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Brief report
Microsatellite-based species identification method for *Drosophila virilis* group species

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Species of the *D. virilis* group are widely used in evolutionary research, but the individuals of different species are difficult to distinguish from each other morphologically. We constructed a fast and easy microsatellite-based identification method for the species of the group occurring sympatrically in northern Europe. The neighbor joining tree based on 14 microsatellite loci also gave a good resolution of the species divergence pattern in the whole group.

The *Drosophila virilis* group species have been extensively used in studies concerning genome structure, speciation and evolution of species-specific behavioural characters (Patterson and Stone 1952; Throckmorton 1982; Hoikkala et al. 2005; Routtu et al. 2007). The group consists of 11-14 species or subspecies, depending on whether *D. canadiana* is included, whether *D. borealis* is separated into eastern and western forms and whether *D. americana americana* and *D. americana texana* are classified as subspecies. Spicer and Bell (2002) have divided the group into four phylads based on the phylogeny of mitochondrial 12S and 16S rRNA genes: the *virilis* phylad (*D. virilis*, *D. americana americana*, *D. a. texana*, *D. novamexicana* and *D. lummei*), the *montana* phylad (*D. montana*, *D. lacicola*, *D. borealis* eastern and *D. borealis* western and *D. flavomontana*), the *littoralis* phylad (*D. littoralis*, *D. canadiana* and *D. ezoana*) and the *kanekoi* phylad represented by *D. kanekoi*.

The species of *D. virilis* group are especially suitable for speciation studies as they represent different levels of divergence with an estimated time of 9.0 ± 0.7 mya between *virilis* and *montana* phylad species and 2.6 ± 0.4 mya between *D. virilis* and the other species in the *virilis* phylad (Nurminsky et al. 1996). Many of these species can be crossed with each other to study the genetic basis of interspecific differences (Throckmorton 1982). Also, geographically isolated populations of some species of the group offer a good opportunity to study the effects of ecological and behavioural factors on population divergence, and to trace the genetic mechanisms underlying the process. For example the divergence time between the *D. montana* populations from different continents is estimated to be from 0.45 to 0.9 mya (Päällysaho et al. 2005; Mirol et al. 2007).

Species of the *D. virilis* group share some morphological characters such as a relatively large size for Drosophilidae and a dark shade around the longest cross-vein of the wing, which make identification at the group level easy. The species are, however, difficult to distinguish from each other. Researchers have used different methods for identifying the species of wild-collected flies, such as classifying the flies on the basis of external male genitalia (Lakovaara and Hackman 1973; Kulikov et al. 2004) female spermathecae (Pitnick et al. 1999), mating flies with laboratory-reared flies of known species (Liimatainen and Hoikkala 1998) and recording and analyzing the songs of wild-caught males (Huttunen et al. 2002). Species-identification has also been made by using protein gel electrophoresis (Lakovaara et al. 1976; Spicer 1991) and RAPD fingerprinting (Mikhailovsky et al. 2007). Unfortunately, these methods are not very accurate and/or they are very time consuming and require special expertise.

Microsatellite markers have been used earlier to explore the phylogeny of the *D. virilis* group species (Orsini et al. 2004) and to map genes that affect male song traits (Huttunen et al. 2004). Orsini et al. (2004) focused their study on the species of the *virilis* phylad. We have used here a similar approach with a special emphasis on North-European species. The main aim of this study was to develop a fast and easy species identification method for the *D. virilis* group species occurring sympatrically in Northern Europe (*D. montana*, *D. littoralis*, *D. ezoana* and *D. lummei*; Aspi et al. 1993; Bächli et al. 2005). For this purpose the microsatellite locus *Vir72ms* appeared to be especially informative. The 14 polymorphic microsatellite loci used in the study amplified in all *D. virilis* group species, which gave us an opportunity also to check how well they cluster the fly strains of different species throughout the group and to identify possible misclassifications.

MATERIAL AND METHODS

50 microsatellite loci (20 loci from Orsini and Schlötterer 2004 and 30 microsatellites designed

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on the basis of the available *D. virilis* scaffolds (SCHÄFER et al. pers.com.)) were screened with PCR for all *D. virilis* group species. The list of strains/individual flies representing the study species with information on their geographical origin is given in Appendix 1. A total of 14 loci could be amplified in all species and therefore they were selected for further analyses (Table 1). Genomic DNA was extracted from the fly samples using the high salt extraction method (MILLER et al. 1988) adapted to *Drosophila* flies. PCR was performed using fluorescent 6-Fam or Yakima yellow (TAGC, Copenhagen, Denmark) labelled forward primer in a 10 μl reaction volume with 50–100 ng genomic DNA, 1 μM of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$ and 1 unit of Taq DNA Polymerase (BioTools). The PCR profile was kept for 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 30 s at 52–59°C and 30 s at 72°C, and finally one cycle of 10 min at 72°C in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). Alleles were separated with formamide and analysed with an ABI 3100 automatic sequencer (Applied Biosystems). The resulting spectrograms were genotyped with GeneMapper 3.7 software (Applied Biosystems).

The Excel Microsatellite Toolkit (PARK 2001) was used to calculate the mean number of alleles (MNA) and allele ranges over all loci for each species. An individual-based microsatellite genetic distance matrix with unequal sample sizes for different species was constructed with GenAlEx 6.031 (PEAKALL and SMOUSE 2006), where a genetic distance for a single locus with i, j, k and l alleles is defined as a set of squared distances; $d^2(ii,ii)=0$, $d^2(ij,ij)=0$, $d^2(ii,ij)=1$, $d^2(ij,ik)=1$, $d^2(ij,kl)=2$, $d^2(ii,jk)=3$ and $d^2(ii,jj)=4$. Genetic distances were visualised with MEGA 3.1 (KUMAR et al. 2004) (Fig. 1). The program STRUCTURE (PRITCHARD et al. 2000) with a Bayesian model-based clustering method was used to assign individuals to homogenous clusters (species). This method estimates the fraction of individual multilocus genotypes belonging to each cluster. The number of clusters is inferred by calculating the probability P(X|K) of the data, given a certain prior value of K (number of clusters) over a number of Markov chain Monte Carlo (MCMC) iterations. The scores of individuals in the clusters correspond to the probability of ancestry in any one of them. An admixture model with correlated allele frequencies was used for

Table 1. *Microsatellite loci used in this study with chromosome position, expected size, locus name, annealing temperature, repeat type and primer sequences. Microsatellites developed by SCHÄFER et al. (pers.com.), except for Mon26, Mon6, Mon17a and Mon20, developed by ORSINI and SCHLÖTTERER (2004).*

Chromosome	Expected size	Locus	Ta°C	Microsatellite repeat	Primer sequences (5′–3′)
X	152	*Vir99ms*	55	CA	F: acaatgcttgcacaatgacg R: ccatgcaaattgtgaactgc
X	140	*Vir72ms*	55	TA	F: ggtccctgctcagaacaact R: cgctgcttaagccaacaata
2	204	*Vir32ms*	55	CA	F: gggtgttgatgtcgagtgtg R: aagaagtccaaagcgctcaa
2	155	*Vir4ms*	55	GT	F: ttgcaatattcccccatttc R: gcggcagaaatgacattgac
2	245	*Vir38ms*	55	CA	F: gaattcgcaatgcacgtaaa R: cgacgtatctgtgagccact
3	157	*Vir90ms*	55	GT	F: caattaaaaggaccgcctga R: tcattatgcggaaatgctga
3	139	*Vir93ms*	55	GA	F: acgtggtccaagcaatttgt R: tgagctcccgaccagtttag
3	149	*Vir11ms*	55	TA	F: gcaaaacatgaataatgcgaac R: ctttgacaatggcaccacac
4	162	*Vir17ms*	55	GT	F: tgcaacgttcggtagtcaag R: cggctttgggtcttagattg
5	149	*Vir74ms*	55	TA	F: ggcaggttttatcaggcaac R: tggcatctcgatacgcataa
X	114–144	*Mon26*	52	TG	F: gagtggcagacacaacctca R: gccaacagtgcacgtaatttt
2	156–168	*Mon6*	59	GTT	F: gtccgaaccacgcaataact R: gctgttgatgatgatgaggc
Unknown	134–162	*Mon17a*	56	GT	F: atatctgtgcagaggcaggg R: tgaaattcaagtgcagcgac
Unknown	144–148	*Mon20*	58,5	TG	F: gcagcagccacaatatcaaa R: ggctgctgttgttaaaggct

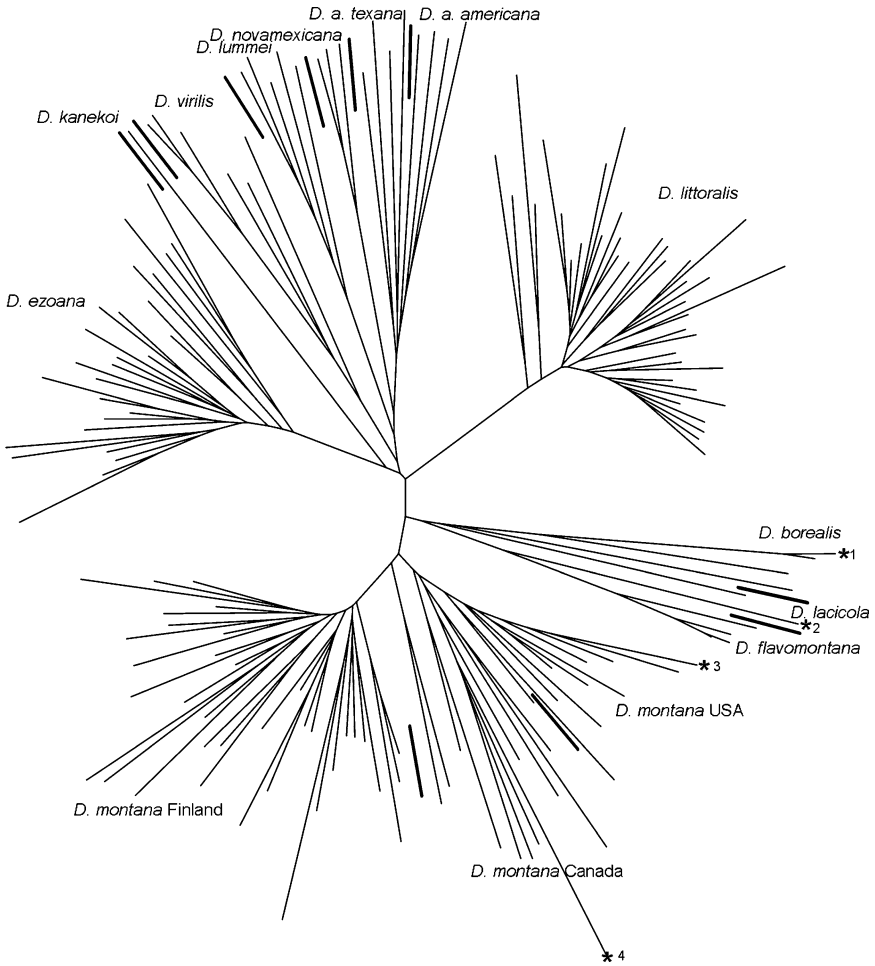

Fig. 1. Unrooted neighbor joining tree based on 14 microsatellite loci. Misclassified individuals: *1; *D. flavomontana* strain 15010-0981.00, *2; *D. lummei* strain 1100, *3; *D. borealis* strain 15010-0961.03 and *4; *D. lacicola* strain 15010-0991.13.

the clustering analysis. Several runs of various lengths (10 000) were performed for each number of clusters (K), testing K values from 7 to 14. Finally, to choose the best value of K, two independent runs (200 000 iterations, 200 000 steps) were done using K values 12 and 13.

RESULTS AND DISCUSSION

Species of the *D. virilis* group are difficult to identify at the species level and several of them could be classified as cryptic species. This causes a lot of difficulties in research, especially as the populations of many of these species occur sympatrically in the wild. Our aim was to make the identification of the flies collected in northern Europe easier, as well as to study species clustering at the group level and to detect possible misclassifications in laboratory strains. The new species identification method was based on a set of 14 polymorphic microsatellite loci, which can be amplified in all *D. virilis* group species (except one locus which did not amplify in *D. borealis*). Samples for the three species found in the wild in northern Europe (*D. ezoana*, *D. littoralis* and *D. montana*) and *D. flavomontana* included wild-caught flies in addition to the flies of laboratory strains of the species. Other species were represented by the flies of one to six laboratory strains (Table 2).

The mean number of alleles across all microsatellite loci ranged from 1.1 to 9.3 (Table 2). A distribution of allele frequencies among loci showed species-specific variation, which was most pronounced in the *Vir72ms* locus with several species specific diagnostic alleles with no overlap in size (Table 3). This locus separated *D. kanekoi*, *D. littoralis, D. canadiana*, *D. ezoana*, *D. novamexicana* and *D. virilis* into their own groups. Interestingly, *D. a. americana* shared one allele with *D. a. texana* and one with *D. lummei*. In addition, *D. montana*, *D. flavomontana*, *D. borealis* and *D. lacicola* shared one fixed or nearly fixed allele. The

Table 2. *Species of D. virilis group with information on their sample size, the number of microsatellite loci typed and the mean number of alleles over all studied loci.*

Species	No. of strains	No. of wild-caught individuals	No. of loci	Mean no. of alleles
D. a. americana	4		14	2.8
D. a. texana	4		14	3.1
D. borealis	3		13	2.3
D. canadiana	1		14	1.1
*D. ezoana**	3	24	14	5.8
D. flavomontana	2	3	14	2.3
D. kanekoi	1		14	1.0
D. lacicola	2		14	2.0
*D. littoralis**	5	32	14	6.8
*D. lummei**	5		14	2.1
*D. montana**	32	25	14	9.3
D. novamexicana	3		14	1.5
D. virilis	6		14	3.1

*species occurring sympatrically in northern Europe (*D. lummei* has not been found in the wild for several years).

microsatellite locus *Vir72ms* appeared to be especially informative for identification of the North-European species of the group (*D. montana*, *D. littoralis*, *D. ezoana* and *D. lummei*); all of these species had diagnostic allele or alleles at this locus (Table 3). This locus will be especially valuable for detecting rare species like *D. lummei* which, to our knowledge, has not been collected and identified in Fennoscandia during the last 20 years.

The neighbor joining tree based on 14 microsatellite loci (Fig. 1.) gave a good resolution of an overall species divergence pattern. It clustered *D. virilis* phylad species in one branch close to *D. ezoana*. Contrary to the groupings in Orsini et al. (2004) and Spicer and Bell (2002), *D. a. americana* grouped together with *D. a. texana*. This grouping is supported by Schäfer et al. (2006), suggesting that the two subspecies are just chromosomal forms of a single species. *D. montana* populations were clustered in one branch subdivided into three different clades according to the geographical origin of the strains/individuals (Finland, Canada and USA). Other species of the *montana* phylad clustered close to *D. montana* as expected. One *D. lacicola*, one *D. flavomontana*, one *D. lummei* and one *D. borealis* strain clustered among nonconspecifics. *D. kanekoi* had its own well differentiated branch which is similar to phylogenies based on mtDNA 12S and 16S rRNA (Spicer and Bell 2002) and nuclear *adh* gene (Nurminsky et al. 1996).

Multilocus genotypes were clustered into groups which were similar to those constructed with the neighbor joining tree in this study (Fig. 1). The assignment success was high ($>81\%$) in almost all samples (Table 4). The model that explained the data best ($P \sim 1.000$) partitioned the *D. virilis* group species into twelve clusters, while all of the other models were inadequate ($P < 0.001$). Clustering of multilocus genotypes placed all *D. virilis* group species except

Table 3. *Allele frequencies (%) in the Vir72ms locus for all species in the D. virilis group. The frequencies of the alleles separating the species of the North-European species community are shown in bold. Abbreviations are AME (D. a. americana), BOR (D. borealis), CAN (D. canadiana), EZO (D. ezoana), FLA (D. flavomontana), KAN (D. kanekoi), LAC (D. lacicola), LIT (D. littoralis), LUM (D. lummei), MON (D. montana), NOV (D. novamexicana), TEX (D. a. texana) and VIR (D. virilis).*

	VIR	AME	TEX	LUM	NOV	LIT	CAN	MON	FLA	BOR	LAC	EZO	KAN
84													100.0
86						**100.0**	100.0						
88								**97.4**	100.0	100.0	100.0		
90												**100.0**	
92					100.0								
94								**2.6**					
100		66.7		**100.0**									
102	100.0												
104		33.3	100.0										

Table 4. *Minimum percentage of D. virilis group species belonging to the 12 clusters inferred by the multilocus clustering method. Numbers of individuals assigned to a certain cluster are given in the parentheses.*

Species	n	1	2	3	4	5	6	7	8	9	10	11	12
D. a. americana	4								81.4 (4)				
D. a. texana	4								91.3 (4)				
D. borealis	3	98.5 (1)										98.7 (2)*	
D. canadiana	1										98.6 (1)		
D. ezoana	27		84.7 (27)										
D. flavomontana	4											98.6 (1)	97.8 (3)
D. kanekoi	1					98.2 (1)							
D. lacicola	2									97.8 (1)		74.0 (1)	
D. littoralis	36										89.8 (36)		
D. lummei	6			97.6 (5)									78.7 (1)
D. novamexicana	3				98.6 (3)								
D. virilis	5							89.1 (5)					
D. montana	57	87.4 (9)					83.6 (36)			77.4 (11)			

*one *D. borealis* individual was assigned to this cluster with 62.9% success.

D. montana strains, one *D. borealis* strain, one *D. lummei* strain, two *D. flavomontana* strains and two *D. lacicola* strains into a single cluster. *D. montana* flies were assigned into three separate clusters according to their geographic origin (Finland, Canada and USA), except for the three Canadian individuals that had admixture origins of Canadian and USA clusters. As in the neighbor joining tree, one western *D. borealis* strain (0961.03) clustered with *D. montana* from Colorado, USA (with 98.5% success), *D. canadiana* clustered with *D. littoralis* (with 98.6% success), and *D. a. americana* clustered together with *D. a. texana* to a single cluster with 81.4% and 91.3% success, respectively.

The species status for some members of the *D. virilis* group has been recently under debate (Nurminsky et al. 1996; Schlötterer 2000; Spicer and Bell 2002; Orsini and Schlötterer 2004; Schäfer et al. 2006). To get more information on the species status of *D. canadiana* and to clarify the misclassified *D. borealis* strain, we performed some interstrain crosses (data not shown). Both the neighbor joining tree and the clustering method clustered *D. canadiana* with *D. littoralis*. Interspecific hybrids between these two species appeared to be fully fertile showing no significant pre- and postzygotic barriers. Also the male courtship songs of the two species are indistinguishable (Hoikkala unpubl.). Thus, the species status of *D. canadiana* is questionable. The *D. borealis* strain, which clustered with *D. montana* (Colorado) strains in the neighbor joining tree, mated and produced offspring with a *D. montana* (Colorado) strain without any pre- or postzygotic barrier symptoms, which suggested it to be *D. montana*. The fact that the flies of this strain did not produce offspring with the flies of western or eastern *D. borealis* strains gives further support to this conclusion.

Finally, we tested our species identification method for the flies collected in Jyväskylä, Finland (62°15′N, 25°45′E) in spring 2007. The *Vir72ms* locus separated the flies of different species accurately and fast: among thirty genotyped individuals, 22 flies were *D. littoralis* and 8 flies *D. montana.*

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APPENDIX 1. The strains/individuals of the *Drosophila virilis* group species with their ID number, strain code, geographical origin and collection year, if known.

Species	ID	Strain/individual	Origin	Year
D. a. americana	AME1	G 96.47	Gary, Indiana, USA	
	AME2	G 96.48	Gary, Indiana, USA	
	AME3	NN 97.2	Niobrara, Nebraska, USA	
	AME4	NN 97.8	Niobrara, Nebraska, USA	
D. a. texana	TEX1	ML 97.3	Monroe, Louisiana, USA	
	TEX2	ML 97.5	Monroe, Louisiana, USA	
	TEX3	LP 97.7	Lone Star, Texas, USA	
	TEX4	CD 97.5	Lone Star, Texas, USA	
D. borealis	BOR1	C3F7	Crested Butte, Colorado, USA	2003
	BOR2	0961.00	Itasca Park, Minnesota, USA	1950
	BOR3	0961.03	Chester, Idaho, USA	1949
	BOR4	0961.05	Quebec, Canada	1949
D. canadiana	CAN1	1091.00	British Columbia, Canada	
D. ezoana	EZO1	EZO 2	Kemi, Finland	1995
	EZO2	E 04.3	Oulanka, Finland	2004
	EZO3	EZO/OU	Oulu, Finland	2003
	EZO4	O 25*	Oulanka, Finland	2002
	EZO5	O 59*	Oulanka, Finland	2002
	EZO6	O 70*	Oulanka, Finland	2002
	EZO7	O 71*	Oulanka, Finland	2002
	EZO8	O3F13*	Oulanka, Finland	2003
	EZO9	O3F17*	Oulanka, Finland	2003
	EZO10	03F22*	Oulanka, Finland	2003
	EZO11	O3F29*	Oulanka, Finland	2003
	EZO12	O3F46*	Oulanka, Finland	2003
	EZO13	O3F49*	Oulanka, Finland	2003
	EZO14	O3F55*	Oulanka, Finland	2003
	EZO15	O3F62*	Oulanka, Finland	2003
	EZO16	O3F69*	Oulanka, Finland	2003
	EZO17	O3F70*	Oulanka, Finland	2003
	EZO18	O3F75*	Oulanka, Finland	2003
	EZO19	O3F76*	Oulanka, Finland	2003
	EZO20	O3F84*	Oulanka, Finland	2003
	EZO21	O3F88*	Oulanka, Finland	2003
	EZO22	O3F102*	Oulanka, Finland	2003
	EZO23	O3F108*	Oulanka, Finland	2003
	EZO24	O3F115*	Oulanka, Finland	2003
	EZO25	O3F118*	Oulanka, Finland	2003
	EZO26	O3F119*	Oulanka, Finland	2003
	EZO27	O3F124*	Oulanka, Finland	2003
D. flavomontana	FLA1	0981.00	Chester, Idaho, USA	1949
	FLA2	0981.02	Craig, Colorado, USA	1949
	FLA3	COL 1*	Colorado, USA	2003
	FLA4	COL 3*	Colorado, USA	2003
	FLA5	COL 5*	Colorado, USA	2003
D. kanekoi	KAN1	1061.00	Sapporo, Japan	
D. lacicola	LAC1	0991.13	Manitoba, Canada	1949
	LAC2	0991.12	Fenske Lake, Minnesota, USA	1947
D. littoralis	LIT1	LITTO 1	Jyväskylä, Finland	2006
	LIT2	LI 10	Don-river, Rostov, Russia	2000
	LIT3	1052	Batumi, Georgia, Russia	1973
	LIT4	OU 7	Oulanka, Finland	2004
	LIT5	KU 05.5	Kuopio, Finland	2005
	LIT6	LIT 2*	Jyväskylä, Finland	2006
	LIT7	LIT 3*	Jyväskylä, Finland	2006
	LIT8	LIT 4*	Jyväskylä, Finland	2006
	LIT9	LIT 5*	Jyväskylä, Finland	2006

APPENDIX 1 (*Continued*)

Species	ID	Strain/individual	Origin	Year
	LIT10	LIT 1*	Jyväskylä, Finland	2006
	LIT11	04.1198*	Jyväskylä, Finland	2006
	LIT12	04.1241*	Jyväskylä, Finland	2006
	LIT13	04.1254*	Jyväskylä, Finland	2006
	LIT14	04.1264*	Jyväskylä, Finland	2006
	LIT15	04.1281*	Jyväskylä, Finland	2006
	LIT16	04.1304*	Jyväskylä, Finland	2006
	LIT17	04.1308*	Jyväskylä, Finland	2006
	LIT18	04.1323*	Jyväskylä, Finland	2006
	LIT19	04.1326*	Jyväskylä, Finland	2006
	LIT20	04.1335*	Jyväskylä, Finland	2006
	LIT21	04.1346*	Jyväskylä, Finland	2006
	LIT22	04.1351*	Jyväskylä, Finland	2006
	LIT23	04.1401*	Jyväskylä, Finland	2006
	LIT24	04.1455*	Jyväskylä, Finland	2006
	LIT25	04.1491*	Jyväskylä, Finland	2006
	LIT26	04.1506*	Jyväskylä, Finland	2006
	LIT27	04.1514*	Jyväskylä, Finland	2006
	LIT28	04.1555*	Jyväskylä, Finland	2006
	LIT29	04.1580*	Jyväskylä, Finland	2006
	LIT30	04.1591*	Jyväskylä, Finland	2006
	LIT31	K 34*	Kemi, Finland	2002
	LIT32	K 47*	Kemi, Finland	2002
	LIT33	L 2*	Lappajärvi, Finland	2002
	LIT34	L 3 *	Lappajärvi, Finland	2002
	LIT35	L 4*	Lappajärvi, Finland	2002
	LIT36	L 5*	Lappajärvi, Finland	2002
	LIT37	L 6*	Lappajärvi, Finland	2002
D. lummei	LUM1	200	Moscow, Russia	1988
	LUM2	LU 1	Kemi, Finland	1990
	LUM3	1100	Vaajasalo, Kuopio, Finland	1969
	LUM4	1101S, W;60	Överkalix, Sweden	1970
	LUM5	1143	Sakata, Yamagata, Japan	1974
	LUM6	LuJapFu	Hokkaido, Japan	1974
D. montana	MON1	C3F6	Crested Butte, Colorado, USA	2003
	MON2	CAN3F4	Vancouver, Canada	2003
	MON3	03F66	Oulanka, Finland	2003
	MON4	MO 4	Oulu, Finland	2000
	MON5	JKL	Jyväskylä, Finland	2006
	MON6	1021.13	Kawasaki, Japan	
	MON7	C3F2	Crested Butte, Colorado, USA	2003
	MON8	C3F13	Crested Butte, Colorado, USA	2003
	MON9	CAN3F1	Vancouver, Canada	2003
	MON10	CAN3F2A	Vancouver, Canada	2003
	MON11	CAN3F2B	Vancouver, Canada	2003
	MON12	CAN3F3	Vancouver, Canada	2003
	MON13	CAN3F5	Vancouver, Canada	2003
	MON14	CAN3F8	Vancouver, Canada	2003
	MON15	CAN3F12	Vancouver, Canada	2003
	MON16	CAN3F13	Vancouver, Canada	2003
	MON17	CAN3F14	Vancouver, Canada	2003
	MON18	CAN3F15	Vancouver, Canada	2003
	MON19	O3F20	Oulanka, Finland	2003
	MON20	O3F39	Oulanka, Finland	2003
	MON21	O3F43	Oulanka, Finland	2003
	MON22	O3F52	Oulanka, Finland	2003
	MON23	O3F63	Oulanka, Finland	2003
	MON24	O3F77	Oulanka, Finland	2003
	MON25	O3F79	Oulanka, Finland	2003
	MON26	O3F80	Oulanka, Finland	2003
	MON27	O3F94	Oulanka, Finland	2003

APPENDIX 1 (*Continued*)

Species	ID	Strain/individual	Origin	Year
	MON28	O3F97	Oulanka, Finland	2003
	MON29	C3F1*	Crested Butte, Colorado, USA	2003
	MON30	C3F5*	Crested Butte, Colorado, USA	2003
	MON31	C3F8*	Crested Butte, Colorado, USA	2003
	MON32	C3F9*	Crested Butte, Colorado, USA	2003
	MON33	L 1*	Lappajärvi, Finland	2002
	MON34	L 5*	Lappajärvi, Finland	2002
	MON35	L 7*	Lappajärvi, Finland	2002
	MON36	L 7.2*	Lappajärvi, Finland	2002
	MON37	L 7.3*	Lappajärvi, Finland	2002
	MON38	K 10.1*	Kemi, Finland	2002
	MON39	K 12.2*	Kemi, Finland	2002
	MON40	K 14.1*	Kemi, Finland	2002
	MON41	K 15.1*	Kemi, Finland	2002
	MON42	K 22.1*	Kemi, Finland	2002
	MON43	JKL 1*	Jyväskylä, Finland	2006
	MON44	JKL 2*	Jyväskylä, Finland	2006
	MON45	JKL 3*	Jyväskylä, Finland	2006
	MON46	JKL 4*	Jyväskylä, Finland	2006
	MON47	JKL 5*	Jyväskylä, Finland	2006
	MON48	K 21*	Kemi, Finland	2002
	MON49	K 36*	Kemi, Finland	2002
	MON50	K 48*	Kemi, Finland	2002
	MON51	O 61*	Oulanka, Finland	2002
	MON52	O3F26	Oulanka, Finland	2003
	MON53	O3F42	Oulanka, Finland	2003
	MON54	O3F106	Oulanka, Finland	2003
	MON55	O3F112	Oulanka, Finland	2003
	MON56	COL 2*	Colorado, USA	2003
	MON57	COL 4*	Colorado, USA	2003
D. novamexicana	NOV1	1031.00	Grand Junction, Colorado, USA	
	NOV2	1031.04	Moab, Utah, USA	1949
	NOV3	1031.08	San Antonio, New Mexico, USA	1947
D.virilis	VIR1	V-WW-03	Wuwei, China	2002
	VIR2	V-QUFU	Qufu, China	2002
	VIR3	TOYAMA-1	Toyama, Japan	2004
	VIR4	TOYAMA-11	Toyama, Japan	2004
	VIR5	DNH	Dunghuang, China	2002
	VIR6	HUNAN	Hunan, China	2002

*wild-caught individual.