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ORIGINAL ARTICLE

Behavioral and genetic characteristics of a new species of *Nasonia*

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Nasonia (Hymenoptera: Pteromalidae) is a genus of parasitoid wasps, which is fast emerging as a model system for evolutionary, genetic, developmental and host–endosymbiont interaction studies. In this study, we report a new species, *Nasonia oneida*, distinguish its behavioral, genetic and morphological features, and characterize its pre-mating and post-mating isolation with the other *Nasonia* species. Phylogenetic analyses indicate that *N. oneida* is the sister species to *Nasonia giraulti* with its own uniquely distinct cuticular hydrocarbon profiles, behavioral characteristics and subtle morphological differences. An important characteristic of *N. oneida* is the strong mate discrimination shown by the females against all the other *Nasonia* species. A genetic analysis of this phenotype by interspecies hybridization indicates that this strong discriminating phenotype is recessive. A formal species description of *N. oneida* Raychoudhury & Desjardins is also provided.
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Introduction

The parasitoid wasp *Nasonia* has been used for genetic research for over 50 years (Whiting, 1967), and with the recent genome sequencing for three species (Werren *et al*., 2010), methods for systemic RNA interference (Lynch and Desplan, 2006), and additional genetic tools, it is now emerging as a genetic model system (Werren and Loehlin, 2009). Evolutionary genetic studies of *Nasonia* have been conducted on sex ratio (Werren, 1980; Skinner, 1982), interspecific differences in morphology (Gadau *et al*., 1999; Weston *et al*., 1999; Clark *et al*., 2010), hybrid breakdown (Breeuwer and Werren, 1995; Gadau *et al*., 1999, 2002; Niehuis *et al*., 2008; Clark *et al*., 2010), host–endosymbiont interactions (Breeuwer *et al*., 1992; Bordenstein *et al*., 2001, 2006), courtship and mating behavior (Beukeboom and van den Assem, 2002; Velthuis *et al*., 2004; Burton-Chellew *et al*., 2007), and early development (Lynch *et al*., 2006; Rosenberg *et al*., 2009). Any additional species in the genus would broaden and strengthen this model system, especially for evolutionary genetic studies.

Until the 1990s, only one species was described in the genus, the cosmopolitan *Nasonia vitripennis*, which is a generalist parasitoid attacking a variety of calyptrate flies in the families Sarcophagidae, Muscidae and Calliphoridae. Darling and Werren (1990) subsequently described two additional sibling species (*Nasonia giraulti* and *Nasonia longicornis*) indigenous to North America. *N. longicornis* is found in the western United States and *N. giraulti* in the northeastern United States, and both are specialists on the calliphorid genus *Protocalliphora* (bird blowflies). In this study, we describe a fourth species of *Nasonia*, *Nasonia oneida*. We present the morphological, genetic and behavioral features of *N. oneida* and also comment on the population genetics of this new species and its sister species *N. giraulti*.

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Materials and methods

Nasonia strains used

Nasonia are usually collected from bird's nests where the wasps parasitize the blowfly pupae, which in turn parasitize altricial nestlings. Nests were collected following the fledgling of the nestlings and the blowfly pupae were then sorted from the nest. These were then placed individually in vials and emergence of parasitoids, if any, were recorded. Emerging *Nasonia* or other parasitoids were collected from them for further analysis. *N. oneida* was first obtained in 2005 from Brewerton, NY, USA, and subsequently in 2008 from Springville, which is 295 km southwest from Brewerton. Initially, this species was thought to be *N. giraulti* because the males of *N. oneida* have large forewings, which distinguishes *N. giraulti* from its sympatric relative *N. vitripennis*, in which the males have small forewings (Darling and Werren, 1990). Moreover, *N. oneida* and *N. giraulti* have very similar *Wolbachia* and mitochondria, reflecting introgression and subsequent sweep of cytotype (Raychoudhury *et al*., 2009). Thus, *N. oneida* isolates from the field were initially believed to be *N. giraulti* until other biological features

became apparent. Initial behavioral observations revealed pre-mating discrimination with a standard *N. giraulti* strain. This led to the discovery of additional phenotypic characteristics of *N. oneida* and additional field collections, confirming it as a distinct species.

In all, 11 different strains of *N. oneida* have been collected from two locations in the New York State. The standard laboratory strains of *N. oneida* are NONY11/36 (O1), which is from Brewerton, NY (USA), whereas NONYSP1C (O2) is the strain obtained from Springville, NY (USA). Both these strains were used for behavioral observations against standard laboratory strains from the other three species. As, in our preliminary analysis, *N. giraulti* seemed to be a very close relative of *N. Oneida*, we used two different strains from both *N. oneida* and *N. giraulti* for the behavioral analysis to rule out the possibility that features unique to these two taxa are not strain specific but are characteristic of the species. Thus, two *N. oneida* strains (O1 and O2) were used against two different *N. giraulti* strains, NGRV2X (G1), which is the standard strain for the species, and a recently collected strain called NGNY6A5 (G2), which was obtained in 2005 from New York. For *N. vitripennis* and *N. longicornis*, strains AsymCX and NLCA9304 were used, respectively. For phylogenetic analysis of the nuclear data, we used multiple strains from all the four species (summarized in Supplementary Table 1). O1 was cured of its *Wolbachia* infection to produce the strain O1U and was used for hybridization studies with strains from the other three species.

Evolutionary and phylogenetic analysis of DNA sequences

DNA was extracted from a single female insect per strain using the DNAeasy kit (Qiagen, Valencia, CA, USA). Nine nuclear genes were sequenced for this study: casein kinase, lipase, arp 2/3 complex, opsin I, phosphoglucose isomerase, cAMP-dependant protein kinase, ATP synthase coupling factor F, fumarylacetoacetate and a sugar transporter. The primer sequences and conditions are described in Raychoudhury *et al*. (2009). To test for divergence in the mitochondria, the *cox1* region was used with the primers and conditions described in Oliveira *et al*. (2008). The NCBI accession numbers for each fragment from all the strains are summarized in Supplementary Table 1. To clean the reactions before sequencing, amplified reactions (8 μl) were incubated with 0.5 U of shrimp alkaline phosphatase and 1.0 U of exonuclease I (Amersham, Piscataway, NJ, USA) along with the supplied buffer. Sequencing was performed directly from the amplified products using a BigDye v3.0 terminator sequencing kit and an ABI 3700 or 3730*xl* (Applied Biosystems, Foster City, CA, USA) automated sequencer. The chromatograms generated were manually inspected and cleaned with Sequencher (Gene Code Corporation, Ann Arbor, MI, USA) and the sequences were aligned with Bioedit vs 7.0.1 (Hall, 1999). The entire sets of nuclear sequences were concatenated and indels were removed. Bayesian maximum-likelihood trees were constructed using this concatenated data for the nuclear genes with MrBayes v 3.1.2 (Ronquist and Huelsenbeck, 2003). We used the web-based application Find Model (http://hcv.lanl.gov/content/hcvdb/findmodel/findmodel.html) to find the best fitting model for sequence evolution, which was the general time-reversible model with gamma-distributed rate variation (GTR + Γ). For phylogenetic analysis with MrBayes, the Markov chains were run for at least 1 000 000 generations, sampling every 10 generations until the s.d. of the split frequencies were <0.01. The first 25% of the generations were discarded as burn-in and the resultant 50% majority-rule trees were visualized in Treeview v1.6.6 (Page, 1996). Analyses of the different molecular genetic parameters were carried out using DNAsp vs 4.10.2 (Rozas *et al*., 2003). A median-joining network of sequences was constructed with Network (http://www.fluxus-engineering.com/sharenet.htm) for the *cox1* sequences, as well as the concatenated data set for the nuclear markers.

Mate discrimination

A series of no-choice assays with *N. oneida* females were carried out to establish the levels of pre-mating isolation with the other three species. Single males and females of fixed age (1–2 days postemergence) were put together in 12-mm glass culture vials for 10 min in a no-choice trial and mating were observed directly. Female rejection was scored when the male initiated courtship display but the female did not become receptive (Velthuis *et al*., 2004). If no courtship display was performed by the males during the 10 min of observation, then these data were removed from the final analysis. We also tested the mate preference of interspecies hybrid females. F1 hybrid females were produced by interspecies crosses between males from O1U and females from the other three species and vice versa. These reciprocal crosses resulted in F1 hybrid females with the cytoplasm of both *N. oneida* and each of the other three species. As the acceptance of *N. oneida* females for heterospecific males was very low, we used Fisher's exact test (FET), in preference to χ^2-test, for statistical analysis of these data.

F2 hybrid male breakdown and *Wolbachia*-induced postzygotic incompatibility

In *Nasonia*, there have been previous reports of F2 hybrid male mortality due to interspecific genetic incompatibility (Breeuwer and Werren, 1995). To test whether such incompatibility exists with *N. oneida* and the other species, we conducted reciprocal hybrid crosses between *N. oneida* and the other three species and quantified the number of F2 males. Because of its haplodiploid sex determination, *Nasonia* females produce only haploid sons as virgins. As these males are haploid, any recessive hybrid incompatibility loci causing inviability will result in a reduction in the number of F2 male offspring. Thus, the mean number of adult male offspring was used to determine whether *N. oneida* show any F2 hybrid male breakdown with the other species. Females of O1U who successfully mated with the other three species in the mate discrimination assay were hosted individually. F1 hybrid virgin females from these crosses were each hosted singly with two *Sarcophaga bullata* pupae for 3 days and the F2 males were allowed to emerge and were then counted.

We tested whether *Wolbachia* induces any postzygotic incompatibility between *N. oneida* and *N. giraulti*, as it does with the other species of *Nasonia* (Breeuwer and Werren, 1990; Bordenstein *et al*., 2001). Infected males and females from both the species were crossed and the females who successfully mated were hosted individually

with two *S. bullata* pupae for 2 days. The females were then removed and the progeny were allowed to emerge and then counted. All the *Nasonia* strains were maintained in standard laboratory conditions of 25 °C, 24 h light for 2 weeks.

Morphological measurements

N. oneida males and females were examined for species-specific morphological features. Females of all *Nasonia* species are difficult to distinguish, whereas males are morphologically distinct (Darling and Werren, 1990). This is also true for *N. oneida* because the male antennae and wings are relatively easy to distinguish. Wing measurements were carried out with a protocol refined from Weston *et al*. (1999). Individuals were reared in uncrowded conditions and single females were provided with two *S. bullata* hosts for 48 h. The forewings were dissected at the hinge and mounted dry on glass slides under coverslips. Heads were mounted on double-sided cellophane tape on the mandible. Wings were digitized using a Zeiss AxioImager Z1 and Zeiss AxioVision 4.6 software (Carl Zeiss, Thornwood, NY, USA) at $\times 10$ as mosaic images. Heads were digitized at $\times 4$ taking advantage of the green autofluorescence of the ocelli (Loehlin *et al*., 2010).

The forewing length was measured from digital images as the distance between the notch that forms at the proximal edge of the hinge and the distal tip of the wing. Width was measured as the width of a box drawn perpendicular to the length axis that contained the most anterior and posterior points of the forewing. The head width, an approximation of body size (Weston *et al*., 1999), was measured as the distance between the inner edges of the compound eyes at the posterior edge of the paired ocelli. A composite measurement, the normalized wing multiple, avoids the observed correlation between the wing and body sizes (Weston *et al*., 1999; Gadau *et al*., 2002). Normalized wing multiple is calculated as (wing length $\times$ wing width/head width). Length to width ratios of male antennal flagella were also measured.

CHC profiling with gas chromatography/mass spectrometry

Cuticular hydrocarbons (CHCs), secreted on the insect cuticle primarily for desiccation prevention (Gibbs, 1998), have been shown to have major roles in inter- and intraspecific chemical communication in various insect species (Ayasse *et al*., 2001; Howard and Blomquist, 2005). In many cases, CHC profiles clearly show sex- and species-specific differences, and distinct CHC components serve as signals for species recognition (Singer, 1998; Thomas and Simmons, 2008) and sexual communication (Ferveur, 2005). We tested the CHC profiles in both sexes of *N. oneida* and *N. giraulti* in an effort to determine whether the profiles support separation of these two as distinct species. Males and females from three strains of each species (*N. oneida* strains: NONYBR6A, NONYBR6B, NONY11/32; *N. giraulti* strains: NGNY6X, NGNY7B, NGVA7B) were analyzed amounting to 119 individuals in total. The wasps were freeze-killed and stored individually in glass vials at -20 °C after emergence from their *S. bullata* hosts. For CHC extraction, each wasp was placed for 10 min in 10 µl of hexane. The extracts obtained were then transferred into a fresh vial and reduced by evaporating the solvent with gaseous nitrogen to $\sim$1 µl. The concentrated extracts were subsequently injected into a gas chromatograph coupled with a mass spectrometer (GC: 7890A; MS: 5975C; Agilent Technologies, Waldbronn, Germany) operating in electron impact ionization mode. The entire sample was injected in a splitless injector in the splitless mode for 60 s with an injector temperature of 250 °C. Separation of compounds was carried out on a fused silica capillary column (HP-5ms; Agilent Technologies) coated with a 0.25 µm (5%-phenyl)-methylpolysiloxane stationary phase with temperature program starting from 60 °C and increasing by 40 °C per min to 200 °C, followed by an increase in 5 °C per min to 320 °C. Peak area integration and calculation were carried out using the data analysis software from Enhanced Chemstation G1701AA, Version A.03.00 (Hewlett-Packard Company, Palo Alto, CA, USA).

For data analysis, the absolute peak area values were divided by the total sum of all peaks (44 CHC peaks detected in total) to normalize the data and eliminate the effects of the fluctuations in the extracted quantities. Thus, peak ratios relative to the total CHC peak area sum were obtained for each peak in each individual, which were used for all subsequent analysis. A discriminant analysis was carried out using SPSS 11.01 for windows (SPSS, Chicago, IL, USA) to access the potential of the CHC profile differences to discriminate strains, species and sexes, amounting to 12 predefined groups. Wilks' λ and the percentage of correct assignments of individuals to the respective groups, as well as the percentage of correct classification in leave-one-out cross-validation were used as measurement of the quality of the discriminant analysis.

Results

Population genetics and divergence

Nuclear sequences were obtained from 11 different *N. oneida* strains, 13 different *N. giraulti* strains from 4 different states in the United States, along with 16 different *N. longicornis* strains and 2 *N. vitrpennis* strains (Supplementary Table 1). Sequences from nine nuclear markers were concatenated to give a data set of 5408 bp. The tree of these concatenated sequences (Figure 1) clearly shows *N. oneida* to be a distinct, reciprocally monophyletic lineage of *Nasonia*, most closely related to *N. giraulti* (posterior probability 100). Moreover, the nuclear haplotype network also clearly delineates *N. oneida* from its closest relative *N. giraulti* (Figure 2a). We estimated the total pairwise divergence between *N. oneida* and *N. giraulti* to be 0.80%, between *N. oneida* and *N. longicornis* to be 0.88% and between *N. vitripennis* and *N. oneida* to be 2.65% (summarized in Table 1).

The *Nasonia* lineage is presumed to have split relatively recently. Campbell *et al*. (1993) used the differences in the internal transcribed spacer 2 region of the *28S* rDNA locus and estimated the divergence between *N. longicornis* and *N. giraulti* to be 0.4 million years ago (MYA). Raychoudhury *et al*. (2009) used a rate of 2.2% synonymous divergence per million years (from Tamura *et al*., 2004) and estimated the same split to be 0.51 MYA. Using the same rate of synonymous divergence, the *N. oneida* and *N. giraulti* split is estimated to be around 0.41 MYA, and the *N. oneida* and *N. longicornis*

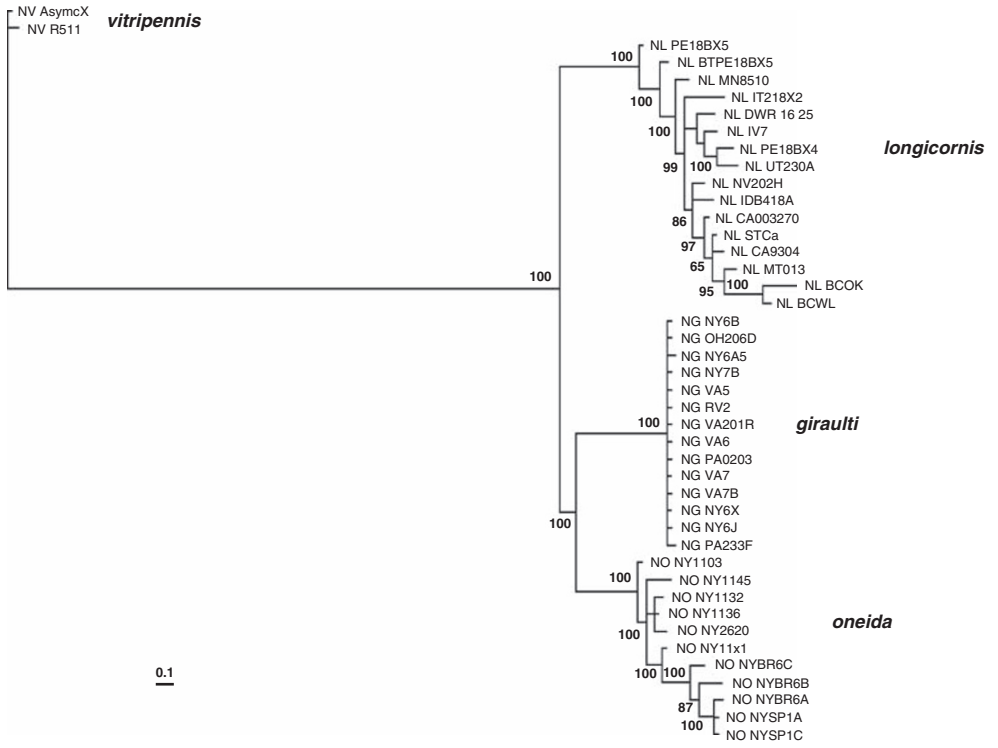

Figure 1 The MrBayes tree of the concatenated nuclear data set comprising of nine genes (total of 5408 bp), showing *N. oneida* to be an independent but closely related lineage of *Nasonia*.

split is estimated to be 0.46 MYA. Clearly, these three are closely related species with relatively recent divergences.

The total pairwise divergence in the mitochondria between *N. oneida* and *N. longicornis* is 8.69%, whereas the difference between *N. oneida* and *N. giraulti* is low, 1.24% (Table 1). There is evidence of a prior interspecies mitochondrial introgression between *N. oneida* and *N. giraulti* (Raychoudhury *et al*., 2009). Nevertheless, the mitochondrial haplotypes can be sorted by species (Figure 2b) and there are no shared mitochondrial haplotypes between the two species. Therefore, there has been sufficient time since the mitochondrial introgression for unique changes to accumulate in the *N. oneida* mitochondria, including six different synonymous changes that are fixed between the species.

Differences in wing size and male antennae

N. oneida forewings are slightly different in size from *N. giraulti*, in a pattern of elevated sexual dimorphism. Using the composite measurement of normalized wing multiple, which corrects the wing area for head size, *N. oneida* male forewings are significantly smaller than *N. giraulti* males ($n=20$, Student's *t*-test, $P<0.01$), with a mean difference of 5%. In contrast, *N. oneida* female forewings are 3% larger than *N. giraulti* ($n=20$, Student's *t*-test, $P<0.01$). This result is consistent with the pattern of differential sexual dimorphism for forewing size observed between the other *Nasonia* species (Darling and Werren 1990). *N. oneida* male antennal flagella also differed significantly in their length to width ratios from those of *N. giraulti* ($n=12$, Student's *t*-test, $P<0.0001$). The mean ± 1 s.d. for *N. oneida* was 10.43 ± 0.52, whereas it was 8.30 ± 0.27 for *N. giraulti*.

CHC profiles

We conducted a CHC profile comparison of males and females from *N. giraulti* and *N. oneida* using three different strains from each species. The CHCs of *N. oneida* and *N. giraulti* show a strong separation in both sexes (summarized in Figure 3).

The discriminant analysis carried out on the relative abundances of all the identified 44 CHC compounds significantly differentiated the 12 predefined groups according to sex and species (Wilks' $\lambda < 0.0001$, $\chi^2 = 1671.79$, $P<0.0001$). Function 1 accounted for 54% of the total variance, clearly discriminating the sexes of each species, whereas function 2, accounting for 29.1% of the total variance, separated the six different strains into sharply distinguishable species clusters (Figure 3).

Mate discrimination

One of the primary evidences that speciation has occurred is the presence of pre-mating isolation, which in most cases, is the first barrier to evolve (Coyne and Orr, 2004). *N. oneida* shows a distinct pattern of female mate discrimination against the males of the other species. This was the first character that alerted us to its difference with *N. giraulti*. In Figure 4a, we summarize the data for the behavioral isolation seen with *N. giraulti*. Females show a strong sexual isolation against heterospecific males. O1 and O2 females accept only 12 and 13% of G1 males, significantly less than in the reciprocal cross, in which G1 females accept 87 and 96% of O1 and O2 males (FET, $P<10^{-6}$). Acceptance of G2 males by the two *N. oneida* strains was also low, 12 and 15%, whereas G2 female accepted 86 and 92% males from O1 and O2, respectively (compared with within-species controls,

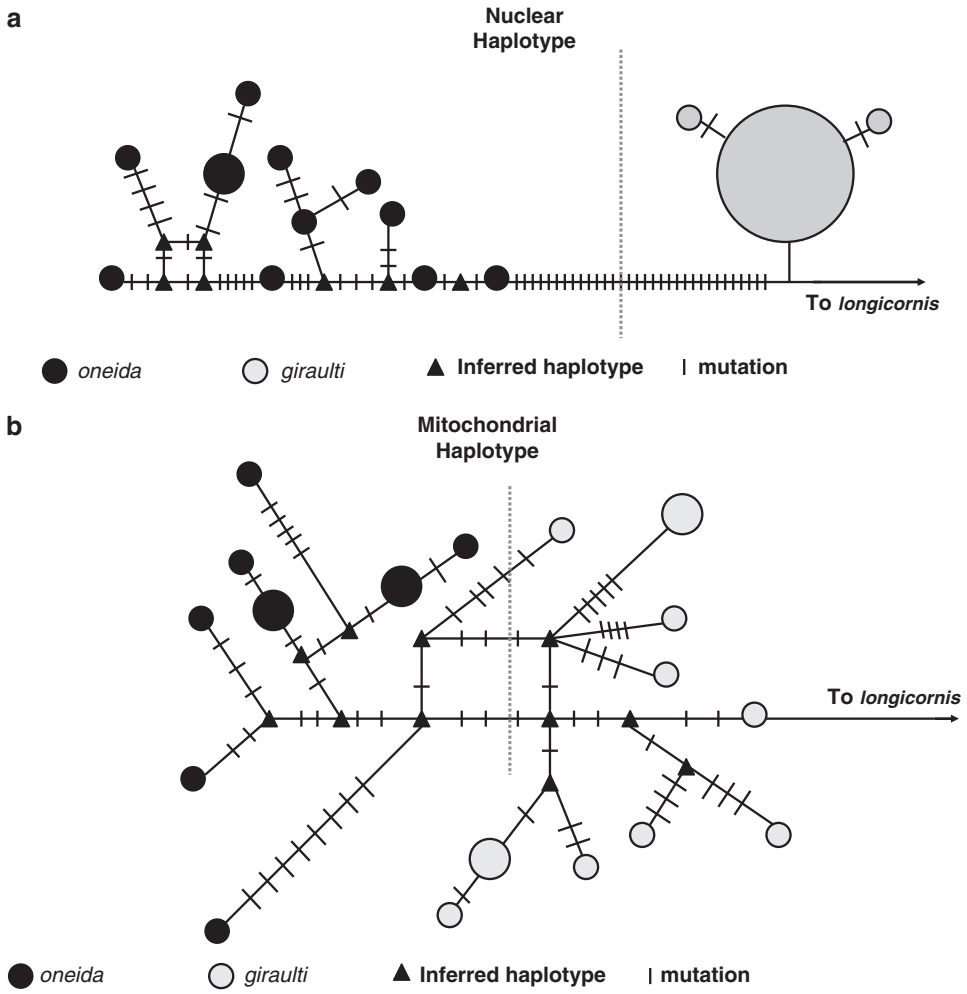

Figure 2 Haplotype networks of concatenated sequences for the nuclear genes (**a**) and the mitochondrial *cox1* gene (**b**). The size of each node represents its frequency (not to scale). The haplotypes representing the two species are separated by a dashed line.

Table 1 Measure of the divergence of *Nasonia oneida* with the other three species in synonymous, nonsynonymous and intronic sequences for both nuclear and mitochondrial DNA

	O/*V*	*O*/*L*	*O*/*G*
Nuclear genes			
Synonymous sites	0.0470	0.0101	0.0091
Nonsynonymous sites	0.0053	0.0088	0.0036
Intronic sites	0.0474	0.0125	0.0126
All sites	0.0265	0.0088	0.0080
Mitochondrial cox1			
Synonymous sites	0.6963	0.4660	0.0570
Nonsynonymous sites	0.0148	0.0054	0.0001
All sites	0.1231	0.0869	0.0124

Abbreviations: G, *N. girault*i; L, *N. longicornis*; O, *N. oneida*; V, *N. vitripennis*.
Results are on the basis of 2652 bp of coding sequences and 2424 bp of intronic sequences obtained from nine nuclear markers and 1000 bp of the mitochondrial *cox1* gene. All values are corrected with Jukes and Cantor method (Jukes and Cantor, 1969).

FET in both, $P<10^{-6}$). Thus, *N. oneida* females show strong sexual isolation against *N. girault*i males, but *N. girault*i females do not. Similarly, with males of *N. longicornis* and *N. vitripennis*, O1 and O2 also show strong discrimination and only accept 8 and 21% of *N. longicornis* males and 2 and 7% of *N. vitripennis* males, respectively (Figures 4b and c). Therefore, *N. oneida* females show strong sexual isolation against the males of the other three species. In its native range in New York State, *N. oneida* is sympatric with *N. vitripennis* and *N. girault*i, and this high level of mate discrimination probably has a role in maintaining genetic integrity of the species.

The genetic nature of the mate discrimination phenotype of *N. oneida* females was further investigated using F1 females from successful interspecies crosses. Results indicate that F1 hybrid females lose their discriminating phenotype. F1 hybrid females (with *N. oneida* cytoplasm) accept *N. girault*i males 75% of the time, significantly greater than the 12% acceptance seen in *N. oneida* females (compared with the acceptance of *N. oneida*, FET, $P<10^{-6}$). Hybrid females with *N. girault*i cytoplasm also do not discriminate against *N. girault*i males. Similarly, F1 females with both types of cytoplasm did not discriminate significantly against *N. oneida* males. This indicates that the mate discrimination phenotype is recessive because female discrimination against heterospecific males significantly decreases in heterozygotes.

A similar pattern is also seen with *N. longicornis* and *N. vitripennis* (summarized in Figures 4b and c, respectively).

Postzygotic F2 male hybrid breakdown

Nasonia species, although mostly interfertile after treatment with antibiotics to cure the resident *Wolbachia* infections, do show partial F2 hybrid male breakdown (Breeuwer and Werren, 1995) and thus some degree of intrinsic postzygotic isolation. Postzygotic isolation is most pronounced between *N. vitripennis* and *N. girault* (Breeuwer and Werren, 1995) and is visible as a reduction in the number of F2 male progeny of the hybrid females. Postzygotic isolation of *N. oneida* with all the other three species was investigated by crossing *Wolbachia*-free O1U females with *Wolbachia*-free males of the other three species and vice versa, and then checking for reduction in the mean number of F2 hybrid sons of the hybrid virgin females (Figure 5). There is significant hybrid breakdown between *N. vitripennis* and *N. oneida* irrespective of the cytoplasm of the F1 females (Mann–Whitney *U*-test (MWU), for *N. oneida* cytoplasm $U = 599$, $P < 0.001$, and in *N. vitripennis* cytoplasm, $U = 1225.5$, $P < 0.001$). With *N. girault*, there was significant difference in mean number of adult hybrid males relative to the control (MWU, for *N. oneida* cytoplasm $U = 351.5$, $P < 0.001$, and for *N. girault* cytoplasm, $U = 478$, $P < 0.001$). However, the effect was in the opposite direction from what was expected, as there was a significant increase in the number of hybrid males relative to the within species controls. Crosses between *N. longicornis* and *N. oneida* show an asymmetric effect on the F2 offspring number. No reduction in hybrid F2 male number is seen in the *N. oneida* cytoplasm relative to pure *N. oneida* virgins (MWU, $U = 210$, $P = 0.59$), but in the *N. longicornis* cytoplasm, there is a significant reduction in F2 hybrid males relative to pure *N. longicornis* (MWU,

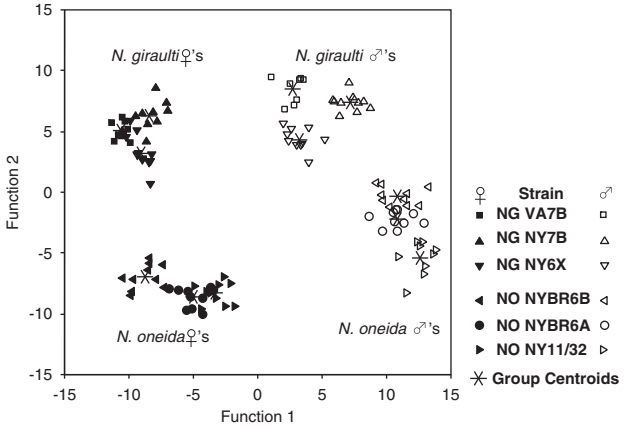

Figure 3 Discriminant analysis of cuticular hydrocarbon profiling of *N. oneida* and *N. girault*, showing clear separation by sex and species.

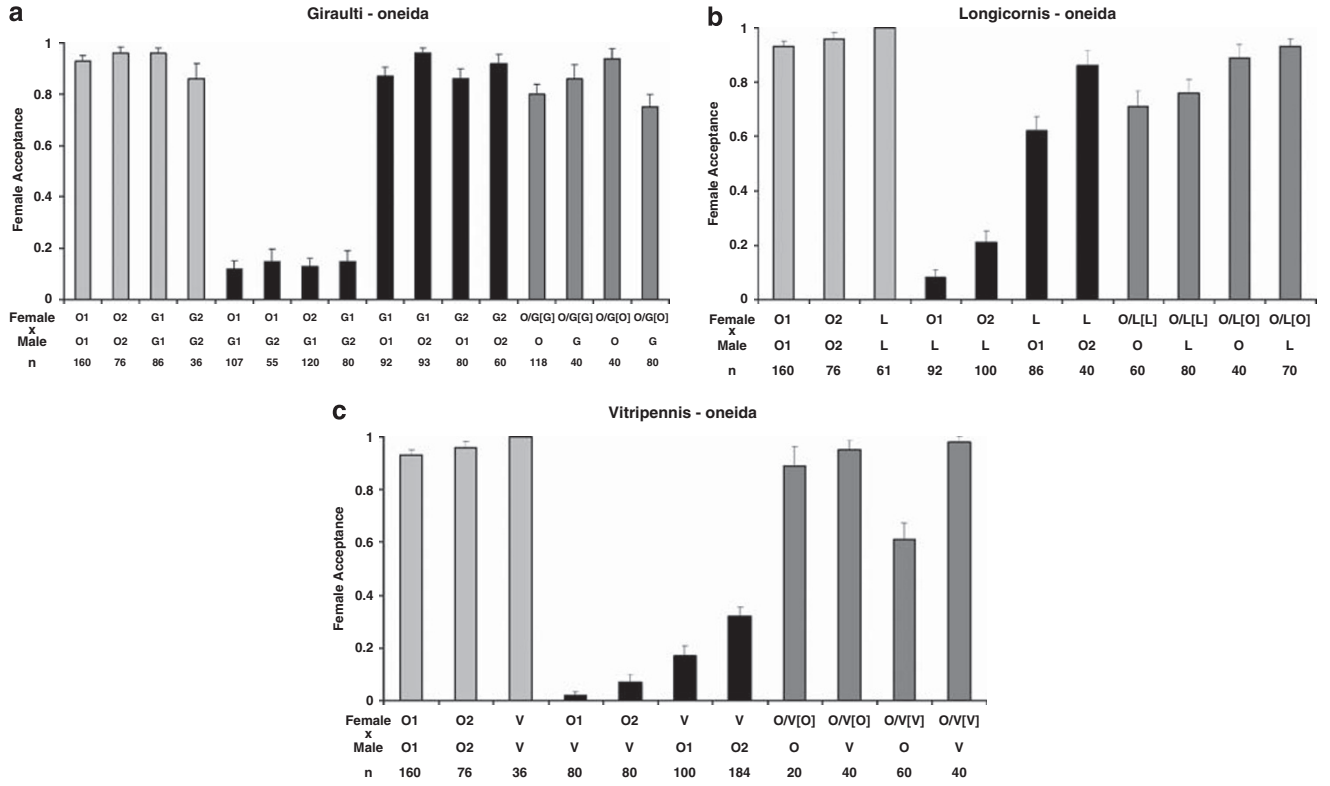

Figure 4 Behavioral observations of *N. oneida* (O) with (**a**) *N. girault* (G), (**b**) *N. longicornis* (L) and (**c**) *N. vitripennis* (V), showing *N. oneida* females to be strongly isolated from the other three species. F1 hybrids between species have significantly reduced levels of mate discrimination. Hybrid crosses were performed in both directions and the cytoplasm of F1 hybrids are indicated by [ ]. Two different *N. oneida* and *N. girault* strains were used for these observations (O1, O2 and G1, G2, respectively), whereas hybrid females were established only with the *Wolbachia*-free strain O1U. (Light gray bars: acceptance of conspecific males. Black bars: acceptance of heterospecific males. Dark gray bars: hybrid female acceptance.) See text for details.

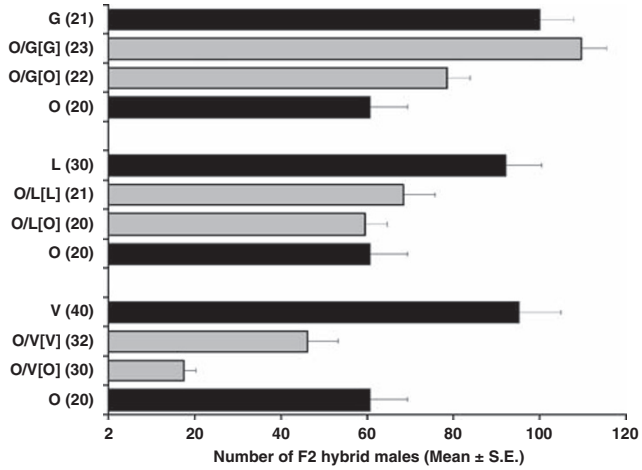

Figure 5 Mean numbers of male offspring obtained from virgins of the four species and their hybrids. Family sizes of hybrid females are indicated with gray bars. (V, G, L and O are *N. vitripennis*, *N. giraulti*, *N. longicornis* and *N. Oneida*, respectively). Hybridizations were carried out in both directions and the cytoplasm is indicated by [ ]. See text for details.

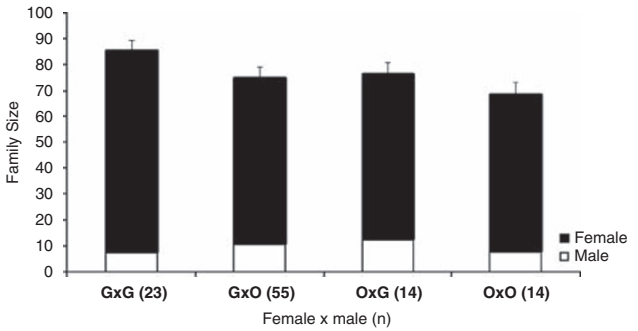

Figure 6 Crosses between *Wolbachia*-infected *N. oneida* (O) and *N. giraulti* (G) showing no effects of cytoplasmic incompatibility between the species.

$U = 531$, $P < 0.001$). Thus, we can conclude that *N. oneida* has significant levels of intrinsic postzygotic isolation with *N. vitripennis*, asymmetric and unidirectional isolation with *N. longicornis* and little or none with *N. giraulti*.

Wolbachia-induced postzygotic isolation

One of the main isolating barriers among *Nasonia* species is the endosymbiont *Wolbachia*, which causes postmating isolation among the species (Breeuwer *et al*., 1992; Bordenstein *et al*., 2001). Raychoudhury *et al*. (2009) previously showed that the three strains of *Wolbachia* in *N. oneida* are identical in sequence to *N. giraulti*, and probably were introduced by hybrid introgression between the species. We tested for *Wolbachia*-induced cytoplasmic incompatibility by crossing infected *N. oneida* and *N. giraulti*. As expected, there is no detectable level of incompatibility (Figure 6) due to the presence of *Wolbachia* (MWU, $U = 606$, $P = 0.07$). This indicates that *Wolbachia*-induced cytoplasmic incompatibility does not have a major role in postzygotic isolation between *N. oneida* and *N. giraulti*, at least under laboratory conditions.

N. oneida: species diagnosis

Both sexes of *N. oneida* differ from *N. vitripennis* and *N. longicornis* by antennal structure; *N. oneida* has an angulate antennal scape (as seen in Figure 7 of male antennae) similar to *N. giraulti*, whereas *N. vitripennis* has a spindle-shaped scape and *N. longicornis* has a cylindrical scape. Females of *N. oneida* can also be distinguished from *N. vitripennis* by a lack of setae on the stigma and distal margin of the forewing, although *N. giraulti* and *N. longicornis* also lack these setae to varying extents. Females of *N. oneida* are difficult to distinguish from its closest sister species *N. giraulti* by morphology. *N. oneida* females tend to have a slightly curved stigmal vein, which is more similar to the straight stigmal vein of *N. vitripennis* than to the strongly arched stigmal vein of *N. giraulti* or the elbowed stigmal vein of *N. longicornis* (see Figure 7), although limited overlap does occur between all the three species. Males of *N. oneida* are easily distinguished from *N. vitripennis* by their broad and rounded forewings, whereas *N. vitripennis* has small forewings and *N. longicornis* has triangular forewings (see Figure 7). *N. oneida* males can be additionally distinguished from *N. longicornis* by their long funicular setae (see Figure 7). *N. oneida* males can also be distinguished by their long and slender antennal flagellum ($> 9.5\times$ as long as wide), whereas *N. giraulti* and *N. vitripennis* male flagellum are shorter and wider ($< 8.8\times$ and $< 8.5\times$ as long as wide, respectively; see Figure 7). *N. longicornis* males have an intermediate flagellum, which is difficult to distinguish from any of the other species. *N. oneida* males can also be distinguished from *N. giraulti* by the coloration of the dorsal surface of the mesosoma, which is green in *N. oneida* and reddish in *N. giraulti*, although the colors sometimes fade after death.

Type material

All type material comes from strain NONYSP1C, which was derived from female wasps that emerged from a single *Protocalliphora* pupa collected from a tree swallow nest in Springville (NY, USA) on 8 January 2008 by Richard Wells of the New York State Bluebird Society. One holotype female and seven paratype females and four paratype males have been deposited in the United States National Museum in Washington, DC. Two paratype females and one paratype male are deposited at each of the British Museum of Natural History, London, UK, and the Royal Ontario Museum, Toronto, Ontario, Canada.

Etymology

N. oneida was named after Lake Oneida, on the shores of which the first specimens of the species were collected.

N. oneida type description

A detailed description of genus-level characters is given in Darling and Werren (1990). *Female*: length, 2.3 mm; head:antenna:scape cylindrical in lateral view; scape:pedicel:F1:F2:club:flagellum 5.9:2.1:1.2:1:2.7:9.7; F1 and F2 $0.7\times$ as long as wide. Mesosoma: dorsellum with upper margin not scalloped, sinuately emarginated along midline. Forewing: $2.2\times$ as long as wide, with marginal setae only along posterior wing margin, marginal vein:postmarginal vein:stigmal vein 1.8:1.2:1, stigmal

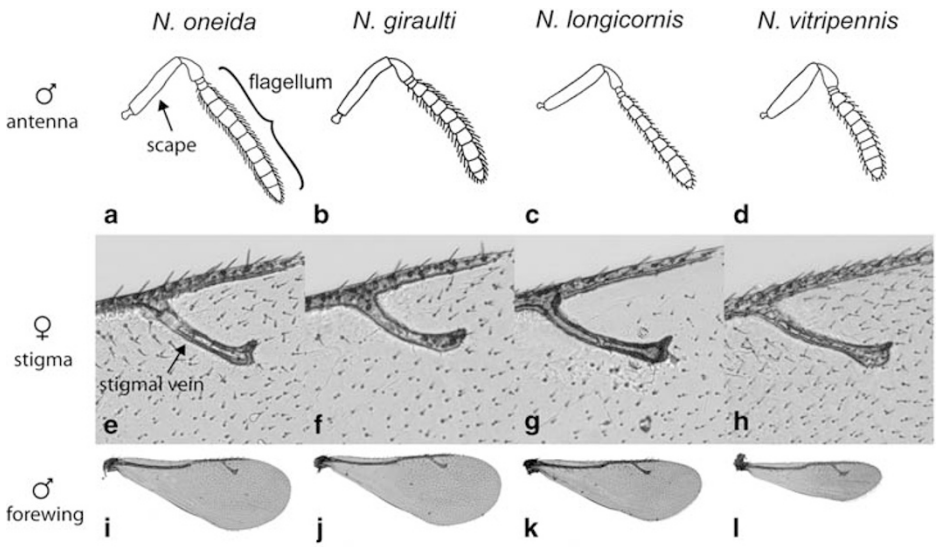

Figure 7 Morphological differences between *N. oneida* and the other *Nasonia* species. *N. oneida* has an angulate scape (**a**) as does *N. giraulti* (**b**), whereas *N. vitripennis* has a spindle-shaped scape and *N. longicornis* has a cylindrical scape. *N. oneida* has the narrowest male antennal flagellum (**a**), with a length to width ratio of ~10.4:1 (**a**). *N. longicornis*, *N. giraulti* and *N. vitripennis* have progressively wider antennal flagella, with length to width ratios of ~9.5:1, ~8.3:1 and ~8.0:1, respectively (**b**, **c** and **d**). *N. oneida* females tend to have a slightly curved stigmal vein on the forewing (**e**), which is more similar to the straight stigmal vein of *N. vitripennis* (**h**) than to the strongly arched stigmal vein of *N. giraulti* (**f**) or the elbowed stigmal vein of *N. longicornis* (**g**). The forewings of *N. oneida* males are broad and rounded (**i**), similar to those of *N. giraulti* (**j**), whereas the forewings of *N. longicornis* are triangular (**k**) and those of *N. vitripennis* are short and narrow (**l**).

vein relatively straight, only slightly arched toward postmarginal vein, distance from stigma to postmarginal vein 0.6 × length of stigmal vein. *Male*: as female except: length, 2.2 mm; head: antenna, scape: pedicel: F1: F2: club: flagellum 4.6:1.5:1:1:2.8:9.4; flagellum 10.5 × as long as wide, club 3 × as long as wide, antennal setae 0.7 × as long as F2. Malar space expanded lateroventrally, cheek-like. Mesosoma: dorsally with greenish coloration. Forewing: 2.4 × as long as wide, marginal vein:postmarginal vein:stigmal vein 2:1.7:1.

Discussion

In this study, we establish that *N. oneida* is a distinct species in the genus *Nasonia*, and is a sister species to *N. giraulti*. *N. oneida* can be distinguished by genetic divergence from the other three species (Figure 1), distinct CHC profiles in both sexes (Figure 3) separating it from *N. giraulti*, subtle morphological differences (Figure 7) and asymmetric mate discrimination against the other species (Figure 4).

A distinctive feature of *N. oneida* is strong, but mostly, asymmetric pre-mating isolation. Even in no-choice situations *N. oneida* females strongly discriminate against *N. giraulti* males, whereas *N. giraulti* females readily mate with *N. oneida* males. One of the ways such isolation could have evolved is selection against interspecific hybrids, also known as reinforcement (Butlin, 1989). Evidence that hybridization between these two species has occurred in the past comes from the fact that they share similar mitochondria, which could have been a result of *Wolbachia*-mediated introgression (Raychoudhury *et al*., 2009). Moreover, *N. oneida* is sympatric with *N. vitripennis* and *N. giraulti* in its native range and now also with *N. longicornis* (Raychoudhury and Werren, unpublished data). Thus, it seems plausible that such a sympatric distribution increased the probability of hybridization, setting the stage for reinforcement to occur. However, we could not detect any intrinsic postzygotic isolation (hybrid breakdown) with *N. giraulti*, at least under laboratory conditions. Moreover, *Wolbachia* does not seem to be a cause of post-mating isolation between *N. oneida* and *N. giraulti* and can be ruled out as a causal feature currently selecting for reinforcement, in contrast to the role of *Wolbachia* shown in *Drosophila recens* and *Drosophila subquinaria* (Jaenike *et al*., 2006). Therefore, at least in laboratory conditions, we could not detect a significant cost to hybridization with *N. giraulti*. This does not rule out significant levels of reduced hybrid fitness in the ecological context, which would not be detected under laboratory conditions. Several studies indicate that ecological isolation can precede intrinsic isolating effects, such as hybrid sterility and inviability (Schluter, 2001).

Unlike *N. oneida* females, *N. giraulti* females do not show a pronounced mate discrimination against *N. oneida* males. G1 females accept O1 and O2 males 87 and 96% of the time, whereas G2 females accept them 86 and 92% of the time (FET, $P = 0.48$). This lack of mate discrimination is consistent with earlier studies between *N. giraulti* and *N. longicornis* (Bordenstein *et al*., 2001). This, perhaps, can be explained by a unique biological feature of *N. giraulti*. Mating typically takes place inside the host (Drapaeu and Werren, 1999), and therefore there may not be strong selection for female mate discrimination to evolve because opportunities for heterospecific mating are low. More information regarding the biology of *N. oneida* needs to be established before a comprehensive hypothesis is put forward to explain the evolution of this strong mate discrimination.

Despite its recent discovery and collection from only two sites in New York, there is considerably more genetic

variation in *N. oneida* than in all *N. giraulti* collected from throughout the northeastern United States. As Figure 2a indicates there is a very low level of variation in *N. giraulti* compared with *N. oneida*. This, perhaps, is an indication of *N. giraulti* going through a severe bottleneck sometime in its recent past. In contrast, there is some variation in *N. giraulti* mitochondria as indicated by Figure 2b. This can be explained by the rapid mutation rates in *Nasonia* mitochondria relative to the nucleus. Oliveira *et al*. (2008) found that *Nasonia* mitochondrial genes evolve at a rate that is 35 times greater than the nuclear genes. Therefore, looking at the pattern of nuclear and mitochondrial haplotype networks, what we can surmise is that *N. giraulti* experienced a bottleneck sufficiently long ago to allow the accumulation of some level of variation in its mitochondria, but not in its nuclear genes. Severe inbreeding within the host may have maintained an effectively clonal species of *N. giraulti* subsequent to the bottleneck. The data also indicate that *N. oneida* is not an artificial (anthropogenic) introduction in New York, as such an event would produce a genetic haplotype structure with significantly less variation than observed. Levels of nuclear variation in *N. oneida* (Figure 1 and Figure 2a) in just two locations in New York are similar to that found in *N. vitripennis* and *N. longicornis* (Werren *et al*., 2010), indicating comparable effective population sizes. In contrast *N. giraulti*, despite its widespread geographical distribution in eastern North America, seems to be effectively a clonal population.

Raychoudhury *et al*. (2009) estimated the synonymous divergence between *N. longicornis* and *N. giraulti* to be 1.12%, and in this study we estimate the synonymous divergence between *N. longicornis* and *N. oneida* to be 1.01% and that between *N. giraulti* and *N. oneida* to be 0.91%. Thus, the levels of synonymous divergence between these three species are very similar. This indicates that these species diverged at approximately the same time. Nevertheless, phylogenetic analysis indicates that *N. oneida* and *N. giraulti* are sister species with strong bootstrap support (Figure 1), because of a number of shared fixed mutations present in *N. oneida* and *N. giraulti* but absent in *N. longicornis*. A slight asymmetric F2 hybrid breakdown also occurs between *N. oneida* and *N. longicornis*, but not between *N. oneida* and *N. giraulti* (Figure 5), which is a further evidence that *N. oneida* is biologically closer to *N. giraulti* than it is to *N. longicornis*. It is possible that the similarity between *N. oneida* and *N. giraulti* is due to an ancient hybridization event between these species. A recent hybridization event between *N. longicornis* and either *N. oneida* or *N. giraulti* to create a the third 'hybrid species' is unlikely because seven of nine genes show higher similarity between *N. oneida* and *N. giraulti*, one is ambiguous (due to low level of polymorphism) and only one joins *N. oneida* and *N. longicornis*. In none of the cases do individual gene sequences from *N. oneida* and *N. giraulti* fall within the *N. longicornis* clade, which would be expected if hybridization occurred recently. We conclude that the nuclear sequence information indicates that these three species diverged approximately at the same time (0.4–0.5 MYA), but that *N. giraulti* and *N. oneida* are sister species.

In contrast to the nuclear, data the mitochondrial data suggest a more recent mitochondrial sweep between *N. oneida* and *N. giraulti*, probably due to *Wolbachia*-induced cytoplasmic incompatibility. There are no divergence among six *Wolbachia* genes of these three species and the mitochondrial sequences are similar (Raychoudhury *et al*., 2009). This *Wolbachia*-mitochondrial hybridization event is estimated to have occurred $\sim$0.06–0.07 MYA, on the basis of the rates of synonymous mitochondrial divergence for *Nasonia* (76.3–94.9% per MY, Raychoudhury *et al*., 2009) and the level of synonymous mitochondrial divergence between the species (5.7%, Table 1). We conclude that the hybridization leading to the mitochondrial-*Wolbachia* sweep did not lead to a general nuclear genetic admixture of the species, as also argued in Raychoudhury *et al*. (2009). Given the strong cytoplasmic drive caused by *Wolbachia*, the sweep could readily have occurred from a single or few hybridization events, which would result in little genetic admixture. However, complete genome sequencing of *N. oneida* (underway) will reveal whether there are signatures of low levels of hybridization within the genome.

A key question about the biology of *N. oneida* is 'where did it come from?' The first specimens were found in Brewerton, NY, USA, in 2005, a site that has been relatively well investigated by field collections for *Nasonia* since 1987. Therefore, the sudden appearance of *N. oneida* in the Brewerton collection site is surprising. This suggests that this species is a recent migrant to the area. An alternate explanation is that we had previously incorrectly identified *N. oneida* collected from Brewerton as *N. giraulti* because, as mentioned above, these two species have similar morphological features. However, no existing *N. giraulti* field strains in the laboratory, collected before 2005, are *N. oneida*, indicating that *N. oneida* was not collected before 2005. Field collections performed in New York in 2005 yielded both species, but since then, we have failed to obtain *N. giraulti* from New York. This also coincides with the discovery of *N. longicornis* as an introduced population in New York (Raychoudhury and Werren, unpublished). Thus, there is a complex scenario of *Nasonia* distribution in New York. All the four species now seem to be sympatric here, contrary to the earlier estimated presence of just two, *N. vitripennis* and *N. giraulti* (Darling and Werren, 1990). Whether the introduction of *N. longicornis* had any role in our inability to obtain *N. giraulti* and the discovery of *N. oneida* is difficult to say, but these two phenomena may not be coincidental. A thorough field study in Upstate New York, extending to Canada and other areas, is necessary to determine the geographical range and history of *N. oneida*.

Nasonia belongs in the family Pteromalidae, which has $>$3500 described species (Noyes, 2003) and is a member of the super family Chalcidoidea, a group containing $>$22 000 described species (Noyes, 2003). Thus, it is not surprising that new species are being found. But what is counterintuitive is that studies of *Nasonia* in such well-investigated sites, such as those in New York State, can still yield such novelties. Our understanding of the distribution and diversity of *Nasonia* is clearly incomplete and is in flux. New species are being found in previously well-investigated sites and past distributions are being challenged. Upstate New York remains a well-characterized area for *Nasonia* field studies but future study must investigate areas that have not been as well

investigated. These sites are midwestern United States extending to Canada, south and southeastern United States and Eurasia.

Nasonia is fast emerging as a model for the evolutionary genetics of speciation (Breeuwer and Werren, 1995; Werren and Loehlin, 2009), and has been further facilitated by the recent sequencing of genomes of three species, *N. giraulti*, *N. longicornis* and *N. vitripennis* (Werren *et al*., 2010); *N. oneida* sequencing is now underway. The ability to cross species boundaries by genetic crosses is a particularly useful tool for evolutionary genetic investigations, allowing the introgression, fine-scale mapping and cloning of genes involved in phenotypic differences between the species (Loehlin *et al*., 2010; Werren *et al*., 2010). The discovery of *N. oneida* opens up further opportunities for these microevolutionary genetic investigations. For example, *N. oneida* differs from the other *Nasonia* in CHCs, male mating behavior, antennal and wing morphology, diapause tendency and female mate preference. *N. oneida* is characterized by strong female mate discrimination that acts as a recessive character to acceptance of heterospecific males. Introgression of a *N. oneida* male wing-size gene into an *N. vitripennis* background has already been carried out successfully (Loehlin *et al*., 2010). The search for genes for mate preference has had a long and tortuous history (Ritchie, 1992). Besides *Drosophila*, in which some answers to the genetic basis of mate preference are forthcoming (Doi *et al*., 2001), very little is known in other species. *N. oneida*, with its haplodiploidy, interfertility and a strong mate preference phenotype represents a strong new candidate to investigate the genetic basis of female mate discrimination.

Conflict of interest

The authors declare no conflict of interest.

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