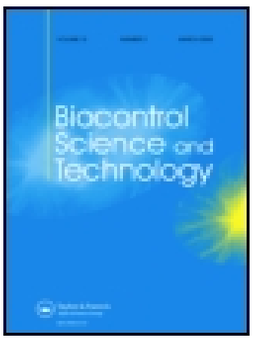

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Isolation of 12 polymorphic tetranucleotide microsatellite markers of the leaf beetle *Ophraella communa,* a promising *Ambrosia* biocontrol agent also in Europe

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ABSTRACT

Following its first record in Europe in 2013, the North American ragweed leaf beetle *Ophraella communa,* used already as a most successful biocontrol agent against common ragweed in China, is spreading rapidly, asking for a detailed analysis of the potential benefit and risk of this introduction for Europe. Here, we report twelve specific and polymorphic tetranucleotide microsatellite markers, which can be used for redrawing its global invasion history and spread across native and introduced ranges. The high level of polymorphism (i.e. from 4 to 18 alleles per locus) and the genetic variation detected within and between one native and two introduced populations provide adequate statistical power for elucidating the beetle's invasion process.

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The North American common ragweed, *Ambrosia artemisiifolia L.* (Asteraceae), is one of the most prominent plant invaders worldwide (Essl et al., 2015). In Europe, the plant is the cause of high economic losses due to severe impacts on human health resulting from its huge amount of highly allergenic pollen and because it is an important and hard-to-control crop weed (Essl et al., 2015; Müller-Schärer et al., 2014). In its native range, common ragweed is the preferred host plants of *Ophraella communa* LeSage 1986 (Coleoptera: Chrysomelidae), an oligophagous leaf beetle endemic to North America, distributed from Mexico to the Canadian Prairies. This leaf beetle was accidentally introduced in Eastern Asia almost two decades ago, where it is now used in China as a most successful biological control agent against common ragweed (Müller-Schärer et al., 2014). Since its recent and accidental introduction into Western Europe, with first records in Southern Switzerland and the Milano area (Italy) in 2013, local aerial pollen concentrations of *A. artemisiifolia* have significantly dropped by 80% compared to non-infested areas (Bonini et al., 2015). This steep decline in pollen concentration, where *O. communa* is present, made this insect a most promising candidate for long-term management of common ragweed also in Europe (Müller-Schärer et al., 2014). Following its fast spread south of the Alps (Zadravec, Horvatic, & Prpic, 2019), multiple studies have been initiated to determine, whether this beetle should be considered as a troublesome

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introduction or whether it is likely to become the first case of a successful biological control of an invasive weed in continental Europe.

To identify the source population(s) of the introduction in Europe and to monitor the future spread of the ragweed leaf beetle genotypes, it is essential to know the extent and structure of the neutral genetic diversity of *O. communa* populations in both the native and the introduced ranges. Here, we report a set of twelve polymorphic tetranucleotide microsatellite markers that will be useful to investigate the invasion processes of *O. communa*.

The whole genome sequence (ca. 92,800 raw sequences) of a genomic DNA pool of two males and two females, collected in the two introduced ranges, i.e. China (Shenzhen: 22° 32'10.032"N, 114°03'44.459"E; Wuhan: 30°32'42.029"N, 114°25'14.739"E) and Italy (Pavia: 45°06'490"N, 9°07'480"E; Parma: 44°33'47.131"N; 10°13'47.128"E), was obtained from paired-end reads of 250 base pairs (bp) sequenced on a MiSeq® Next Generation Sequencer v.2 (Illumina) (Microsynth platform, Balgach, Switzerland). Following bioinformatics processing (i.e. *de-novo* assembly, detection of microsatellite motifs and primer pairs performed by the Microsynth platform), a total of 5,338 sequences containing microsatellite motifs were delivered, including 306 di-, tri- and tetra-nucleotide loci with suitable primer pairs. Although the number of alleles is usually reduced in tetra-nucleotide loci compared to di- and tri-nucleotide loci, they are generally easier to detect and display less slippage artifacts. Primer pairs of 53 tetra-nucleotide loci, displaying at least 7 repeat units, were therefore tested for amplification, polymorphism and congruence of motif repetitions using a 6-FAM M13 tail (cf. Schuelke (2000) for details of the procedure). Tests were performed on a set of 16 individuals collected in both native and introduced ranges (Appendix 1). As a result, eight of the 53 loci tested were monomorphic, nine loci did not yield amplification products and 16 displayed uninterpretable amplification patterns. The remaining 20 loci were then tested for allele inheritance using two leaf beetle couples collected in Italy and 12 of their offspring (i.e. six per couple, produced in the quarantine facilities at the University of Fribourg, Switzerland). At the end of the selection procedure, twelve promising tetra-nucleotide microsatellite markers were chosen for developing multiplex PCRs. Multiplex PCRs are simultaneous amplifications of several sequences by using multiple primer pairs, each branded by a fluorescent dye, in a reduced number of reaction mixtures. To allow the annealing of multiple primer pairs, we used a touchdown PCR method by programming a range of temperatures in the PCR protocols. In summary, we developed three multiplexes and three simplexes in this study, each with specific temperature conditions and primer concentrations (see Table 1 for more details).

Once the development of the PCRs was achieved, polymorphisms of the 12 loci were estimated in one native and two introduced populations (Table 2). To do so, genomic DNA of 20–24 individuals per population were extracted using a customised sbeadex™ kit (LGC). Locus amplification was performed following the optimised PCRs developed upstream (Table 1). PCR reactions were performed in a total volume of 15 µL containing 2 µL of template DNA (ca. 20 ng of genomic DNA), 1X GoTaq® Reaction Buffer with 0.5 U of GoTaq® G2 DNA polymerase (Promega), 0.25 mM of each dNTP, 1 mg/mL of bovine serum albumin, as well as reverse primers at 0.2 µM and forward primers in locus-specific concentrations (Table 1). All PCRs were performed with a TProfessional thermocycler (Biometra) using the following conditions: an initial denaturation phase at 95°C for

Table 1. Details of the 12 microsatellite loci of *Ophraella communa* and amplification conditions of multiplex PCRs.

Locus name[#]	GenBank Accession Number	Primer sequences (5′->3′)F: Forward, R: Reserve	Repeat motif	Size range (bp)	Multiplex PCR Number	Temperature conditions	Fluorescent dye (*)	Forward primer final concent-rations	
								F* (μM)	F (μM)
Ocom_Q51	MN167543	F: GCACAATAGGTCTCATAGATCGC R: AGCCATGGTGGAGGTTACTG	$(CATA)_{13}$	132–242	1	TD: 62–56°C (6 c.,−1°C/c.) AM: 56°C (20 c.)	Atto532	0.12	0.08
Ocom_Q38	MN167542	F: TTTTATAGACACAGCTGAACTCC R: ACATGCCATTCTTTTTAGGTTTTG	$(AATA)_{7}$	121–145	1		Atto550	0.15	0.05
Ocom_Q18	MN167537	F: TTTTATCTTGGCACTGGCGG R: GGACTTTAGGAGGCAAAAAGTGG	$(TCTT)_{9}$	128–204	1		FAM	0.12	0.08
Ocom_Q05	MN167534	F: CGTGTTCTACATTTTATTACGTTTTGC R: CATGGCAAGTAAGGGGACAC	$(ATGT)_{8}$	169–181	1		Atto565	0.10	0.10
Ocom_Q37	MN167541	F: AGGGATTTCTAAAATGCAGTTGG R: GAACATGGCAAGTAAGGGGG	$(TATG)_{15}$	155–251	2	TD: 65–55°C (10 c., −1°C/c.) AM: 55°C (20 c.)	Atto532	0.08	0.12
Ocom_Q25	MN167538	F: GCTGCTAGGACCTGTACCATC R: GCATCTGGACCCGTATTCTTG	$(TATC)_{12}$	192–220	2		Atto550	0.05	0.15
Ocom_Q02	MN167532	F: AGCCCGACGATGTCCTAAAC R: CCAACGTGGGGTTTATACGAAG	$(ACAT)_{9}$	208–232	–	AM: 52°C (23 c.)	FAM	0.12	0.08
Ocom_Q31	MN167540	F: GAACATGGCAAGTAAGGGGG R: GAACTTGGGCCGTAAACCAG	$(CATA)_{10}$	230–306	–	AM: 52°C (23 c.)	Atto565	0.10	0.10
Ocom_Q27	MN167539	F: ACAAATGGTAAGGCGTTTGC R: TGCCTGGAATCCGTAAGAGG	$(TGTA)_{13}$	151–223	3	TD: 64–54°C (10 c., −1°C/c.) AM: 54°C (20 c.)	Atto532	0.12	0.08
Ocom_Q07	MN167535	F: TCGCAGTGTACTGATCACCC R: TACTCACGAAGCGTCTCCAG	$(CATA)_{8}$	208–240	3		Atto550	0.05	0.15
Ocom_Q08	MN167536	F: TCACGTTCAATGTCATAGCG R: AGCAATTTATGTACTGAACTCTTGTG	$(TAGA)_{8}$	158–178	3		FAM	0.10	0.10
Ocom_Q04	MN167533	F: AAGAGCTTTCGCATGTTGTG R: AACTTCTTTGGAGGCGTCGG	$(TATC)_{7}$	202–222	–	AM: 52°C (23 c.)	Atto565	0.10	0.10

[#]The names of loci were defined by the abbreviation of the species name (Ocom for *Ophraella communa*), the number of base pairs in the repeat motifs (Q for "quattuor") and a number between 1 and 53 corresponding to the number of primer pairs tested in this study. Both touchdown (TD), amplification (AM) temperatures and number of cycles (c.) are given for each multiplex PCR. The two temperatures given for the touchdown correspond to the initial and final temperatures. The concentrations of fluorescent (F*) and non-fluorescent (F) forward primers are marker-specific.

Table 2. Genetic polymorphisms of the 12 microsatellite loci of *Ophraella communa* in one native and two introduced populations, and cross-amplification success on *Ophraella slobodkini*.

		Native population				Introduced populations								Cross-amplification (*O. slobodkini*)
		Falmouth, USA ($n = 20$)38°20′15.684″N, 77° 29′6.180″W				Wanjia, China ($n = 24$)29°27′50.868″N, 13° 25′27.551″E				Ponte San Pietro, Italy ($n = 22$)45°41′40.236″N, 9°35′29.759″E				Ferndale, USA ($n = 11$) 28°37′45.300″N, 81° 41′43.908″W
Locus name	Total N_A	N_A	Ho	He	F_{IS}	N_A	Ho	He	F_{IS}	N_A	Ho	He	F_{IS}	N_A
Ocom_Q51	18	14	0.35	0.91	0.621*,‡	4	0.21	0.59	0.654*,‡	7	0.14	0.78	0.820*,‡	0
Ocom_Q38	7	7	0.85	0.73	−0.166	2	0.04	0.04	0.000	4	0.48	0.58	0.184	1
Ocom_Q18	16	16	0.53	0.93	0.441*,‡	4	0.14	0.53	0.748*,‡	7	0.73	0.81	0.102	0
Ocom_Q05	4	4	0.17	0.70	0.768*,‡	2	0.18	0.24	0.250	3	0.27	0.42	0.349	0
Ocom_Q37	14	12	0.85	0.90	0.056	3	0.63	0.52	−0.206	8	0.68	0.80	0.149	0
Ocom_Q25	7	5	0.26	0.67	0.612*,‡	3	0.17	0.44	0.629*,‡	2	0.10	0.18	0.465	0
Ocom_Q02	6	5	0.29	0.64	0.548*,‡	4	0.21	0.63	0.670*,‡	5	0.26	0.67	0.612*,‡	0
Ocom_Q31	12	11	0.60	0.91	0.349*,‡	1	NA	NA	NA	6	0.65	0.79	0.181	0
Ocom_Q27	13	9	0.45	0.89	0.502*,‡	5	0.29	0.67	0.573*,‡	5	0.30	0.75	0.605*,‡	0
Ocom_Q07	6	6	0.39	0.71	0.460*,‡	2	0.05	0.48	0.898*,‡	3	0.45	0.61	0.254	0
Ocom_Q08	6	6	0.58	0.75	0.237*	2	0.39	0.45	0.132	4	0.33	0.59	0.444*,‡	0
Ocom_Q04	4	3	0.21	0.57	0.636*,‡	2	0.39	0.48	0.182	2	0.11	0.29	0.636	0
Average (± SD)	**9.4 ± 4.9**	**8.2 ± 4.2**	**0.46 ± 0.23**	**0.78 ± 0.13**	–	**2.8 ± 1.2**	**0.25 ± 0.17**	**0.46 ± 0.18**	–	**4.7 ± 2.0**	**0.38 ± 0.22**	**0.61 ± 0.21**	–	–

Notes: GPS coordinates, number of individuals (n), allelic richness (N_A), expected (He) and observed (Ho) heterozygosity rates and Weir and Cockerham estimates (F_{IS}) are provided for each locus and population. * indicates significant deviation from Hardy-Weinberg equilibrium (p-value < 0.05). ‡ indicates evidence for null alleles according to Micro-Checker (p-value < 0.05). Loci are sorted in the same order as in Table 1.

5 min, followed by 6–10 touchdown cycles, with each cycle consisting of a denaturation at 95 °C for 30 s, annealing for 45 s with specific step-downs temperatures (Table 1), and elongation at 72°C for 45 s; touchdown cycles were immediately followed by 20–23 amplification cycles, with each cycle consisting of a denaturation at 95 °C for 30 s, annealing at primer-specific temperatures for 45 s (Table 1), and elongation at 72°C for 45 s; a final elongation phase at 72°C for 10 min finished the PCR programmes. For the genotyping, 1 µL of PCR products was added to a mix containing 9.7 µL of formamide and 0.3 µL of DNA Size Standard 500 Orange (Nimagen). An ABI 3130xl capillary sequencer (Applied Biosystems) was used to separate PCR fragments. The sizes of fragments, corresponding to the alleles, were scored using GeneMarker v.2.7.2 (SoftGenetics, State College, Pennsylvania, USA). Finally, following the same procedure, cross-priming was tested on 11 individuals of *Ophraella slobodkini* Futuyma, 1991, a species sharing host association of *A. artemisiifolia* with *O. communa* and living in sympatry in Georgia and South Carolina, USA (Futuyma, 1991).

For each locus and each of the three *O. communa* populations, allele scoring allowed us to determine both allelic richness (i.e. number of allele) and allele frequencies, as well as the number of heterozygote individuals (i.e. individuals displaying two different alleles at a given locus). Then, using the R package Genepop v.1.1.2 (Rousset, 2008), observed (Ho) (Eq. 1) and expected (He) (Eq. 2) heterozygosity rates were computed for each locus and population, as:

$$\mathrm{Ho} = \frac{\text{Number of heterozygotes}}{\text{Number of individuals}} \tag{1}$$

$$\mathrm{He} = 1 - \sum_{i=1}^{k} pi^2 \tag{2}$$

where p_i is the frequency of the ith of k alleles for a given locus.

Linkage disequilibrium (i.e. non-random association of alleles at different loci) and deviation from Hardy-Weinberg equilibrium (i.e. constancy of genotype and allele frequencies over generations), based on Weir and Cockerham estimate, F_{IS} (Weir & Cockerham, 1984), were also tested per locus and population using the Genepop R package (Rousset, 2008). For both tests, p-values were adjusted based on the false discovery rate to account for multiple testing errors. The presence of null alleles was then examined per locus and population with Micro-Checker v.2.2.3 (Van Oosterhout, Hutchinson, Wills, & Shipley, 2004).

Across all populations, the allelic richness per locus ranged from 4 to 18, with an average of 9.4 ± 4.9 alleles per locus (see Table 2 for more details). At a regional level, allele richness per locus tended to be higher in the native population compared to the introduced ones. For instance, 11 alleles were reported at the Ocom_Q31 locus in the native population, which dropped to six and one allele in the Italian and Chinese population, respectively. The same pattern was found for both observed and expected heterozygosity rates: for instance, on average across all loci, expected heterozygosity rates amounted to 0.78 ± 0.13 for the North-American population and decreased to 0.61 ± 0.21 and to 0.46 ± 0.18 for the Italian and Chinese population, respectively. These findings indicate a reduced number of population sources and/or introduction events in the introduced ranges and are in line with the results of Nishide et al. (2015) for Japanese

populations of *O. communa* using mitochondrial sequences. Post-introduction genetic bottlenecks or founding events commonly occur in introduced populations and might have further contributed to the reduced genetic diversity observed in our two introduced populations. Further genetic studies using the microsatellite markers developed here and including additional native and introduced Asian and European populations will allow to better understand the invasion history of the ragweed leaf beetle worldwide.

Significant linkage disequilibrium was detected for only one pair of loci out of 179 (for Ocom_Q37 & Ocom_Q38 in the Italian population). Interestingly, several microsatellites showed a significant deviation from Hardy-Weinberg equilibrium. These deviations, due to a deficit in heterozygosity, were found both in native (10 out of 12 loci) and introduced populations (4 and 6 out of 12 loci for the Italian and Chinese population, respectively). Such heterozygosity deficits have been reported for several Coleopteran species (e.g. McKeown et al., 2018) and may result from biological processes, such as selection, inbreeding or Wahlund effects (i.e. sampling of individuals from genetically distinct groups). Knowing that all populations were randomly sampled (i.e. leaf beetles were collected on more than 50–100 *A. artemisiifolia* plants using sweep nets), inbreeding and Wahlund effects are unlikely, but cannot be excluded. While both reasons do not impair the development of microsatellite markers, upcoming genetic population studies must implement an appropriate sampling design to avoid genetic relatedness and local genetic clustering. A more reliable explanation of heterozygosity deficits is the presence of null alleles resulting from nucleotide variations of flanking regions that can prevent primer annealing during PCR amplification. Here, Micro-checker results confirmed evidence of null alleles for the majority of the markers deviating from Hardy-Weinberg equilibrium (Table 2). Null alleles are often unavoidable in species showing large effective population sizes and short generation time, such as *O. communa* (Müller-Schärer et al., 2014), resulting in an overestimation of population differentiation (Chapuis & Estoup, 2007). However, the occurrence of null alleles varies across geographic regions (i.e. 75%, 50% and 33% of microsatellite null alleles were detected with Micro-checker in the native and in the introduced Chinese and Italian population, respectively), allowing to choose region-specific microsatellite markers for further population genetic studies.

Cross-priming amplification failed for almost all of the microsatellite markers (Table 2). Therefore, these markers cannot be used to assess phylogenetic relationships and population genetic diversity and structure of other *Ophraella* species. Only the Ocom_Q38 locus successfully amplified in *O. slobodkini* with a single allele detected. Interestingly, this allele (113 bp) was not observed in the studied *O. communa* populations (from 121 to 145 bp), suggesting that this marker may be used as a diagnostic to separate the two species and to evaluate potential hybridizations, in addition to the genetic markers already developed by Futuyma (1991).

The development of these microsatellite markers offers an efficient and cost-effective tool to assess the genetic diversity and structure of native and introduced populations of *O. communa,* as well as identifying the sources of introduced populations, the number of introduction events and the process of expansion following introductions. In addition, the set of markers will be useful for pre- (e.g. identification of host associations) and post-release assessments (e.g. evolutionary changes), and thus help scientists and decision-makers to implement optimal management strategies using this promising biocontrol candidate against the notorious and widespread common ragweed.

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Disclosure statement

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Appendix 1. Populations selected to test primer pairs of the 53 tetranucleotide loci, using a set of 16 individuals (with 2 individuals per population).

Location	Country	Date of sampling	Collector(s)	GPS coordinates (latitude, longitude)
Wuhan	China	2015-08-31	Zhang Jialiang	30°32′42.029″N, 114°25′14.739″E
Shenzhen	China	2017-07-29	Heinz Müller-Schärer, Yan Sun	22°32′10.032″N, 114°03′44.459″E
Pavia	Italy	2015-08-13	Peter Toth	45°06′49.000″N, 9°07′48.000″E
Parma	Italy	2015-08-31	Benno Augustinus	44°33′47.131″N, 10°13′47.128″E
Providence (SC)	USA	2016-10-01	Heinz Müller-Schärer, Yan Sun	33°22′56.244″N, 80°30′49.680″W
Gordonville (VA)	USA	2016-10-04	Heinz Müller-Schärer, Yan Sun	38°08′42.396″N, 78°11′0.455″W
Baltimore (MD)	USA	2016-10-05	Heinz Müller-Schärer, Yan Sun	39°14′29.724″N, 76°34′47.892″W
Springfield (MA)	USA	2016-10-06	Heinz Müller-Schärer, Yan Sun	42°08′57.084″N, 72°29′46.391″W