Molecular Biology Reports (2018) 45:203–209
https://doi.org/10.1007/s11033-018-4153-4

SHORT COMMUNICATION

Polymorphic nuclear markers for coastal plant species with dynamic geographic distributions, the rock samphire (*Crithmum maritimum*) and the vulnerable dune pansy (*Viola tricolor* subsp. *curtisii*)

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Received: 13 October 2017 / Accepted: 31 January 2018 / Published online: 5 February 2018

Abstract
Identifying spatial patterns of genetic differentiation across a species range is critical to set up conservation and restoration decision-making. This is especially timely, since global change triggers shifts in species' geographic distribution and in the geographical variation of mating system and patterns of genetic differentiation, with varying consequences at the trailing and leading edges of a species' distribution. Using 454 pyrosequencing, we developed nuclear microsatellite loci for two plant species showing a strictly coastal geographical distribution and contrasting range dynamics: the expanding rock samphire (*Crithmum maritimum*, 21 loci) and the highly endangered and receding dune pansy (*Viola tricolor* subsp. *curtisii*, 12 loci). Population genetic structure was then assessed by genotyping more than 100 individuals from four populations of each of the two target species. Rock samphire displayed high levels of genetic differentiation ($F_{ST}=0.38$), and a genetic structure typical of a mostly selfing species (F_{IS} ranging from 0.16 to 0.58). Populations of dune pansy showed a less pronounced level of population structuring ($F_{ST}=0.25$) and a genotypic structure more suggestive of a mixed-mating system when excluding two loci with heterozygote excess. These results demonstrate that the genetic markers developed here are useful to assess the mating system of populations of these two species. They will be tools of choice to investigate phylogeographical patterns and variation in mating system over the geographical distribution ranges for two coastal plant species that are subject to dynamic evolution due to rapid contemporary global change.

Keywords Gene flow · Geographic range shifts · Global change · Nuclear microsatellites · Plant mating system · Population genetic structure

Introduction

In an age of rapid contemporary global change, understanding the processes and the factors that shape geographical patterns of genetic differentiation is a critical prerequisite to acquire biologically meaningful predictions of plant evolution, and for designing efficient conservation and restoration practices [1, 2]. Indeed, global environmental changes induce shifts in the geographical range of many plant species owing to habitat change and/or climate change [3, 4]. Species respond differently to these rapid environmental changes, according to their life-history attributes such as dispersal and competitive abilities, and their capacity to evolve by phenotypic plasticity or by genetic adaptation, which is dependent on standing genetic variation [5].

Towards the edges of a species' distribution, populations are generally thought to be characterized by reduced population size compared to well-established populations localized at the center of the range, although this feature is not always observed [6]. Low effective population size could result in increased random effect of genetic drift and a subsequent reduced level of genetic diversity that can impede adaptive ability to respond to environmental changes, a common pattern observed in many taxa [7–9]. Therefore, at the leading edge of a species' distribution, newly settled populations are founded by a few migrants, and are thus expected to harbor low levels of genetic diversity and large dispersal abilities

Data availability Nuclear DNA sequences used to design the 33 microsatellite loci primers have been assigned the following GenBank Numbers: MG009034–MG009066.

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[10]. On the other hand, at the trailing edge of a species' distribution, remnant populations are expected to exhibit larger levels of genetic diversity, a testimony of formerly larger population size, and their dispersal abilities may be lower. With respect to the evolution of reproductive attributes, small population sizes may drive a shift towards more inbred mating systems at both the leading and the trailing edge of a species' distribution [11]. Investigating how spatial patterns of population genetic features vary across species geographical range needs efficient polymorphic molecular markers to understand a species demographic history and to set up broadscale predictions facilitating conservation decision-making [7]. Here, we describe the first nuclear microsatellite markers for two coastal plant species facing rapid environmental changes, one common species expanding its geographical range northwards, and one endangered species of high conservation value with a contracting geographical distribution.

The rock samphire, *C. maritimum* (2n = 20 in most of its geographic distribution, except maybe in the Canaries islands [12]), is a perennial Apiaceae, found on rocky shores, cliffs and man-made environments such as harbors and dikes along most of the shores of the Black Sea, the Mediterranean basin, the Atlantic coast and up to the North Sea (Fig. 1a). The species appeared on the coasts of Belgium and the Netherlands only at the beginning of the twentieth century, where most populations are found in disturbed man-made environments, which suggests a recent origin [13]. The distribution of this species is limited by temperature and is likely currently expanding northwards [14, 15]. Leaves are rich in vitamin C and were used by sailors to prevent scurvy. Seeds may drift over long distances through major sea currents [16]. The dune pansy, *V. tricolor* subsp. *curtisii* (2n = 26 chromosomes [17]), is a perennial Violaceae found on young dunes along the coast of the North Sea, from northern islands in the Netherlands to Northern France (Fig. 1b). In Northern France, where the species reaches its trailing edge, populations are receding due to habitat loss. Seeds are mostly dispersed over short distances by various ant species [18].

Due to their strictly coastal—i.e. linear—geographic distribution, both species are perfect models to investigate the variation of genetic diversity, mating system and dispersal abilities throughout a gradient from geographical range center to geographical range edge (northward leading edge for rock samphire and southward trailing edge for dune pansy).

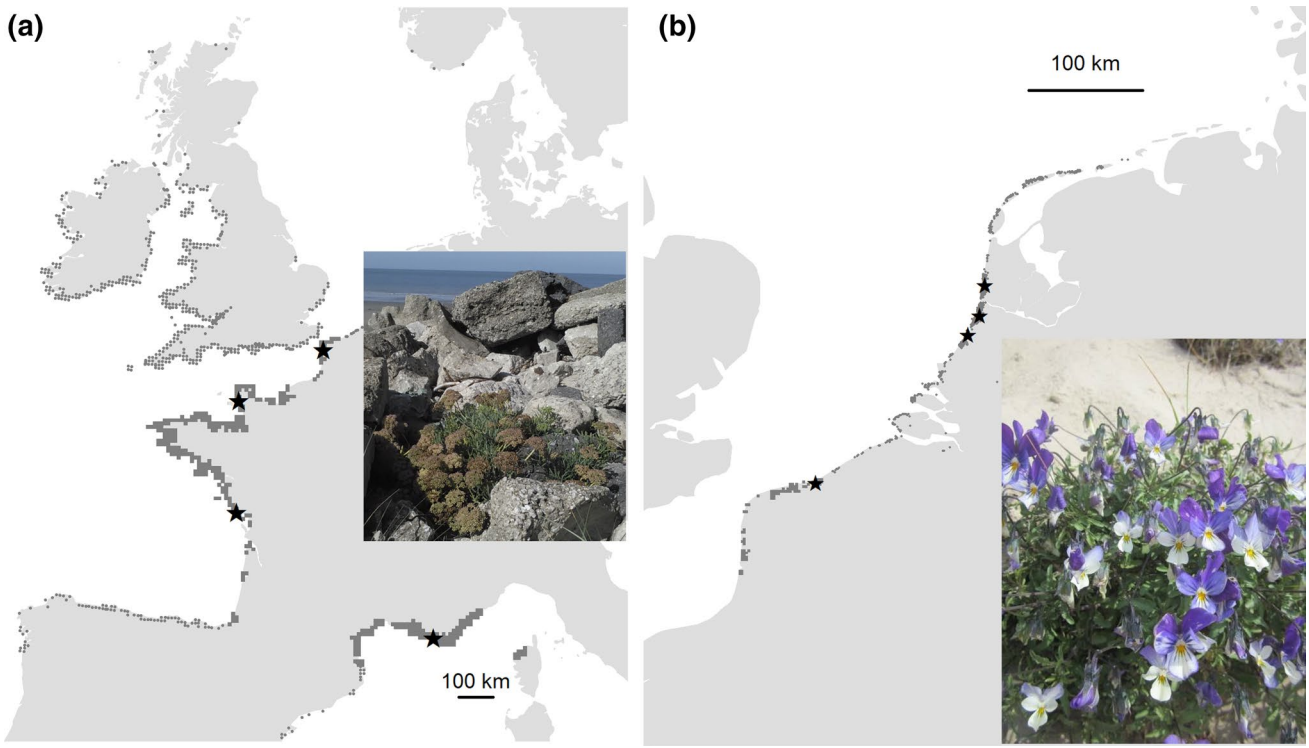

Fig. 1 Distribution ranges of **a** rock samphire (*Crithmum maritimum*), **b** dune pansy (*Viola tricolor* subsp. *curtisii*). Dark grey indicates occurrences according to the Federation of French botanical conservatories (http://siflore.fcbn.fr; larger squares) and the Global Biodiversity Information Facility (http://www.gbif.org/; smaller squares). Black stars indicate the locations of the populations genotyped in this study

Materials and methods

Total genomic DNA from *C. maritimum* and *V. tricolor* subsp. *curtisii* was isolated using the NucleoSpin 96 plant II kit (MACHEREY-NAGEL, Duren, Germany) following the manufacturer instructions and was sent to the GenoScreen genomic platform, Lille, France (http://www.genoscreen.fr). A random pool of ten individuals was chosen for Shotgun sequencing of 38.88 and 22.82 ng genomic library for *C. maritimum* and *V. tricolor* subsp. *curtisii*, respectively. A high-throughput method for isolating high-quality microsatellite markers for non-model organisms was then used as described in Malausa et al. [19]. One microgram of genomic DNA was mechanically fragmented and enriched for the following eight probes: TG, TC, AAC, AAG, AGG, ACG, ACAT and ACTC repeat motifs. Enriched fragments were subsequently amplified. PCR products were purified, quantified and GS-FLX libraries were then realized according to manufacturer's protocols and sequenced on a GS-FLX Titanium PicoTiterPlate (Roche Diagnostics, Mannheim, Germany), as in Faucher et al. [20]. Of 33,284 and 33,931 randomly fragmented sequences, 2151 and 1408 non-compound sequences containing microsatellite motifs were retained for *C. maritimum* and *V. tricolor* subsp. *curtisii*, respectively. A total of 134 and 146 sequences likely to contain suitable highly polymorphic markers, excluding microsatellite motif shorter than five repeats, were then kept for *C. maritimum* and *V. tricolor* subsp. *curtisii*. Among these, 47 loci with the longest repeat sequences (at least eight repeat motifs) were tested for efficient low-cost genotyping.

PCR reactions were performed in optimized cost-effective multiplexes that comprised sets ranging from four to nine microsatellite loci in a single PCR reaction (see Table 1). Forward primers for successfully amplified microsatellite loci were labelled with AT565™ (Eurofins Scientific, Luxembourg), HEX™, AT550™ (Eurofins Scientific, Luxembourg), or 6-FAM™ fluorescent dye (Applied Biosystems, Foster City, California, USA). We used 10 µL volume including 1× multiplex PCR master mix (QIAGEN Hilden, Germany), 3 µL (5–20 ng) of genomic DNA, and 0.2 µM of labelled forward and reverse primer. The PCR cycling program had an initial denaturation of 95 °C for 15 min; 28–32 cycles of 94 °C for 30 s, annealing temperature of 55 °C for 90 s, and 72 °C for 1 min; and a final extension at 60 °C for 30 min. PCR was conducted on a Eppendorf Mastercycler pro 384 (Applied Biosystems). 1.5 µL of PCR amplicon was pooled with 0.25 µL of GeneScan 500 LIZ size standard (Applied Biosystems) and 9.75 µL of deionized formamide (Applied Biosystems), electrophoresed and finally sized using an ABI PRISM 3130 Sequencer (Applied Biosystems) and the software GeneMapper version 5 (Applied Biosystems), respectively.

To screen the polymorphism of most suitable microsatellite loci, four populations were sampled for each species, totalizing 107 and 113 individuals with sampling size ranging from 19 to 35 and from 23 to 30 individuals for *C. maritimum* and *V. tricolor* subsp. *curtisii*, respectively. Species geographical distribution and locations of collected populations are shown in Fig. 1. Using FSTAT version 2.9.3 [21], linkage disequilibrium among loci and the following standard population genetic parameters were estimated for each locus across the surveyed populations: the number of alleles (A_n), the allelic richness (A_r) corrected for minimum sampling size, the observed (H_o) and expected (H_e) heterozygosity under Hardy–Weinberg equilibrium, and the unbiased fixation index (F_{IS}). Statistical significance of single-locus F_{IS} estimates were tested using 10,000 random permutations of alleles among individuals. Mean levels of genetic differentiation (F_{ST}) among populations were also calculated for each locus using FSTAT. Significance of single-locus F_{ST} values were assessed using a G test by randomly permuting genotypes among populations, as suggested by Goudet et al. [22] when populations are suspected to deviate from random mating.

Results and discussion

Out of 47 microsatellite loci tested for each species, 21 (*C. maritimum*) and 12 (*V. tricolor* subsp. *curtisii*) were polymorphic with easily readable electrophenograms, no dubious genotypes, and clear PCR amplification product sizing. The remaining loci showed non-specific amplifications or unreadable stutter peaks. No significant linkage disequilibrium was observed for any pairs of loci for either species, after Bonferroni corrections [23]. Primer pairs were successfully combined into multiplexes to reduce time and genotyping costs: three (*C. maritimum*) and two (*V. tricolor* subsp. *curtisii*) multiplexes were optimized, each including from four to nine loci successfully amplified (Table 1).

Substantial polymorphism was observed for both species: for *C. maritimum* the number of alleles ranged from 3 to 12 among loci for a total of 110 alleles; for *V. tricolor* subsp. *curtisii* 2 to 15 alleles were observed among loci for a total of 88 alleles. Allelic richness ranged from 1.98 to 9.30 and from 1.46 to 10.71 for *C. maritimum* and *V. tricolor* subsp. *curtisii* respectively.

Crithmum maritimum unambiguously displayed a genotypic structure typical of what can be observed in predominantly selfing species [24, 25]: H_o and H_e ranged from 0.036 to 0.434 and from 0.061 to 0.622 respectively, with all but one locus showing significant F_{IS} estimates ranging from 0.162 to 0.583 (Table 1). In *V. tricolor* subsp. *curtisii*, two loci were characterized by heterozygote excess (loci VTC 37 and VTC 41, see Table 1). Within each population, these

Table 1 Name, species identity, primer sequence (5′–3′), repeat motif from the original sequence, allelic size range (bp), multiplex number, dye used, the total number of allele (A_n), the allelic richness (A_r) adjusted for a minimum sample size of 18–21 individuals, mean observed (H_o) and expected (H_e) heterozygosity, mean intra-population fixation index (F_{IS}, a measure of departure from panmixia), mean population differentiation (measured by F_{ST}), and the accession numbers for 21 and 12 highly polymorphic microsatellite markers isolated in the common rock samphire, *Crithmum maritimum* (CM), and the endangered dune pansy, *Viola tricolor* subsp. *curtisii* (VTC), respectively

Locus name	Species	Primer sequences (5′–3′)	Repeat motif	Allelic size range	Multiplex number	Dye	A_n	A_r	H_o	H_e	F_{IS}	F_{ST}	Accession no.
CM03	CM	F: GCTTACTTAGTTGAGATCCAGTGTTT R: TCCACGACTACGAGAGAGGG	$(AAG)_{19}$	105–159	1	HEX	12	9.30	0.434	0.622	0.311***	0.321***	MG009034
CM04	CM	F: AAAACATTCATCTCCTGCACTC R: TGAAAATTTTGAACTGTGAGTTTGA	$(TTC)_{18}$	157–200	1	AT550	10	8.13	0.332	0.644	0.441***	0.217***	MG009035
CM06	CM	F: ATTACCGGCTGACTCGTGAT R: AGGACTTGTGTGACTTGCAGAA	$(AC)_{15}$	185–189	1	AT565	3	1.98	0.036	0.061	0.474*	0.035*	MG009036
CM07	CM	F: GCGTGATTCAAAGATCAAGAAC R: GGAAGCTTTCTTCTACCATTGC	$(AAC)_{15}$	262–288	1	6-FAM	4	3.14	0.068	0.153	0.583***	0.756***	MG009037
CM19	CM	F: TGATGAGACCAAGAAGGGAGA R: CACTCACATCAACCTGGTTCT	$(AG)_{11}$	177–181	1	6-FAM	3	2.87	0.160	0.226	0.317**	0.068***	MG009042
CM20	CM	F: AGCTGCTGCCTCCCTACTTT R: TGAACACTCGGTTAAGCAGTTC	$(AG)_{11}$	283–291	1	HEX	4	3.31	0.101	0.147	0.326*	0.709***	MG009043
CM35	CM	F: ATGTGCATCATAAGTCGATTCC R: TTGATCGGGAGCTTTGGA	$(AG)_{10}$	282–296	1	AT565	5	3.66	0.227	0.342	0.319***	0.448***	MG009051
CM36	CM	F: CGAGTCAAGGAATGAGAGAGG R: CGCACTGGTTCTTCCATAGAGT	$(AC)_{10}$	326–335	1	AT550	3	2.99	0.159	0.367	0.538***	0.142***	MG009052
CM11	CM	F: GAAATAAAGAAAGCAACTGGTGG R: TGGATCAAGATCAGAGCACA	$(AG)_{13}$	140–153	2	6-FAM	6	5.35	0.282	0.346	0.162*	0.535***	MG009038
CM12	CM	F: TGTGATTCTTGTTTGTTTGCTGT R: GAACACACATGGCATGAACAA	$(AG)_{13}$	166–186	2	HEX	7	4.48	0.089	0.209	0.570***	0.598***	MG009039
CM14	CM	F: CACCTCAATTCCCGAATACAA R: TTGCCCTATCTTCTGTTCTTCA	$(AC)_{13}$	181–209	2	AT550	7	4.70	0.311	0.436	0.287**	0.430***	MG009040
CM15	CM	F: CATGGAGCAACACATCAAGAA R: TGGTCCTAAGCCTATTCAGGTC	$(CA)_{13}$	99–110	2	AT565	7	4.46	0.236	0.397	0.409***	0.390***	MG009041
CM21	CM	F: GCAAAGCTTTCAGCAGACCT R: GCATACATAGCAGGTAGAGCAA	$(AC)_{11}$	296–302	2	AT565	4	3.24	0.124	0.217	0.407**	0.166***	MG009044
CM27	CM	F: TCAAGTTCTTTCCAAGATTTCAA R: CAGGAGAGCAAGTGATAATCAGAG	$(AG)_{10}$	231–235	2	6-FAM	3	2.68	0.127	0.139	0.075^{NS}	0.704***	MG009045
CM28	CM	F: GCTCGTACGATCTTGTAAACATACC R: AACAGGCTCGGCAAAGATAA	$(AC)_{10}$	284–288	2	HEX	3	2.97	0.156	0.222	0.302**	0.548***	MG009046
CM30	CM	F: GTGCCATTGGAGTCCAGTTC R: TTGAATCTGAGTTTGAGCCG	$(AG)_{10}$	253–259	2	AT565	4	3.18	0.303	0.409	0.286***	0.006^{NS}	MG009047
CM33	CM	F: TCTCTGATTGCTTTGTCTCTATGC R: CAAGATTTACATCGGGTTCTTG	$(AG)_{10}$	301–318	2	NED	6	4.47	0.336	0.513	0.378***	0.214***	MG009049

Table 1 (continued)

Locus name	Species	Primer sequences (5′–3′)	Repeat motif	Allelic size range	Multiplex number	Dye	A_n	A_r	H_o	H_e	F_{IS}	F_{ST}	Accession no.
CM31	CM	F: TGATGGGTCGGGTTTAATTT R: CATGGCTTCTCTTCTCGCTC	$(AG)_{10}$	253–259	3	NED	4	3.04	0.284	0.430	0.474***	0.003^{NS}	MG009048
CM34	CM	F: ACCTAGGCAAGTGATCAGTTTATTTC R: ACTATCATCATTTCGCATCCC	$(AG)_{10}$	289–312	3	6-FAM	7	4.56	0.083	0.171	0.517***	0.656***	MG009050
CM38	CM	F: TTCAGCTTGGCATTCACATC R: TGATCAAAGTTTGCCATCCA	$(CAT)_{10}$	128–132	3	AT565	3	2.99	0.300	0.464	0.336***	0.240***	MG009053
CM41	CM	F: CACACCGGATCCACACAGT R: TGATTGAGTTGAAGTGATGGTG	$(AG)_9$	113–121	3	HEX	5	3.21	0.292	0.459	0.354***	0.158***	MG009054
VTC02	VTC	F: AACATGCGCCAGTTCATT R: CTGAATGGAAAGGTACATCAA	$(AAC)_{15}$	110–168	4	6-FAM	15	10.71	0.600	0.780	0.232***	0.092***	MG009055
VTC03	VTC	F: AGTTGTCGCCAGTTTACGAT R: AGAAATGGCACACAATCCTT	$(AAAG)_{14}$	259–321	4	6-FAM	14	8.45	0.538	0.664	0.193***	0.205***	MG009056
VTC07	VTC	F: TTTGATTCTCAGGAGGAAGA R: GACAGCAGAGCTATGACCAG	$(AGG)_{11}$	106–132	4	AT550	7	5.32	0.416	0.440	0.063^{NS}	0.107***	MG009057
VTC08	VTC	F: CGCAGGTTAGCCAATGTAT R: GAATTCGGGCAGATGATTT	$(AG)_{10}$	168–206	4	AT565	9	5.45	0.557	0.605	0.093^{NS}	0.238***	MG009058
VTC13	VTC	F: TAGTACGTTGCTTGCTGGAG R: GCTTTGCCTCTCTCCTTACA	$(AG)_8$	156–169	4	HEX	8	6.49	0.357	0.489	0.282***	0.410***	MG009060
VTC15	VTC	F: GCAGTCCAATCACTATAAGCC R: TCGTTTGTCACCTTCATCTG	$(AG)_8$	301–307	4	AT565	4	3.02	0.250	0.253	0.007^{NS}	0.065***	MG009061
VTC23	VTC	F: GAGCTTCTCTACAATTACTTTTGTCC R: AGATCATTGAGTTGGTGGGC	$(CT)_7$	316–319	4	HEX	3	3	0.242	0.320	0.224**	0.508***	MG009063
VTC09	VTC	F: CAAAGTTGTTCCAGCGATGA R: AAATCTGACCCGGTTCTCG	$(TGA)_{10}$	82–151	5	6-FAM	15	9.32	0.373	0.586	0.368***	0.324***	MG009059
VTC22	VTC	F: GCCTCAGAGTCATCCAAAGG R: GCTGTGCATCTTCCTCCCT	$(AGA)_8$	88–100	5	AT565	5	4.17	0.539	0.498	-0.054^{NS}	0.124***	MG009062
VTC34	VTC	F: CTGGATCATGAAATCGGGTC R: GAGTGAAAGCGCAACAATCA	$(TA)_6$	269–271	5	HEX	2	1.46	0.009	0.025	0.659**	0.022^{NS}	MG009064
VTC37	VTC	F: GGCTTGCGATCCAATGTTAT R: AACCCTACGACAGGTTGCAT	$(AGA)_6$	159–168	5	AT565	2	1.99	0.287	0.179	−0.579***	0.340***	MG009065
VTC41	VTC	F: CCATGATCAGTTCTCCAGCA R: GAAAGTGGGATGAATGACTTGA	$(AG)_6$	127–135	5	HEX	4	2.89	0.379	0.279	−0.377***	0.171***	MG009066

All results were estimated over four populations in each species ($N = 107$ and 113 individuals for *Crithmum maritimum* and *Viola tricolor* subsp. *curtisii*, respectively)

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

^{NS}Non-significant

two loci were either fixed at a monomorphic state, or at a heterozygous state. Given that the other loci showed consistent F_{IS} values, this pattern rules out clonality. *V. tricolor* is known to show karyological instability, with sometimes different ecotypes sharing the right chromosome number (2n = 26) but exhibiting different chromosome architecture [26]. These two loci were thus not true microsatellite loci useful to infer the levels of inbreeding, but probably resulted from the duplication of a locus that no longer behaves as a microsatellite. When excluding these two loci, *V. tricolor* subsp. *curtisii* exhibited a genotypic structure suggestive of a mixed-mating system, with H_o and H_e varying from 0.009 to 0.600 and from 0.025 to 0.780, and with F_{IS} values being either suggestive of significant heterozygote deficiencies or perfect match with Hardy–Weinberg expectations.

As a result, genetic differentiation was significant for *C. maritimum* for all loci but one, with F_{ST} values ranging from 0.035 to 0.756 with a mean multilocus value of 0.384 ($P < 10^{-3}$). Genetic differentiation was less pronounced for *V. tricolor* subsp. *curtisii*, but still significant except for one locus: single-locus F_{ST} estimates varied from 0.092 to 0.508 (all at $P < 0.05$, Table 1) with a mean multilocus value of 0.250 ($P < 10^{-3}$).

Overall, low-cost high throughput genotyping showed that these newly nuclear microsatellite loci exhibited substantial polymorphism and significant genetic divergence among populations that make them makers of choice for determining inbreeding levels, patterns of gene flow among populations and contrasts in intraspecific genetic diversity over the geographical distribution of both species. Further studies of the genetic diversity distribution, large-scale population structuring, paternity analyses and evolution of mating system at the leading and trailing edges of species distribution in these two species of contrasting life-history traits will help understand the demography of shifting distribution range edges and the adaptive evolution imposed by selective pressures due to rapid changing environment [5, 9].

Acknowledgements We wish to thank Vincent Comor, Chloé Ponitzki and Eric Schmitt for help in collecting populations. This work was funded by the "Région Nord–Pas-de-Calais" (AREOLAIRE project). This work was also supported by a PhD fellowship from the French Research Ministry and from the "Région Nord–Pas-de-Calais" (AREOLAIRE project) to Mathilde Latron. This work is also a contribution to the CPER research project CLIMIBIO. The authors thank the French Ministry for Higher Education and Research, the Hauts de France Regional Council and the European Regional Development Fund for their financial support to this project.

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