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MOLECULAR SYSTEMATICS OF THE GENUS *PIMEPHALES* (TELEOSTEI: CYPRINIDAE)

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ABSTRACT—Mitochondrial DNA (mtDNA) restriction sites were surveyed among geographic samples representing the four extant species of the North American cyprinid genus *Pimephales*. Maximum-parsimony analysis of restriction-site data using unordered character states provided support for a sister-group relationship between *P. promelas* and the three remaining species. Maximum-parsimony analysis using Dollo criteria yielded a completely resolved tree with sister-group relationships between *P. notatus* and *P. promelas* and between *P. tenellus* and *P. vigilax*. Fitch-Margoliash analysis of a pairwise nucleotide-sequence-divergence matrix yielded the following distance relationships: (*P. promelas* (*P. notatus* (*P. tenellus, P. vigilax*))). The hypothesis of relationships based on Dollo parsimony is identical to one proposed on the basis of parsimony analysis of morphological characters. A sister-group relationship between *P. notatus* and *P. vigilax,* as suggested by analysis of chromosomal nucleolus organizer region (NOR) phenotypes, was not supported. Mitochondrial DNA divergence among geographic samples of *P. vigilax* corresponded to the two nominal subspecies: *P. v. vigilax* and *P. v. perspicuus*. A sample of *P. notatus* from Michigan was divergent in mtDNA sequence from samples of *P. notatus* from Kansas and Oklahoma; whereas a sample of *P. promelas* from Michigan was very similar in mtDNA sequence to a sample of *P. promelas* from Nebraska. Glacial isolation or indiscriminate introductions may explain this difference in geographic pattern of mtDNA divergence/similarity among populations of the two species.

The North American cyprinid genus *Pimephales* consists of four extant species distributed east of the Rocky Mountains (Lee et al., 1980). Three species, *P. notatus, P. promelas,* and *P. vigilax,* are widespread and found in many central North American drainages. The fourth, *P. tenellus,* is restricted to the Ozarkian and Ouachita highlands of Arkansas, Kansas, Missouri, and Oklahoma. Monophyly of the four species of *Pimephales* has been inferred from morphological, allozyme, and chromosomal characteristics (Mayden, 1987, pers. comm.; Li and Gold, 1991; Coburn and Cavender, 1992).

Hypotheses of relationships among species of *Pimephales* have been proposed by several authors. Hubbs and Black (1947) suggested that *P. tenellus* and *P. vigilax* were closely related. Mayden (1987), based on a cladistic analysis of morphological character variation, hypothesized that *P. tenellus* and *P. vigilax* were sister species, and that a *P. tenellus—P. vigilax* clade was sister to a clade comprised of *P. promelas* and *P. notatus*. Li and Gold (1991) examined trypsin G-band patterns of nucleolus organizer region (NOR) bearing chromosomes and hypothesized that *P. notatus* and *P. vigilax* shared a derived NOR chromosome character state, whereas *P. tenellus* and *P. promelas* possessed a primitive NOR state. Hubbs and Black (1947) separated *P. vigilax* into two species: *P. vigilax,* occurring from the Trinity River in east Texas westward into New Mexico; and *P. perspicuus,* occurring north and east of Texas. Hubbs (1951) later incidentally synonymized the two into a single species which Cross (1953) formally arranged into two subspecies (*P. v. vigilax* and *P. v. perspicuus*).

In this paper, we present data on restriction-enzyme site variation in the mitochondrial DNA (mtDNA) of the four species of *Pimephales,* including both recognized subspecies of *P. vigilax*. Our purpose was to infer a molecular hypothesis of phylogenetic relationships among the species of *Pimephales*.

MATERIALS AND METHODS—Specimens of *Pimephales* were obtained by seine from natural populations.

Collection localities, number of individuals, and catalogue numbers for voucher specimens are given in Appendix 1. Specimens of *Opsopoeodus emiliae* (Appendix 1) were obtained for use as an outgroup in phylogenetic inference. The use of *O. emiliae* as an outgroup was based on morphological, allozyme, and chromosomal evidence (Cavender and Coburn, 1986; Li and Gold, 1991; Coburn and Cavender, 1992; Mayden, pers. comm.) that suggest monotypic *Opsopoeodus* is sister to *Pimephales*.

DNA preparation, restriction-enzyme digestion, transfer hybridization, and autoradiography primarily followed methods in Gold and Richardson (1991). Several assays were carried out using end-labelling of gradient-purified mtDNA as described in Dowling et al. (1990). Digestions of mtDNA were performed with the following restriction enzymes: *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Nhe*I, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Xba*I, and *Xho*I. The hybridization probe was an intact mtDNA molecule from *Cyprinella lutrensis* cloned into bacteriophage lambda (Richardson and Gold, 1991). Mapping of individual restriction sites was carried out using double digestions (Dowling et al., 1990). Additional mapping information was acquired from digestions of polymerase chain reaction (PCR) amplified products in a manner similar to that described by Schmidt and Gold (1992).

A restriction-site presence-absence (binary) matrix was generated for sample localities. Restriction sites were coded as present if they occurred in any specimen from a given sample locality. Because restriction-site losses are more subject to convergence than are restriction-site gains (Debry and Slade, 1985; Swofford and Olsen, 1990), this coding scheme reduces the effect of convergent restriction site losses (Dowling and Brown, 1989). Maximum-parsimony analysis of the binary matrix employed version 3.1.1 of the Phylogenetic Analysis Using Parsimony (PAUP) package of Swofford (1993). Initial searches were carried out using unordered character-state transformations. Subsequent searches employed Dollo character coding (Farris, 1977) to further mitigate effects of convergent restriction site losses (Debry and Slade, 1985; Swofford and Olsen, 1990). Bootstrap replications (500) were performed on both analyses. Estimates of nucleotide sequence divergence among pairwise combinations of samples were generated from restriction sites (Nei and Li, 1979) using equations in Nei and Tajima (1981). A distance tree was generated from the resulting pairwise-sample distance matrix using the Fitch-Margoliash (FITCH) procedure in version 3.4 of the Phylogeny Inference Package (PHYLIP) of Felsenstein (1991).

RESULTS—A total of 103 unique restriction sites were assayed among 35 individuals and 12 sample localities (Appendix 2). No obvious variation in mtDNA genome size was observed and total size of mtDNA genomes in all individuals assayed was estimated to be 16.8 ± 0.2 *SE* kilobases.

Maximum-parsimony analysis of unordered character states resulted in five equally-parsimonious trees (CI = 0.65, RI = 0.74) of 129 steps. Strongest support (Fig. 1A) was indicated for monophyly of geographic samples in each of three species: *P. promelas, P. tenellus,* and *P. vigilax*. Monophyly of the three geographic samples of *P. notatus* was not supported in ≥50% of bootstrap replicates. Monophyly of samples of *P. notatus* from Kansas and Oklahoma was supported (96% of bootstrap replicates), whereas the sample of *P. notatus* from Michigan formed part of a polytomy that included the other samples of *P. notatus, P. tenellus,* and *P. vigilax*. Within *P. vigilax,* bootstrapping strongly supported a sister group relationship between a clade comprising samples from Kansas and Oklahoma (*P. v. perspicuus*) and the sample from Texas (*P. v. vigilax*). Weaker support indicated *P. promelas* to be sister to a clade of the three remaining species. These three species (*P. notatus, P. tenellus,* and *P. vigilax*) formed an unresolved trichotomy.

Increased resolution was obtained using Dollo character coding where a single, fully-resolved tree of 146 steps was found (Fig. 1B). Monophyly of all samples of *P. notatus* was supported in 68% of bootstrap replicates, where the sample of *P. notatus* from Michigan was sister to a clade of the samples of *P. notatus* from Kansas and Oklahoma. Sister group relationships between *P. notatus* and *P. promelas* and between *P. tenellus* and *P. vigilax* were supported in ≥50% of bootstrap replicates. Inferred relationships among samples of *P. vigilax* were the same as when using unordered character states.

The topology of the distance tree produced by the Fitch-Margoliash method (Fig. 2) had similarities with both trees produced by maximum parsimony: (1) samples from each of the four species of *Pimephales* grouped together; (2) within *P. notatus,* the sample from Michigan was the most divergent; (3) within *P. vigilax,* samples from Kansas and Oklahoma (*P. v. perspicuus*) were more similar to one another than either was to the sample from Texas (*P. v. vigilax*); and (4) *P. vigilax* grouped with *P. tenellus*. Levels of similarity among all nominal *Pimephales* suggested by Fitch-Margoliash analysis were: (*P. promelas* (*P. notatus* ((*P. v. perspicuus, P. v. vigilax*) *P. tenellus*))). Lengths of branches defining the group-

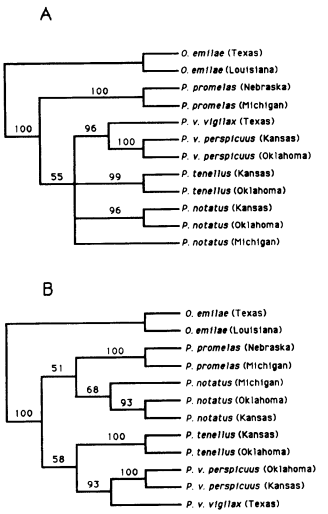

FIG. 1—(A) Strict-consensus tree based on maximum-parsimony analysis of 103 restriction sites using unordered character-state transformations. (B) Most parsimonious tree based on maximum-parsimony analysis of 103 restriction sites using the Dollo parsimony model. Numbers above internodes represent percent of 500 bootstrap replicates in which a node was supported; branch lengths are not representative of evolutionary distances.

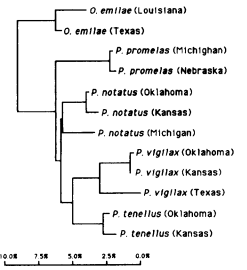

FIG. 2—Distance tree generated using the Fitch-Margoliash method. Branch lengths are proportional to estimates of nucleotide sequence divergence.

ing of all three samples of *P. notatus* and the grouping of *P. notatus, P. vigilax,* and *P. tenellus* were short in relation to other branches.

The average nucleotide sequence difference between the sample of *P. notatus* from Michigan and those from Kansas and Oklahoma was more than four-fold greater than that between the samples of *P. notatus* from Kansas and Oklahoma (4.8% vs. 1.1%). This difference is similar to that observed between the two subspecies of *P. vigilax* (5.31%). In contrast, the sample of *P. promelas* from Michigan differed in nucleotide sequence from the sample of *P. promelas* from Nebraska by only 0.6%. Except for the previously noted cases, nucleotide sequence divergence was much larger in comparisons between samples. Individuals from the same sample differed by an average of only 0.4%.

DISCUSSION—Debry and Slade (1985) and Swofford and Olsen (1990) have pointed out that it is more likely for a restriction-site character to be lost, rather than gained, at any particular restriction-site location, and that the Dollo parsimony model may be more appropriate than other models for restriction-site data. In the *Pimephales* data set, the shortest tree derived from maximum-parsimony analysis using the Dollo criterion, ((*P. promelas, P. notatus*) (*P. tenellus, P. vigilax*)), was identical to the one proposed by Mayden (1987) based on parsimony analysis of morphological characters. The congruence of trees derived from mtDNA restriction-site data (using Dollo parsimony) and from morphological data provide support for this phylogenetic hypothesis of relationships among species of *Pimephales.*

The alternative hypothesis (*P. promelas* (*P. notatus* (*P. tenellus, P. vigilax*))), however, cannot be unequivocally disregarded. Bootstrap resampling (using the Dollo criterion) of the mtDNA restriction-site data provided only 51% support for the clade comprised of *P. promelas* and *P. notatus,* and the alternative hypothesis was only a single step longer than the most parsimonious hypothesis. The alternative hypothesis also was consistent with Fitch-Margoliash analysis of the nucleotide sequence divergence matrix. Finally, in the study of morphological variation by Mayden (1987), only a single synapomorphy (shape of the pharyngeal pad of the basioccipital) supported the clade of *P. promelas* and *P. notatus,* whereas three independent characters supported the clade of *P. tenellus* and *P. vigilax.* Modification of the pharyngeal pad of the basioccipital shows extensive homoplasy in other cyprinids (Mayden, 1989; Coburn and Cavender, 1992; Schmidt, 1994).

A sister-group relationship between *P. notatus* and *P. vigilax,* as hypothesized by Li and Gold (1991) on the basis of chromosomal nucleolus organizer region (NOR) phenotypes, was not supported by either of the phylogenetic hypotheses inferred from mtDNA restriction-site data. The chromosomal hypothesis, however, was con-

strained by the possibility that one of the chromosomal NORs represented an ancestral polymorphism rather than a "fixed" NOR character state (Li and Gold, 1991). Assuming that *P. tenellus* and *P. vigilax* are sister species, as inferred from both morphological and mtDNA restriction-site data (Mayden, 1987; this paper), the *C'* NOR in *P. notatus* and *P. vigilax* is best interpreted as a phylogenetically uninformative ancestral polymorphism.

Geographic patterns of mtDNA divergence differed within species of *Pimephales*. In *P. vigilax*, differences among geographic samples corresponded to the nominal subspecies *P. v. vigilax* and *P. v. perspicuus*. The extent of mtDNA nucleotide-sequence divergence (5.31%) between *P. v. vigilax* and *P. v. perspicuus* approximates levels of mtDNA divergence observed between several, closely-related cyprinid species (Dowling et al., 1992; Dowling, unpubl.; Richardson and Gold, unpubl.) and suggests that further study of morphological and ecological variation in *P. vigilax* may be warranted.

Within *P. notatus*, the sample from Michigan differed substantially in mtDNA sequence from samples of *P. notatus* from Kansas and Oklahoma; whereas samples of *P. promelas* from Michigan and Nebraska differed in mtDNA sequence by less than the difference between the samples of *P. notatus* from Kansas and Oklahoma. The extent of mtDNA nucleotide-sequence divergence (4.8%) between Michigan and Kansas-Oklahoma samples of *P. notatus* implies long-term separation of mtDNA lineages, and is compatible with the hypothesis that these mtDNA lineages may have been isolated in separate glacial refugia. The mtDNA sequence similarity between samples of *P. promelas* from Michigan and Nebraska indicates that separation of mtDNA lineages is recent and is compatible with the possibility that one or both samples could be the result of incidental introductions from the same source population. This possibility is not unlikely, as *P. promelas* is easily transported and is a popular and commonly-marketed bait fish in the central United States. Alternatively, the mtDNA lineages in *P. promelas* could be the result of widespread dispersal following the most recent Pleistocene glaciations. One last point to note is that the extent of mtDNA nucleotide sequence divergence between or among samples of *P. notatus* and *P. vigilax* suggests that analysis of mtDNA among geographic samples of these two species may be useful in testing hypotheses of biogeography throughout the central United States.

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APPENDIX 1

Collection localities, drainages (in parentheses), number of individuals examined in the study, and catalogue numbers of voucher specimens. Voucher specimens are housed in either the Texas Cooperative Wildlife Collections (TCWC) at Texas A&M University or the collections at Arizona State University (ASU).

P. notatus—Middle Creek (Missouri R.), Franklin Co., Kansas, 3, TCWC 7244.01; Saline River (Raisin R.), Washtenaw Co., Michigan, 5, ASU 12792; Salt Creek (Arkansas R.), Osage Co., Oklahoma, 2; *P. promelas*—South Platte River (Platte R.), Kieth Co., Nebraska, 3, TCWC 7241.01; unnamed tributary (Rouge R.), Oakland Co., Michigan, 3; *P. tenellus*—Neosho River (Neosho R.), Lyon Co., Kansas, 3, TCWC 7245.01; Salt Creek (Arkansas R.), Osage Co., Oklahoma, 2; *P. v. vigilax*—Brazos River (Brazos R.), Brazos Co., Texas, 3, TCWC 7242.01; *P. v. perspicuus*—Kansas River (Kansas R.), Douglas Co., Kansas, 3, TCWC 7243.01; Salt Creek (Arkansas R.), Osage Co., Oklahoma, 2; and *O. emiliae*—Navasota River (Brazos R.), Brazos Co., Texas, 3, 7246.01; Hog Creek Branch (L. Pontchatrain), Livingston Par., Louisiana, 3, TCWC 7247.01.

APPENDIX 2

Matrix of restriction sites by sample locality. 1 = presence of a restriction site; 0 = absence of a restriction site. A *Sac*I site was arbitrarily assigned to map location 0.0. Acronyms for sampling localities are as follows: ETX (*Opsopeodus emilae,* Texas); ELA (*O. emilae,* Louisiana); PNE (*Pimephales promelas,* Nebraska); PMI (*P. promelas,* Michigan); VTX (*P. v. vigilax,* Texas); VKS (*P. v. perspicuus,* Kansas); VOK (*P. v. perspicuus,* Oklahoma); TKS (*P. tenellus,* Kansas); TOK (*P. tenellus,* Oklahoma); NKS (*P. notatus,* Kansas); NOK (*P. notatus,* Oklahoma); NMI (*P. notatus,* Michigan).

Restriction enzyme	Map location	Locality											
		ETX	ELA	PNE	PMI	VTX	VKS	VOK	TKS	TOK	NKS	NOK	NMI
*Sac*I	0.00	0	0	0	0	1	1	1	1	1	1	1	1
*Bgl*II	0.09	1	1	1	1	1	1	1	1	1	1	1	1
*Nhe*I	0.33	1	1	1	1	1	1	1	1	1	1	1	1
*Xba*I	0.80	1	1	1	1	1	1	1	1	1	1	1	1
*Sac*II	1.12	1	1	1	1	1	1	1	1	1	1	1	1
*Eco*RI	1.21	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*dIII	1.32	0	0	0	0	1	1	1	0	0	0	0	0
*Nhe*I	1.44	0	0	1	1	1	1	1	1	1	1	1	1
*Eco*RI	1.52	0	1	0	0	0	0	0	0	0	0	0	0
*Nhe*I	1.66	1	0	0	0	0	0	0	0	0	0	0	0
*Bcl*I	1.78	0	0	0	0	0	0	0	0	0	1	1	0
*Xba*I	2.00	0	0	1	1	1	1	1	0	1	1	1	0
*Xba*I	2.35	0	0	0	0	0	0	0	0	0	1	0	0
*Eco*RI	2.56	0	0	1	1	0	0	0	0	0	0	0	0
*Hin*dIII	2.79	1	1	0	0	0	0	0	0	0	0	0	0
*Pst*I	2.80	0	0	1	1	0	0	0	0	0	0	0	0
*Nhe*I	3.09	1	1	1	1	0	0	0	1	1	0	0	1
*Bgl*II	3.13	0	0	1	1	0	0	0	0	0	0	0	0
*Eco*RV	3.17	0	0	1	1	0	1	1	1	0	0	0	0
*Eco*RV	3.50	1	1	0	0	1	1	1	1	1	1	1	1
*Bam*HI	3.50	1	0	1	1	1	1	1	1	1	0	0	1
*Bam*HI	3.86	0	0	0	0	0	1	1	0	0	0	0	0
*Pst*I	4.10	0	0	0	0	0	0	0	1	1	0	0	0
*Bcl*I	4.53	1	1	0	0	0	0	0	0	0	0	0	0
*Hin*dIII	4.53	0	0	0	0	0	0	0	0	0	0	0	1
*Nhe*I	4.60	0	0	1	1	0	0	0	1	1	1	1	0
*Eco*RV	4.60	0	0	0	0	1	0	0	0	0	0	0	0
*Bcl*I	4.69	0	0	1	0	0	0	0	0	0	0	0	0
*Pvu*II	5.03	0	0	0	0	0	1	1	0	0	0	0	0
*Xba*I	5.14	1	1	0	0	0	0	0	0	0	0	0	0
*Pst*I	5.15	1	0	1	1	1	1	1	0	0	1	1	1
*Xho*I	5.16	0	0	0	0	1	0	0	0	0	0	0	0
*Nhe*I	5.40	0	0	0	0	0	0	0	0	0	0	0	1
*Eco*RV	5.70	0	0	0	0	1	0	0	0	0	0	0	0
*Bcl*I	5.88	1	1	1	1	1	1	1	1	1	1	1	1
*Hin*dIII	6.08	0	0	0	0	1	1	1	0	0	0	0	0
*Xho*I	6.80	0	0	1	1	1	1	1	1	1	1	1	1
*Pvu*II	6.82	0	0	1	1	0	0	0	0	0	0	0	0
*Eco*RI	6.83	1	1	0	0	1	0	0	0	0	1	1	0
*Pst*I	6.85	0	0	0	0	1	1	1	1	1	1	1	1
*Bam*HI	7.00	0	0	1	1	0	0	0	0	0	1	1	1
*Bam*HI	7.20	1	1	1	1	0	1	1	0	0	0	0	0
*Bgl*II	7.29	0	1	0	0	1	0	0	0	0	0	0	0
*Hin*dIII	7.54	0	0	1	1	0	0	0	0	0	1	1	1
*Nhe*I	7.60	0	0	0	0	0	0	0	0	0	0	0	1

APPENDIX 2

Continued

Restriction enzyme	Map location	Locality											
		ETX	ELA	PNE	PMI	VTX	VKS	VOK	TKS	TOK	NKS	NOK	NMI
*Pvu*II	7.71	0	0	0	0	1	1	1	1	1	0	1	1
*Bcl*I	7.80	1	1	0	0	0	0	0	0	0	0	0	0
*Bgl*II	7.89	0	0	0	0	1	0	0	0	0	0	0	0
*Hin*dIII	7.99	1	1	1	1	1	1	1	0	0	1	1	1
*Nhe*I	8.11	1	0	1	1	0	0	0	0	0	0	0	0
*Bcl*I	8.31	0	0	0	0	1	1	1	0	0	0	0	0
*Sac*I	8.97	1	1	1	1	0	1	1	1	1	0	0	1
*Xho*I	9.19	0	0	0	0	1	0	0	0	0	0	0	0
*Xba*I	9.48	1	1	0	0	1	1	1	1	1	1	1	1
*Pst*I	9.75	1	1	1	1	1	1	1	1	1	1	1	1
*Pvu*II	9.79	0	0	1	1	1	0	0	0	0	1	1	0
*Pvu*II	9.96	0	0	0	0	1	0	0	1	1	0	0	0
*Eco*RI	10.04	1	1	1	1	0	0	0	0	0	0	0	1
*Hin*dIII	10.08	1	1	1	1	0	0	0	1	1	1	1	1
*Xba*I	10.60	1	1	0	0	0	0	0	1	1	1	1	1
*Bgl*II	10.70	0	0	1	1	1	1	1	1	1	1	1	1
*Xba*I	11.00	0	0	0	0	1	1	1	1	1	1	1	0
*Bgl*II	11.10	0	0	0	0	1	0	0	0	0	0	0	0
*Sac*I	11.96	0	0	0	0	0	0	0	0	0	1	1	1
*Pst*I	12.31	0	0	1	1	0	0	0	0	0	0	0	0
*Bgl*II	12.41	0	0	0	0	0	0	0	0	0	1	1	1
*Pst*I	12.47	0	0	0	0	1	1	1	0	0	0	0	0
*Nhe*I	12.50	0	0	0	0	1	1	1	0	0	1	1	0
*Bcl*I	12.50	1	1	0	0	1	0	0	0	0	0	0	0
*Hin*dIII	12.50	1	1	1	1	1	1	1	1	1	1	1	1
*Bgl*II	12.65	0	0	1	1	0	0	0	0	0	0	0	0
*Xba*I	13.00	1	1	1	1	1	1	1	1	1	1	1	1
*Sac*I	13.20	1	0	0	0	1	0	0	1	0	0	0	0
*Bcl*I	13.28	0	0	1	1	0	1	1	1	1	1	1	1
*Pst*I	13.61	1	0	1	1	1	1	1	1	1	1	1	1
*Pvu*II	13.73	0	0	1	1	0	0	0	0	0	0	0	0
*Hin*dIII	13.80	0	0	1	1	0	0	0	1	1	1	1	1
*Bgl*II	13.89	0	0	0	0	1	1	1	0	0	0	0	0
*Nhe*I	13.89	0	0	0	0	0	0	0	0	0	0	0	1
*Hin*dIII	13.90	0	0	0	0	0	1	1	0	0	0	0	0
*Eco*RV	14.00	1	0	0	0	0	1	1	0	0	0	0	0
*Pvu*II	14.06	0	0	1	1	0	0	0	0	0	1	0	0
*Pst*I	14.06	0	0	1	1	1	1	1	1	1	1	1	1
*Eco*RI	14.31	0	0	0	0	0	0	0	1	0	0	0	0
*Hin*dIII	14.33	1	1	1	1	1	1	1	1	1	1	1	1
*Nhe*I	14.46	0	0	0	1	0	0	0	0	0	0	0	0
*Sac*II	14.59	0	0	0	0	0	0	0	0	0	0	0	1
*Pvu*II	14.68	1	1	0	0	0	0	0	0	0	0	1	1
*Bcl*I	15.00	0	0	1	0	0	0	0	0	0	1	1	0
*Hin*dIII	15.05	0	0	0	0	1	0	0	1	1	0	0	0
*Sac*I	15.09	0	0	0	0	0	0	0	1	1	0	0	0
*Sac*I	15.19	0	0	0	0	0	0	0	0	0	0	0	1
*Eco*RV	15.75	0	0	0	0	0	1	1	0	0	0	0	0
*Hin*dIII	16.03	1	1	0	0	0	0	0	0	0	0	0	0
*Sac*II	16.15	1	1	1	1	1	1	1	1	1	1	1	1

APPENDIX 2

Continued

Restriction enzyme	Map location	Locality											
		ETX	ELA	PNE	PMI	VTX	VKS	VOK	TKS	TOK	NKS	NOK	NMI
*Sac*I	16.32	1	1	1	1	0	0	0	0	0	0	0	0
*Eco*RV	A	0	0	0	0	0	1	1	0	0	0	0	0
*Eco*RV	B	0	1	0	0	0	0	0	0	0	0	0	0
*Eco*RV	C	0	0	0	0	0	0	0	1	0	0	0	0
*Nhe*I	A	0	0	0	0	0	0	0	0	1	0	0	0
*Sac*II	A	0	0	0	0	0	0	0	1	1	0	0	0
*Hin*dIII	A	0	0	0	1	0	0	0	0	0	0	0	0
*Hin*dIII	B	0	0	0	0	0	0	0	0	0	1	0	0

Note: Exact locations of the last seven restriction sites listed were not determined because of limited tissue supply and/or absence of conserved reference sites in close proximity to the seven sites.