Isolation and characterization of 11 polymorphic microsatellite markers in the highly invasive Western conifer seed bug, Leptoglossus occidentalis (Heteroptera, Coreidae)

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Word count (including title, abstract, keywords, main text and table captions): 799

Abstract:

Eleven polymorphic microsatellite markers were developed from enriched DNA libraries for the invasive Western conifer seed bug, *Leptoglossus occidentalis*. The number of alleles ranged from two to 11 and observed heterozygosities from 0.038 to 0.933. Additional results of cross-species amplifications are reported for two congeneric species. This set of microsatellite markers, the first one available for *L. occidentalis*, enables further investigations of population structure of this species which represents a serious threat for European conifer regeneration.

Keywords: genetic diversity; invasion; microsatellites.

The Western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Heteroptera, Coreidae), was accidentally introduced in Europe and first reported in Italy in 1999 (Fent and Kment 2011). Then, the bug colonized most of Europe within just a decade (Fent and Kment 2011). Adults and nymphs feed on cones of a wide range of conifer species. Consequently, this introduction represents a risk not only for commercial seed crops but also for conifer ecosystems, impacting natural regeneration (Lesieur et al. 2014). The impact of *L. occidentalis* can also be enhanced through a newly established association between the alien insect and a native fungal pathogen, *Diplodia pinea* (Luchi et al. 2012). In order to set up an appropriate management program in Europe, microsatellites are needed to reveal the origin of newly established populations. We report here the isolation and characterization of 11 microsatellite loci useful for estimating genetic diversity in *L. occidentalis*.

Total genomic DNA was isolated from one pooled sample of individuals collected from four localities situated in France (Lavercantière, Southwestern France and Serre-Ponçon, French Alps) and in the native range (two different sites in British Columbia, Canada). The extraction was then sent to GenoScreen, France (www.genoscreen.com). A total of 1 µg was used for the development of microsatellite libraries through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries, as described in Malausa et al. (2011). Among 907 sequences comprising a microsatellites motif, 298 primer sets were designed and a sub-group of 48 primers pairs was tested for amplification. Primer sets were discarded if they failed to amplify or led to multiple fragments. Then, 12 microsatellites loci were selected from validated ones for polymorphism study. PCR amplifications were performed in a volume of 25 µl containing 20 ng of template DNA, 1 U of Dream-Taq DNA Polymerase (Thermo Scientific), 1.875 µL of 10x Dream Taq Green Buffer (including 20 mM of $MgCl_2$), 6 pmol of dNTPs, 0.5 µl of Bétaine and 10 pmol of each primer. The PCR cycling consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles : denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

Another set of 10 microsatellites was tested in our laboratory for subsequent PCR from which only two were polymorphic. Amplifications were performed in a 10 µl reaction volume containing 20 ng of template DNA, 0.5 U of Dream-Taq DNA Polymerase, 0.48 µl of 10x Dream Taq Green Buffer, 0.2 µl of Bétaine, 4 pmol of dNTPs and 8 pmol of each primer for MSLO07 and 4 pmol for MSLO15. The cycling conditions were the same than described previously except the primer annealing temperature (52°C).

Consequently, 11 markers were selected and tested on 30 individuals obtained from a seed orchard located at Lavercantière. Fragments were run on an ABI 3500 Genetic Analyzer using GeneScan™ - 600 LIZ® Size Standard, and sized with GeneMapper® v4.1 software (Life Technologies - Applied Biosystems).

Deviations from Hardy-Weinberg equilibrium (HWE), expected and observed heterozygosity and linkage disequilibrium were calculated using ARLEQUIN 3.11 (Excoffier et al. 2005). The existence of null alleles was tested using MICROCHECKER (http://www.microchecker.hull.ac.uk/).

The number of alleles ranged from two to 11 and the expected heterozygosity from 0.337 to 0.872 (Table 1). Significant departures from HWE in the direction of heterozygote deficiency were detected for three loci (Lep04, Lep05 and Lep31) probably due to the presence of null alleles (detected in Lep 04 and Lep05) or sampling biases. There was no case of linkage disequilibrium among loci after applying sequential Bonferroni corrections for multiple tests. Cross-species amplifications were performed in two North American congeneric species, *L. phyllopus* and *L. corculus*; a highly polyphagous pest of various angiosperms and a pine seed pest, respectively. Three loci failed to amplify in *L. corculus* and only one (Lep04) in *L. phyllopus* (Table 2). With regards to *L. occidentalis*, these loci are promising for further analyses intended to study the dispersal patterns and the invasion routes of this highly invasive pest now discovered in Asia.

Acknowledgement:

We greatly acknowledge support from the European FP7 projects ISEFOR (*Increasing Sustainability of European Forests: Modelling for Security Against Invasive Pests and Pathogens under Climate Change* – collaborative project no. 245268) and QBOL (*Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health"* no. 226482, KBBE-2008-1-4-01), the COST Action PERMIT (*Pathway Evaluation and pest Risk Management In Transport*- FP1002-181110-06882), and a grant from the French Ministry of Agriculture, Food, Fisheries, Rural Affairs and Spatial Planning (Agreement no. E 01/09). The Titanium pyrosequencing was funded by the grant AIP BioRessources EcoMicro from the Institut National de la Recherche Agronomique (INRA). We gratefully thank Magally Torres-Leguizamon and Géraldine

Roux-Morabito for their helpful advices and Ward Strong, Christian Blazy, Paula Mitchell and Alex Mangini for their help in collecting insect samples.

References:

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evol Bioinform 1:47-50

Fent M, Kment P (2011) First record of the invasive western conifer seed bug *Leptoglossus occidentalis* (Heteroptera: Coreidae) in Turkey. North-West J Zool 7 (1):72-80. doi:111106

Lesieur V, Yart A, Guilbon S, Lorme P, Auger-Rozenberg M-A, Roques A (2014) The invasive *Leptoglossus* seed bug, a threat for commercial seed crops, but for conifer diversity? Biol Inv:1-17. doi:10.1007/s10530-013-0630-9

Luchi N, Mancini V, Feducci M, Santini A, Capretti P (2012) *Leptoglossus occidentalis* and *Diplodia pinea*: a new insect-fungus association in Mediterranean forests. Forest Pathol 42 (3):246-251. doi:10.1111/j.1439-0329.2011.00750.x

Malausa T, Gilles A, Meglecz E, Blanquart H, Duthoy S, Costedoat C, Dubut V, Pech N, Castagnone-Sereno P, Delye C, Feau N, Frey P, Gauthier P, Guillemaud T, Hazard L, Le Corre V, Lung-Escarmant B, Male PJG, Ferreira S, Martin JF (2011) High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. Mol Ecol Resour 11 (4):638-644. doi:10.1111/j.1755-0998.2011.02992.x

Table captions:

Table 1. Microsatellite data and polymorphism characterization of the *Leptoglosssus occidentalis* population from Lavercantière.

Table 2. Cross-species amplification results for *Leptoglossus* spp.

Locus	Primer sequences (5'-3')	Repeat motif	5' Dye	Multiplex marker set	Size range	*Na*	*Ho*	*He*
Lep04	F: GTGGCTTGCGCTGTGTATAG	$(GT)_6$	NED	1	118-122	3	0,038	**0,446*** **
	R: TGACTCAGGAATAACAACAACACA							
Lep05	F: GGGACGAATTTCCCGTAGAT	$(AT)_7$	PET	1	128-134	3	0,226	**0,495*** **
	R: GCGGGAGGTCTGACTTATGA							
Lep07	F: TCTTCCTCATCTTCATCAGAATCA	$(TCA)_7$	NED	2	140-149	2	0,290	0,337
	R: GGTGAAGTTAGCGCAGAGTCA							
Lep16	F: GGAGATGTTCCTCTGCCGT	$(AC)_9$	VIC	2	162-198	8	0,839	0,829
	R: AGTATGATTTAAAAGGCTGCATAGTA							
Lep17	F: ACCCAGCTTCCGCTATTTAT	$(GT)_9$	VIC	1	114-118	3	0,452	0,564
	R: TGCGTAAAACATACTCCCACA							
Lep25	F: ACGAAAACGTTTGCTGTTTG	$(AG)_8$	6FAM	2	99-107	3	0,484	0,524
	R: AACATTCTTTAATCGTCGGCT							
Lep31	F: TAAAAATGTTTTCTCTTTACTGCG	(GT)8	6FAM	1	132-152	6	0,500	**0,733*** **
	R: CCAAATTTCTGTATGTTTGCTTG							
Lep36	F: TGTACATAACAGAATGAGACATGCAC	$(CA)_{13}$	PET	2	145-179	8	0,742	0,829
	R: CATGAACACATCCTCTCGGA							
Lep43	F: CAATTTCAACAACCTCGGGA	$(GT)_{10}$	PET	1	207-255	11	0,933	0,872
	R: GTAGGATCCTGCGTGAGAGC							
MSLO07	F: TTCCTCAATATTAAGTTGGTTCTCTG	$(CA)_{14}(TA)_4$	6FAM	/	125-155	7	0,767	0,728
	R: TTACCCAGCAAGACAAACCC							
MSLO15	F: ACCAATTGGCATGAAGTCCT	$(CT)_{10}(CA)_8$	6FAM	/	204-234	3	0,467	0,495
	R: GCTTCATGGGCTAGTGAGGT							
					mean	5,182	0,522	0,647
					SD	2,960	0,276	0,194

N_A: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity; values in bold indicate significant deviation from Hardy-Weinberg equilibrium (***: P < 0.01 ; **: 0.01 < P < 0.05 ; *: 0.05 < P < 0.1).

Species	Tested individuals	Locus										
		Lep04	Lep05	Lep07	Lep16	Lep17	Lep25	Lep31	Lep36	Lep43	MSLO07	MSLO15
L. corculus	4	0	128 (2)	146	0	108 (1)	101-119	132-144	0	294-298	125-175	204-206
L. phyllopus	4	0	128	137-146	136-216	108-110	103-129	132-142	137-147 (3)	304-322	125-175	210-234 (3)

1 Numbers refer to the size (bp) of the PCR products. 0 refers to unsuccessful amplifications. When partial amplification, number of amplified individuals are within brackets.