A core set of microsatellite markersfor western corn rootworm (Coleoptera: Chrysomelidae) population genetics studies

Kyung Seok Kim, Uwe Stolz, Nicholas J. Miller, Eric R. Waits, Thomas Guillemaud, Douglas V. Sumerford, Thomas W. Sappington

A Core Set of Microsatellite Markers for Western Corn Rootworm (Coleoptera: Chrysomelidae) Population Genetics Studies

KYUNG SEOK KIM,[1] UWE STOLZ,[2,3] NICHOLAS J. MILLER,[1,4] ERIC R. WAITS,[2] THOMAS GUILLEMAUD,[4] DOUGLAS V. SUMERFORD,[1] AND THOMAS W. SAPPINGTON[1,5]

ABSTRACT Interest in the ecological and population genetics of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, has grown rapidly in the last few years in North America and Europe. This interest is a result of a number of converging issues related to the increasing difficulty in managing this pest and the need to characterize and understand gene flow in the context of insect resistance management. One of the key components needed for successful population genetics studies is the availability of suitable molecular markers. Using a standard group of microsatellite markers enables researchers from different laboratories to directly compare and share their data, reducing duplication of effort and facilitating collaborative work among laboratories. We screened 22 candidate microsatellite loci against five criteria to create a core set of microsatellite markers for *D. v. virgifera* population genetics studies. The criteria for inclusion were moderate to high polymorphism, unambiguous readability and repeatability, no evidence of null alleles, apparent selective neutrality, and no linkage between loci. Based on our results, we recommend six microsatellite markers to be included as a core set in future population genetics studies of *D. v. virgifera* along with any other microsatellite or genetic markers. As more microsatellites are developed, those meeting the criteria can be added to the core set. We encourage other groups of researchers with common interests in a particular insect species to develop their own core sets of markers for population genetics applications.

KEY WORDS *Diabrotica*, western corn rootworm, microsatellites, population genetics, DNA markers

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, causes huge economic losses annually in the United States (Metcalf 1983, Sappington et al. 2006) and is now responsible for increasing damage in Europe as it continues to expand its range after its initial detection in the early 1990s (Barčić et al. 2003, Hemerik et al. 2004, Miller et al. 2005). In addition, this pest is becoming more difficult to manage in parts of its range in North America because of the development of resistance to chemical insecticides (Meinke et al. 1998, Wright et al. 2000) and to crop rotation (Levine et al. 2002). Transgenic *Bt* corn expressing the Cry3Bb1 toxin from *Bacillus thuringiensis* has proven very effective in the United States (Al-Deeb and Wilde 2005), but there is concern that *D. v. virgifera* could evolve resistance to this product as well. An insect resistance management (IRM) strategy has been mandated in the United States to prolong the effective life of *Bt* corn, but the effectiveness of the IRM plan depends in part on rates and patterns of movement among rootworm populations, something that is not well understood. Indeed, the rate of resistance evolution will depend in part on both movement and gene flow, as well as the size of populations. The availability of robust molecular markers for rootworms will be instrumental in population genetics studies to determine effective population sizes, genetic structuring, and rates of gene flow.

Any type of genetic marker that is neutral and polymorphic can be used for studying the population genetics of *Diabrotica* or any other insect. Microsatellites enjoy a number of advantages making them a popular genetic marker system, widely used to test ecological and evolutionary hypotheses in natural or experimental populations (Zhang and Hewitt 2003, Selkoe and Toonen 2006). They are highly polymorphic, codominant, and usually effectively neutral. In addition, this marker system is reliable and amenable to multiplexing, high-throughput genotyping, and automated scoring. Although developing new microsatellite markers

[1] USDA–ARS Corn Insects and Crop Genetics Research Unit, Genetics Laboratory, Iowa State University, Ames, IA 50011.

[2] U.S. Environmental Protection Agency, Molecular Ecology Research Branch, National Exposure Research Laboratory, 26 W Martin Luther King Dr., Cincinnati, OH 45268.

[3] Current address: College of Public Health, The Ohio State University, M-006 Starling-Loving Hall, 320 West 10th Ave., Columbus, OH 43210.

[4] Equipe "Biologie des Populations en Interaction," UMR 1112 R.O.S.E. INRA-Université de Nice-Sophia Antipolis, 400 Route des Chappes, BP 167 06903 Sophia Antipolis cedex, France.

[5] Corresponding author, e-mail: Tom.Sappington@ars.usda.gov.

by sequencing clones from repeat-enriched genomic libraries usually involves considerable time and significant cost (Zane et al. 2002), once they are developed, genotyping is rapid and cost effective. The establishment of an international *Diabrotica* Genetics Consortium was fueled in large part by the desire of scientists in several laboratories to avoid costly duplication of effort in developing microsatellite markers for this insect (Sappington et al. 2006). *Diabrotica* microsatellite markers have been developed by three collaborating laboratories (Kim and Sappington 2005b, Kim et al. 2008, Waits and Stolz 2008). Several of these markers have been successfully used to assess genetic structure of both natural populations (Kim and Sappington 2005a, Miller et al. 2006, 2007, Kim et al. 2008) and laboratory colonies (Kim et al. 2007) of *D. v. virgifera*, and to identify routes of invasion in Europe (Miller et al. 2005).

One of the priorities established by consortium scientists meeting at the International *Diabrotica* Genetics Conference in December 2004 was to develop a core set of microsatellite markers to facilitate future *D. v. virgifera* population genetics studies (Sappington et al. 2006). Use of the same microsatellites in different laboratories will allow direct comparisons among data sets, including studies of temporal changes in genetic structure within a region, spatial differences in structure across regions, and meta-analyses. Moreover, common use of a core set of markers allows direct sharing of *D. v. virgifera* genotype datasets across laboratories, thereby reducing unnecessary duplicate genotyping of overlapping populations. For example, the Agricultural Research Service provided raw microsatellite genotype data from U.S. populations analyzed for one study (Kim and Sappington 2005b) to the French team using the same markers to analyze invasion routes of *D. v. virgifera* in Europe (Miller et al. 2005), thus saving the latter significant resources and time to the benefit of all.

Here, we report development of a core set of microsatellite markers for *D. v. virgifera* that we recommend for any future population genetics studies of this insect where microsatellites are to be used. We describe the criteria used for inclusion of a marker in the core set, the methods for testing candidate markers, and standardization of the chosen markers across laboratories. We encourage other laboratories to consider collaborating in the development of core sets of microsatellites for population genetics applications in their target species as well, and hope this case study with *D. v. virgifera* will provide a useful template for developing such panels.

Materials and Methods

Microsatellites and Fragment Analysis

A total of 22 rootworm microsatellites were considered for inclusion in the *D. v. virgifera* core set of microsatellites for population genetic studies. These candidate loci were initially isolated by the USDA–ARS, Corn Insects and Crop Genetics Research Unit, Ames, IA, from a *D. v. virgifera* genomic DNA using biotin-enrichment methods (Kim and Sappington 2005b), and the U.S. Environmental Protection Agency, Molecular Ecology Research Branch (EPA), Cincinnati, OH. The EPA laboratory developed microsatellites from genomic DNA pooled from Mexican corn rootworms, *D. v. zeae* Krysan and Smith, and northern corn rootworms, *D. barberi* Smith and Lawrence, by an enrichment method using core repeat motifs different from the ARS team (Waits and Stolz 2008). *D. v. virgifera* and *D. v. zeae* are subspecies that differ morphologically and are partially reproductively isolated, but which hybridize in nature (Krysan et al. 1980, Giordano et al. 1997). Cross-amplification of loci in these subspecies is usually successful (Kim and Sappington 2005b, Waits and Stolz 2008).

Genotyping of individuals was conducted using adults of mixed sexes that were frozen after collection. Genomic DNA was extracted from individual rootworms using Bio-Rad's Aqua Pure isolation kit (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. Each microsatellite locus was amplified in multiplexed or single polymerase chain reactions (PCR) in three laboratories: ARS, EPA, and the Institut National de la Recherche Agronomique (INRA), Sophia Antipolis, France. The PCR fragments were analyzed by capillary gel electrophoresis on two different automated sequencing systems: the EPA and INRA laboratories each used an ABI 3100 (Applied Biosystems, Foster City, CA), and ARS used a Beckman-Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Genotypes were determined using Applied Biosystems' Genemapper software (EPA) or SoftGenetics' GeneMarker program, version 1.40 (SoftGenetics, State College, PA) (INRA) for data from the ABI sequencers and using CEQ 8000 Software, version 5.0 (ARS), for data from the Beckman-Coulter CEQ 8000.

Evaluating Microsatellite Loci for Inclusion in the Core Set

The following criteria were used to guide the selection of microsatellite markers for the core set: moderate to high level of polymorphism; unambiguous readability and repeatability across laboratories; no evidence of null alleles; apparent selective neutrality; and not linked with other core markers.

Polymorphism. Polymorphism of each microsatellite marker was examined for a sample of 61 *D. v. virgifera* adults collected from Ankeny, IA, in 2003. Measures of polymorphism include the number of alleles per locus (A), observed heterozygosity (H_O), and unbiased estimates of expected heterozygosity (H_E) under Hardy-Weinberg assumptions (Nei 1987), all of which were calculated using the program CERVUS 2.0 (Marshall et al. 1998). We considered moderate to high polymorphism ($0.4 \leq H_E < 0.9$) necessary for possible inclusion in the core set of microsatellites. Markers of low polymorphism may have insufficient power to detect structure among populations unless they are highly divergent. Con-

versely, hypervariable microsatellites are at increased risk of size homoplasy, where alleles that are identical in state are not identical by descent, thus leading to underestimates in the degree of between-population divergence (Estoup et al. 1995). Such markers will underestimate measures of population differentiation such as F_{ST} (Estoup et al. 1995; Hedrick 1999), although they can be very useful for applications such as population assignment or parentage analysis.

Readability and Repeatability. DNA from the same set of 16 *D. v. virgifera* individuals collected from Pennsylvania in 2004 was amplified and genotyped independently at three laboratories (ARS, EPA, INRA) and evaluated for readability and repeatability at each locus. The laboratories used different combinations of automatic sequencers and software for allele size calls and genotyping, as indicated above. The program MICRO-CHECKER (Oosterhout et al. 2004) was used to screen for technical errors that can occur during PCR amplification, such as stuttering, large allele dropout, and null alleles.

Null Alleles. A null allele arises from a mutation in a primer binding site that prevents PCR amplification of the microsatellite locus. If one of two alleles at a locus in an individual does not amplify, the genotype at that locus will be scored as homozygous for the allele that does amplify, when in fact the true identity of the unamplified allele is unknown. The presence of null alleles in the population thus biases estimates of population genetics parameters through overestimation of homozygosity, so it is important to exclude markers harboring such alleles from the core set. To screen for null alleles, controlled single-pair crosses of *D. v. virgifera* were made as described by Kim et al. (2008). Genomic DNA was extracted from the parents and ≈50 of the F_1 offspring from each cross. At each microsatellite locus, we checked for distortions to expected Mendelian segregation of alleles among offspring based on parental genotypes. All 22 microsatellite loci were examined across 10 families. The frequency (p) at which a null allele occurs and can be expected to be detected with 95% confidence from a given number of genes (n) can be calculated from the equation $Y = (1 - p)^n$, where Y is the chosen probability level of not detecting a null allele (here, 0.05). Thus, for 10 families, assuming the parents in the single-pair matings were unrelated, we expected with 95% confidence to detect a null allele present in the population at a frequency ≥0.072. The potential occurrence of null alleles also was tested in the Iowa sample of 61 adults using the program MICRO-CHECKER (Oosterhout et al. 2004). MICRO-CHECKER infers the presence of a null allele when significant excess homozygosity is distributed evenly across all of the alleles at a locus.

Selective Neutrality. Population genetics structure analyses are based on the premise that genetic variation and evolutionary mechanisms have identical effects across neutral loci. Thus, it is imperative that the markers used are selectively neutral. Absence of selection at each locus was examined by the Ewens-Watterson homozygosity test (Watterson 1978) and the Ewens-Watterson-Slatkin exact test (Slatkin 1994, 1996) using allele frequency distribution, as implemented with the software package Arlequin 3.1 (Schneider et al. 2000). In these tests, the null distribution of the homozygosity statistic (F_{exp}) under the infinite-alleles model and the sampling theory of neutral alleles of Ewens (1972) are obtained from computer simulations and are compared with the observed homozygosity (F_{obs}) from the original sample. Significantly low P values ($P < 0.05$) indicate the null hypothesis of selective neutrality should be rejected and imply the presence of selection, which can take the form of balancing selection in favor of heterozygotes or directional selection of advantageous alleles. Although these tests cannot prove the absence of selection, they are the best available at this time, and any loci they flag should be removed from consideration.

Linkage Relationships. If two marker loci do not assort independently, estimates of population genetics parameters will be biased. Thus, even when two microsatellite loci are suitable for population studies in every other way, if they are statistically linked, only one should be used in a given analysis. Parents and ≈50 offspring from four controlled families were genotyped and used to test for linkage between the candidate microsatellite loci. Potential linkage between each locus pair was examined with χ^2 goodness-of fit tests between the observed numbers of genotypes in the offspring and those expected under free recombination of the loci. We also tested for linkage disequilibrium between pairs of loci for a sample of 61 *D. v. virgifera* adults collected from Ankeny, IA, in 2003 based on the Fisher method, using the genotypic disequilibrium option implemented in the program GENEPOP (Raymond and Rousset 1995).

Results

Polymorphism

Of the 22 *D. v. virgifera* candidate microsatellites screened for inclusion in the core set, 13, 8, and 1 had dinucleotide, trinucleotide, and tetranucleotide repeat motifs, respectively (Kim and Sappington 2005b, Waits and Stolz 2008). In the survey of 61 individuals, the number of alleles per locus ranged from 2 to 20, and H_E values ranged from 0.192 to 0.886. Three different measures showed the highest polymorphism in *DVV-D3* and the lowest in *Dba01.* Five markers had a high level of polymorphism, with $0.8 \leq H_E < 0.9$. The majority of markers had a moderate level of polymorphism, with $0.4 \leq H_E < 0.8$, whereas four showed low levels of polymorphism with $H_E < 0.4$ (Kim and Sappington 2005b, Waits and Stolz 2008).

Readability and Repeatability

Most of the markers were easy to score and were repeatable across the three laboratories. However, readability of a few of the markers—*DVV-D9, DVV-D10, DVV-D11,* and *DVV-T1*—was variable across laboratories. Possible scoring errors caused by stuttering

Table 1. Genotyping characteristics of each *D. v. virgifera* microsatellite based on MICRO-CHECKER analysis and evidence of null alleles based on family analysis

Locus	MICRO-CHECKER				Family analysis[a]
	Homozygote excess	Scoring error from stuttering	Large allele dropout	Null allele (frequency)	Null allele (no. parents with null allele)
DVV-D1	No	No	No	No (0.045)	Yes (5)
DVV-D2	No	No	No	No (0.054)	No
DVV-D3	Yes	No	No	Yes (0.062)	Yes (3)
DVV-D4	No	No	No	No (−0.038)	No
DVV-D5	No	No	No	No (−0.000)	Yes (1)
DVV-D6	Yes	No	No	Yes (0.163)	Yes (3)
DVV-D7	Yes	Yes	No	Yes (0.153)	Yes (5)
DVV-D8	No	No	No	No (−0.000)	No
DVV-D9	No	No	No	No (0.061)	No
DVV-D10	No	No	No[b]	No (0.025)	No
DVV-D11	No	No	No	No (0.008)	No
DVV-D12	Yes	No	No	Yes (0.132)	Yes (5)
DVV-D13	Yes	No	No	Yes (0.098)	Yes (9)
DVV-T1	No	No	No[b]	No (0.069)	Yes (1)[c]
DVV-T2	No	No	No	No (−0.009)	No
DVV-T3	No	No	No	No (0.011)	No
DVV-T4	Yes	Yes	No	Yes (0.163)	Yes (2)
Dba01	No	No	No	No (−0.113)	No
Dviz11	No	No	No	No (0.029)	No
Dba03	No	No	No	No (−0.044)	No
Dba05	No	No	No	No (−0.022)	No
Dba07	No	No	No	No (−0.016)	No

[a] Based on a survey of Mendelian inheritance of offspring and parental genotypes in 10 unrelated families.
[b] Marker showed substantial difference in band intensity between the small and large alleles by manual examination.
[c] Might be caused by the poor readability of large allele size in one family.

were detected in *DVV-D7* and *DVV-T4* (Table 1). The MICRO-CHECKER analysis showed no evidence of large-allele dropout. However, differences in band intensity between the small and large alleles was observed for *DVV-T1* and *DVV-D10*, making scoring of alleles at these loci difficult in some individuals.

Null Alleles

For 9 of 22 loci examined by family analysis, alleles of the offspring did not match one of the parents in at least one family, showing abnormal segregation and therefore the presence of a null allele. Six of these loci also were flagged by the MICRO-CHECKER analysis as having null alleles and homozygote excess (Table 1). Thus, three loci had null alleles that were too rare in the Iowa population to cause substantial deviations from Hardy-Weinberg expectations and be detected by MICRO-CHECKER. Although family analysis is the most sensitive approach for detecting null alleles, it is not always a practical option, and MICRO-CHECKER offers the best alternative.

Selective Neutrality

The homozygosity test of selective neutrality showed that observed homozygosity for two loci, *DVV-T1* and *Dba03*, departed significantly ($P < 0.05$) from expected values (Table 2). The *DVV-D3* locus also showed evidence of non-neutrality in one of two neutrality tests (i.e., the Ewens-Watterson-Slatkin exact test). Thus, the null hypothesis of selective neutrality was not rejected for 19 of the candidate loci.

Linkage Relationships

Linkage analysis using controlled families showed that the locus pairs *DVV-D8*/*DVV-D9*, *Dba01*/*Dviz11*,

Table 2. Results of selective neutrality tests of the 22 microsatellite loci in a western corn rootworm population

Locus	F_{obs}[a]	F_{exp}[b]	Watterson F *P* value	Slatkin's exact *P* value
DVV-D1	0.265	0.318	0.413	0.270
DVV-D2	0.232	0.314	0.261	0.083
DVV-D3	0.122	0.187	0.082	**0.038**
DVV-D4	0.323	0.384	0.399	0.315
DVV-D5	0.779	0.673	0.650	0.507
DVV-D6	0.552	0.508	0.662	0.682
DVV-D7	0.218	0.259	0.418	0.479
DVV-D8	0.133	0.135	0.604	0.370
DVV-D9	0.422	0.501	0.390	0.645
DVV-D10	0.179	0.265	0.152	0.333
DVV-D11	0.162	0.236	0.125	0.142
DVV-D12	0.166	0.238	0.154	0.098
DVV-D13	0.164	0.215	0.258	0.331
DVV-T1	0.285	0.585	**0.010**	**0.012**
DVV-T2	0.500	0.682	0.219	0.134
DVV-T3	0.625	0.819	0.196	0.196
DVV-T4	0.445	0.501	0.455	0.313
Dba01	0.810	0.811	0.420	0.420
Dviz11	0.632	0.681	0.428	0.198
Dba03	0.233	0.495	**0.005**	**0.005**
Dba05	0.503	0.508	0.547	0.881
Dba07	0.384	0.439	0.445	0.753

Significant deviation from neutrality ($P \leq 0.05$) indicated in bold.
[a] The observed homozygosity calculated by the sum of the squares of allele frequencies.
[b] The expected homozygosity under neutrality generated by simulating random neutral samples.

Table 3. Summary of performance of *D. v. virgifera* microsatellites relative to each criterion for inclusion in the core set

Locus	Polymorphism[a]	Readability and repeatability[b]	Evidence of null alleles[c]	Selective neutrality[d]	Linkage between loci
DVV-D1	M	C	Yes	Yes	—
DVV-D2	M	C	No	Yes	*Dba03*[e]
DVV-D3	H	C	Yes	No	—
DVV-D4	M	C	No	Yes	—
DVV-D5	L	V	Yes	Yes	—
DVV-D6	M	C	Yes	Yes	—
DVV-D7	M	C	Yes	Yes	—
DVV-D8	H	C	No	Yes	*DVV–D9*[e], *DVV–D10*[f]
DVV-D9	M	V	No	Yes	*DVV–D8*[e]
DVV-D10	M	V	No	Yes	*DVV–D8*[f]
DVV-D11	H	V	No	Yes	—
DVV-D12	H	C	Yes	Yes	—
DVV-D13	H	C	Yes	Yes	—
DVV-T1	M	V	Yes	No	—
DVV-T2	M	C	No	Yes	—
DVV-T3	L	C	No	Yes	—
DVV-T4	M	C	Yes	Yes	—
Dba01	L	C	No	Yes	*Dviz11*[e]
Dviz11	L	C	No	Yes	*Dba01*[e]
Dba03	M	C	No	No	*DVV-D2*[e]
Dba05	M	C	No	Yes	—
Dba07	M	C	No	Yes	—

[a] Based on the expected heterozygosity (H_E); 0 < low (L) < 0.4, 0.4 ≤ moderate (M) < 0.8, 0.8 ≤ high (H) < 0.9.
[b] C, clear; V, variable across laboratories.
[c] Presence of null allele based on family analysis.
[d] Yes denotes the null hypothesis of selective neutrality against the presence of selection was not rejected for that locus at $P = 0.05$, and no denotes the null hypothesis of neutrality was rejected for that locus.
[e] Linkage analysis from controlled family.
[f] Linkage disequilibrium based on Fisher's exact test for genotypic linkage disequilibrium using the program GENEPOP 3.3 (Raymond and Rousset 1995).

and *Dba03*/*DVV-D2* are tightly linked to one another. The exact tests for genotypic linkage disequilibrium between pairs of loci (231 tests) showed significant linkage disequilibrium for *DVV-D8*/*DVV-D10*, after correction for multiple tests. *DVV-D8* was implicated by both tests but as part of different locus pairs. Thus, seven loci were implicated in linkage relationships by at least one test.

Recommended Core Set and Standardization

Performance of the 22 loci relative to each of the criteria for inclusion in the core set of *D. v. virgifera* microsatellites (i.e., moderate to high polymorphism, easily scored alleles, no evidence of null alleles, apparent selective neutrality, and no linkage with other core loci) is summarized in Table 3. There were six microsatellites that satisfied all of the above criteria and are recommended as the core set for use in future population genetics studies of this species: *DVV-D2*, *DVV–D4*, *DVV–D8*, *DVV-T2*, *Dba05*, and *Dba07* (Table 4).

In addition to the six core microsatellites, five additional loci should be usable in studies of *D. v. virgifera* populations but are excluded from the core set because of an H_E value <0.4 (*DVV-T3* and *Dviz11* or

Table 4. Recommended core set of *D. v. virgifera* microsatellite markers for population genetics studies and characteristics from a survey of 61 wild individuals[a]

Locus name (repeat motif)	Primer sequences (5′–3′)	Size range (bp)	No. of alleles	GenBank accession no.
DVV-D2 (dinucleotide)	F: CACGCAGCACTTAATTGGTTT R: CTATGCCTCCCAATTCGTGT	182–208	9	AY738532
DVV-D4 (dinucleotide)	F: TGTGTGCAGTGTCCCGTTAT R: GTGGCCAGTATTCACGACCT	221–239	7	AY738534
DVV-D8 (dinucleotide)	F: AAGGCAGGTAGTAATGTTGGTGA R: TCATCACTAATGGGGAAACGA	211–249	20	AY738538
DVV-T2 (trinucleotide)	F: ATCGGTTTTGGCTGGATATG R: GTTCAACAACTCGCAAACCA	212–224	3	AY738546
Dba05 (trinucleotide)	F: GCTGAGGAGGCTTATGTC R: CAATGGAGGTTGGCTATT	215–235	5	EF524280
Dba07 (tetranucleotide)	F: ATCGGTGTAACTTTTCCACA R: CACATCGGCATAGGATAGAC	215–235	6	EF524282

[a] Data for *DVV* loci are from Kim and Sappington (2005b). *Dba* loci were described by Waits and Stolz (2008), but data for size range and no. of alleles were determined independently in this study from the same set of 61 wild individuals as the *DVV* loci.

Table 5. Genotypes of 16 western corn rootworm individuals for the core set of *D. v. virgifera* microsatellites from the EPA laboratory and relative offset in allele size for genotypes from other laboratories

Individual ID	*DVV-D2*			*DVV-D4*			*DVV-D8*			*DVV-T2*			*Dba05*			*Dba07*		
	EPA	INRA	ARS	EPA	INRA	ARS	EPA	INRA	ARS	EPA	INRA	ARS	EPA	INRA	ARS	EPA	INRA	ARS
1	184/186	3/3	2/2	231/237	6/6	4/4	223/247	5/5	6/6	214/226	4/4	2/2	224/227	5/5	3/3	217/221	4/4	2/2
2	186/186	3/3	2/2	237/237	6/6	4/4	221/247	5/5	6/6	214/226	4/4	2/2	224/227	5/5	3/3	221/221	4/4	2/2
3	186/210	3/3	2/2	225/229	6/6	4/4	223/227	5/5	6/6	214/226	4/4	2/2	224/227	5/5	3/3	221/221	4/4	2/2
4	186/186	3/3	2/2	233/239	6/6	4/4	245/251	5/5	6/6	226/226	4/4	2/2	224/224	5/5	3/3	217/237	4/4	2/2
5	184/186	3/3	2/2	229/233	6/6	4/4	217/249	5/5	6/6	226/226	4/4	2/2	227/230	5/5	3/3	221/221	4/4	2/2
6	184/186	3/3	2/2	231/233	6/6	4/4	221/225	5/5	6/6	214/223	4/4	2/2	224/227	5/5	3/3	221/237	4/4	2/2
7	184/190	3/3	2/2	229/229	6/6	4/4	249/249	5/5	6/6	226/226	4/4	2/2	227/227	5/5	3/3	221/237	4/4	2/2
8	186/204	3/3	2/2	225/231	6/6	4/4	221/223	5/5	6/6	214/226	4/4	2/2	227/227	5/5	3/3	221/221	4/4	2/2
9	186/208	3/3	2/2	229/231	6/6	4/4	221/223	5/5	6/6	226/226	4/4	2/2	227/227	5/5	3/3	221/237	4/4	2/2
10	184/204	3/3	2/2	229/237	6/6	4/4	223/245	5/5	6/6	223/226	4/4	2/2	224/227	5/5	3/3	221/237	4/4	2/2
11	186/204	3/3	2/2	231/231	6/6	4/4	217/249	5/5	6/6	226/226	4/4	2/2	224/227	5/5	3/3	217/237	4/4	2/2
12	186/208	3/3	2/2	229/239	6/6	4/4	223/245	5/5	6/6	216/226	4/4	2/2	227/227	5/5	3/3	221/221	4/4	2/2
13	184/186	3/3	2/2	225/231	6/6	4/4	219/223	5/5	6/6	226/226	4/4	2/2	224/227	5/5	3/3	217/217	4/4	2/2
14	186/186	3/3	2/2	231/231	6/6	4/4	221/223	5/5	6/6	226/226	4/4	2/2	227/227	5/5	3/3	221/237	4/4	2/2
15	186/186	3/3	2/2	231/233	6/6	4/4	223/227	5/5	6/6	223/226	4/4	2/2	227/227	5/5	3/3	217/221	4/4	2/2
16	186/192	3/3	2/2	231/231	6/6	4/4	223/227	5/5	6/6	226/226	4/4	2/2	227/227	5/5	3/3	221/221	4/4	2/2

Each genotype from the INRA and ARS laboratories were subtracted from corresponding EPA genotypes.

Dba01), linkage disequilibrium with a core microsatellite (*DVV–D10*), or they exhibited variable readability across laboratories (*DVV-D9, DVV–D10, DVV–D11*). Although variability across laboratories disqualifies the latter markers from the core set, they probably will be consistent and work well within any particular laboratory. *DVV-T3* and *Dviz11* exhibited only two and three alleles, respectively, in the Iowa population surveyed, but more alleles may yet be discovered in other populations.

Apparent allele sizes can differ depending on the equipment and software used, so standardization of allele calls using reference DNA from a common panel of individuals is important (Roslin Institute 2002). For the selected core set of microsatellites, we compared genotypes obtained from 16 *D. v. virgifera* individuals in three different laboratories (ARS, EPA, INRA) to test the consistency of genotyping using different electrophoresis platforms and analysis software. Allele calls differed by up to six bases, depending on the laboratory and the locus. However, there was a consistent offset in allele size between the laboratories, so that samples showed the same genotypes across the three laboratories after correcting for these offsets in allele calls (Table 5).

Discussion

Although a standardized set of microsatellite markers is desirable for studying the population genetics of any organism, it is especially useful for organisms that are the subject of research in multiple laboratories and countries. Obvious candidates include organisms of economic significance and wide geographic distribution, including insect pests such as *D. v. virgifera.*

The concept of a core set, or universal panel, of microsatellite markers for use across all laboratories for a given organism was pioneered by researchers concerned with conserving genetic diversity in domestic farm animals (FAO 1995, Bradley 1996, Baumung et al. 2004, Hoffmann et al. 2004). However, despite widespread familiarity with this concept within the livestock genetics community and virtually unanimous acknowledgment of its benefits, adoption of the recommended markers has been patchy depending on the species (Baumung et al. 2004). Failure to adopt the recommended markers most often occurs when a laboratory already has developed its own markers and does not want to switch to new ones for various reasons (Baumung et al. 2004). In other words, inconsistent adoption arises from an embarrassment of riches: hundreds of microsatellite markers had been developed for various species of farm animals in multiple laboratories by the mid-1990s when the first list of recommended markers was compiled (FAO 1995). With a few exceptions, such as *Drosophila* (Wilder et al. 2002) and other insects whose genomes have been sequenced (Archak et al. 2007), there is no such embarrassment of riches for insect species. Ironically, the relative lag in progress in developing microsatellites in insects provides entomologists with a valuable window of opportunity to standardize markers from the outset for their favorite species. Such an opening now exists for the western corn rootworm, as recognized by *Diabrotica* Genetics Consortium participants (Sappington et al. 2006).

Based on our results, we propose a core set of six *D. v. virgifera* microsatellites that have met stringent criteria for usefulness in population genetics applications. We identified five additional markers that are also generally suitable for population genetics studies, although they did not meet all the proposed criteria. On the basis of a survey of 16 *D. v. virgifera* individuals, the core set of microsatellites gave the same genotypes across three laboratories after correcting for systematic offsets in allele size estimates. Therefore, we recommend that at least this core set of markers be used in future population genetic studies of *D. v. virgifera.*

When such a core set of markers is constructed for other insects, it will be important to survey polymorphism from more than one population. In the case of *D. v. virgifera,* previous studies using the four core

DVV loci in multiple populations over extensive geographic areas in the United States and Europe showed that polymorphism in the Iowa sample of 61 individuals is representative of the other populations (Kim and Sappington 2005a). Together with the observed genetic homogeneity of populations across much of the United States (Kim and Sappington 2005a), this indicates that the two core *Dba* markers can be expected to show similar polymorphism across space as well.

The criteria used to identify the core set of microsatellites can be applied to any additional microsatellites that might be developed for this species, and new markers meeting the criteria can be added to the core set in the future. For example, a potentially large number of microsatellites could be developed inexpensively through mining *D. v. virgifera* expressed sequence tag (EST) databases (Kim et al. 2008). Although EST-derived microsatellites loci are part of an expressed gene and thus may be subject to direct selection, background selection, or genetic hitchhiking (Li et al. 2002), polymorphisms in EST-derived microsatellites often behave as effectively neutral markers and can provide valid information about the genetic structure of natural populations (Woodhead et al. 2005, Kim et al. 2008).

To take the best advantage of the core set of markers for *D. v. virgifera*, the next step will be to create a publicly accessible central database to archive all genotypes at core-set loci generated from any study that uses them. Researchers would either directly upload their genotype data or submit it for uploading. The laboratory responsible for managing the database also should maintain the reference standards for free sharing with any laboratory intending to use the core set. Until such a database is established, the reference DNA will be maintained by the corresponding author and shared with any interested laboratories.

Because of its adaptability to new environments and invasiveness, the evolution of insecticide and crop rotation resistance, potential for evolving resistance to rootworm-active *Bt* corn, considerable but not well-defined dispersal capacity, and economic importance in a growing number of countries, *D. v. virgifera* has drawn the attention of an international community of researchers interested in its ecological genetics (Sappington et al. 2006). Researchers from >20 institutions from five countries currently are participating in the *Diabrotica* Genetics Consortium (Sappington et al. 2006), and microsatellite markers are now being used in at least eight different laboratories in the United States, Canada, France, the United Kingdom, and Australia. Clearly, corn rootworm genetics researchers will benefit from the standardization of microsatellite markers and sharing of reference DNA. This resource will facilitate synergistic cooperation, resulting in significant savings in research time and resources, as well as accelerating progress in understanding and managing this difficult pest.

We encourage other groups of entomologists with shared interests in the ecological genetics of a particular insect species to establish a core set of markers as well. It is never too late to do so. However, the earlier in the process of marker development such an effort is initiated, the higher the future adoption rate of the recommended markers will be, and the fewer will be the lost opportunities for direct comparisons among datasets.

Acknowledgments

We thank J. Gibson and M. Ciosi for technical assistance at the USDA–ARS and INRA, respectively, and M. Bagley and M. Antolin for helpful comments and encouraging this endeavor. N.J.M. and T.G. were partly funded by the Department Santé des Plantes et Environnement of the French Institut National de la Recherche Agronomique. The International *Diabrotica* Genetics Conference was made possible by USDA-CSREES NRI Competitive Grant 2004-35607-15062 to T.W.S. and B.D.S, and by a grant to T.G. from the Office of Science and Technology of the French Consulate in Chicago, IL.

References Cited

Al-Deeb, M. A., and G. E. Wilde. 2005. Effect of *Bt* corn expressing the Cry3Bb1 toxin on western corn rootworm (Coleoptera: Chrysomelidae) biology. J. Kans. Entomol. Soc. 78: 142–152.

Archak, S , E. Meduri, P. S. Kumar, and J. Nagaraju. 2007. InSatDb: a microsatellite database of fully sequenced insect genomes. Nucleic Acids Res. 35 (Suppl 1): D36–D39.

Barčić, J. I., R. Bažok, and M. Maceljski. 2003. Research on the western corn rootworm (*Diabrotica virgifera virgifera* LeConte, Coleoptera: Chrysomelidae) in Croatia (1994–2003). Entomol. Croat. 7: 63–83.

Baumung, R., H. Simianer, and I. Hoffmann. 2004. Genetic diversity studies in farm animals—a survey. J. Anim. Breed. Genet. 121: 361–373.

Bradley, D. G. 1996. Genetic diversity in cattle: selected DNA markers. (http://www.projects.roslin.ac.uk/cdiv/markers.html).

Estoup, A., C. Taillez, J. M. Cornuet, and M. Solignac. 1995. Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apis mellifera* and *Bombus terrestris* (Apidae). Mol. Biol. Evol. 12: 1074–1084.

Ewens, W. J. 1972. The sampling theory of selectively neutral alleles. Theor. Popul. Biol. 3: 87–112.

[FAO] Food and Agriculture Organization of the United Nations. 1995. Global project for the maintenance of domestic animal genetic diversity (MoDAD). (http://dad.fao.org/).

Giordano, R., J. J. Jackson, and H. M. Robertson. 1997. The role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. Proc. Natl. Acad. Sci. U.S.A. 94: 11439–11444.

Hedrick, P. W. 1999. Perspective: highly variable loci and their interpretation in evolution and conservation. Evolution 53: 313–318.

Hemerik, L., C. Busstra, and P. Mols. 2004. Predicting the temperature-dependent natural population expansion of the western corn rootworm, *Diabrotica virgifera.* Entomol. Exp. Appl. 111: 59–69.

Hoffmann, I., P. A. Marsan, J. Stuart, F. Barker, E. G. Cothran, O. Hanotte, J. A. Lenstra, D. Milan, S. Weigend, and H. Simianer. 2004. New MoDAD marker sets to be used in diversity studies for the major farm animal species:

recommendations of a joint ISAG/FAO working group. (http://dad.fao.org/).

Kim, K. S., and T. W. Sappington. 2005a. Genetic structuring of western corn rootworm (Coleoptera: Chrysomelidae) populations in the U.S. based on microsatellite loci analysis. Environ. Entomol. 34: 494–503.

Kim, K. S., and T. W. Sappington. 2005b. Polymorphic microsatellite loci from the western corn rootworm (Insecta: Coleoptera: Chrysomelidae) and cross-amplification with other *Diabrotica* spp. Mol. Ecol. Notes 5: 115–117.

Kim, K. S., B. W. French, D. V. Sumerford, and T. W. Sappington. 2007a. Genetic diversity in laboratory colonies of western corn rootworm (Coleoptera: Chrysomelidae), including a nondiapause colony. Environ. Entomol. 36: 637–645.

Kim, K. S., S. T. Ratcliffe, B. W. French, L. Liu, and T. W. Sappington. 2008. Utility of EST-derived SSRs as population genetics markers in a beetle. J. Hered. (Doi: 10.1093/j hered/esm104.)

Krysan, J. L., R. F. Smith, T. F. Branson, and P. L. Guss. 1980. A new subspecies of *Diabrotica virgifera* (Coleoptera: Chrysomelidae): description, distribution, and sexual compatibility. Ann. Entomol. Soc. Am. 73: 123–130.

Levine, E., J. L. Spencer, S. A. Isard, D. W. Onstad, and M. E. Gray. 2002. Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. Am. Entomol. 48: 94–107.

Li, Y. C., A. B. Korol, T. Fahima, A. Beiles, and E. Nevo. 2002. Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. Mol. Ecol. 11: 2453–2465.

Marshall, T. C., J. Slate, L. Kruuk, and J. M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7: 639–655.

Meinke, L. J , B. D. Siegfried, R. J. Wright, and L. D. Chandler. 1998. Adult susceptibility of Nebraska western corn rootworm populations to selected insecticides. J. Econ. Entomol. 91: 594–600.

Metcalf, R. L. 1983. Implications and prognosis of resistance to insecticides, pp. 703–733. *In* G. P. Georghiou, and T. Saito (eds.), Pest resistance to pesticides. Plenum, New York.

Miller, N., A. Estoup, S. Toepfer, D. Bourguet, L. Lapchin, S. Derridj, K. S. Kim, P. Reynaud, L. Furlan, and T. Guillemaud. 2005. Multiple transatlantic introductions of the western corn rootworm. Science 310: 992.

Miller, N. J., K. S. Kim, S. T. Ratcliffe, A. Estoup, D. Bourguet, and T. Guillemaud. 2006. Absence of genetic divergence between western corn rootworms (Coleoptera: Chrysomelidae) resistant and susceptible to control by crop rotation. J. Econ. Entomol. 99: 685–690.

Miller, N. J., M. Ciosi, T. W. Sappington, S. T. Ratcliffe, J. L. Spencer, and T. Guillemaud. 2007. Genome scan of *Diabrotica virgifera virgifera* for genetic variation associated with crop rotation tolerance. J. Appl. Entomol. 131: 378–385.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Oosterhout, C. V., W. F. Hutchinson, D P.M. Wills, and P. F. Shipley. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4: 535–538.

Raymond, M., and F. Rousset. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86: 248–249.

Roslin Institute. 2002. Projects based at Roslin Institute. (http://www.projects.roslin.ac.uk.

Sappington, T. W., B. D. Siegfried, and T. Guillemaud. 2006. Coordinated *Diabrotica* genetics research: accelerating progress on an urgent insect pest problem. Am. Entomol. 52: 90–97.

Schneider, S., J. M. Kueffer, D. Roessli, and L. Excoffier. 2000. ARLEQUIN manual, version 2.0. A software for population genetic analysis. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Selkoe, K. A., and R. J. Toonen. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. Ecol. Lett. 9: 615–629.

Slatkin, M. 1994. An exact test for neutrality based on the Ewens sampling distribution. Genet. Res. 64: 71–74.

Slatkin, M. 1996. A correction to the exact test based on the Ewens sampling distribution. Genet. Res. 68: 259–260.

Waits, E. R., and U. Stolz. 2008. Polymorphic microsatellite loci from northern and Mexican corn rootworms (Insecta: Coleoptera: Chrysomelidae) and cross-amplification with other *Diabrotica* spp. Mol. Ecol. Resources. (Doi: 10.1111/j.1471-8286.2007.02056.X.)

Watterson, G. 1978. The homozygosity test of neutrality. Genetics 88: 405–417.

Wilder, J. A., T. Diaz, R.J.W. O'Neill, J. Kenney, and H. Hollocher. 2002. Characterization and isolation of novel microsatellites from the *Drosophila dunni* subgroup. Genet. Res. 80: 177–185.

Woodhead, M., J. Russell, J. Squirrell, P. M. Hollingsworth, K. Mackenzie, M. Gibby, and W. Powell. 2005. Comparative analysis of population genetic structure in *Athyrium distentifolium* (Pteridophyta) using AFLPs and SSRs from anonymous and transcribed gene regions. Mol. Ecol. 14: 1681–1695.

Wright, R. J., M. E. Scharf, L. J. Meinke, X. Zhou, B. D. Siegfried, and L. D. Chandler. 2000. Larval susceptibility of an insecticide-resistant western corn rootworm (Coleoptera: Chrysomelidae) population to soil insecticides: laboratory bioassays, assays of detoxification enzymes, and field performance. J. Econ. Entomol. 93: 7–13.

Zane, L., L. Bargelloni, and T. Patarnello. 2002. Strategies for microsatellite isolation: a review. Mol. Ecol. 11: 1–16.

Zhang, D. X , and G. M. Hewitt. 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. Mol. Ecol. 12: 563–584.