RESEARCH NOTE

Development of a microsatellite primer set to investigate the genetic population structure of *Armadillidium nasatum* (Crustacea, Oniscidea)

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Introduction

An essential tool in molecular ecology studies, the microsatellite markers allow the investigation of the genetic structure of populations. Here, we developed a panel of 12 polymorphic microsatellite markers isolated from a combination of two approaches: 454 pyrosequencing of a repeat-enriched genomic library and cross-species amplification. These microsatellite markers were isolated from *Armadillidium nasatum* for which they represent a promising tool regarding genetic studies. Moreover, this study increases the available number of microsatellite markers for *A. vulgare* and *A. depressum* for which some of these markers have also been amplified.

A. nasatum is a terrestrial isopod (Crustacea, Oniscidea) belonging to the family Armadillidiidae (Vandel 1962). Terrestrial isopods are sensitive to environmental disturbances (Paoletti and Cantarino 2002) and pollutions (Sastrodihardjo and Van Straalen 1993). Thus, they provide information on the environmental quality of agroecosystems and are indeed considered as bioindicators (Souty-Grosset *et al.* 2005a). For example, grassland habitats in western France are managed in relation to woodlouse biodiversity (Souty-Grosset *et al*. 2005b). Terrestrial isopods are members of the detritivore guild promoting decomposition processes and nutrient cycling (Paoletti and Hassall 1999), consequently playing a key role in ecosystem services (Berg *et al.* 2010; David and Handa 2010). They constitute an interesting model because they are indispensable for biochemical ecosystem balance (Paoletti and Hassall 1999) and because of their suitability for measuring the direct effects of human activities on their habitats. Human practices such as soil tillage, pesticide application, chemical pollution, along with soil acidification adversely affect the soil macrofauna abundance and biodiversity (Zimmer and Topp 1997; Natal da Luz *et al.* 2004; Souty-Grosset *et al.* 2005a, b). It has been observed that the specific diversity and abundance of terrestrial isopods decrease in intensive agricultural systems (Paoletti and Hassall 1999). Landscape modifications include enlargement of fields to make them amenable to mechanization. Such increases in plot size may entail habitat fragmentation through disruption of continuous structures. However, if the influence of landscape structural elements on *A. nasatum* community structure (presence and abundance) (Altieri 1999) is known at regional scale, no molecular tool is available for studying the resulting genetic structure of *A. nasatum* populations in agroecosystems. Currently, microsatellite markers are the most relevant markers for molecular ecology studies including dynamics, evolution and demographic history of wild populations.

As we intend to expand the use of *A. nasatum* for estimating the impact of changes in agricultural practices on agroecosystems and their associated isopod diversity, we developed, in this report, a set of 12 polymorphic microsatellite markers isolated from *A. nasatum* using two approaches: 454 pyrosequencing of a repeat-enriched genomic library and cross-species amplification. Some of these molecular markers were also tested in other woodlouse species belonging to the Armadillidiidae family such as *A. vulgare* and *A. depressum*. Taking into account its polymorphism, this microsatellite marker set will be efficient for studying the influence of landscape features and agricultural practices on genetic structure and demographic history of *A. nasatum* populations.

Materials and methods

Microsatellite isolation

We isolated microsatellites using both 454 pyrosequencing and cross-species amplification.

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From 454 pyrosequencing: From eight individuals of *A. nasatum*, collected in field populations (La Mothe Saint Héray; France; 46.3596°N, –0.1123°E), we extracted total genomic DNA using standard phenol–chloroform extraction (Kocher *et al.* 1989). From these DNA samples, Genoscreen (Lille, France) constructed a microsatellite-enriched genomic library, as previously described in Malausa *et al.* (2011). This library was sequenced by Genoscreen in a partial 454 GS FLX sequencer run with Titanium chemistry, as previously described in Malausa *et al.* (2011). The resulting reads were analysed using QDD software (Meglecz *et al.* 2010) to detect microsatellite motifs and design primers for PCR amplification. From 245 reads containing microsatellite motifs, Genoscreen selected and tested on gel electrophoresis (agarose 2%) the amplification of 23 primer pairs (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Priority was given to primer pairs amplifying fragments higher than 100 bp and showing a high number of repeated microsatellite motifs. PCR conditions were 1 U Taq polymerase (FastStart Taq kit, Roche, Basel, Switzerland), 6 pmol dNTP (FastStart Taq kit), 37.5 pmol $MgCl_2$, 1 pmol primers and 2 μL of extracted DNA. Concerning the amplification cycles, an initial denaturation of 10 min at 95°C was followed by 40 cycles of 30 s at 95°C, 30 s at the annealing temperature of 55°C, and 1 min at 72°C and a final extension of 10 min at 72°C. For microsatellite markers yielding an amplification band on agarose gel, we ordered forward primers with labelled dyes (6_FAM, HEX or NED).

From cross species amplification: We tested the cross amplification on *A. nasatum* of 50 microsatellite primer pairs previously characterized in *A. vulgare* (Giraud *et al.* 2013) (table 2 in electronic supplementary material). We performed these tests using eight individuals of *A. nasatum* whose DNA was isolated as mentioned above. First, each locus was amplified by PCR and fluorescently labelled using the M13(-21) primer genotyping protocol (Schuelke 2000) to minimize genotyping costs. This PCR method uses three primers: a locus-specific forward primer with M13(-21) tail at its 5′ end, a locus-specific reverse primer and a universal 6_FAM-labelled M13(-21) primer. PCR amplification was performed in 10 μL reactions, using 0.5 μM of both 6_FAM-M13(-21) and reverse primers, 0.125 μM forward primer, 0.25 U GoTaq DNA polymerase (Promega, Madison, USA), 1× PCR reaction buffer (Promega), 0.2 mM dNTPs (Promega) and 1 μL DNA template. PCR thermal conditions were as previously described in Schuelke (2000). Subsequently, amplification products were resolved by electrophoresis on 1.5% agarose gel. For loci revealing an amplification band on agarose gel, we added 0.5 μL PCR products to 9 μL formamide and 0.35 μL ROX standard (Life Technologies, St Aubin, France), to resolve them by electrophoresis on an ABI PRISM 3130 Genetic Analyzer. Product sizes were determined using the GeneMapper® software (Applied Biosystems,

Table 1. Raw microsatellite data.

			Population 1 (41)				Population 2 (51)				Population 3 (29)				All:121				
Loci name	Isolation approach	Selected	No. genotyped	*A*	H_e	F_{is}	Null allele	No. genotyped	*A*	H_e	F_{is}	Null allele	No. genotyped	*A*	H_e	F_{is}	Null allele	(n)	*A*	H_e	F_{is}	Range (bp)
An5	454 pyrosequencing	Yes	32	3	0.57	0.40	yes	39	3	0.61	–0.21	no	28	3	0.61	–0.35	no	99	3	0.60	–0.06	107–111
An7	454 pyrosequencing	Yes	33	3	0.12	***0.49***	yes	30	6	0.45	***0.56***	yes	21	3	0.18	0.48	yes	84	5	0.25	0.51	210–222
An12	454 pyrosequencing	Yes	32	3	0.50	0.13	no	36	3	0.58	–0.05	no	27	3	0.49	0.17	no	95	3	0.52	0.08	74–86
An16	454 pyrosequencing	Yes	36	4	0.48	***0.71***	yes	42	5	0.37	***0.55***	yes	26	5	0.50	***0.62***	yes	104	5	0.45	0.63	106–114
An17	454 pyrosequencing	Yes	35	6	0.75	0.39	yes	43	6	0.74	0.28	yes	26	6	0.80	***0.42***	yes	104	6	0.76	0.36	103–113
An20	454 pyrosequencing	Yes	38	5	0.71	0.33	yes	44	5	0.73	***0.41***	yes	27	5	0.77	0.32	yes	109	5	0.73	0.35	100–108
Av0009	Cross species	Yes	40	3	0.38	***0.67***	yes	49	3	0.39	***0.05***	no	27	3	0.44	0.41	yes	116	3	0.40	0.38	113–117
Av0018	Cross species	Yes	41	3	0.45	0.02	no	50	3	0.51	–0.13	no	25	3	0.51	–0.19	no	116	3	0.49	–0.10	99–105
Av0024	Cross species	Yes	21	3	0.47	–0.11	no	33	4	0.67	***0.82***	yes	15	3	0.61	0.45	yes	69	5	0.58	0.39	214–272
Av0028	Cross species	Yes	41	3	0.51	***0.52***	yes	50	3	0.53	0.29	no	26	3	0.48	0.04	no	117	3	0.51	0.28	208–216
Av0037	Cross species	Yes	41	3	0.57	–0.03	no	51	3	0.54	0.09	no	27	3	0.56	0.08	no	119	3	0.56	0.05	165–171
Av0049	Cross species	Yes	41	3	0.54	0.09	no	51	3	0.55	0.00	no	29	3	0.59	0.01	no	121	3	0.56	0.03	162–171
An15	454 pyrosequencing	Amplification success rate <50%	14	5	0.76	0.24	NA	23	6	0.76	***0.77***	NA	16	4	0.69	0.46	NA	53	6	0.74	0.49	244–277
Av0005	Cross species	Amplification success rate <50%	24	3	0.45	0.17	no	15	2	0.49	-0.36	no	15	3	0.54	–0.61	no	54	3	0.49	-0.27	98–102

We computed the statistics of labelled loci and of three populations with FSTAT ver. 2.9.3.2 software (Goudet 2001). These statistics include the allele number (*A*), the expected heterozygosity (H_e), and F_{is} values. In italic and bold: the F_{is} values significantly different from 0 ($P < 0.001$, significance threshold adjusted with the Bonferroni procedure for 48 tests). We used Micro-checker ver. 2.2.3 (Oosterhout *et al.* 2004) to detect signs of null alleles. No. genotype indicates the number of individuals finally genotyped and taken into account for the computation of number of alleles, heterozygosities and F_{is}. Isolation approach and range size of the amplified fragment were also shown for each tested microsatellite marker. Also, in brackets: the population sizes. Loci An_11 and An_14 are monomorphic and consequently do not appear in the table. NA, not available.

Table 2. Transferability of the tested microsatellite markers. No cross-amplification was detected in *Porcellio dilatatus dilatatus* and *Porcellionides pruinosus* and for the loci: An_7 and An_11 whatever focal species. Parameters are as described in the legend to table 1.

	Armadillidium vulgare ($n = 10$)					*Armadillidium depressum* ($n = 10$)				
Loci name	No. genotyped	*A*	H_e	F_{is}	Range (bp)	No. genotyped	*A*	H_e	F_{is}	Range (bp)
An_5	0	NA	NA	NA	NA	5	2	0.6	0.67	107–109
An_12	10	1	0	–	80	0	NA	NA	NA	NA
An_16	10	2	0.43	–0.39	108–112	10	1	0	–	108
An_17	10	2	0.39	–0.29	105–109	10	1	0	–	105
An_20	10	2	0.52	0.04	100–102	0	NA	NA	NA	NA
An_14	6	2	0.5	0.33	116–119	10	2	0.28	0.64	119–125
An_15	7	1	0	–	362	10	2	0.44	0.1	244–268

Saint Aubin, France), followed by eye verification. For microsatellite markers yielding a specific amplification (i.e. two alleles maximum per individual), we ordered forward primers with labelled dyes (6_FAM, HEX or NED).

Microsatellite genotyping

For each microsatellite locus, we then verified amplification in simplex conditions in eight individuals of *A. nasatum* whose genomic DNA was isolated as mentioned. All PCR reactions were carried out using the Qiagen, Venlo, The Netherlands, multiplex kit according to the manufacturer's standard microsatellite amplification protocol in a final volume of 10 μL, with an annealing temperature of 58°C and a final concentration of 0.2 μM for each primer. We added 1 μL of PCR product to 9 μL formamide and 0.5 μL ROX standard (Life Technologies) and resolved by electrophoresis on an ABI PRISM®3130 Genetic Analyzer. Amplification product size was determined using GeneMapper®software (Applied Biosystems), followed by visual verification. We then, selected microsatellite markers yielding a specific amplification to investigate genetic polymorphism.

Statistical analysis

Genetic variation was estimated from 121 DNA samples isolated as above from three distinct wild populations located in western France close to La Mothe Saint Héray (population 1: 46.405°N, –0.193°E; population 2: 46.368°N, –0.135°E; population 3: 46.367°N, –0.127°E).

We used Microchecker ver. 2.2.3 (Oosterhout *et al.* 2004) to detect signs of null alleles or scoring errors due to stuttering. We tested *A. nasatum* populations for departures from Hardy–Weinberg expectations, using the method implemented in the software Genalex (Peakall and Smouse 2006). Linkage disequilibrium (LD) was assessed for each specific microsatellite marker using exact tests (1000 permutations) as implemented in FSTAT ver. 2.9.3.2 (Goudet 2001). We adjusted the levels of significance for multiple tests using standard Bonferroni correction (Rice 1989). Further, we assessed genetic polymorphism with the number of alleles (*A*), expected heterozygosity (H_e) (Weir and Cockerham 1984) and F_{IS} using FSTAT ver. 2.9.3.2 (Goudet 2001) with 1000 permutations. To compare the polymorphism rate of microsatellite markers retained from both isolation approaches, we performed a nonparametric Wilcoxon test between H_e and *A* from the three *A. nasatum* populations. This test was performed with GraphPad InStat software (http://graphpad.com/scientific-software/instat/).

Locus transferability in terrestrial isopod species

We tested the transferability of the nine microsatellite markers identified using the 454 approach and yielding an amplification band in *A. nasatum*. Transferability was tested using 10 individuals from each of four terrestrial isopod species:

A. vulgare (sampled from Helsingør; Denmark; 56.0346°N, 12.5923°E), *A. depressum* (sampled from Sainte Marie, île de Ré; France 46.1528°N, −1.3278°E), *Porcellio dilatatus dilatatus* (sampled from Rom; France 46.2945°N, 0.1153°E) and *Porcellionides pruinosus* (sampled from Saint Martin du Fouilloux; France 46.5914°N, −0.1216°E). Total genomic DNA was obtained for each individual by standard phenol–chloroform extraction (Kocher *et al.* 1989). Transferability analyses were realized with amplification performed using forward fluorescent-labelled primers and the Multiplex Kit in the conditions described above. PCR products were resolved by electrophoresis on an ABI PRISM® 3130 Genetic Analyzer and their sizes were determined using GeneMapper® software (Applied Biosystems), followed by eye verification.

Results

From 454 pyrosequencing isolation

Among the 23 microsatellite markers tested by Genoscreen, 14 were successfully amplified. However, only nine out of the 14 markers revealed a specific amplification through electrophoresis on an ABI Genetic Analyzer. Among the nine loci, one showed an amplification success rate lower than 50% and two were monomorphic. Thus, we retained six loci that were polymorphic (i.e. >2 alleles) and showing an amplification success rate greater than 50% (table 1 in electronic supplementary material).

From cross species isolation

Among the 50 tested loci, 20 revealed an amplification band on agarose gel, seven of which showed a specific amplification after electrophoresis on an ABI Genetic Analyzer. However, one locus revealed an amplification success rate lower than 50%, thus we retained six loci that were polymorphic (i.e. >2 alleles) and showed an amplification success rate greater than 50% (table 2 in supplementary material).

From the two approaches, we finally validated 12 microsatellite markers. In this microsatellite markers' set, no evidence of LD was detected between all pairs of loci ($P<0.0004$, significance threshold adjusted with the Bonferroni procedure for 120 tests). However, we detected deviations from Hardy–Weinberg expectations and signs of null alleles among several pairs of loci/populations ($P < 0.001$, significance threshold adjusted with the Bonferroni procedure for 48 tests). Microchecker analysis indicated that null alleles do not result from technical problems (i.e. allele dropout or stuttering errors), but rather has a biological explanation, i.e. a general excess of homozygotes for most allele size classes. It is difficult to know if this result is linked to intrinsic characteristics of the loci or the populations. Indeed, such a homozygote excess could be explained by our quite small sampling or a Wahlund effect resulting from a sampling which does not represent real genetic populations. Concerning genetic polymorphism of the selected microsatellite markers, the number of alleles per locus ranged from two to six and gene diversity ranged from 0.28 to 0.76 (table 1).

Although pairwise Wilcoxon tests revealed that the number of alleles was significantly higher for microsatellite markers isolated from the 454 approach relative to those isolated from cross-species amplification (Wilcoxon tests: $P = 0.002$), no significant differentiation was revealed for heterozygosity (Wilcoxon tests: $P = 0.26$).

Locus transferability in terrestrial isopod species

Four of the nine microsatellite markers identified by the 454 sequencing approach and yielding an amplification band in *A. nasatum*, were successfully amplified in *A. vulgare* and three out of the nine in *A. depressum*, in both of which they are polymorphic (i.e. more than two alleles). However, no marker was amplified in *P. pruinosus* and *P. d. dilatatus* (table 2).

Discussion

In this study, we highlighted a polymorphic microsatellite marker set useful for *A. nasatum*. We isolated 12 microsatellite markers using two different approaches: 454 pyrosequencing of a repeat-enriched library and cross-species amplification. Among these 12 microsatellite markers one: AV0018, has already amplified alleles, in other conditions, in *A. nasatum* (Giraud *et al.* 2013). Thus, the 11 new loci increase the microsatellite marker number available in *A. nasatum*. The six microsatellite markers obtained from 454 pyrosequencing showed higher allele number than those isolated from cross-species amplification. We hypothesize that molecular markers with a high mutation rate are less conserved across the phylogeny than those showing low mutation rate, hence the lower polymorphism rate for markers originally isolated from *A. vulgare* (Giraud *et al.* 2013). Moreover, the cross-species amplification rate found in this study (i.e. 6/50 = 12% from *A. vulgare* to *A. nasatum*, 4/9 = 44% from *A. nasatum* to *A. vulgare* and 3/9 = 33% from *A. nasatum* to *A. depressum*) is consistent with results reported by Barbarà *et al.* (2007) from a bibliographic survey, revealing an average cross-species amplification success of 38% in arthropod groups. Cross-species amplification success depends mainly on the genetic distance between tested species. Thus, this could explain the failure of cross-amplification between the distantly related isopod families Armadillidae (i.e. *A. nasatum*, *A. vulgare* and *A. depressum*) and Porcellionidae (i.e. *P. pruinosus* and *P. dilatatus dilatatus*).

This study provides promising tools for *A. nasatum* and increases the number of microsatellite markers available for *A. vulgare* (Verne *et al.* 2006; Giraud *et al.* 2013). Further, these markers represent the first microsatellite marker set available for *A. depressum*. An important tool in molecular ecology studies, these microsatellite markers will allow to

investigate the genetic diversity and population structuring of *A. nasatum* and to improve molecular studies on *A. vulgare* and *A. depressum*. We are currently using the polymorphism of this microsatellite marker set to investigate the genetic structure of *A. nasatum* populations occurring in an agropastoral landscape located in western France. This study aims to investigate the influence of landscape features on genetic structure and the gene flow (rate and direction) of *A. nasatum* populations in a human-dominated landscape.

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