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Species-Diagnostic Single-Nucleotide Polymorphism and Sequence-Tagged Site Markers for the Parasitic Wasp Genus *Nasonia* (Hymenoptera: Pteromalidae)

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ABSTRACT Wasps of the genus *Nasonia* are important biological control agents of house flies and related filth flies, which are major vectors of human pathogens. Species of *Nasonia* (Hymenoptera: Pteromalidae) are not easily differentiated from one another by morphological characters, and molecular markers for their reliable identification have been missing so far. Here, we report eight single-nucleotide polymorphism and three sequence-tagged site markers derived from expressed sequenced tag libraries for the two closely related and regionally sympatric species *N. giraulti* and *N. vitripennis*. We studied variation of these markers in natural populations of the two species, and we mapped them in the *Nasonia* genome. The markers are species-diagnostic and evenly spread over all five chromosomes. They are ideal for rapid species identification and hybrid recognition, and they can be used to map economically relevant quantitative trait loci in the *Nasonia* genome.

KEY WORDS *Nasonia*, species identification, Ecotilling, genome mapping

Wasps of the genus *Nasonia* Ashmead and its close relatives *Muscidifurax* Girault & Sanders, *Spalangia* Latreille, and *Trichomalopsis* Crawford are pupal parasitoids of the common house fly, *Musca domestica* L., and related filth flies, which breed in accumulated animal wastes and garbage (Patterson and Rutz 1986, Legner 1995). These flies are regarded as disease vectors of major significance to human health (Greenberg 1973). The common house fly, for example, is known to transmit >100 human pathogens, including those responsible for coxsackie disease, conjunctivitis, and many enteric diseases (cholera, salmonellosis, coli and amoebic dysentery) (Greenberg 1973). Therefore, it is not surprising that *Nasonia* received considerable attention in applied entomology as a biological control agent of the common house fly, and it is sold routinely by commercial insectaries for control of house flies in dairy farms, feedlots, and poultry rearing operations (Axtel and Rutz 1986). Beside its importance for con-

trolling fly populations, *Nasonia* has become an important model system for behavioral, evolutionary, developmental, and genetic research (Whiting 1967, Pultz and Leaf 2003; also see van Opijnen et al. 2005, Lynch et al. 2006, Beukeboom et al. 2007, Gadau et al. 2007, Shuker et al. 2007). Key features for receiving this recognition are its parasitic life cycle, haplodiploid sex determination, and the ease of interspecific crossbreeding (Beukeboom and Desplan 2003).

Because adult females of *Nasonia* are highly similar morphologically (Darling and Werren 1990), studies on natural populations have been hampered by a lack of molecular markers that allow reliable species identification and hybrid recognition. The extensive use of *Nasonia* species in biological control and as model system to study the genetics of economically important traits (e.g., host recognition, host preference, egg clutch size) requires describing molecular genetic markers to aid the identification of individuals in the genus *Nasonia*. Here, we describe and characterize eight single-nucleotide polymorphism (SNP) and three sequence-tagged site (STS) markers that cover all five chromosomes of *Nasonia* spp. The markers can be used for rapid species identification and reliable detection of hybrids in field populations of *N. giraulti* and *N. vitripennis*; both species occur in the eastern United States in microsympatry. The markers can further be applied for mapping quantitative trait loci (QTL) of commercially relevant traits in the *Nasonia* genome.

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Materials and Methods

Potentially species-specific SNP and STS markers were identified for 23 randomly chosen genes in expressed sequenced tag (EST) libraries for *Nasonia vitripennis* and *N. giraulti* made from prepupal to adult stages: actin (ACT), apolipoprotein III (apoLp-III), Cu/Zn superoxide dismutase, cytochrome *c* oxidase subunit Va, elongation factor (EF)-1 α (F1 copy), heat shock protein 83, 26-kDa lectin, prophenoloxidase activating factor 3, ribosomal protein S2, troponin C, and ubiquinol-cytochrome *c* reductase (Rieske iron-sulfur polypeptide 1). The primers to amplify the selected markers were designed with the aid of Primer3 on the Web at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

We studied the SNP and STS markers in two different sets of *Nasonia* samples: 1) 50 individuals of the two *Nasonia* species collected in the field from distant locations as well as from sites where both occur in microsympatry; specifically, 16 samples of *N. giraulti* collected from five sites (located in New York, Pennsylvania, and Virginia) within its restricted range in the eastern United States and 34 samples of *N. vitripennis* collected in Canada, France, The Netherlands, Russia, Sweden, and the United States (Idaho, Indiana, Minnesota, New York, Ohio, Oregon, Pennsylvania, and Utah); and 2) a mapping population consisting of 120 F₂ hybrid males obtained from a laboratory cross between *N. giraulti* and *N. vitripennis* (see below). The first set was used to survey marker variation within and between species. The second set was used to map the markers in the *Nasonia* genome with the aid of chromosome-specific microsatellite and sequence-tagged site markers (Pietsch et al. 2004, Rütten et al. 2004).

For obtaining the mapping population, we crossed a female of the *Nasonia* strain AsymCX with a male of the strain RV2X(U) and let the emerging F₁ hybrid females lay unfertilized eggs, which developed into the (haploid) F₂ hybrid males. The laboratory strain AsymCX is derived from the *Wolbachia*-infected *N. vitripennis* wild-type strain LBii; its geographical origin is Leiden, The Netherlands (Breeuwer and Werren 1990). RV2X(U) is descended from the *Wolbachia*-infected *N. giraulti* wild-type strain RV2, which had been collected in Rochester, NY (Breeuwer and Werren 1995). Both strains are available from us upon request.

DNA was extracted from adult wasps by using a Chelex protocol. Each wasp was homogenized with a sealed 1-ml pipette tip in 100 μ l of 5% Chelex suspension (Chelex 100; Bio-Rad, Hercules, CA) in TE buffer, pH 8.0. After adding 1 μ l of proteinase K (5 mg/ml), the samples were incubated for 1 h at 57°C and then for 5 min at 95°C. All sample vessels were finally centrifuged for 10 min at 21,000 \times *g*, and the supernatant was then transferred into sterile 1.5-ml tubes. Quantity and quality of all extracts were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For detecting SNP polymorphism, we adopted the recently described Ecotilling technique (Comai et al. 2004). The polymerase chain reaction (PCR) product with a

SNP site of unknown genotype is mixed with an equivalent PCR product of known genotype (=reference DNA). After heating and annealing the DNA strands, heteroduplex DNA is selectively cut at the mismatch site by an endonuclease, thus indicating a difference in the genotype between the sample and the reference DNA. Length polymorphism of STS markers was directly inferred by separating PCR products on a denaturing polyacrylamide gel.

PCRs were performed in 12.5- μ l volumes (1 \times Colorless GoTaq reaction buffer, 0.625 U of GoTaq polymerase; Promega, Madison, WI; 1.6 mM dNTP mix, 0.4 μ M of each primer, and 10 ng of DNA) and using an Eppendorf epGradient Mastercycler (Eppendorf, Hamburg, Germany). To enable the detection of the DNA fragments, we used fluorescently (IR700) labeled primers. For Ecotilling, we used differently labeled primers to amplify sample (F primer, IR700) and reference (R primer, IR800) DNA. As reference DNA, we used PCR products of the inbred strains RV2X(U) and AsymCX. The PCR temperature profile started with an initial 5-min denaturation step at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The profile ended with a 10-min extension step at 72°C.

For heteroduplex formation, we mixed 4 μ l of the amplified product from the samples with 4 μ l of the amplified product from the reference DNA. After denaturation at 95°C for 10 min., the mixtures were cooled down from 95 to 85°C with a rate of -2°C/s. The temperature was subsequently decreased from 85 to 25°C at -1°C/s and finally chilled to 4°C.

Mung bean endonuclease digestions were performed in 20- μ l volumes consisting of 7.5 μ l of annealed PCR-amplified product and 12.5 μ l of endonuclease buffer mix (16 mM MgSO₄, 320 μ M ZnSO₄, 32 mM Bis-Tris, pH 6.5, 0.032% Triton X-100, 0.32 μ g/ml bovine serum albumin, and 2 U of mung bean nuclease; New England Biolabs, Ipswich, MA) (LI-COR Technical Note/modified; LI-COR, Lincoln, NE). The reaction mix was incubated for 30 min at 60°C. We terminated the endonuclease activity by adding 2 μ l of 0.2% sodium dodecyl sulfate to each sample.

To separate and visualize the fluorescently labeled fragments, we used a LI-COR 4300 DNA Analysis System (LI-COR). We took 2 μ l of the endonuclease-treated PCR products and 2 μ l of the 1:10 diluted STS marker PCR products, respectively, and mixed them with 1 μ l of loading buffer (95% formamide, 20 mM EDTA, 40 mg of bromphenol blue). The DNA was then denatured at 95°C for 2 min. After chilling the samples on ice, the DNA fragments were separated on a denaturing polyacrylamide gel using 6.5% KB^{Plus} Gel Matrix (LI-COR). Fragment lengths were finally estimated with the SAGA Generation 2 software (LI-COR).

For mapping the markers in the *Nasonia* genome, we used the program MultiPoint (<http://www.multipoint.com>) (Mester et al. 2003a, 2003b, 2004). The putative function of ESTs associated with the analyzed STS and SNP markers was determined by similarity

Table 1. Oligonucleotide primers for the amplification of species-diagnostic SNP and STS markers in *N. giraulti* and *N. vitripennis*

| Locus | Type | Chr ^a | Primer sequences (5'-3') | Amplicon length ^b | Indel/SNP position ^c | Indel/SNP identity ^b | GenBank accession no. ^b |
|------------------|------|------------------|---|------------------------------|---------------------------------|---------------------------------|------------------------------------|
| EF-1 α F1 | STS | 1 | F: GGTCATCGTCTCAATCATC R: TTGTTTTACCTTTTCCCAACC | 219/214 | 154-158 | TCGTT/- | EF190561 EF190560 |
| Cu/Zn SOD | SNP | 1 | F: AGTTCATGCCTTTGTGATGC R: CACCGACAAACAGATTCCAGC | 253 | 152 | A/G | EF190557 EF190556 |
| TpnC | SNP | 2 | F: CTGAGGCTCTGGAGAAGGAG R: AAGTCGACGGTGCCAGAG | 257 | 121 | A/G | EF190573 EF190572 |
| UQCRFS1 | SNP | 2 | F: GCAACCTGAATGGTTGATTG R: TGGACCTTTCCTAATTCCTCC | 142 | 82 | A/T | EF190571 EF190570 |
| apoLp-III | STS | 3 | F: CCTTCCTGGAACTGCTTCTG R: TTCGTCAACAACGTCCAGAG | 271/270 | 136-141 177-181 | CGTAAA/- -/GAAAG | EF190555 EF190554 |
| RPS2 | SNP | 3 | F: TCAGCCTACTCGTTTCAAGG R: GCTTACCGATCTTTGTTAACC | 256 | 141 | T/C | EF190553 EF190552 |
| Hsp83 | SNP | 3 | F: GCTCTTCCTCGTCGATTCC R: GAAGCACCTCGAGATCAACC | 215 | 178 | T/C | EF190563 EF190562 |
| ACT | STS | 4 | F: TCCGAAACCAATTTCTTCTG R: AATGTGTATTAATAAGCACTTTCCG | 86/90 | 1-4 | -/GCTG | EF190569 EF190568 |
| PPAF-III | SNP | 4 | F: TGTCAAGCTCAAGGTCAACG R: TCCATACACAGGGTGACG | 237 | 49 | C/T | EF190559 EF190558 |
| COXVa | SNP | 4 | F: AATTCAATGTAAGGCTCGTTTC R: TGGATGAACTGGGTATCAGC | 144 | 77 | C/G | EF190567 EF190566 |
| 26-kDa lectin | SNP | 5 | F: CGTATTTTTCGAAGCCTCTCG R: TCCGAGATAAAAAGCTCGTTC | 153 | 35 | C/T | EF190565 EF190564 |

F, forward; R, reverse.

^a Positions of markers on the chromosomes (=Chr) are shown by Gadau et al. (2007).

^b The two sizes/alleles/accession numbers are for *N. giraulti* and *N. vitripennis*, respectively.

^c Position in the alignment of the two sequences that are deposited in GenBank.

search in GenBank. All sequences have been submitted to GenBank (accession numbers shown in Table 1).

Results and Discussion

Of the 23 primer pairs that had initially been selected for amplification of the EST-derived STS and SNP markers, six did not amplify and one amplified unreliably. The remaining 17 primer pairs amplified the expected products. However, only 11 of the amplified markers proved to be conserved within *N. giraulti* and *N. vitripennis* but diagnostically differed between them; they are listed and characterized in Table 1.

Intraspecific variation of all markers was assessed in *N. vitripennis* and *N. giraulti* by studying samples from various locations throughout their distribution. Because we found no evidence for intraspecific variation, each of the markers can be used for molecular species identification. However, we recommend studying a combination of markers to counteract potentially undetected intraspecific variation that could confound identification, in particular because the sample size for one of the species in our study (i.e., *N. giraulti*) was small. The markers EF-1 α F1, apoLp-III, and ACT are ideally suited for this purpose, because their amplicon length consistently differs between *N. giraulti* and *N. vitripennis*, facilitating high-throughput genotyping by simply separating PCR products on a denaturing polyacrylamide gel. If the genotype of one of the STS markers is different from the genotype of the other two markers, the remaining SNP markers should be analyzed to assess whether previously undetected intraspecific variation or whether hybridization (see below) could be the cause.

Genetic research in *Nasonia* has been promoted by the ease with which its species can be crossed in the laboratory after curing them from their *Wolbachia* endosymbionts, which cause cytoplasmic incompatibility (Breeuwer and Werren 1990, 1995). However, to what extent such hybrids also occur in nature is currently not well understood. Mitochondrial markers, which had previously been used to identify the cytoplasm of interspecific F₂ hybrids in laboratory experiments, cannot be used because of the maternal inheritance of the mitochondria (Gadau et al. 1999, van Opijnen et al. 2005). The nuclear markers reported in this article have the required properties to study the occurrence of hybrids in nature; they are spread over all five chromosomes and they allow the recognition of F₁ and F₂ hybrids. We expect F₁ hybrid females to be heterozygotic for all species-specific markers. Hybrid males, which first occur in the F₂ generation because of the haplodiploid sex determination in Hymenoptera, should exhibit on average a 1:1 ratio of the parental alleles at the species diagnostic markers. The same is expected for the (diploid) F₂ females.

Finally, we point out that the species-specific markers add to the available set of microsatellite and sequence-tagged site markers (Pietsch et al. 2004, Rütten et al. 2004), and they can be used in combination with these markers for mapping QTL of economically important traits, such as host recognition or egg clutch size, by studying hybrids between *N. giraulti* and *N. vitripennis* (Gadau et al. 2007).

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