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A fine line between sex and unisexuality: the phylogenetic constraints on parthenogenesis in lacertid lizards

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A phylogeny of Caucasian rock lizards (genus *Darevskia*, formerly *Lacerta*) was reconstructed using mitochondrial DNA sequence and allozyme data. All 15 bisexual species grouped into three major clades: the *caucasica*, *saxicola* and *rudis* clades. Unisexual *Darevskia* originate from inter-clade hybridization, never from within clades. Only two clades, the *caucasica* clade and the *rudis* clade, were involved in forming unisexuals; the *saxicola* clade was never involved. Furthermore, the hybridization is directional in that the *caucasica* clade contributed only maternal parents and the *rudis* clade only paternal parents. The formation of unisexual species is best explained by sexually directional phylogenetic constraints. We hypothesize that the causative agents are likely to be genes linked with the sex chromosomes within the parental sexual species.

ADDITIONAL KEY WORDS:—*Darevskia* – unisexuality – sex chromosomes – allozymes – mtDNA sequences – hybridization.

CONTENTS

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INTRODUCTION

Not only is the evolution of unisexuality a captivating topic, it is of great significance to evolutionary biologists. Much has been learned about genetic mechanisms from unisexual organisms. In addition, insight into ecological and behavioural attributes of these species has been gained. Yet interest has not waned, but rather increased, as more unisexual species have been described and novel reproductive modes discovered (Dawley & Bogart, 1989).

The field of phylogenetic systematics has made a colossal impact on the study of evolutionary biology. With the appearance of numerous sound phylogenies, much of evolutionary biology has abandoned erudite story-telling and entered the domain of forming and testing falsifiable hypotheses (Brooks & McLennan, 1991; Harvey & Pagel, 1991). Phylogenetic methods are beginning to have their impact, although the full significance of the approach remains to be realized. One application of phylogenetic methods is an examination of the phylogenetic constraints on the evolution of unisexuality.

Few groups of reptiles have made as strong an impact on our understanding of evolutionary mechanisms and reproduction as have the rock lizards of the Caucasus Mountains, genus *Darevskia* Arribas, 1997 (formerly *Lacerta*) of the family Lacertidae. Within this group of lacertid lizards, unisexuality in amniotes was first detected (Darevsky, 1957, 1958, 1967). Numerous other investigations have followed this discovery, for example, studies of unisexuality of North American whiptail lizards, genus *Cnemidophorus*. As a group, perhaps no other vertebrates have been so thoroughly studied from ecological and behavioural perspectives for all inclusive species as have the Caucasian rock lizards.

Recent molecular studies have greatly extended our knowledge about this group of lizards, both in clarifying the species boundaries of the sexuals and confirming the hybrid origin and the parentage of the unisexuals. Four former subspecies, *D. caucasica alpina*, *D. c. daghestanica*, *D. saxicola lindholmi* and *D. s. saxicola* have been elevated to species status based on morphological and allozymic data (Fu *et al.*, 1995; MacCulloch *et al.*, 2000). Bobyn *et al.* (1996) and Fu, Murphy & Darevsky (2000) considered *D. nairensis* conspecific with *D. raddei* based on allozyme and mitochondrial DNA data, respectively. Using allozymes in combination with mitochondrial DNA (mtDNA) data, four sexual species, *D. raddei*, *D. mixta*, *D. valentini* and *D. portschinskii*, were identified as the parental species of the seven currently known unisexual species,

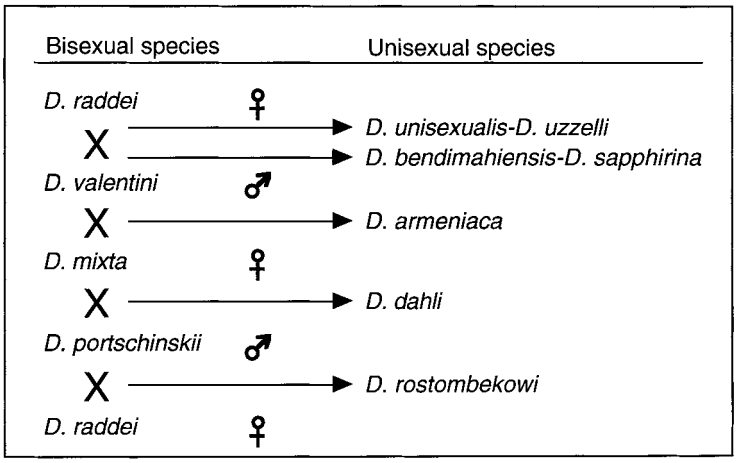

Figure 1. Parentage of unisexual *Darevskia*.

which originated from interspecific hybridization (Fig. 1; Moritz *et al.*, 1992a; MacCulloch *et al.*, 1995b, 1997c; Murphy *et al.*, 1997; Fu, Murphy & Darevsky, 1999a; Fu *et al.*, 1998, 2000, in press). Furthermore, mitochondrial DNA data revealed that the hybridization between *D. raddei* and *D. valentini* occurred at least two times, which led to the formation of two distinctive unisexual lineages, *D. unisexualis—D. uzzelli* and *D. bendimahiensis—D. sapphirina* (Fu *et al.*, 2000). Hybridization resulting in persistent unisexual lineages has occurred at least five times in Caucasian rock lizards (Fu *et al.*, 2000).

The formation of unisexual species in vertebrates is highly constrained and two hypotheses have been promoted regarding the constraints in lizards. Darevsky, Kupriyanova & Uzzell (1985) argued that parthenogenesis was phylogenetically restricted because of lineage-dependent genetic factor(s) that determine clonal reproduction in hybrids. Alternatively, Moritz *et al.* (1989) put forward a "balance hypothesis", based on genetic distance, which proposes a more generalized genetic interaction that leads to the production of unreduced eggs, yet does not reduce offspring viability.

Moritz, Wright & Brown (1992b) examined the competing hypotheses and found the balance hypothesis preferable in explaining the formation of parthenogenetic species of *Cnemidophorus*. They concluded that phylogeny was not a major component in the formation of unisexuality. However, for the two hypotheses to be tested, it is necessary to examine not only the phylogeny of the parental species involved in hybrid formation of the unisexuals, but also to place the parental phylogeny in the context of the phylogenetic relationships of other bisexual species of the group. The phylogeny of Caucasian rock lizards has been previously evaluated (Darevsky, 1967; Murphy *et al.*, 1996a; Fu, Murphy & Darevsky, 1997). In this study, we extended the phylogenetic evaluation based on all molecular data from previous population and phylogenetic studies, using a substantial amount of new data. The recent thorough population studies provided a firm foundation for our phylogenetic estimation. Furthermore, we summarized all information on the parentage of the seven unisexuals. With this phylogenetic framework and parentage information, we tested competing hypotheses of hybrid origins for unisexual *Darevskia*.

TABLE 1. Species of *Darevskia* examined for allozyme in this study. N_{pop} = number of populations examined; N_{sp} = number of specimens examined

Species	N_{pop}	N_{sp}	References
D. alpina	1	4	Fu *et al.* (1995); this study*
D. armeniaca	8	117	MacCulloch *et al.* (1995b); Fu *et al.* (1999a)
D. bendimahiensis	1	25	Fu *et al.* (in press)
D. brauneri	6	48	MacCulloch *et al.* (2000)
D. caucasica	1	11	Fu *et al.* (1995)
D. clarkorum	1	4	this study*
D. daghestanica	3	78	Fu *et al.* (1995)
D. dahli	6	161	Murphy *et al.* (1997)
D. derjugini	3	22	MacCulloch *et al.* (1997a)
D. lindholmi	2	20	MacCulloch *et al.* (2000)
D. mixta	2	14	this study*
D. parvula	1	7	this study*
D. portschinskii	4	82	MacCulloch *et al.* (1995a, 1997b)
D. praticola	2	64	MacCulloch *et al.* (1997a)
D. raddei	11	246	Bobyn *et al.* (1996); this study*
D. rostombekovi	4	65	MacCulloch *et al.* (1997c)
D. rudis	1	26	MacCulloch *et al.* (1995a)
D. sapphirina	1	27	Fu *et al.* (in press)
D. saxicola	1	31	MacCulloch *et al.* (2000)
D. unisexualis	4	58	Fu *et al.* (1998, in press)
D. uzzelli	1	26	Fu *et al.* (in press)
D. valentini	4	95	MacCulloch *et al.* (1995a); this study*

* Specific location data and genotypic data are available upon request.

MATERIAL AND METHODS

Specimens examined

Fieldwork in the Caucasus Mountains was conducted from 1992 to 1996. Some additional material was made available to us by colleagues. The voucher specimens are deposited in the herpetological collection of the Royal Ontario Museum (specific data available on request). A summary of the taxa examined for allozyme electrophoresis, sample sizes, and the original references are provided in Table 1. Parts of the data have been published in the previous studies.

Due to the commonplace occurrence of polymorphism in *Darevskia*, two cases of suspicious species identification might confound the phylogenetic evaluation of Murphy *et al.* (1996a) and Fu *et al.* (1997). First, the '*D. saxicola*' used by Murphy *et al.* (1996a) and Fu *et al.* (1997) is likely an anatomical variant of *D. raddei*. Both morphologically and allozymically, the population assembles *D. raddei*, although its status needs to be further investigated. Second, Fu *et al.* (1997) hypothesized that the '*D. alpina*' used in their study is likely a hybrid. Subsequently, two precautions

were taken in this study. First, all morphologically identified specimens were screened by allozyme electrophoresis and the species identities were confirmed using fixed allelic markers. Second, multiple specimens of most species were sequenced for mtDNA. The sample sizes are as follow: *D. alpina* (4; including the '*D. alpina*' used by Fu *et al.*, 1997), *D. brauneri* (3), *D. caucasica* (1), *D. clarkorum* (5), *D. daghestanica* (2), *D. derjugini* (2), *D. lindholmi* (1), *D. mixta* (2) *D. parvula* (2), *D. portschinskii* (2), *D. praticola* (2), *D. raddei* (4), *D. rudis* (2), *D. saxicola* (2; including one '*D. saxicola*' from Murphy *et al.*, 1996a and Fu *et al.*, 1997), *D. valentini* (2).

Laboratory protocols

Two types of data were examined. Allozymes were used to define species boundaries, identify parentage, and reconstruct phylogenetic relationships. MtDNA sequence data were used to identify parentage and evaluate the phylogeny of the lizards.

Protein electrophoresis, staining protocols, enzyme and locus nomenclature and allelic designations follow Murphy *et al.* (1996b). Buffer combinations for resolving locus products are given in Fu *et al.* (1995), MacCulloch *et al.* (1995a), and Bobyn *et al.* (1996). Wherever possible each locus product was resolved on two buffer systems to minimize hidden variation. At least one allelic product at a given locus was required to have a minimum of 1 cm migration from the origin before scoring.

Mitochondrial DNA sequences of cytochrome *b*, ATPase 6 and 16S genes were obtained using the following protocols. Whole DNA was extracted from muscle or liver tissues using the standard phenol/chloroform method; polymerase chain reaction (PCR) was used to amplify the target DNA fragments, with annealing temperature varied from 42–50°C (Palumbi, 1996); PCR products were directly sequenced using P^{33} labelled terminator cycle sequencing protocols (Amersham). Primers used for PCR and sequencing are listed in Appendix 1.

Phylogenetic analysis

The allozyme data and the mtDNA sequence data were analysed separately using the parsimony principle. Outgroup selection was based on current phylogenetic hypotheses of the family Lacertidae (Harris, Arnold & Thomas, 1998; Fu, 1998, 1999). All tree searches, nodal evaluations, and genetic distance calculations were conducted using PAUP* (ver. 4.01b; Swofford, 1998). All data editing and tree manipulations were conducted using MacClade (ver. 3.04; Maddison & Maddison, 1992).

Allozyme data were coded using the mutation model, as recommended by Murphy (1993) and exemplified by Murphy *et al.* (1996a) and Murphy and Doyle (1998). Under this model, the locus was treated as the character and only mutation events are considered. *Lacerta media*, *L. strigata* and *L. vivipara* were used as outgroups. To facilitate the analysis, a hypothetical ancestor was constructed to represent the ancestral allelic composition.

Algyroides fitzingeri, *Lacerta media*, *L. monticola* and *L. vivipara* were used as outgroups for the DNA analysis. The alignment of DNA sequence data (16S rRNA gene) was accomplished using ClustalW with the following parameters: gap = 10.00; gap

extension = 0.05 (Version 1.6, Thompson, Higgins & Gibson, 1994). Minor modifications were made by eye and sites with ambiguous alignment were excluded from the phylogenetic analysis, because the homology cannot be assumed confidently (Hillis & Dixon, 1991). Each base site was treated as an unordered character. Analysis of the mtDNA sequence data involved both unweighted and weighted parsimony evaluations.

Bootstrap proportion (Felsenstein, 1985) and decay index (Bremer's branch support; Bremer, 1988) were used to evaluate the recovered nodes. Because of commonplace hybridization among species, a congruence analysis was subsequently conducted based on nodal support analysis. A preferred tree was chosen based on Lanyon's (1993) phylogenetic framework concept using consensus methods. Where conflicts between the two data sets occurred, the preferred tree reflected the well-supported alternative. Where conflicting arrangements were each well supported by mtDNA sequence and allozyme data, the preferred tree reflected the nuclear-based allozyme data because the mtDNA data may reflect the maternal lineage of past hybridization events.

Based on the phylogenetic analysis, we summarized the allozyme data which confirmed the parentage of all seven unisexual *Darevskia*. Confirmation of a unisexual's parentage required the same conditions as the unequivocal identification of bisexual hybrids: uniquely derived marker alleles restricted to both maternal and paternal lineages (Murphy & Crabtree, 1988), and a minimum of two diagnostic loci (Baverstock & Moritz, 1996). Lastly, based on the resulting preferred phylogeny, we conducted an analysis of the phylogenetic constraints on unisexuality.

Genetic distance analysis

The 'balance hypothesis' (Moritz *et al.*, 1989) regards the amount of genetic divergence as the major constraint in the origin of parthenogenesis in lizards. Therefore, to test this hypothesis, the pairwise distances between all species based on the DNA sequence data were calculated. The Kimura 2-parameter model was empirically chosen based on properties of the data.

RESULTS

MtDNA phylogeny

A 450 bp fragment of the cytochrome *b* gene was sequenced for all specimens except four of the five *D. clarkorum*, for a total of 31 individuals representing 15 ingroup species. One specimen of each outgroup species was also sequenced, except *Lacerta vivipara*, whose sequence was obtained from GenBank (accession number U69834). A preliminary phylogenetic analysis was conducted to examine the monophyly of the currently recognized species. All species were confirmed to be monophyletic groups except *D. alpina* and *D. saxicola*. The specimen of '*D. alpina*' used in Fu *et al.* (1997) was nested between the two samples of *D. mixta* while the other three *D. alpina* formed a clade. The '*D. saxicola*' used by Murphy *et al.* (1996a) fell, as expected, within the clade of *D. raddei*. These two individuals were excluded

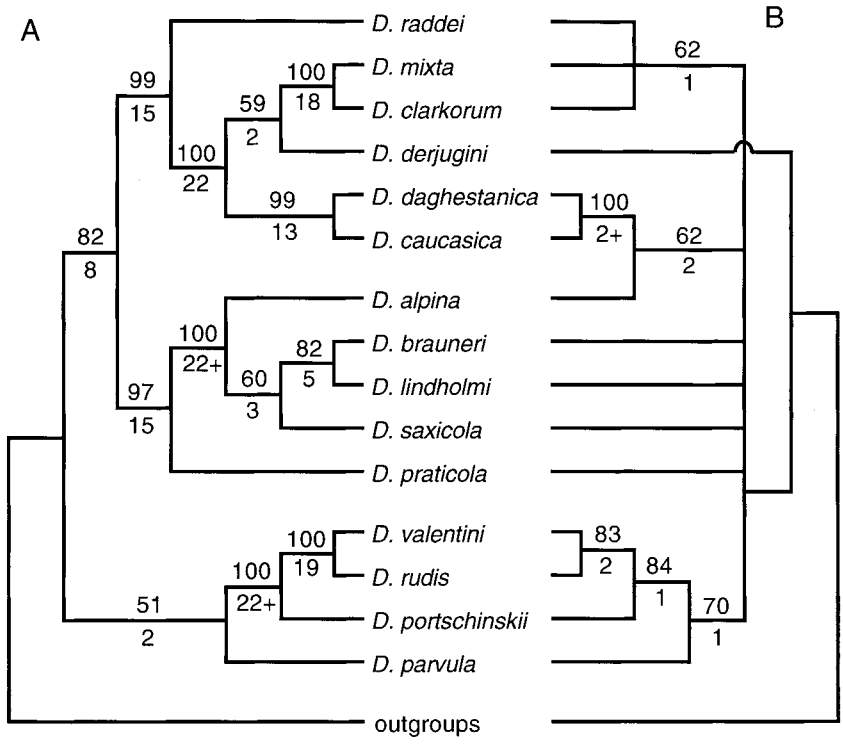

Figure 2. Phylogenies of the genus *Darevskia*. Numbers above the lines are boostrap proportions calculated from 1000 replicates; numbers below the lines are decay indices. A, the most parsimonious tree from mtDNA sequence data. B, strict consensus tree of 89 equally most parsimonious trees from the allozyme data.

from subsequent analyses. All five specimens of *D. clarkorum* were sequenced for ATPase 6, which confirmed the monophyly of the species.

With species monophyly confirmed, one specimen from each species was sequenced for the remaining portion of cytochrome *b* (a total of 1044 bp), 16S (a total of 1173 bp aligned) and ATPase 6 (a total of 596 bp) genes. A fragment of $tRNA^{Thr}$ (37 bp), which is adjacent to the cytochrome *b* gene, was also sequenced. Attempts to sequence ATPase 6 in three ingroup species (*D. valentini*, *D. rudis*, and *D. alpina*) and three outgroup members (*Algyroides fitzingeri*, *L. vivipara* and *L. monticola*) failed, and only a shorter fragment (403 bp) of *D. portschinskii* was sequenced. A 27 base pair fragment of the 16S gene was excluded from the phylogenetic analysis due to ambiguous alignment.

The total of 2851 bp of mtDNA sequence data yielded 769 potentially cladistically informative characters. A single most parsimonious tree was found with 2393 steps, CI of 0.463 and RI of 0.522 (Fig. 2). The tree is well resolved and well supported. Ten nodes received bootstrap proportions greater than 0.70, and they were exactly the same ten nodes which received the highest decay indices. All species were arranged into three groups: the *caucasica* group containing *D. caucasica*, *D. daghestanica*, *D. derjugini*, *D. mixta*, *D. clarkorum*, and *D. raddei*; the *saxicola* group containing *D. saxicola*, *D. brauneri*, *D. lindholmi*, *D. alpina*, and *D. praticola*; and the *rudis* group

containing *D. parvula*, *D. rudis*, *D. valentini* and *D. portschinskii*. The *caucasica* group and the *saxicola* group are sister groups. The tree is similar to that of Fu *et al.* (1997) in many aspects, such as the association of *D. caucasica*, *D. daghestanica*, *D. derjugini*, *D. mixta*, *D. clarkorum*, and the association of *D. rudis*, *D. valentini*, *D. portschinskii*, but different in the placements of *D. parvula* and *D. praticola*.

A weighted parsimony analysis, in which only transversions from the 16S rRNA gene and the 1st and 2nd codon positional substitutions of the protein genes (cytochrome *b* and ATPase 6) were used, resulted in four equally most parsimonious trees. All taxa grouped into three clades exactly the same as above, and the monophyly of the three groups and the sister group relationship of the *saxicola* group with the *caucasica* group were well supported. However, the relationships within the *saxicola* and *caucasica* groups were not well resolved.

Allozyme phylogeny

A total of 35 loci were resolved (Appendix 2). Among them, 11 are invariant among the ingroup members, and 6 are autapomorphic, making these phylogenetically uninformative. Mutation model coding resulted in 18 informative characters, producing 89 equally parsimonious trees with 56 steps, CI of 0.7143 and RI of 0.7714. The strict consensus tree is shown in Figure 2. Most resolved nodes received bootstrap values greater than 50. The decay indices were also mapped on the consensus tree. Herein we consider that the low bootstrap proportions and decay indices do not represent a lack of confidence, but rather reflect the shortage of informative characters (Felsenstein, 1985; Fu & Murphy, 1999).

The allozyme data confirm the results of the mtDNA data regarding the monophyly of the *rudis* group and the relationships within the group. All nodes are supported by at least one unique derived allele (i.e. inclusively shared by the members of the clade). The data also strongly unite *D. caucasica* and *D. daghestanica*, which together constitute the sister group of *D. alpina*. The monophyly of the clade containing these three species is supported by four synapomorphies, including two unique derived alleles (at *mAat-A* and *mSod-A*). Furthermore, *D. mixta*, *D. raddei* and *D. clarkorum* form a clade. Although the association of *D. clarkorum* with *D. mixta* and *D. raddei* may only be because of the locus *Ck-C*, the close relationship of the latter two species is strongly supported by three unique derived alleles (at *sAcoh-A, Ldh-B* and *sMdhp-A*). The allozyme data place *D. derjugini* as the basal taxon to all other *Darevskia*. However, we found no unique allele supporting this placement. The resulting tree is essentially the same as in Murphy *et al.* (1996a), but includes more taxa.

Parentage of unisexuals

Table 2 summarizes the occurrence of apomorphic alleles shared among the unisexual species and their parents. All species share from 6 to 8 derived alleles with the patriarchal parent, and from 8 to 10 with the matriarch. In every case a greater proportion of derived alleles has its origins from the matriarch. In addition, from 2 to 5 of the fixed heterozygotes are composed of derived alleles present in both parents. The large number of heterozygotes and shared apomorphic alleles unequivocally establish the hybrid origin and parentage of the unisexual species.

TABLE 2. Summary of alleles resolved in seven unisexual species of *Darevskia* and their bisexual parents. Only informative loci are listed. Bold letters indicate derived alleles. Abbreviations are as follows: *mixt* = *D. mixta*, *arme* = *D. armeniaca*, *vale* = *D. valentini*, *unis* = *D. unisexualis*, *uzze* = *D. uzzelli*, *bend* = *D. bendimahiensis*, *sapp* = *D. sappirhina*, *radd* = *D. raddei*, *rost* = *D. rostombekowi*, *port* = *D. portschinskii*, and *dahl* = *D. dahli*, F = female, M = male, U = unisexuals

	Species and sex											
Locus	**F** *mixt*	U *arme*	**M** *vale*	U *unis*	U *uzze*	U *bend*	U *sapp*	**F** *radd*	U *rost*	**M** *port*	U *dahl*	**F** *mixt*
sAat-A	**d**	**c/d**	**c**	**c/d**	**c/d**	**c/d**	**c/d**	**d**	**c/d**	**c**	**c/d**	**d**
sAcoh-A	**a**,c,**h**	**a/e**	**e**	**e/f**	**e/f**	**e/f**	**e/f**	c,**f,h**	**f/g**	**g**	**a/g**	**a**,c,**h**
				e[1]								
mAcoh-A	**b**	**b/i**	**i**	d[1]	d/**i**	d[1]	d[1]	d/**e**	d	d	**b**/d	**b**
Acp-B	**k**	**h/k**	**h**	**c/h**	**c/h**	**c/h**	**c/h**	**c**	**c/i**	**i**	**i/k**	**k**
Cat-A	**a**	**a**/b	b	b	b	b	b	b	b/d	d	**a**/d	**a**
Ck-C	b	b/**c**	**c**	b/**c**	b/**c**	b/**c**	b/**c**	**a**/b	**a**/b	b	b	b
		c[1]										
Gpi-A	b	b	b	b/**c**	b/**c**	b/**c**	b/**c**	b/**c**/**e**	b/**c**	b	b	b
Gpi-B	**b**	**b/h**	**h**	**h/j**	**h/j**	**h/j**	**h/j**	**j**	**h/j**	**h**	**b/h**	**b**
Ldh-B	**d**	**d/f**	**f**	**d/f**	**d/f**	**d/f**	**d/f**	**d**	**d/f**	**f**	**d/f**	**d**
					d[1]							
sMdhp-A	**g**	f/**g**	f	f/**g**	f/**g**	f/**g**	f/**g**	**g**	f/**g**	f	f/**g**	**g**
mMdhp-A	c	c/**f**	**f**	**f/h**	**f/h**	**f/h**	**f/h**	**h**	**e/h**	**e**	c/**e**	c
Mpi-A	**e**	**e/h**	**h**	**h/i**	**h/i**	**h/i**	**h/i**	**i**	**h/i**	**h**	**e/h**	**e**
Pep-B	**m**	c/**m**	c	c/**j**	c/**j**	c/**j**	c/**j**	d/**j**	d[1]	**b**/c	c/**m**	m
				j[1]		c[1]						
Pnp-A	d	d/**e**	**e**	d/**e**	d/**e**	d/**e**	d/**e**	**b/c**/d	d/**e**	**e**	d/**e**	d
		e[1]									d[1]	
											e[1]	
											a[1]	
mSod-A	**b**	**b**[1]	c	c	c	c	c	c	c	c	**b**/c	**b**
sSod-A	**c**	a/**c**	a	a/**c**	a/**c**	a/**c**	a/**c**	**c**	a/**c**	a	a/ **c**	**c**

[1] Unisexuals do not express both alleles resolved in putative origin.

Genetic distance

To evaluate the genetic divergence among the species and the three major clades, a pairwise distance comparison of the mtDNA data was conducted. *Darevskia alpina* was excluded from the calculation because of the problematic origin of its mtDNA (see Discussion). A transversion bias was observed, so the Kimura 2-parameter distance was used. The results are in Table 3; not surprisingly, intra-clade distances are smaller than inter-clade distances. Although phylogenetically the *caucasica* group and the *saxicola* group are closer to each other than either is to the *rudis* group, the genetic divergence showed no significant difference among the three ($P \leq 0.05$). Given variation among sample sizes, the sensitivity of allozyme genetic distance measures to the choice of loci surveyed, required assumptions about equal rates of

TABLE 3. The intra- and inter-clade genetic distances among the Caucasian rock lizards. The Kimura 2-parameter distances are calculated based on the mtDNA sequence data, and presented in forms of averages and ranges of pairwise distances between species. The bold numbers are inter-clade distances

	The *caucasica* group	The *rudis* group		The *saxicola* group
		portschinskii	*valentini*	
The *caucasica* group	0.08026 (0.03297–0.11009)	**0.12949 (0.11144–0.14622)**		**0.12817 (0.10982–0.14807)**
mixta	—	**0.13019**	**0.12212**	—
raddei	—	**0.13730**	**0.12865**	—
The *rudis* group		0.07944 (0.01434–0.12896)		**0.13372 (0.12134–0.15589)**
The *saxicola* group				0.07633 (0.03810–0.13768)

change, and the lack of resolving power of gene frequency data (Murphy *et al.*, 1996b; Murphy & Doyle, 1998), we avoided calculating genetic distance measures.

DISCUSSION

Preferred phylogeny of the genus Darevskia

The poor resolution of the allozyme tree is not surprising. To be able to fully resolve the tree, empirically at least twice as many informative loci as the number of taxa are needed (Murphy *et al.*, 1996a, b). Clearly, this was not the case here.

Both the mtDNA and allozyme data supported the monophyly of the three groups and found the same relationships within the *rudis* group (Fig. 2). Congruence from independent data sets provides the best support for relationships (Hillis, 1987; Miyamoto & Fitch, 1995). The sister group relationship of *D. daghestanica* and *D. caucasica* is also supported by both data sets.

Few cases of conflict occur between the mtDNA and allozyme phylogenies, and historical interspecific hybridization (gene introgression) may account for some of the conflicts (Fig. 2). *Darevskia alpina* might have acquired mtDNA by gene introgression from *D. mixta* as well as from the ancestor of *D. saxicola*, *D. brauneri* and *D. lindholmi*, and therefore has two mtDNA lineages. Fu *et al.* (1997), as well as this study, found that the mtDNA of one *D. alpina* specimen is closer to *D. mixta* than to the other *D. alpina* examined; the single specimen nests between the two *D. mixta* samples on the cytochrome *b* tree. MtDNA of the other three specimens of *D. alpina* revealed their close genealogical relationship with the *D. saxicola* complex (*D. saxicola*, *D. brauneri* and *D. lindholmi*) (Fig. 2). However, the allozyme data show that all four *D. alpina* specimens share the same derived allelic properties and therefore form a single evolutionary lineage. Furthermore, the association of *D. alpina* with *D. daghestanica* and *D. caucasica* is strongly supported by the allozyme data (two unique synapomorphies) as well as by morphological data (Darevsky, 1967), which more likely reflects the genealogy of the species. The placement of *D. raddei* possibly represents another case of gene introgression. Although both allozyme data and mtDNA data suggest that *D. raddei* and *D. mixta* belong to the *caucasica* clade, the mtDNA data placed *D. raddei* at the base of the clade while the allozyme data united it with *D. clarkorum* and *D. mixta*. Both solutions are strongly supported: a 100% bootstrap proportion and high decay index of 20 from the mtDNA and three unique

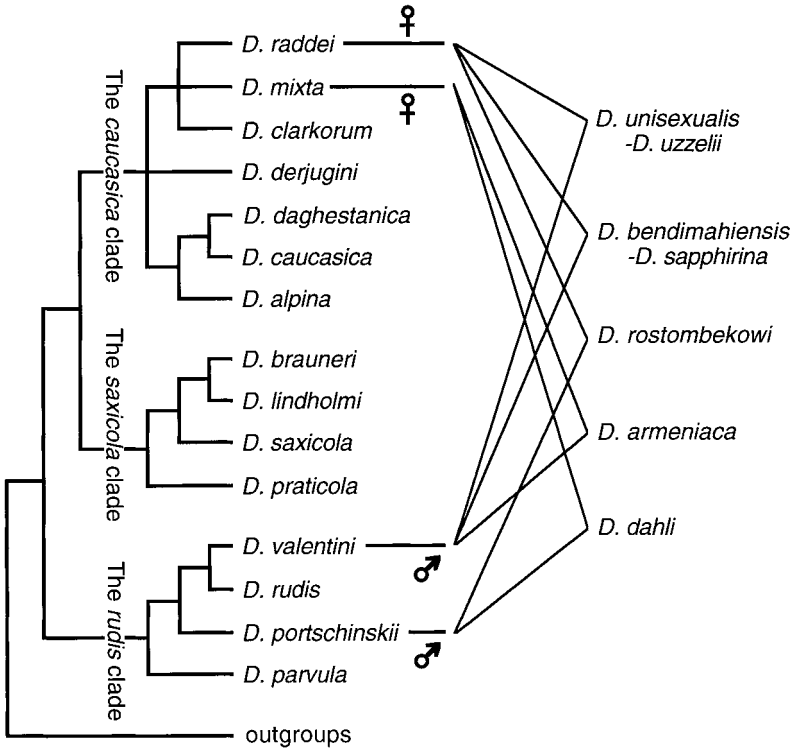

Figure 3. Phylogenetic constraints of the origin of unisexuality in *Darevskia*. The left side is the preferred phylogeny of the genus and the right side are the hybrid origins of the five hypothetical unisexual lineages. The limited involvement of the sexual species and directional hybridization reveal that the formation of unisexual species is phylogenetically constrained.

derived alleles from the allozymes (Fig. 2). In this case, we consider the allozyme resolution more likely represents the genealogy of the species.

We do not believe the conflicting placements of *D. derjugini* result from gene introgression. The mtDNA data strongly united *D. derjugini* with the *caucasica* group, while the allozyme data placed it basely, although there is no single unique allele supporting the basal placement. In this case, we accept the mtDNA placement.

Our preferred phylogeny is a consensus of the trees from both data sets (Fig. 3). We did not perform a combined data analysis because the mtDNA data would undoubtedly have swamped the allozyme data.

Confirmed parentage of unisexuals

Parentage of the unisexual species was previously reviewed by Uzzell & Darevsky (1975), Darevsky *et al.* (1985), and Darevsky (1992), and reiterated by Moritz *et al.* (1992a). In general, the early estimates, based on intermediate morphology (Darevsky & Danielyan, 1977; Darevsky *et al.*, 1985; Schmidtler, Eiselt & Darevsky, 1994) and correlation with karyology (Kupriyanova, 1986, 1989, 1992), have been concordant with the recent molecular studies (e.g. Moritz *et al.*, 1992a; Fu *et al.*, 2000). The

scope of the allozyme evaluations summarized in this study, in numbers of both species and loci, enable us to undeniably confirm the parents of the unisexual species. For example, *D. valentini* and *D. rudis* were very similar, with fixed allozyme differences at only 3 loci (Table 2; MacCulloch *et al.*, 1995a). However, the presence of fixed marker alleles in *D. valentini* clearly eliminates *D. rudis* as a possible parent to the unisexuals. Similarly, the sister taxa *D. mixta* and *D. raddei* could be differentiated by 9 of 35 loci (Table 2; Appendix 2); in this case the determination of parentage is clear.

The only exception to this concordance is the maternal parent of *D. uzzelli*. Originally, Darevsky & Danielyan (1977) proposed *D. parvula* and *D. raddei nairensis* as the parental species of *D. uzzelli*. Moritz *et al.* (1992a) identified *D. valentini* as the maternal parent using mtDNA restriction enzyme data. However, our recent allozyme data and DNA sequence data positively demonstrate that *D. valentini* is the paternal parent while *D. raddei* is the maternal parent of *D. uzzelli* (Fu *et al.*, 2000; in press).

Phylogenetic perspective

Figure 3 shows our preferred phylogeny of 15 bisexual species of Caucasian *Darevskia* and the hybrid origins of the unisexuals. Mapping the unisexuals and their parental species on the tree reveals clear patterns. First, the hybridization events leading to unisexual species occurred only between the major clades, and never within clades. Only two clades, the *caucasica* clade and the *rudis* clade, were involved in forming unisexuals; the *saxicola* clade was never involved. Although some uncertainties about intraclade phylogenetic relationships remain, both allozyme and mtDNA data agree that *D. mixta* and *D. raddei* belong to one clade, while *D. valentini* and *D. portshinskii* belong to another. Second, the hybridization is directional in that the *caucasica* clade contributed only maternal parents and the *rudis* clade only paternal parents. Repetition from five independent unisexual lineages proves that these patterns are not random ($P = 0.5^5 = 0.03 < 0.05$). The patterns are best explained by the existence of phylogenetic constraints, i.e. lineage-dependent factor(s) that restrict the origin of unisexuality.

The formation of unisexual vertebrates always involves interspecific hybridization. However, any understanding of the potential constraints on the formation of unisexual species must consider both intra- and inter-clade hybridization. The occurrence of ongoing and hypothesized past interspecific bisexual hybrid individuals is given in Table 4. Such hybridization is common among *Darevskia*, although the offspring are typically fertile bisexuals, never unisexuals.

Within-clade hybridization occurs among most sympatric species. For instance, among lizards in the *caucasica* clade, *D. alpina* is sympatric with *D. caucasica*, and *D. caucasica* with *D. daghestanica*. In both cases, individual interspecific hybrids are known from regions of overlap even though the species are not broadly syntopic (Darevsky, 1967; Fu *et al.*, 1995; Roitberg, 1994). Also, *D. derjugini* hybridizes with *D. mixta* producing viable hybrids and backcrosses (Darevsky, unpublished data). In contrast, sympatric *D. derjugini* and *D. clarkorum* are not known to hybridize. In all instances of hybridization, it appears as though the offspring are viable, and where investigated (Fu *et al.*, 1995; Murphy *et al.*, unpublished data) backcrossing occurs.

Inter-clade hybridization occurs among members of all three major clades (Table

TABLE 4. The documented inter- and intra-clade hybridization events among Caucasian rock lizards

Hybrid cross	References
intra-clade	
D. alpina × *D. caucasica*	Darevsky (1967); Fu *et al.*, 1995
D. caucasica × *D. daghestanica*	Darevsky (1967); Fu *et al.*, 1995
D. mixta × *D. derjugini*	Darevsky (1967)
D. mixta × *D. alpina*	Fu *et al.* (1997); this study
D. parvula × *D. rudis*	Darevsky (1967)
D. saxicola × *D. brauneri*	Darevsky (1967)
inter-clade	
D. alpina × *D. brauneri*	Darevsky (1967)
D. caucasica × *D. saxicola*	Darevsky (1967)
D. derjugini × *D. parvula*	Darevsky (1967)
D. raddei × *D. portschinskii*	Darevsky (1967)
D. rudis × *D. clarkorum*	Darevsky & Tuniyev (1997)

4), although not in all possible combinations. Some members of the *caucasica* clade hybridize with species from the *saxicola* clade, for example, *D. caucasica* with *D. saxicola*, and *D. alpina* and *D. brauneri*. The offspring from the hybridization events appear as F_1 individuals only, although backcrossing may occur (unpublished observations). *Darevskia raddei* of the *caucasica* clade is sympatric with *D. portschinskii* of the *rudis* clade in Armenia and an excessive number of unique alleles in Armenian populations of *D. raddei* is indicative of hybridization (Murphy *et al.*, unpublished data). Hybrids of *D. clarkorum* and *D. rudis* are also common; however, other species of the *caucasica* clade are also sympatric with *D. rudis* and in these cases hybrids are not known (Darevsky, unpublished data). This may be because of differences in body size; the members of the *caucasica* clade are small ($\bar{x} = 59$ mm SVL) and *D. rudis* ($\bar{x} = 75$ mm SVL) is the largest Caucasian *Darevskia*. In contrast, although *D. mixta* occurs sympatrically with both *D. parvula* and *D. rudis* of the *rudis* clade, as do *D. raddei* and *D. valentini*, no hybrids have been observed. In the former instance size may prevent breeding, and in the latter case, differences in breeding season prevent hybridization (Danielyan, 1965). Finally, although the ground lizard *D. derjugini* of the *caucasica* clade is broadly sympatric with *D. praticola* of the *saxicola* clade, and with *D. parvula* and *D. rudis* of the *rudis* clade, hybrids are unknown. None of these hybridizations have given rise to parthenogenetic lizards.

Why is the *saxicola* clade not involved in the formation of unisexual species? Species sympatry in the Caucasus provides abundant opportunities for hybridization. In particular, there are cases of sympatry between members of the *saxicola* clade and of the other two clades, and interspecific hybridization is documented. Abundant opportunities for hybridizations forming unisexual species occurred when distributions were compressed sympatrically into a few Pleistocene refugia (e.g. Darevsky, 1959). However, it is possible that members of the *saxicola* clade were isolated in refugia separate from other *Darevskia*, precluding hybridization. Given that (1) most members of the *saxicola* clade occur in the northern Caucasus, (2) the northern Caucasus were completely glaciated about 12 000 years ago, and (3) unlimited opportunities to hybridize may be required for successful formation of unisexuality, then it would not be too surprising that this clade has not contributed to the formation of unisexual species. However, the *saxicola* clade is involved in hybridization

events; extensive hybridization and backcrossing occurs between *D. alpina* and *D. brauneri*, and between *D. caucasica* and *D. saxicola*. This would seem to refute isolation as an explanation for the clade not being involved in the origin of unisexual species.

Clearly, the hybrid formation of unisexual species is phylogenetically constrained. But the question remains: what are the constraints?

We believe that sex chromosomes may play a key role in the formation of unisexual species. *Darevskia* has a chromosomal mechanism of sex determination and the female is the heterogametic sex (ZW). *Darevskia dahli* and *D. armeniaca* express the derived micro-heteromorphic W chromosome present in their maternal lineage, *D. mixta* (Kupriyanova, 1989, 1992). Similarly, *D. unisexualis* expresses the derived micro-heteromorphic chromosome present in its maternal lineage, *D. raddei* (Kupriyanova, 1989; Darevsky *et al.*, 1985). *Darevskia rostombekowi* may express its matriarch's state, but if so, then the W chromosome in *D. raddei* is polymorphic since the W chromosome of *D. rostombekowi* is more similar in size and heterochromatin pattern to *D. portschinskii* than it is to the matriarchal species (Kupriyanova, 1989; Darevsky *et al.*, 1985). Possibly, genes are linked with the highly derived W chromosome and the possession of these genes might be a prerequisite for unisexuality. This is especially likely given the probable sister taxon relationship of the two maternal parental species. Moreover, one of the Z chromosomes of the *rudis* group likely possesses some control factors as well, although we have not found evidence confirming this. In any case, the combination between genes from the W chromosome of the maternal (*caucasica*) clade and genes from the Z chromosome of the paternal (*rudis*) clade breaks down the normal meiotic process and produces unreduced but viable eggs.

Phylogenetic constraints, however, do not fully explain all aspects of the process of formation of unisexuals. Laboratory experiments involving crossing the parental species failed to produce viable unisexual lineages. Darevsky (unpublished data) attempted to cross *D. raddei* and *D. portschinskii*, the parents of the unisexual *D. rostombekowi*, under laboratory conditions but was unsuccessful. However, he achieved interspecific mating between *D. valentini* and *D. armeniaca*, producing sterile 3n hybrids (all triploid *Darevskia* are sterile). Danielyan (1981) successfully crossed *D. mixta* and *D. valentini*, the parents of *D. armeniaca*, by introducing females of the former species into populations of the latter, and then recapturing bred females. The progeny, although *D. armeniaca*-like in appearance, were weak, some were deformed, and all soon perished. Evidently, simply repeating the historical hybridization event does not guarantee parthenogenetic offspring.

The formation of unisexual species in *Darevskia* is likely under genetic, as well as phylogenetic, constraints. The balance hypothesis states that there is a narrow range of genetic divergence between the parental species within which F_1 hybrids have a reasonable probability of establishing unisexual lineages. Within this range, the genetic difference is hypothesized to be great enough to break down the normal meiotic process, but not so great as to effectively lower the fecundity and viability of the offspring (Moritz *et al.*, 1989). Our distance data showed that the divergences between the pairs of parental species are moderate in amount and narrow in range (Table 3). However, similar genetic distance values exist between members of the *saxicola* clade, which is not involved in the formation of unisexuals, and members of the other two clades. Thus, genetic distance alone cannot account for the formation of unisexual offspring. Furthermore, the balance hypothesis cannot explain why maternal parents are constrained to one clade, and paternals to the other.

Ecological constraints may also affect unisexuals. For example, a successful hybridization event might give rise to a unisexual lineage which may not persist because of competition or other ecological factors. The 'weed hypothesis' states that, as a consequence of competition with their parental species, unisexual species often occupy disturbed or other less preferable habitats, acting as 'weed species' (Wright & Lowe, 1968). Our observations indicate that unisexual species of *Darevskia* occur in a wide spectrum of habitats, ranging from optimal to marginal, and in some cases vastly outnumber syntopic bisexual species. Many mesic or xeric habitats, however, are occupied only by unisexuals, with all sympatric bisexuals absent (Darevsky, 1992). However, in some other cases it appears that unisexuals can exclude bisexuals from optimal, more humid habitats. At Muradiye Waterfall, Turkey, only unisexual *D. bendimahiensis* occur in the immediate vicinity of the waterfall, where humidity is high, while its maternal parental species *D. raddei* only occurs in more mesic habitats up- or downstream. The ecological constraints in *Darevskia* may not be as severe as in other groups.

Even if two species have the right combination of genes, the chance of producing parthenogenetic offspring via interspecific hybridization is infinitesimally small. The explanation for the formation of unisexuals may be more complex than simply choosing between the hypotheses of Darevsky *et al.* (1985) and Moritz *et al.* (1989). It appears that although the former explanation is more relevant than the latter, it still does not fully explain the phenomenon. The fine-line for forming unisexual species in hybridization events is phylogenetically constrained. Furthermore, these constraints are directional. These constraints form the best explanation for the formation of unisexual species of Caucasian *Darevskia*.

Numerous questions about unisexuality in many taxa remain unanswered. Nonetheless, we are convinced that these questions will be better understood when viewed in the light of defendable phylogenetic hypotheses, and only those that consider the unisexuals' parents in their phylogenetic context relative to other bisexual species. *Darevskia* provides an excellent system for study and further research on this topic will likely reveal the key elements to the origin of unisexuality in these lizards. Much more work will be needed to evaluate their applicability to other unisexual vertebrates.

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APPENDIX 1

Primers used for DNA amplification and sequencing. Primers are designed by their 3′ ends, which correspond to the position in the human mitochondrial genome (Anderson *et al.*, 1981) by convenience. H and L designate heavy- and light-strand primers, respectively.

L1921 5′ CCC GAA ACC AAA CGA GCA A 3′ (This study)
L2510 5′ CCG ACT GTT TAC CAA AAA CAT 3′ (Modified from Palumbi, 1996)
H2568 5′ CTA CCT TTG CAC GGT TAG GAT ACC GCG GC 3′ (This study)
H3060 5′ CCG GAT CCC CGG CCG GTC TGA ACT CAG ATC ACG 3′ (Palumbi, 1996)
L8552 5′ ATG AAC CTA AGC TTC TTC GAC CAA TT 3′ (Haddrath, pers. comm.)
H8956 5′ ATA AAA AGG CTA ATT GTT TCG AT 3′ (Haddrath, pers. comm.)
H9148 5′ ACG AAT ACG TAG GCT TGG ATT A 3′ (Fu *et al.*, 1999a)
L14841 5′ CCA TCC AAC ATC TCA GCA TGA TGA AA 3′ (Kocher *et al.*, 1989)
H15149 5′ GCC CCT CAG AAT GAT ATT TGT CCT CA 3′ (Kocher *et al.*, 1989)
L15153 5′ TGA GGA CAA ATA TCC TTC TGA GG 3′ (Complementary of H15149)
H15488 5′ TTG CTG GGG TGA AGT TTT CTG GGT C 3′ (Haddrath, pers. comm.)
L15369 5′ CAT GAA ACT GGA TCA AAC AAC CC 3′ (Fu *et al.*, 2000)
H15915 5′ GTC TTC AGT TTT TGG TTT ACA AGA C 3′ (Haddrath, pers. comm.)

APPENDIX 2

The alleles in [ ] are either apomorphic or pleisemorphic, which are phylogenetically uninformative, thus were ignored from the analyses. Alleles in ( ) are considered as plesiomorphic after the first level evaluation, thus ignored from the final analysis.

	Loci							
Species	*sAat-A*	*mAat-A*	*sAcoh-A*	*mAcoh-A*	*Acp-B*	*Ada-A*	*Cat-A*	*Cbp-1*
Ingroup taxa								
1. *D. alpina*	f	b	c	a	c	a	e	a
2. *D. brauneri*	[a]c	c	c	d	?	a	c	a
3. *D. caucasica*	d	b	d	g	j	a	e	a
4. *D. clarkorum*	d	c	d	d	?	a	d	a
5. *D. daghestanica*	d	b	d	g	j	a	e	a
6. *D. derjugini*	f	c	d	d	d	a	f	a
7. *D. lindholmi*	c[e]	c	b	d	?	a	c	a
8. *D. mixta*	d	c	[a,c]h	b	k	a	a	a
9. *D. parvula*	f	c	c	d	i	a	e	a
10. *D. portschinskii*	c	c	g	d	i	a	e	a
11. *D. praticola*	f	c	c	a	?	a	d	a
12. *D. raddei*	d	c	[c,f]h	d[e]	c	a	c	a
13. *D. rudis*	c	c	e	d	g	a	e	a
14. *D. saxicola*	c	c	d	d	?	a	c	a
15. *D. valentini*	c	c	e	i	h	a	b	a
Outgroup taxa								
16. *L. strigata*	f	c	c	c	f	b	c	a
17. *L. vivipara*	b	a	c	h	d	b	c	a
18. *L. media*	f	c	c	f	e	c	c	a
Hypothetical ancestor	f	c	c	?	d	?	c	a
Level of consideration	1	1	1	2	1		1	
Character type	unord	ord	unord	unord	unord	Del	unord	Del

APPENDIX 2—*continued*

	Loci									
Sp.	*Ck-A*	*Ck-C*	*Est-D*	*Gda-A*	*bGlus-A*	*bGlur-A*	*Gpi-A*	*Gpi-B*	*Gtdh-A*	*G6pdh-A*
Ingroup taxa										
1.	b	d	a	a	a	d	b	e	a	b
2.	b	b[d]	a	a	a	d	b	a	a	b
3.	b	b[d]	a	a	a	d	[b]d	d	a	b
4.	b	a[b]	a	a	a	d	b	m	a	b
5.	b	b	a	a	a	d	[b]d	d	a	b
6.	c	d	a	a	a	d	c	i	a	b
7.	b	b	a	a	a	d	b	a	a	b
8.	b	b	a	a	a	d	b	b	a	b
9.	b	b	a	a	a	d	b	k	a	b
10.	b	b	a	a	a	d	b	h	a	b
11.	b	b	a	a	a	d	f	a	a	b
12.	b	a[b]	a	a	a	d	[b]c[e]	j	a	b
13.	b	c	a	a	a	d	[a]b	h	a	b
14.	b	b	a	a	a	d	b	a	a	b
15.	b	c	a	a	a	d	b	h	a	b
Outgroup taxa										
17.	a	d	a	a	b	c	b	c	a	a
18.	b	d	a	a	c	b	b	f	a	b
19.	b	d	a	a	b	a	b	i	a	c
HA	b	d	a	a	?	?	b	i	a	b
LC		l					l	l		
CT	Del	ord[1]	Del	Del	Del	Del	unord	unord	Del	Del

APPENDIX 2—*continued*

	Loci									
Sp.	*bGa-1*	*mIdh-A*	*sIdh-A*	*Ldh-A*	*Ldh-B*	*sMdh-A*	*mMdh-As*	*Mdhp-Am*	*Mdhp-A*	*Mpi-A*
Ingroup taxa										
1.	a	b	e	b	g	a	d	f	c	g
2.	a	b	e	b	(e)g	a	d	d	c	f
3.	a	b	e	b	g	a	d	f	c	b
4.	a	b	e	b	e	a	d	e	d	c
5.	a	b	e	b[c]	g	a	d	f	c	b
6.	a	b	e	b	a	a	d	h	d	g
7.	a	b	e	b	(e)g	a	d	d	c	f
8.	a	b[c]	e	b	d	a	d	g	c	e
9.	a	e	b	b	f	a	d	f	e	d
10.	a	b	e	b	f	a	d	f	e	h
11.	a	b	e	b	g	a	d	f	g	g
12.	a	[a]b	[a]e	[a]b	d	a	d	g	h	i
13.	a	b	e	b	f	a	d	f	f	h
14.	a	b	e	b	(e)g	a	d	d	c	f
15.	a	b	e	b	f	a	d	f	f	h
Outgroup taxa										
16.	a	f	d	d	c	a	c	a	a	c
17.	a	b	g	b	c	a	a	c	b	c
18.	a	e	e	d	b	a	b	b	c	a
HA	a	b	e	b	?	a	?	?	c	c
LC					2			2	1	1
CT	Del	Del	Del	Del	unord	Del	Del	unord	unord	unord

APPENDIX 2—*continued*

	Loci						
Sp.	*Pep-B*	*Pgm-A*	*Pk-A*	*Pnp-A*	*sSod-A*	*mSod-A*	*Tpi-A*
Ingroup taxa							
1.	e[g]	d	a	b[d]	a	a	b
2.	k	d	a	d	a	?	b
3.	k	d	a	b[d]	c	a	e
4.	k	d	a	d	c	?	b
5.	[h]k	[b]d	a	b[d]	c	a	e
6.	d	d	b	d	a	c	d
7.	(d)c	d	a	d	b	?	b
8.	m	d	a	d	c	b	b
9.	f	d	a	d	a	c	b
10.	[b]c	[b]d	a	e	a	c	b
11.	k	d	a	d	a	c	b
12.	d[j]	[b]d	a	b[c,d]	c	c	b
13.	c	d	a	e	a	c	b
14.	c	d	a	d	a	c	b
15.	c	[a]d	a	e	a	c	b
Outgroup taxa							
16.	i	b[c]	a	a	a	d	a
17.	a	b	a	d	a	d	c
18.	l	b	a	d	a	d	a
HA	?	b	a	d	a	?	b
LC	2			l	l	2	2
CT	unord	Del	Del	unord	ord	ord	ord

[1] Ck-A is partially ordered. The polarization and codes are as follows: dd(0)→db→bb(1)→ba(2). c was coded as 3. From either 0, 1 or 2 to 3 requires only one step.