . . . . . N N N N N N N N N N N N N N N
J	. . . . G . . . . C G
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M	. . . . T . . . . G . . . . C G
N	. . . . G . . . . C G
O	. . . . G . . . . C G
P	. . . . . . G . . . . G . . . . T . . . T G
Q	. . . . G . . . . C G
R	. . . . C . . . . G . . . . C G
S	. . . . N . N . . . . T . . . . G . . . . N N N N N
T	. . . . A . . . . N . . . . C G
U	. . . . G . . . . C G
V	T . . . . T . . . . . C . . . . T . . C . . . . . C . . . . . . G . T . . . . . T . . . . C G
W	T . . . . T . . . . N C . . . . T . . C . . . . . C . . . . . . G . T . . . . . T . . . . C G
X	. . . . N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
crucifer1	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
crucifer2	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
triserita1	T T . A . . . . . C . . . . . A C . C . . . . . C . . T . . G . . T . . . . N G . . . . A N . T . . . . . . N N N N N N N
triseriata2	T T . A . . . . . C . . . . . A G . C . . . . . C . . T . . G . . T . . . . T G . . . . A N . T . . . . T . . . C G
versicolor1	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
versicolor2	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N