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G. A. Duncan
Nebraska Wesleyan University, gduncan@nebrwesleyan.edu

P. H. Alder
Clemson University

K. P. Pruess
University of Nebraska-Lincoln, kpruess@unlnotes.unl.edu

T. O. Powers
University of Nebraska-Lincoln, tpowers1@unl.edu

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Molecular Differentiation of Two Sibling Species of the Black Fly *Simulium vittatum* (Diptera: Simuliidae) Based on Random Amplified Polymorphic DNA

G. A. Duncan,[1] P. H. Adler,[2] K. P. Pruess,[3] and T. O. Powers[4]

1. Nebraska Wesleyan University, Lincoln, Nebraska 68504, USA
2. Department of Entomology, Clemson University, Clemson, South Carolina 29634, USA
3. Department of Entomology, University of Nebraska, Lincoln, Nebraska 68583, USA
4. Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583, USA

Corresponding author – K. P. Pruess, email kpruess@unlnotes.unl.edu

Abstract

Larvae of the black fly morphospecies *Simulium vittatum* from Colorado, Montana, Nebraska, and New Hampshire were cytologically identified as either the IS-7 or the IIIL-1 cytospecies. DNA was PCR amplified from cytotyped larvae using eight 10-mer primers, labeled with ^{33}P, and run on polyacrylamide gels. The entire data set of 96 amplicons produced incomplete separation of the two cytospecies when subjected to neighbor-joining and maximum parsimony analyses. However, when analyzed within geographical regions, separate species status was supported. Bootstrap support for distinctness of the two cytospecies was best in Colorado where they were collected in true sympatry. The IS-7 cytospecies was more polymorphic in the western states, where it differed most from IIIL-1, which was most variable in the eastern states. The frequencies of the 17 most common amplicons in the two cytospecies were inversely correlated. A model of speciation derived from the molecular evidence suggests that IS-7 evolved in the west and spread eastward, whereas IIIL-1 later originated in the east and spread westward.

Keywords: *Simulium vittatum*, cytospecies, RAPD analysis, molecular markers, population structure

Résumé

Des larves de morpho-espèces de la mouche noire *Simulium vittatum* provenant du Colorado, du Montana, du Nebraska ou du New Hampshire ont fait l'objet d'un examen cytologique et ont été classifiées comme appartenant aux cyto-espèces IS-7 ou IIIL-1. L'ADN de ces larves typées a été amplifié par PCR à l'aide de huit amorces décanucléotidiques marquées au 33P et les amplicons ont été séparés sur gel de polyacrylamide. Le jeu de données complet, comprenant 96 amplicons, a été analysé par les méthodes « neighbour-joining » et de parcimonie. Ces analyses ont révélé une séparation incomplète des deux cyto-espèces. L'appui par réitération (« bootstrap ») à l'hypothèse de l'existence de deux cyto-espèces distinctes était le plus élevé au sein de la population du Colorado où elles ont été trouvées en sympatrie véritable. La cyto-espèce IS-7 était plus polymorphe dans les états occidentaux, où elle différait le plus du type IIIL-1, laquelle était plus variable dans les états orientaux. Les fréquences des 17 amplicons les plus communs au sein des deux cyto-espèces étaient inversement corrélées. Un modèle de la spéciation, fondé sur les données moléculaires, suggère que le type IS-7 serait apparu dans l'ouest et se serait répandu vers l'est, tandis que le type IIIL-1 serait survenu plus tard dans l'est et se serait déplacé vers l'ouest.

Mots clés: *Simulium vittatum*, cyto-espèces, analyses RAPD, marqueurs moléculaires, structure de la population

[Traduit par la Rédaction]

Introduction

Black flies are widely distributed blood-sucking insects of the family Simuliidae. Some species are medically and economically important, such as the vectors of filarial worms that cause onchocerciasis. *Simulium vittatum*, which is found throughout North America as well as in Greenland, Iceland, and the Faroe Islands, is a pest of domesticated and wild large mammals (Rothfels and Featherston 1981).

Polytene chromosomes of black flies provide characters useful in revealing sibling species and reconstructing phylogenies (Rothfels 1989). *Simulium vittatum* has been well characterized cytogenetically (Rothfels and Dunbar 1953; Pasternak 1964; Rothfels and Featherston 1981). Pasternak (1964) provided polytene chromosome maps of *S. vittatum* and an analysis of inversion polymorphisms in natural populations, noting significant mutual exclusion of the IIIL-1 and IS-7 inversions. To explain this disequilibrium, he proposed a lethal interaction between the two inversions and argued the existence of a single species. Rothfels and Featherston (1981), however, believed *S. vittatum* to be composed of two cytospecies (= sibling species), IIIL-1 and IS-7 (named for their diagnostic sex-linked inversions), which arose out of sympatric speciation, probably in northeastern North America. Morphological characters have not been found that can distinguish the two cytospecies.

Ecological evidence corroborates the species status of IIIL-1 and IS-7 (Adler and Kim 1984). The IS-7 cytospecies is most common in the northern portion of the continent, where the larvae occur in cool, well-oxygenated streams. The IIIL-1 cytospecies, on the other hand, is more common in the central and southern regions, where the larvae occur in warmer flows and tolerate lower oxygen levels. Seasonal differences also are apparent between the two cytospecies in areas of co-occurrence, with IS-7 comparatively more abundant in the spring and fall (Adler and Kim 1984).

Molecular techniques have uncovered previously unknown or poorly understood relationships within and among species and clarified conflicting evidence. Zhu et al. (1998) surveyed 26 populations of *S. vittatum* and identified 18 mitochondrial haplotypes, with the more common haplotypes being present in both cytospecies. Tang et al. (1996), using directed heteroduplex analysis (DHDA) for the 12S and 16S mitochondrial rRNA genes, found no differences among any of eight *S. vittatum* specimens that had been cytotyped as IS-7 or IIIL-1.

RAPD (random amplified polymorphic DNA) PCR has been used to understand genetic differentiation at species, subspecies, and population levels (Wilkerson et al. 1993; Parker et al. 1998; Espinasa and Borowsky 1998; Suazo et al. 1998; Gouin et al. 2001). Short primers anneal to anonymous regions of the genome, resulting in DNA amplification products (hereafter called amplicons) of various sizes (Welsh and McClellan 1990; Williams et al. 1990). These amplicons are highly variable and can be scored as heritable characters.

We used RAPDs to test the hypothesis that IS-7 and IIIL-1 are distinct species that can be differentiated using molecular characters. Radiolabeling and large polyacrylamide sequencing gels, rather than agarose gels, were used to improve the resolution of amplicons that differ by only one, or a few, base pairs. Radiolabeling also permits the scoring of many amplicons that would not be observed on agarose gels (Wilkerson et al. 1993).

Materials and methods

Black flies

Black flies were collected from six localities in the USA (table 1). Larvae from Manitou Lake outlet (Colorado) and Indian Cave (Nebraska) were cut in the field. The abdomen with its silk glands was fixed in Carnoy's solution and the head and thorax were placed in ethanol. At the other four sites, separate samples were fixed by each method. Preparation of silk-gland polytene chromosomes followed routine procedures for the Feulgen-squash technique (Rothfels and Dunbar 1953). Larvae were identified to cytospecies using the diagnostic chromosomal characters of Rothfels and Featherston (1981). The remainder of the carcass, after removal of the gut in the case of entire larvae, was ground in a homogenization buffer, and genomic DNA for RAPD analysis was extracted with phenol-chloroform and ethanol purification methods as described by Wilkerson (1993).

Table 1. Collection data for larvae of *Simulium vittatum*

			No. of individuals of each cytospecies	
Code	Site	Date	IIIL-1	IS-7
CO1-17	Manitou Lake outlet, Teller Co., Colorado	3 August 1993	6	6
MT2-9	Deer Lodge, Powell Co., Montana	1 June 1993	0	6
NE1-30	Indian Cave, Richardson Co., Nebraska	27 April 1994	4	2
NE32-39	Denton, Lancaster Co., Nebraska	29 April 1994	6	0
NH1-6	Bellamy River, Strafford Co., New Hampshire	29 May 1990	6	0
NH10-15	Colebrook, Coos Co., New Hampshire	17 June 1993	0	6

RAPD-PCR, polyacrylamide gel conditions, and autoradiography

Before amplification, all DNA was diluted to two concentrations: 1:20 and 1:100. Both concentrations were then amplified by PCR. Initial screening for valid RAPD primers involved amplification with 58 10-mer oligonucleotide primers (Operon Technologies, Alameda, California), using the amplification conditions described by Williams et al. (1990). Eight primers (table 2) gave numerous reproducible polymorphic amplicons. PCRs were a total volume of 20 μL containing 1× Stoffel buffer (10 mM Tris–HCl, 10 mM KCl, pH 8.3); 8 μM each of dCTP, dTTP, and dGTP; 0.1 μL ^{33}P-labeled dATP (New England Nuclear, Boston, Massachusetts); 3 mM $MgCl_2$; 400 nM of primer; and 1 U Stoeffel *Taq* polymerase (Perkin Elmer, Boston, Massachusetts). Reactions were run in a Perkin Elmer 9600 thermal cycler for 35 cycles under the following conditions: 94°C for 30 s, 45°C for 30 s, and 72°C for 60 s.

Table 2. RAPD 10-mer primers used for DNA amplification and number of scored amplicons in larvae of *Simulium vittatum*

Primer	Sequence (5′→3′)	Amplicons
A4	AATCGGGCTG	14
C10	TGTCTGGGTG	11
C15	GACGGATCAG	13
E9	CTTCACCCGA	13
E12	TTATCGCCCC	9
F1	ACGGATCCTG	14
F6	GGGAATTCGG	11
F9	CCAAGCTTCC	11

The RAPD products (1.8 μL) were separated in a 30 cm × 40 cm 4% w/v polyacrylamide gel using a Bio-Rad (Hercules, California) Sequi-Gen gel apparatus at 300 V for 4600–5150 V·h, with 1× TBE in the upper and lower buffer chambers. Before electrophoresis, a ^{33}P-radiolabeled standard (1 kb) ladder (Invitrogen, Carlsbad, California) was made using the nick translation method and 0.5 μL was loaded into gel wells. After electrophoresis, the gel was dried at 80°C and exposed to Kodak X-OMAT film for 1–15 days, depending on the radioactivity level, and the film processed in Kodak developer, fixer, and stop bath according to manufacturer's instructions.

Scoring of RAPD-PCR amplicons

Only amplicons that were clearly reproducible and polymorphic were scored (fig. 1). Each of the specimens amplified by PCR was scored on a minimum of two gels. Each gel contained a different arrangement (e.g., specimen 1 might be in lane 7 of the first gel, but in lane 52 of the second gel). When there was a question as to whether or not two bands from different specimens loaded in distant gel lanes were the same, additional gels were run such that those in question were positioned side by side. This procedure increased our confidence that comigrating DNA amplicons among individuals from the same population and from different populations were homologous characters and could be used in phylogenetic analysis.

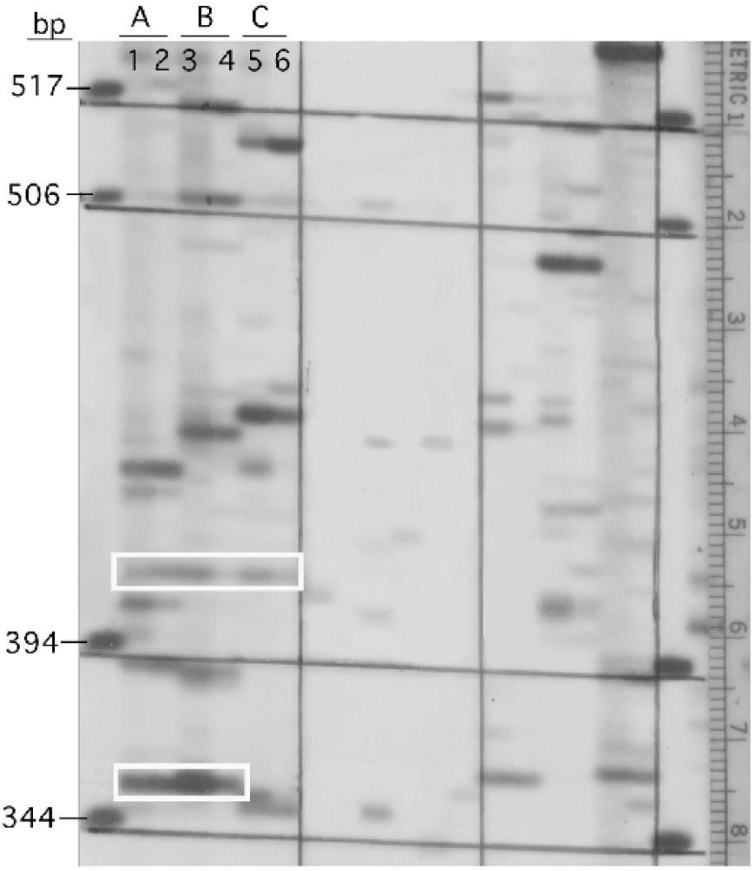

Figure 1. Portion of autoradiogram showing sharing of amplicons in *Simulium vittatum*. A, B, and C are three specimens, each amplified at two DNA concentrations. Those in the top box were scored as having the same amplicon, but only A and B were scored as being the same in the bottom box.

Phylogenetic analysis

Reproducible polymorphic RAPD-PCR bands were scored as present (assigned a value of 1) or absent (assigned a value of 0) in MacClade 3 (Maddison and Maddison 1992) and saved in a NEXUS format. The data then were imported into PAUP (Swofford 2000) for analysis, including both distance (neighbor joining) and maximum parsimony. When using maximum parsimony, analyses with taxon sizes of 12 or less were submitted to exhaustive searches, whereas larger groups were submitted to heuristic searches, using the 50% majority-rule consensus in both cases. Both methods also were subjected to bootstrap analysis (10 000 replicates). In addition to an analysis of the entire data set, the following five subgroups were analyzed: (*i*) IS-7 individuals only; (*ii*) IIIL-1 individuals only; (*iii*) the 12 New Hampshire specimens (6 IS-7, 6 IIIL-1); (*iv*) the 12 Colorado specimens (6 IS-7, 6 IIIL-1) plus the 6 Montana specimens (IS-7); and (*v*) the 12 Colorado specimens plus the 6 Denton, Nebraska, specimens (IIIL-1).

We performed χ^2 tests by summing and comparing the total frequency of presence of amplicons for all individuals of each cytospecies within populations. For this purpose, we combined Colorado and Montana (CO + MT) as representative of western isolates and Nebraska and New Hampshire (NE + NH) for eastern isolates. Correlation of the frequency of amplicons between the two cytospecies was computed using an arcsine transformation.

Results

A total of 96 reproducible polymorphic bands was scored for 42 specimens from 6 different localities. The number of scored polymorphic bands per primer ranged from 9 to 14 (table 2). No single amplicon was present in all individuals of one cytospecies and entirely lacking in the other cytospecies. Thus, only overall similarities could be compared. Only specimens from Colorado (3 males and 3 females of each cytospecies) and Indian Cave, Nebraska, were sexed. These sample sizes were too small to permit conclusions about sex-linked polymorphisms.

When either distance or parsimony analyses were conducted on the entire data set (all 42 specimens), tree topology and bootstrap support (< 50%) did not identify groupings consistent with cytospecies (not shown). In contrast, significant structuring among individuals of the IIIL-1 and IS-7 cytospecies was apparent when the full data set was partitioned according to geography. Within each of the five subgroup analyses, nearly identical tree topologies were observed when comparing trees derived by maximum parsimony and neighbor-joining analyses. All trees shown in figure 2 are from neighbor-joining analyses.

The analysis of IS-7 specimens (fig. 2A) revealed regional differences for the 80 informative RAPD bands. The Montana and Colorado specimens did not form distinct groups but were distinct from the Indian Cave and New Hampshire specimens, as indicated by the tree topology and bootstrap values. However, the analysis of IIIL-1 specimens (fig. 2B) did not show significant regional structuring for the 78 informative RAPD bands, as indicated by the topology of the tree and low bootstrap values.

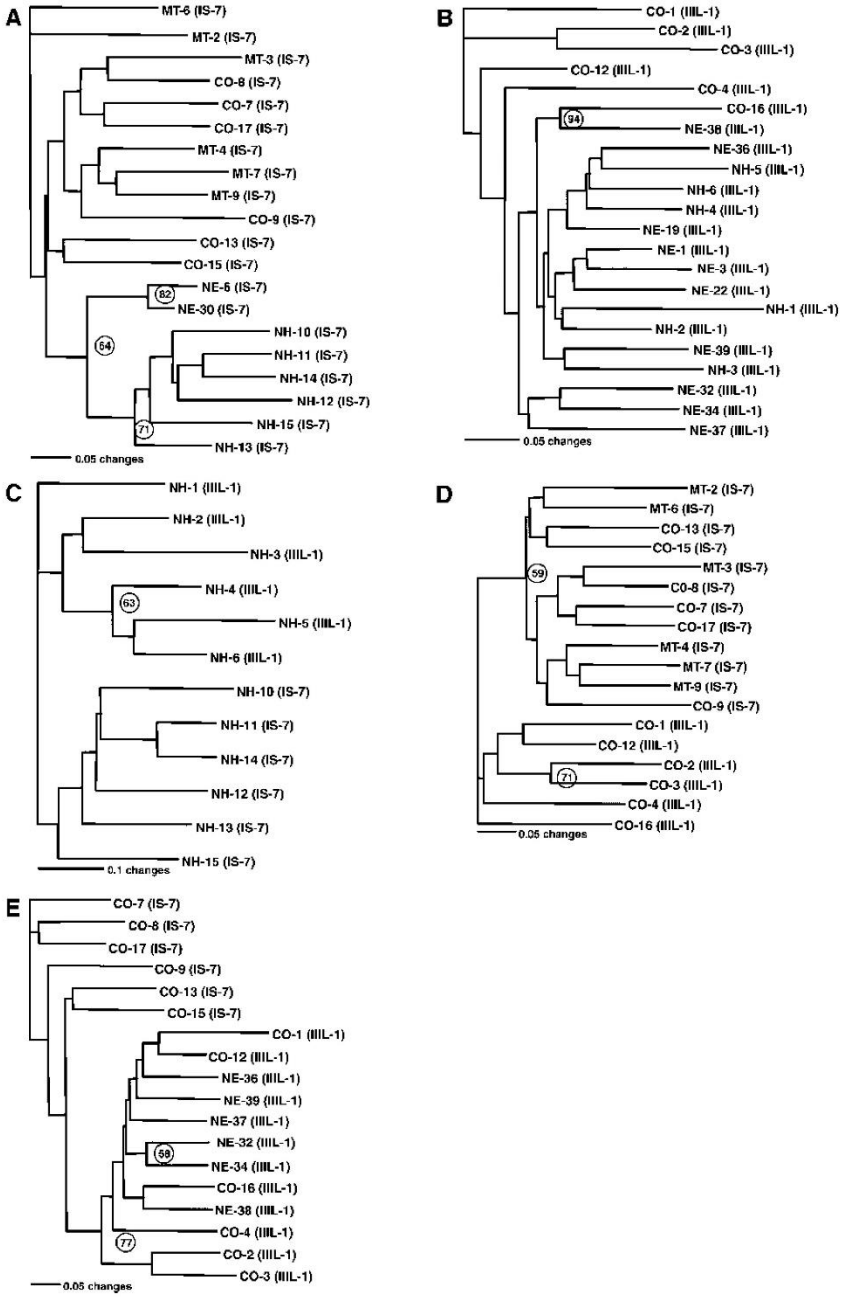

Figure 2. Neighbor-joining phylograms and bootstrap support > 50% for populations of *Simulium vittatum*. (A) All IS-7 specimens. (B) All IIIL-1 specimens. (C) New Hampshire specimens. (D) Colorado and Montana specimens. (E) Colorado and Denton, Nebraska, specimens. Parts C–E illustrate separation of cytospecies IS-7 and IIIL-1 within geographical regions.

The two sets of collections from New Hampshire formed two clades distinguished by locale and cytospecies (fig. 2C), but bootstrap values were not high. Combining the Montana and Colorado specimens (fig. 2D) produced a IS-7 clade distinct from the IIIL-1 specimens collected sympatrically with IS-7 in Colorado. In a similar analysis, combining the Colorado and Denton, Nebraska, specimens (fig. 2E) resulted in the Colorado IIIL-1 and Nebraska IIIL-1 specimens forming a clade (with 77% bootstrap support) distinct from the Colorado IS-7 specimens.

Further combinations of populations within and between geographical areas provided greater statistical support for genetic differences between cytospecies. In IIIL-1, no difference in the frequency of unique amplicons was found between western (5.9%) and eastern (5.8%) larvae ($p > 0.90$). Of shared amplicons, however, 23.0% of western IIIL-1 larvae shared amplicons with eastern IIIL-1 larvae versus 16.4% of eastern IIIL-1 larvae sharing amplicons with western IIIL-1 larvae ($p < 0.01$), suggesting that IIIL-1 is more diverse in the east. Conversely, 14.4% of western IS-7 larvae had unique amplicons versus only 3.0% of eastern IS-7 larvae ($p < 0.01$). For amplicons shared by the IS-7 larvae, 20.5% in the east had amplicons shared in the west, whereas only 15.1% of western larvae had amplicons shared with eastern larvae ($p < 0.05$), suggesting greater diversity of IS-7 in the west.

Contrasting IIIL-1 and IS-7 within geographical areas gave a similar picture. In the east, 10.3% of IIIL-1 larvae had amplicons unique to that cytospecies versus 1.9% for IS-7 ($p < 0.01$). Although not highly significant ($p < 0.10$), a similar picture emerged in the west, where 7.9% of IS-7 had amplicons found only in that cytospecies versus 4.3% for IIIL-1. Overall, 8.7% of the amplicons were unique to IIIL-1 and 9.7% were unique to IS-7. The frequencies of the 96 scored amplicons of IIIL-1 and IS-7 were not significantly correlated ($r = 0.118$, $p > 0.10$). However, for the 17 amplicons present in 50% or more of the individuals of at least one of the cytospecies, the frequencies were significantly and inversely correlated ($r = –0.564$, $p < 0.02$).

Discussion

Our molecular data are concordant with the cytogenetic and ecological data (Rothfels and Featherston 1981; Adler and Kim 1984) that IIIL-1 and IS-7 are distinct species. Although our samples are small and bootstrap support is often weak, the results provide a consistent picture supporting the existence of two distinct species, particularly when comparing IIIL-1 and IS-7 within and between geographical areas. All of our trees show distinct clades, grouped according to cytospecies. The two New Hampshire sites, although separated by some distance, had the lowest bootstrap support, possibly indicating some gene flow.

The strongest molecular evidence that IIIL-1 and IS-7 have undergone speciation comes from samples in which the two taxa are sympatric. IS-7 individuals from Colorado are more similar to IS-7 individuals from Montana than they are to IIIL-1 individuals from the same stream in Colorado. Likewise, IIIL-1 individuals from Colorado are more similar to IIIL-1 individuals from Denton, Nebraska, than they are to IS-7 individuals from the same stream in Colorado. A greater degree of regional structuring is seen among the IS-7 than the IIIL-1 populations. The molecular pattern of greater polymorphism in western versus eastern IS-7 and in eastern versus western IIIL-1 is reflected in the diversity of habitats

occupied by the larvae. Larval habitats occupied by IS-7 are more diverse in the west, whereas those occupied by IIIL-1 are more diverse in the east (Ciborowski and Adler 1990; Adler et al. 2004).

Our molecular data contrast with those of Tang et al. (1996), who found little or no difference in the directed heteroduplex analysis (DHDA) on the 12S and 16S mitochondrial rRNA genes. Although Tang et al. (1996) found identifiable differences among various species, they detected no differences between the two cytospecies of *S. vittatum*. Pruess et al. (2000) found no amino acid differences, and only three silent nucleotide changes in the mitochondrial *cytochrome oxidase II* gene (*COII*) in larvae from Iceland and IIIL-1 larvae from Nebraska. Zhu et al. (1998) identified 14 mitochondrial haplotypes, but the most common haplotype, occurring in 86% of the specimens examined, predominated in both siblings. They also found considerable polymorphism in the mitochondrial *ND4* gene but, again, the common haplotypes were shared by both cytospecies. IIIL-1 and IS-7 might have speciated so recently that genic regions of mitochondrial DNA are too conserved to provide characters for such closely related taxa.

Rothfels and Featherston (1981) found that IS-7 is more cytologically diverse and concluded that it was older. They suggested that undifferentiated sex chromosomes, which occur in Icelandic and other North Atlantic populations, represent the ancestral condition, and they proposed sympatric speciation in northeastern North America from a common ancestor lacking differentiated sex chromosomes.

Although our data do not contradict a model of speciation in northeastern North America, we suggest that alternative hypotheses, based on molecular information, are also plausible. IS-7, for example, could have arisen in western North America where its closest relatives (all other members of the subgenus *Psilozia*) occur, and where its molecular diversity is greatest. Rothfels and Featherston did not examine material from the western United States, where IS-7 is widely distributed and has differentiated sex chromosomes. We accept that IIIL-1 could have arisen from an ancestor lacking differentiated sex chromosomes but suggest that the ancestor was an entity similar to IS-7 that lost its differentiated sex chromosomes as it spread eastward, perhaps during or following the most recent glaciation (about 10 000 years ago). Laboratory colonies of IS-7 derived from a single source population illustrate how rapidly chromosomal changes can occur. Over a period of 18 years (> 100 generations), the frequency of four of the five most common inversions showed significant differences among colonies, and lack of the sex-linked IS-7 inversion in one colony was more pronounced than in any known field population (Brockhouse and Adler 2002). We suggest that populations in extreme northeastern North America and Iceland, which lack differentiated sex chromosomes but share the common autosomal polymorphisms with other North American populations, are derived from an entity similar to IS-7.

The inverse relationship for the frequencies of the more common amplicons of the two cytospecies mimics the cytological pattern in which all major autosomal inversions are shared between the two cytospecies but differ in their frequencies. The amplicons, however, do not provide the degree of cytospecies discrimination that is possible using chromosomal inversions. The explanation for this discrepancy might lie in the role that

chromosomal rearrangements have putatively played in the speciation process. Chromosomal rearrangements, especially in the form of coadapted sex chromosomes (as in *S. vittatum*), are believed to have driven speciation in the Simuliidae (Rothfels 1989), implying that changes in nucleotide sequences need not have occurred.

We interpret the origin of IIIL-1 to be recent, based on the massive sharing of cytological and molecular characters that differ only in frequency. Rapid temperature changes during the Quarternary period (Adams et al. 1999) likely imposed selection pressures. IIIL-1, with its adaptation to warmer water (Adler and Kim 1984), might have evolved during this time and acquired its own differentiated sex chromosomes, based on the IIIL-1 banding sequence. Cytospecies IIIL-1 and IS-7 occur sympatrically over a much broader range than was known to Rothfels and Featherston (Adler et al. 2004). As IIIL-1 spread westward into an area occupied by IS-7, greater molecular and cytological differentiation of the two species resulted. Less differentiation of populations of IIIL-1 might be due not only to its more recent origin but also to the wide diversity of streams occupied by its immature stages (Adler and Kim 1984), thus resulting in fewer bottlenecks in its westward spread. The proliferation of impoundments and irrigation ditches have possibly contributed to an increased abundance and geographical range of the IIIL-1 cytospecies (Adler et al. 2004).

In conclusion, nuclear DNA data (in the form of RAPDs) support the cytogenetic and ecological evidence that *S. vittatum* comprises two genetically distinct entities. Our speciation hypothesis, consistent with the molecular evidence, postulates a western origin for IS-7 and a subsequent eastern origin for IIIL-1.

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