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Individual recognition and individual identity signals in *Polistes fuscatus* wasps vary geographically

Elizabeth A. Tibbetts*, Christian Cely Ortiz, Giorgia G. Auteri, Meagan Simons, Michelle L. Fearon, L. Lacey Knowles

Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, U.S.A

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Geographical variation in animal phenotypes is common, yet we know surprisingly little about how recognition varies across populations. Instead, much recognition research focuses on one or a few populations and assumes recognition behaviour is consistent across a species' range. Here, we show that individual identity signals and individual recognition vary across the geographical range of *Polistes fuscatus* wasps. *Polistes fuscatus* in Michigan and New York, U.S.A., have variable facial patterns that signal individual identity and are used by receivers for individual recognition. However, *P. fuscatus* from Rothrock, Pennsylvania, U.S.A., lack individual identity signals, as they have less variable facial patterns than *P. fuscatus* from Michigan. Furthermore, *P. fuscatus* from Pennsylvania are not capable of individual recognition. Pennsylvania *P. fuscatus* do not learn and remember individual conspecifics during social interactions or during training. The Michigan and Pennsylvania populations are genetically differentiated, but the differentiation is driven by geographical distance, not adaptive differentiation based on recognition differences. Overall, both signals and receiver responses vary across populations of *P. fuscatus*. Our results suggest that recognition systems may rapidly evolve to produce variation in signals and receiver responses across a species' geographical range.

Taxa with intraspecific geographical variation have become important model systems for understanding the factors that mediate the evolution and diversification of animal phenotypes (Foster & Endler, 1999). For example, guppy, *Poecilia reticulata,* coloration differs across populations because there are local differences in predation pressure on bright fish (Endler, 1980; Kemp, Batistic, & Reznick, 2018). Many birds have local song dialects caused by social and ecological differences between populations (Slabbekoorn & Smith, 2002). Geographical variation in sexual ornaments and preferences has attracted substantial attention because there are often genetic associations between signals and preferences that may facilitate speciation (Fowler-Finn & Rodriguez, 2016). However, we have identified relatively few taxa with variation in both nonsexual signals and receiver responses. Instead, most recognition research focuses on one or a few populations and considers the behaviour of the population to represent the entire species (Searcy & Nowicki, 2005).

Individual recognition is one type of communication where we lack evidence of intraspecific geographical variation in both signals and receiver responses. Individual recognition is an essential aspect of social communication across many taxa (fish, birds, crustaceans, mammals and insects) and social contexts (cooperation, reciprocity, social monogamy, parental care, dominance hierarchies) (Tibbetts & Dale, 2007). During individual recognition, receivers learn the unique phenotype of conspecifics (termed individual identity signals or cues), associate the phenotype with individual-specific information and recall the phenotype-information link during subsequent interactions (Tibbetts & Dale, 2007; Tibbetts, Sheehan, & Dale, 2008). For example, humans learn the unique facial features of other humans, associate the facial features with social information (e.g. social relationship, past history of interactions), then recall the information the next time they meet (Wilmer et al., 2010). Previous work on geographical variation in individual identity signals has focused on cases where the average phenotype of individual identity signals varies geographically (Bradbury & Vehrencamp, 1998). For example, many parrots have contact calls with population-specific dialects (Wright & Dahlin, 2018). However, less is known about cases where the presence of individual

* Corresponding author.
E-mail address: tibbetts@umich.edu (E. A. Tibbetts).

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identity signals or the capacity for individual recognition varies across populations (McCulloch & Boness, 2006).

One of the challenges associated with understanding geographical variation in communication is that communication involves adaptations by both senders and receivers (Bradbury & Vehrencamp, 1998; Searcy & Nowicki, 2005). Effective communication depends on senders having variable phenotypes that convey information to receivers and receivers paying attention to these phenotypes and responding appropriately. Either component alone is ineffective. For example, humans with unique facial features that allow individual recognition will benefit if receivers learn and remember them but not if receivers are incapable of recognition. Similarly, the ability to recognize individuals may be beneficial if senders have unique phenotypes, but not if senders appear so similar that individual recognition is impossible. As a result, communication systems provide an opportunity to study how traits evolve when selection on one individual depends on the phenotype of other individuals (Herre, Knowlton, Mueller, & Rehner, 1999; Moore, Brodie, & Wolf, 1997).

Polistes fuscatus paper wasps provide an interesting model system to study geographical variation in individual recognition. In two populations of *P. fuscatus* that have been studied extensively, Ann Arbor, Michigan and Ithaca, New York, U.S.A., nest-founding queens use individual recognition to manage social relationships before and after founding nests. Wasps use individual recognition prior to nest foundation when group membership is flexible and foundresses engage in cooperative and competitive behaviour with many other queens (Sheehan & Tibbetts, 2008; Tibbetts, Wong, & Bonello, 2020). Wasps also use individual recognition to manage relationships in cooperative groups on nests (Tibbetts, 2002). Workers are capable of individual recognition (Injaian & Tibbetts, 2014), although they are less adept at learning and remembering individuals than are nest-founding queens (Tibbetts, Injaian, Sheehan, & Desjardins, 2018).

Previous work indicates that individual recognition in *P. fuscatus* involves adaptations by both receivers and senders. Receivers have adaptations that improve their ability to perceive, process and remember unique individuals. For example, *Polistes* species with face recognition have larger eye facets than species that lack face recognition (Sheehan, Jinn, & Tibbetts, 2014). In addition, *P. fuscatus* are specialized for learning wasp faces; they learn conspecific faces faster and more accurately than other visual information (Sheehan & Tibbetts, 2011). In senders, the primary adaptation that facilitates individual recognition is the highly variable facial patterns that signal individual identity (individual identity signals). Senders with a unique appearance benefit because they are less likely to be confused with other individuals than are senders with a common appearance. As a result, there is negative frequency-dependent selection acting on individual identity signals that results in high phenotypic variation (Fig. 1a) (Sheehan & Tibbetts, 2009). Taxa with individual recognition have more variable phenotypes than taxa that lack individual recognition (Dale, Lank, & Reeve, 2001; Tibbetts, Mullen, & Dale, 2017).

Here, we examine individual identity signals and individual recognition in *P. fuscatus* wasps from a new population in Rothrock, Pennsylvania (PA) and compare the results with *P. fuscatus* from Michigan (MI). *Polistes fuscatus* from Rothrock, PA initially attracted our attention because they appear to lack the highly variable facial patterns that signal individual identity (Fig. 1). First, we compare the extent of facial pattern variation in *P. fuscatus* collected from PA and MI to assess whether there are differences in individual identity signalling between populations. Second, we test whether *P. fuscatus* from PA recognize familiar individuals during social interactions by comparing behaviour towards known and unknown rivals. If *P. fuscatus* from Rothrock, PA are not capable of individual recognition, we predicted that they would treat known and unknown rivals similarly. Previous experiments using the same methods have shown *P. fuscatus* with individual recognition are more aggressive and have fewer nonaggressive interactions during contests with unknown rivals than with known rivals (Injaian & Tibbetts, 2014; Sheehan & Tibbetts, 2008), while *Polistes* that lack individual recognition treat known and unknown rivals similarly (Sheehan & Tibbetts, 2010; Tibbetts, Desjardins, Kou, & Wellman, 2019; Tibbetts et al., 2018). Third, we compare how well PA and MI foundresses learn to discriminate between individual wasp faces during training. *Polistes fuscatus* from MI readily learn to discriminate between individual wasp face images during training (Sheehan & Tibbetts, 2011). If *P. fuscatus* from PA are not capable of

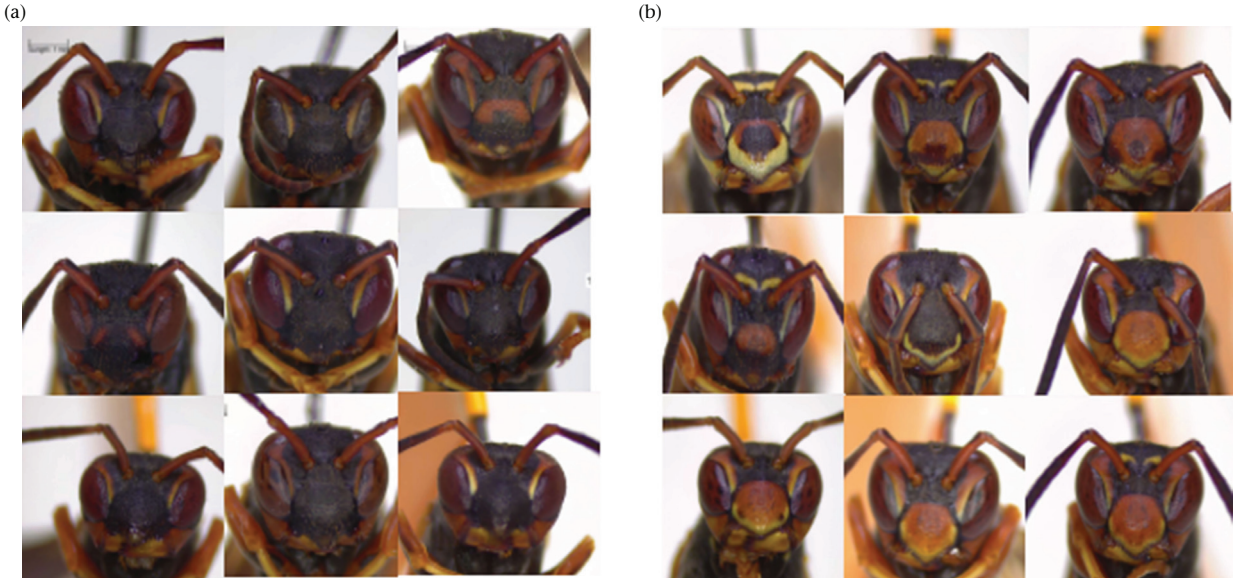

Figure 1. (a) Portraits of nine *P. fuscatus* from Rothrock, Pennsylvania, U.S.A., illustrating the reduced facial pattern variation. (b) Portraits of nine *P. fuscatus* from Ann Arbor, Michigan, U.S.A., illustrating the highly variable facial patterns.

visual individual recognition, we predicted that they would be less adept at learning to discriminate between wasp face images than *P. fuscatus* from MI. Lastly, we use DNA barcoding to confirm that wasps from MI and PA are *P. fuscatus*, and use RADseq to characterize genomic variation of the two populations to evaluate their genetic distinctiveness.

METHODS

Polistes fuscatus foundresses were collected from two locations. Ann Arbor, MI (42°17′59″N, 83°39′46″W) and Roth Rock, PA (40°38′13″N, 78°4′29″W). Wasps were collected from nests and on the wing. After collection, wasps and nests were housed in the laboratory with ad libitum water, sugar and caterpillars.

Facial Pattern Variation

We assessed signals of individual identity by measuring facial pattern variation within each population. We collected and photographed 39 nest-founding queens from Roth Rock, PA and 39 nest founding queens from Ann Arbor, MI, and analysed the photographs in Adobe Photoshop. Facial pattern variation does not differ between queens and workers (Sheehan, Choo, & Tibbetts, 2017). For this study, wasps were collected from nests and only one wasp per nest was analysed. We quantified the proportion of each facial area that was brown, black or yellow. Previous work has shown that these three colours encompass most facial variation in *P. fuscatus* (Tibbetts, 2002). We analysed the three facial areas that vary in *P. fuscatus*: frons, clypeus, inner eye (Appendix, Fig. A1). Then, we compared the variance in coloration between populations.

Social Recognition

We assessed the recognition abilities of *P. fuscatus* wasps by staging contests between pairs of wasps with and without a prior history of social interactions following methods in previous studies (Dreier, van Zweden, & D'Ettorre, 2007; Injaian & Tibbetts, 2014; Sheehan & Tibbetts, 2008, 2010). We performed the experiment twice using two different groups of wasps in two different years. The methods were the same across years.

On the first day (day 0), we placed two wasps who had not interacted previously in a square plastic container (8 × 8 cm) and filmed their interactions. After filming, the wasps were housed together until the next day (day 1), at which point they were separated and returned to their initial housing. On day 2, the same two wasps were filmed interacting again (day 2). To ensure that changes in aggression between days 0 and 2 resulted from recognition and not from decreases in motivation over time, we paired the wasps with other unknown social partners on the day before and after (days 1 and 3). The first half hour of all interactions was videotaped for later analysis of behaviour. Start date was staggered across trials to ensure that differences in behaviour across days were caused by experimental treatment rather than day-specific effects (e.g. any slight differences in temperature, humidity across days). For example, on a particular date, some focal wasps experienced the day 0 treatment, while others experienced the day 3 treatment.

Behaviour in all videos was scored by a research assistant who was blind to day and experimental predictions. Behaviours were ranked as follows, from cooperative to increasingly aggressive: (0) nonaggressive interaction (partners within one body length of each other, but no darts, bites, grapples or mounts occurred); (1) dart (rapid body movement towards partner); (2) dart with open mandibles (rapid body movement towards partner with open mandibles); (3) bite (mandibles closing on body of partner); (4) grapple/mount (wrestling/bodily contact that forces partner to accept submissive positioning) (Sheehan & Tibbetts, 2008). For each trial, we summed the ranks of cooperative and aggressive behaviours. We then divided the sum by the number of total interactions per videotape to calculate an aggression index (Dreier et al., 2007). The aggression index standardized behaviour by taking into account the number and intensity of interactions of each pair, which allowed behaviour to be compared across trials (Dreier et al., 2007; Injaian & Tibbetts, 2015; Sheehan & Tibbetts, 2008, 2010). If the wasps are able to recognize and remember social partners, they should be less aggressive and have more nonaggressive contacts when they interact with a known individual (day 2) than when they interact with an individual they encounter for the first time (days 0, 1, 3).

Face Learning

We quantified wasps' ability to learn and remember conspecific faces using a negative reinforcement training method established in our laboratory (Tibbetts et al., 2018; Tibbetts, Den Uyl, Dwortz, & McLean, 2019; Tibbetts, Desjardins, et al., 2019). Wasps must learn to approach the face image associated with safety to avoid being shocked. The specific face image associated with safety versus shock was varied across trials. Wasps were trained using both PA and MI face stimuli (Appendix, Fig. A2). Face images used for training were printed at life size using a commercially available photo printer.

During training, wasps were placed in a 2.5 × 4 × 0.7 cm wood and Plexiglas box with six identical stimuli glued to the inside walls (2 pictures on the long side and 1 picture on the short side). In half the bouts, the wasp was placed in a box with incorrect stimuli and received a mild electric shock from an electrified pad for 2 min. The electrified pad was made of antistatic conductive foam electrified by two copper wires connected to a Variac transformer, which provided continuous AC current. The mild electric shock is aversive but not harmful to the wasp. In the other half of the bouts, the wasp was placed in a similarly sized box with the correct stimuli and the pad was not electrified for 2 min. Between each bout, the wasp was given a 1 min break in a holding container. For example, a wasp trained to discriminate between face stimuli A and B would experience the following training. First, the wasp was placed with face stimuli A and received a shock for 2 min. The wasp was removed and given a 1 min break. Then, the wasp was placed with face stimuli B and did not receive a shock for 2 min. The wasp was removed and given a 1 min break. This process was repeated three times per wasp, so wasps saw face stimuli A and B three times each. After training, the wasp was given a 45 min break in a holding container with food and water.

Learning was tested by measuring whether the wasp approached the correct or incorrect stimuli over 10 trials. Testing occurred in a 3 × 10 × 0.7 cm rectangle. One end of the rectangle had the correct stimulus (e.g. face B) and the other end of the rectangle had the incorrect stimulus (e.g. face A). At the beginning of each trial, the wasp was placed in the centre of the rectangle between two clear partitions for 5 s, then both partitions were removed simultaneously, and the wasp was free to walk through the rectangle. Wasps who learn usually turn to look at the correct stimulus when they are placed in the rectangle: as soon as the partitions are removed, the wasp quickly walks towards the correct stimulus. A wasp was scored as making a choice when its head and thorax moved beyond a small partition placed 2.5 cm from each

end of the rectangle. After a wasp made a choice, it was removed from the rectangle and given a 1 min break in a holding container. The placement of the stimuli (right or left side) was determined randomly and changed between trials. This ensured that wasps did not associate a particular direction with correct choices.

DNA Barcoding

After using morphological characteristics to identify wasps as *P. fuscatus*, we used 'DNA barcoding' with the mitochondrial *cytochrome c oxidase subunit I* (COI) gene to confirm species identities. DNA barcoding is a commonly used technique to verify the molecular taxonomic identity of insect species (Hebert, Cywinska, Ball, & DeWaard, 2003). We randomly selected 11 wasps from Ann Arbor, MI and 13 wasps from Rothrock, PA for barcoding. DNA was extracted from the abdominal tissues from each wasp using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, U.S.A.), following manufacturer's protocols. DNA was eluted with 100 µl DNAse/RNAse free H_2O, and DNA concentration was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.). We amplified the 658 bp region of the COI gene using the universal animal kingdom primer pair LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3) (Folmer, Black, Hoeh, & Vrijenhoek, 1994; Hebert et al., 2003). All reactions had a total reaction volume of 20 µl, using 0.025 U of Platinum Taq DNA polymerase (Invitrogen), with 4 µl of DNA template, 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 µM dNTPs and 0.5 µM primers (each). Samples were amplified at 95 °C for 1 min, followed by 35 cycles of 95 °C for 45 s, 50 °C for 1 min, 72 °C for 1 min and a final extension step of 72 °C for 7 min. PCR products were verified using gel electrophoresis, then sequenced with Sanger sequencing.

Resulting sequences were edited and aligned in Geneious Prime 2020.0.4 (see Appendix, Table A1 for GenBank accession numbers). Species identity of samples was confirmed by comparing each sequence for the percentage of identity with previously sequenced *Polistes* wasps on the Barcode of Life Database (BOLD v.4, http://www.boldsystems.org/) using the Animal Identification tool (Ratnasingham & Hebert, 2007). Sequences with >98% sequence similarity were identified as *P. fuscatus* in BOLD. The barcodes also identified the recently identified species *Polistes parametricus* in both the MI and PA populations ($N = 2$ and 1, respectively); because of the relatively few *P. parametricus* samples in BOLD, sequences with >97% sequence similarity were identified as *P. parametricus*. All molecular work was completed in the University of Michigan Biodiversity Lab (https://sites.lsa.umich.edu/biodiversity-lab/).

The identity of *P. fuscatus* was also confirmed genomically (see below for details about genomic data generation). Specifically, wasps from the focal populations (Ann Arbor, MI and Rothrock, PA) were combined with a broader geographical sampling of *P. fuscatus* (67 individuals in total). Estimates of phylogenetic relationships using RAxML v.8 (Stamatakis, 2014) with 100 bootstrap replicates clearly showed that seven individuals belonged to a closely related species, *Polistes metricus*, three of which occurred in our focal PA population (see Appendix, Fig. A3) and were excluded from further characterization of genomic variation (detailed below).

Genomic Data Collection and Processing

DNA extracted from thoracic muscles were ground with a mortar and pestle, and DNA was extracted and purified with a DNeasy kit (Qiagen); DNA was quantified using Qubit (Thermo Scientific). A genomic library was prepared using a double-digest restriction site-associated DNA sequencing (ddRADseq) protocol (Peterson, Weber, Kay, Fisher, & Hoekstra, 2012). Briefly, DNA was digested using the restriction enzymes EcoRI and MseI (New England Bioloabs, Ipswich, MA, U.S.A.), digested products were ligated to adaptors containing unique barcodes and 350–450 bp were size-selected using 'pippin prep' (Sage Science, Beverly, MA, U.S.A.). The fragments were amplified by eight PCR cycles to incorporate the Illumina flowcell adaptor. Twenty-two individuals from the focal populations (9 for MI and 13 for PA) were sequenced in a library that contained 74 other individuals as part of a separate project; the library was sequenced in one lane of an Illumina HiSeq2500 to generate single-end 150 bp reads at The Centre for Applied Genomics, Toronto, Canada.

We used Stacks (v.2.1; Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013) for processing the genomic data. Specifically, sequences were demultiplexed using 'process radtags' in Stacks (Catchen et al., 2013), excluding reads with ambiguous barcodes and low quality; 89.2% of reads were retained. A recently released reference genome for *P. fuscatus* (NCBI:txid30207; Miller et al., 2020) was indexed using the Burrows–Wheeler alignment program (v.7.17; Li & Durbin, 2009) and reads were mapped to the reference genome via the MEM algorithm (Li, 2013) using SAMtools (v.1.8–27; Li, 2011; Li et al., 2009) and loci identified in 'gstacks' (Catchen et al., 2013) using the reference-aligned option. Most reads (91.7%) passed quality filters with a mean per-sample coverage of 22.1× (SD ± 15.2×) and consistent phasing for 78.6% of diploid loci (for setting details see Auteri & Knowles, 2020). We ran populations in Stacks (Catchen et al., 2013) with the default settings, excluding single-nucleotide polymorphisms (SNPs) in the last three base pairs of the loci because of unusually high diversity ($\theta > 0.020$; Appendix, Fig. A4) suggestive of sequencing and alignment errors, and required that there be information on a RAD-locus for at least 63% of individuals from each population to be included.

In addition, potentially related individuals were identified using 'related' (Pew, Muir, Wang, & Frasier, 2015) with the Wang estimator option (Wang, 2002) based on analysis of loci with a minor allele frequency (MAF) ≥ 0.01 and less than 25% of missing data per locus. Three individuals (B2, B7 and H5, one from MI and two from PA) were removed due to high estimated levels of relatedness (Wang estimator ≥ 0.06; full sisters or parent–offspring) with another individual in the data set; in each case, we retained individuals with the lowest percentage of missing data. This left us with 992 variant sites across 19 individuals (11 in PA and 8 in MI).

Lastly, to avoid the confounding effects of including loci under selection when characterizing genomic variation between the focal populations, we tested for locus-specific divergence consistent with the signature of selection. Specifically, we used two outlier tests: one based on a threshold of nine standard deviations from AMOVA-corrected F_{ST} values (Excoffier, Smouse, & Quattro, 1992) as output by Stacks (Catchen et al., 2013), and one conducted using the program OutFlank (Whitlock & Lotterhos, 2015) with the output from Stacks converted in PGDSpider v.2.1.1 (Lischer & Excoffier, 2012). Both analyses used adapted R scripts (Auteri & Knowles, 2020; R Core Team, 2015). In the OutFlank analysis, this script also filtered for loci with an expected heterozygosity (HE) of ≥ 0.1, as per program guidelines (Whitlock & Lotterhos, 2015) with trim functions fitted visually (LeftTrimFraction = 0.3 and RightTrimFraction = 0.05) and significance assessed by the 'qvalue' (Storey, Bass, Dabney, & Robinson, 2015). No locus-specific differentiation consistent with the signature of selection was detected between the focal MI and PA populations (Appendix, Fig. A5).

Ethical Note

Training involves low-level electrical shock that is aversive to wasps. However, the level of current was kept low to ensure that wasps were able to learn and were not harmed. Wasps behaved

normally when they were returned to their containers after training. The social recognition experiment involved ritualized aggression between pairs of wasps. Such aggression is common in the wild. No wasps were harmed during the social aggression trials. We rapidly euthanized a relatively small number of wasps on dry ice for genetic analysis.

Analyses of Population Genetic Structure

To evaluate potential geographical structure of genetic variation, we ran a Structure analysis (v.2.3.4; Pritchard, 2000; Pritchard & Falush, 2009) using the admixture model with and without a priori population designation. A burn-in of 50 000 followed by 500 000 repetitions per run, and 10 replicates for K 1–3 genetic clusters, were used to identify the K with the best fit to the data (i.e. the highest maximum likelihood), as opposed to relying on delta K (Evanno, Regnaut, & Goudet, 2005), given the limited number of K values under consideration. We also estimated the rate of genetic drift of each population from an inferred ancestral state in Structure (Pritchard, Stephens, & Donnell, 2000) using the F-model conditioned on a priori population information (Falush, Stephens, & Pritchard, 2003). The mean and standard deviation of the prior on F was set to 0.1, and rates of drift were averaged across runs.

We also performed a principal components analysis (PCA) for 15 individuals and 1880 loci with < 25% missing data, which were identified using Plink v.1.07 (Purcell, 2007). Missing data were replaced with the mean for that locus; missing data were low overall (average of 4%, and not exceeding 8% per individual and 25% per locus). We performed the PCA in R (R Core Team, 2015) using the adegenet (v.2.1; Jombart, 2008; Jombart & Ahmed, 2011) and plyr (v.1.8; Wickham, 2011) packages; results were plotted using ggplot2 (v.3.1; Wickham, 2009).

Statistical Analysis of Individual Recognition and Individual Identity Signals

All data were analysed using IBM SPSS v.26 (IBM, Armonk, NY, U.S.A.).

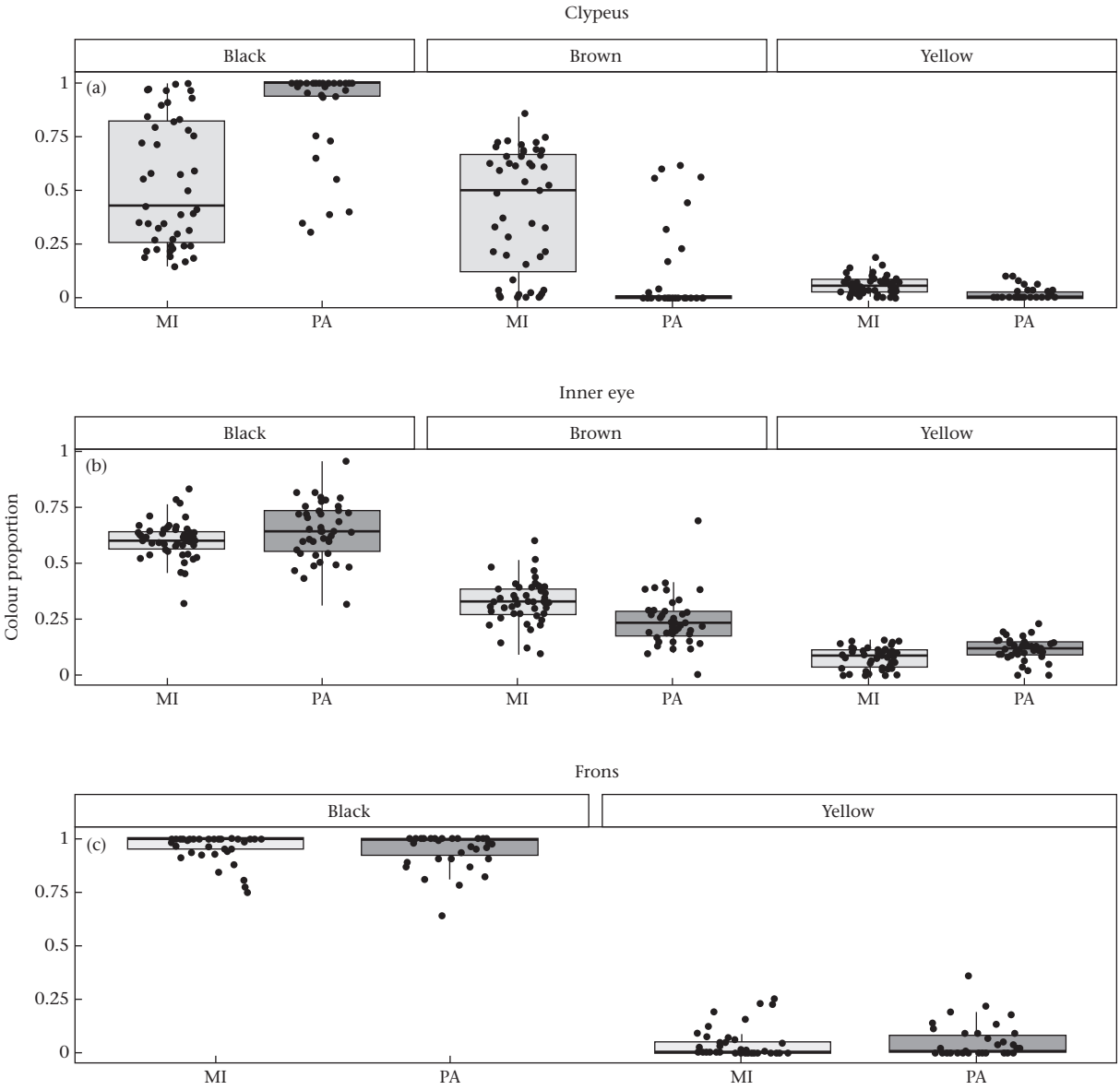

Figure 2. Facial pattern variation in the (a) clypeus, (b) inner eye and (c) frons of *P. fuscatus* collected in Ann Arbor, Michigan (MI) and Rothrock, Pennsylvania (PA), U.S.A. Box plots reflect 1Q, median and 3Q, and whiskers reflect minimum/maximum values.

Facial pattern variation

We compared variance in the proportion of each facial area that had a particular colour with Levene's test for equality of variance ($N = 39$ from Ann Arbor, MI and 39 from Rothrock, PA).

Social individual recognition

We compared aggression index and number of nonaggressive contacts across trials using Friedman's ANOVA nonparametric analysis. Year did not explain any of the variance in behaviour, so was not included in the final analysis. The aggression index or number of nonaggressive contacts were the dependent variables. No post hoc pairwise analyses were performed because there were no significant differences in the main models ($N = 15$ trials in 2013, 19 trials in 2016).

Face learning

We measured learning as the total number of correct choices. We tested whether wasps learned by comparing the number of correct choices versus incorrect choices to the 50:50 random expectation with 2×2 chi-square tests. We used a general linear model to compare the number of correct choices across the four treatment groups (MI wasps on MI faces, PA wasps on MI faces, MI wasps on PA faces, PA wasps on PA faces), followed by a Tukey's HSD post hoc pairwise analysis ($N = 21$ PA on MI faces, 19 MI on MI faces, 13 MI on PA faces, 15 PA on PA faces).

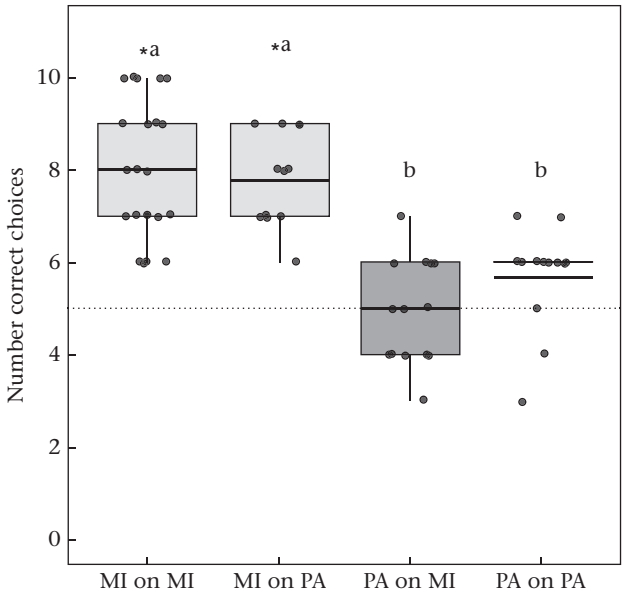

Figure 4. Numbers of correct choices by *P. fuscatus* from Ann Arbor, Michigan (MI) and Rothrock, Pennsylvania (PA), U.S.A. when discriminating between wasp faces from MI and PA populations. Dotted line = random 50:50 expectation. Different letters denote significant differences. *Significantly different from chance. Box plots: 1Q, mean, 3Q. Whiskers: minimum/maximum values.

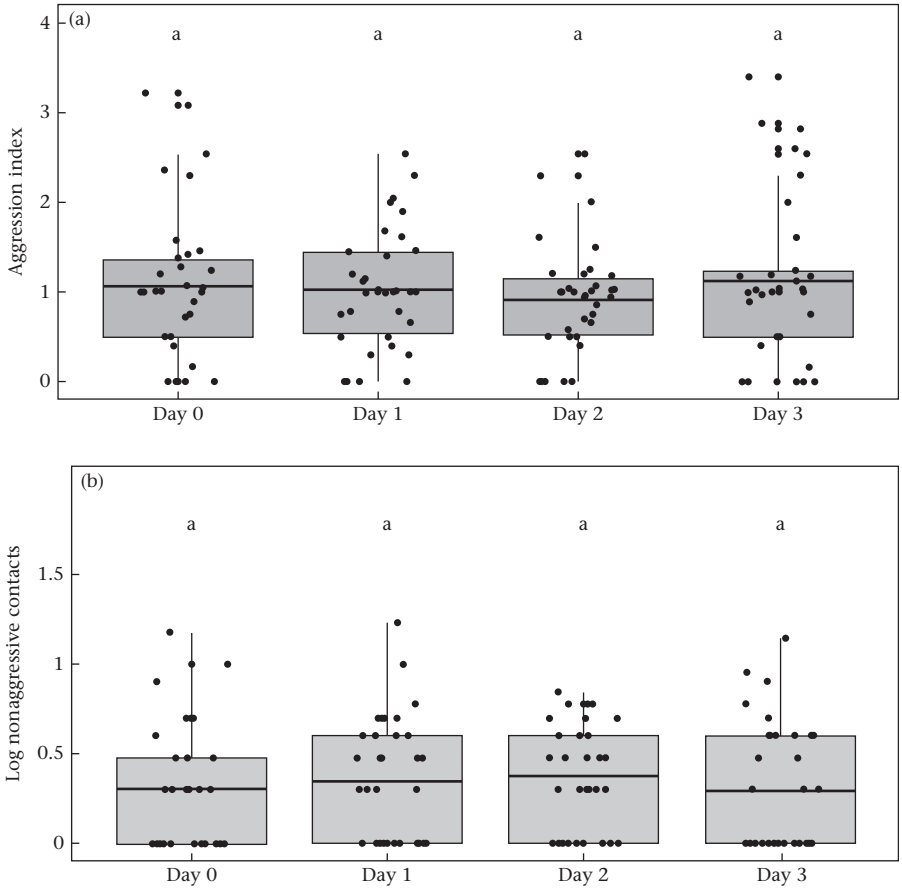

Figure 3. (a) Aggression and (b) nonaggressive contacts of *P. fuscatus* from Rothrock, Pennsylvania, U.S.A. during interactions with known (day 2) and unknown (days 0, 1, 3) individuals. Different letters reflect significant differences ($P<0.05$) between days. Box plots: 1Q, mean, 3Q. Whiskers: minimum/maximum values except outliers >1.5 IQR.

RESULTS

Facial Pattern Variation

Polistes fuscatus from Rothrock, PA had considerably less variation in clypeus coloration than *P. fuscatus* from MI (black: $F_{1,76} = 18.5$, $P < 0.001$; brown: $F_{1,76} = 22.5$, $P < 0.001$; yellow: $F_{1,76} = 7.9$, $P = 0.006$; Fig. 2). However, there are no difference in colour variation in the frons (black: $F_{1,75} = 0.085$, $P = 0.77$; yellow: $F_{1,76} = 0.82$, $P = 0.77$; no individuals with brown on the frons) or inner eye (black: $F_{1,76} = 3.7$, $P = 0.056$; brown: $F_{1,76} = 0.34$, $P = 0.56$; yellow: $F_{1,76} = 0.62$, $P = 0.43$).

Social Recognition

There was no evidence that PA foundresses remembered other individuals during social interactions. Aggressive and nonaggressive behaviours were not influenced by a previous history of social interactions. Wasps were similarly aggressive to new social partners and to individuals that they had interacted with previously ($\chi^2_3 = 2.15$, $P = 0.54$; Fig. 3). In addition, the number of nonaggressive contacts did not differ between known and unknown social partners ($\chi^2_3 = 4.01$, $P = 0.26$). Therefore, foundresses from Rothrock, PA did not respond differently to known and unknown individuals, indicating that they lack the capacity for individual recognition.

Face Learning

MI wasps learnt to discriminate between pairs of MI faces ($\chi^2_1 = 24.8$, $P < 0.0001$; Fig. 4) and pairs of PA faces ($\chi^2_1 = 20.4$, $P < 0.0001$). PA wasps did not learn to discriminate between MI faces ($\chi^2_1 = 0.47$, $P = 0.56$) or PA faces ($\chi^2_1 = 0.48$, $P = 0.49$). Accuracy differed across the four treatment groups ($F_{3,64} = 23.8$, $P < 0.0001$). *Polistes fuscatus* from MI performed better than *P. fuscatus* from PA, but performance was not influenced by whether the training stimuli were pictures of MI or PA wasps (Fig. 4).

Population Genetics

Genetic differentiation between the focal populations was consistently recovered across analyses (Fig. 5); however, note that results from Structure analyses did not consistently recover genetic structure without conditioning analysis a priori population information (although half of runs recovered population structure identical to Fig. 5a, the remaining runs detecting no structure, possibly indicating a lack of power). The structuring of genetic variation appears to be geographical rather than due to selection associated with behavioural differences linked with individual recognition or the absence of individual recognition. Specifically, inclusion of genomic data from a third population also detected evidence of genetic structure (Appendix, Fig. A6), showing that geographical isolation underlies genetic differentiation (as opposed to behavioural differences). Lastly, considering the accumulation of genetic differentiation between the focal populations (Fig. 5), results from the *F*-model implemented in Structure showed similar estimated rates of evolutionary divergence from a common ancestor (i.e. mean $F = 0.24 \pm 0.02$ and 0.35 ± 0.01 for the MI and PA populations, respectively), which again indicates that the shift in individual recognition was not associated with an accelerated rate of genetic differentiation. Mean F_{ST} between the two populations was 0.044 (as reported by Stacks), and additional summary statistics of genomic data can be found in the Appendix, Table A2.

DISCUSSION

This study provides evidence of geographical variation in both individual identity signals and individual recognition in *P. fuscatus*. Individual identity signals differed across populations; *P. fuscatus* from PA had less variable clypeus coloration than *P. fuscatus* from

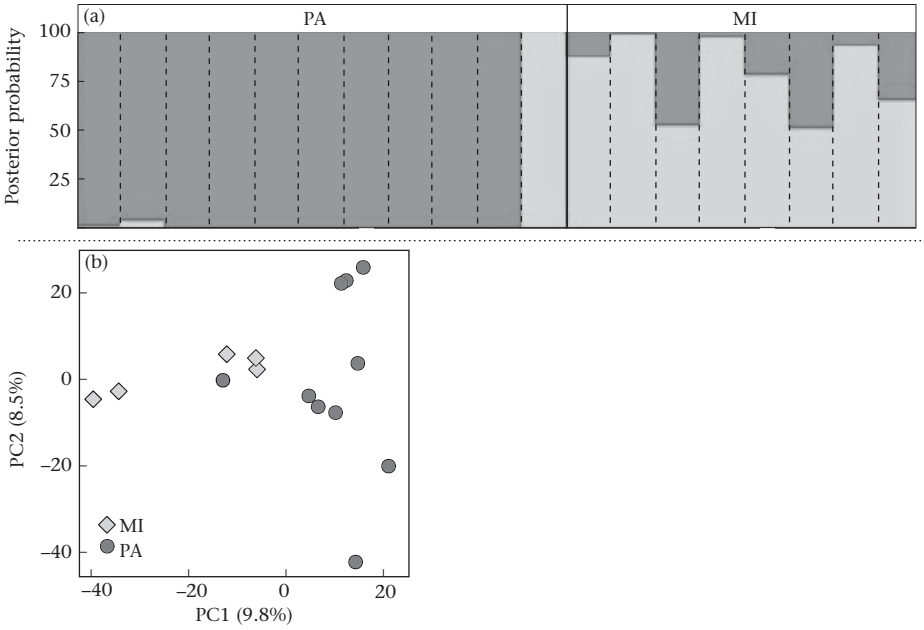

Figure 5. (a) Bayesian estimates of the genetic make-up of individuals (demarcated by dashed lines) for $K = 2$, where the relative contributions of the two ancestral populations are indicated by the two different shaded proportions of each bar; results are from a Structure (Pritchard, Wen, & Falush, 2009) analysis based on conditioning on a priori localities for the focal Pennsylvania and Michigan populations (separated by black boxes). (b) Differentiation between the populations is also reflected in a PCA of the genomic data.

MI, although other aspects of facial pattern coloration did not differ across populations (Fig. 2). Recognition also varied across populations. *Polistes fuscatus* from Rothrock, PA did not learn and remember other individuals during social interactions (Fig. 3), while *P. fuscatus* from Ann Arbor, MI and NY are adept at individual recognition (Injaian & Tibbetts, 2014; Sheehan & Tibbetts, 2008; Tibbetts et al., 2018). *Polistes fuscatus* from Central PA also did not learn to differentiate between conspecific faces during training, while *P. fuscatus* from MI readily learnt to differentiate between conspecific faces from both PA and MI populations (Fig. 4). Finally, DNA barcoding and population genetic analyses showed that wasps from both populations are *P. fuscatus*, so behavioural differences reflect intraspecific geographical variation.

The lack of individual recognition in *P. fuscatus* from Roth Rock, PA is notable, as much previous work has shown that *P. fuscatus* from MI and NY excel at individually recognizing conspecifics. Two key experiments illustrate that PA *P. fuscatus* lack individual recognition. First, the social recognition assay shows that a previous history of social interactions does not influence aggressive or nonaggressive interactions (Fig. 3). The same experiment in Ann Arbor, MI *P. fuscatus* found that wasps remember the fighting ability of specific rivals and are less aggressive to individuals during their second meeting than during their first meeting (Injaian & Tibbetts, 2014; Sheehan & Tibbetts, 2008). However, wasps that lack individual recognition treat individuals the same during their first and second meeting (Sheehan & Tibbetts, 2010; Tibbetts et al., 2018, 2019b). Second, the face-learning experiment shows that PA *P. fuscatus* are less able to differentiate between unique conspecific faces than MI *P. fuscatus*. Within and between species, wasps that are capable of visual individual recognition can learn and remember faces during training, while wasps that are not capable of individual recognition are unable to learn and remember faces (Sheehan & Tibbetts, 2011; Tibbetts et al., 2018, 2019b). For example, *P. metricus* and socially isolated *P. fuscatus* from MI are not capable of individual recognition and are unable to learn to discriminate faces during training (Sheehan & Tibbetts, 2008, 2011; Tibbetts et al., 2019b). In contrast, *P. fuscatus* queens and workers from MI are capable of individual recognition and readily learn to discriminate faces of both MI and PA wasps (Fig. 4) (Sheehan & Tibbetts, 2011; Tibbetts et al., 2018).

Finding geographical variation in individual recognition is somewhat surprising because individual recognition relies upon specific adaptations by both receivers and senders. Receivers have adaptations that facilitate accurate signal discrimination, learning and memory (Sheehan & Tibbetts, 2011; Sheehan et al., 2014; Tibbetts, Den Uyl, et al., 2019). Senders have highly variable phenotypes that facilitate accurate individual discrimination (Sheehan & Tibbetts, 2009, 2010; Tibbetts et al., 2017). Because signals and responses are interdependent, selection on senders depends on the phenotype of receivers and vice versa (Searcy & Nowicki, 2005). For example, highly variable individual identity signals will only benefit senders if receivers have the capacity to discriminate and learn the traits. Similarly, the capacity for individual recognition in receivers will only be favoured if senders have unique phenotypes. Therefore, it is striking to find within-species geographical differentiation in complex, interdependent traits like individual identity signals and individual recognition. While we found some overall genetic differentiation between populations (Fig. 5; similar to Bluher, Miller, & Sheehan, 2020), we did not find exceptionally differentiated genes linked to these adaptations (Appendix, Fig. A5). Broader genomic sampling (given the sparse coverage of RADseq methods) may resolve this, and a false negative is also possible given our conservative outlier-detection approach. It could also be that genetic mechanisms underlying recognition traits are difficult to detect because these traits are polygenic or based on developmental plasticity.

The results are largely consistent with our hypothesis that populations with individual recognition have more variable phenotypes than populations that lack individual recognition. Why do populations differ in both signal (facial pattern variation) and receiver response (recognition behaviour)? One possibility is that differences in recognition across populations alter the selective pressures experienced by senders, thereby altering sender phenotypes. Previous work has shown that MI wasps with unique facial patterns benefit because they are more easily recognized than wasps with a common appearance (Sheehan & Tibbetts, 2009). As a result, there is negative frequency-dependent selection favouring facial pattern diversity in MI. PA wasps lack the capacity for individual recognition, so unique phenotypes are unlikely to benefit senders. As a result, wasps from PA may have lost or never gained the high level of facial pattern variation that signals individual identity. Notably, while many wasps from PA have very similar facial patterns (Fig. 1) and individuals from PA have less facial pattern variation than those from MI (Fig. 2), PA wasps still have some colour variation. As a result, MI wasps learn to differentiate the images of PA individuals during training (Appendix, Fig. A2). Previous work on signal–receiver coevolution has focused on sexual signals, hypothesizing that assortative mating between senders with ornaments and receivers that prefer the ornaments is the key mechanism that links signals and receiver responses (Fowler-Finn & Rodriguez, 2016). Assortative mating does not occur in nonsexual signalling system systems, so additional theoretical and empirical work is important to assess the mechanisms that link signal and receiver response in nonsexual communication systems.

Some previous work on geographical variation in communication has shown that recognition within a population is more effective than recognition between populations. For example, human face recognition improves with experience, so individuals are typically better at differentiating own population faces than different population faces (Tanaka, Kiefer, & Bukach, 2004). Similarly, many birds respond more strongly to songs with the local song dialect than to songs from a distant dialect (Baker, Spitler-Nabors, & Bradley, 1981; Slabbekoorn & Smith, 2002). In contrast, MI *P. fuscatus* learned to discriminate MI and PA facial features with similar accuracy (Fig. 3).

Although many species use individual recognition to manage social relationships, relatively little is known about geographical variation in the capacity for individual recognition (McCulloch & Boness, 2006). Geographical variation commonly occurs in other types of signals and receiver responses (Foster & Endler, 1999; Jennions & Petrie, 1997). For example, male guppy fish vary across populations due to local differences in predation pressure and female preferences for ornaments (Endler, 1980; Kemp et al., 2018). Male barn swallow, *Hirundo rustica*, ornamentation and female preference varies between Europe and North America; long tail feathers are key ornaments in European populations, while bright ventral plumage important in North American populations (Safran & McGraw, 2004; Scordato & Safran, 2014). *Polistes dominula* wasp queens have variable black facial patterns that signal status, rather than individual identity, in the U.S. and some European populations (Tibbetts, 2013; Tibbetts & Lindsay, 2008), while other European populations seem to lack both variable facial patterns and receiver responses to facial patterns (Tibbetts et al., 2011). Geographical variation in communication is likely widespread, although additional research will be important to understand its origin and maintenance of the diversity in communication systems.

Differences in cooperative foundress behaviour may account for the recognition differences between *P. fuscatus* populations. Previous work has shown that a key benefit of individual recognition in

Polistes is that it minimizes conflict among cooperative nest-founding queens (Sheehan & Tibbetts, 2009; Tibbetts, 2002, 2004). In the MI population with individual recognition, *P. fuscatus* foundresses often live in cooperative groups of nest-founding queens (mean = 2.04 foundresses per nest). In contrast, in the PA population that lacks individual recognition, *P. fuscatus* foundresses are less cooperative (mean = 1.2 foundresses per nest) (Sheehan et al., 2015). Reduced cooperation may reduce or eliminate the benefits associated with individual recognition. As a result, the PA population may have lost or never gained individual recognition and individual identity signals.

The analyses of genomic data confirm that MI and PA populations are differentiated, and that this differentiation is most likely a by-product of geographical distance, rather than driven by differences in genes underlying individual recognition per se. For example, differentiation is apparent not only between the focal populations with and without recognition (Fig. 5), but also between focal populations and a third population (Appendix, Fig. A5). With similar genetic distinctiveness among the three populations, the changes in recognition have occurred without any pronounced historical differences (e.g. degrees of geographical isolation) or rates of accumulation in genomic differentiation (as quantified by the *F*-model implemented in Structure).

The individual recognition experiment illustrates that *P. fuscatus* from PA are not capable of remembering specific individuals in any sensory modality (Fig. 3). The individual identity signal analysis (Fig. 2) focuses on visual signals because previous work has shown *P. fuscatus* use only visual signals for individual recognition. For example experimentally altering visual signals prevents individual recognition (Sheehan & Tibbetts, 2009; Tibbetts, 2002). Wasps can learn about individuals by watching conspecifics, even without the tactile interaction needed to assess chemical profiles (Tibbetts et al., 2020). Also consistent with visual recognition, wasps capable of individual recognition are adept at learning conspecific faces, while wasps that lack individual recognition cannot learn conspecific faces (Sheehan & Tibbetts, 2010; Tibbetts, Desjardins, et al., 2019). Wasps do communicate in other sensory modalities (chemical, acoustic) (Izzo, Wells, Huang, & Tibbetts, 2010; Singer & Espelie, 1992; van Zweden, d'Ettorre, Blomquist, & Bagneres, 2010). However, there is no evidence of nonvisual individual recognition in *Polistes*.

Overall, this study demonstrates that individual identity signals and individual recognition vary across the geographical range of *P. fuscatus*. Our results are consistent with growing evidence that geographical variation in communication is common. Moreover, by coupling the behavioural assays with genomic assays, we are able to show that the recognition differences evolved without pronounced genomic differentiation. As such, the combined results highlight that communication is not a stable evolutionary end point. Instead, communication systems may rapidly evolve, leading to extensive variation within and between species.

Data Accessibility

Genomic data are available on Genbank (SRA accession: PRJNA642112). Behavioural data and files associated with genomic data are available on Dryad (https://doi.org/10.5061/dryad.9cnp5hqfs).

Author Contributions

E.A.T. conceived the study, analysed the behavioural data and led writing of the manuscript. C.C.O. collected some of the behavioural and genomic data, G.G.A. and L.L.K. cleaned and analysed the population genetic data, M.F. and M.S. collected the bar-coding data. All authors critically revised the manuscript, gave approval for publication and agree to be held accountable for the work performed.

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Appendix

Table A1

GenBank accession	Species name	Population/State
MT585107	*Polistes parametricus*	Roth Rock, PA
MT585108	*Polistes fuscatus*	Roth Rock, PA
MT585109	*Polistes fuscatus*	Roth Rock, PA
MT585110	*Polistes fuscatus*	Ann Arbor, MI
MT585116	*Polistes fuscatus*	Ann Arbor, MI
MT585117	*Polistes fuscatus*	Ann Arbor, MI
MT585118	*Polistes parametricus*	Ann Arbor, MI
MT585119	*Polistes fuscatus*	Roth Rock, PA
MT585120	*Polistes fuscatus*	Roth Rock, PA
MT585121	*Polistes fuscatus*	Roth Rock, PA
MT585122	*Polistes fuscatus*	Roth Rock, PA
MT585123	*Polistes fuscatus*	Roth Rock, PA
MT585124	*Polistes fuscatus*	Roth Rock, PA
MT585125	*Polistes fuscatus*	Ann Arbor, MI
MT585126	*Polistes fuscatus*	Ann Arbor, MI
MT585127	*Polistes fuscatus*	Ann Arbor, MI
MT585128	*Polistes fuscatus*	Roth Rock, PA
MT585129	*Polistes fuscatus*	Roth Rock, PA
MT585130	*Polistes fuscatus*	Roth Rock, PA
MT585131	*Polistes fuscatus*	Roth Rock, PA
MT585132	*Polistes fuscatus*	Ann Arbor, MI
MT585133	*Polistes parametricus*	Ann Arbor, MI
MT585134	*Polistes fuscatus*	Ann Arbor, MI
MT585135	*Polistes fuscatus*	Ann Arbor, MI

Table A2

Population genetic summaries using variant positions as output by the program Stacks (Catchen et al., 2013), including the inbreeding coefficient (FIS), nucleotide diversity (Pi), number of private alleles in the population, observed heterozygosity (Obs het), observed homozygosity (Obs hom), expected heterozygosity (Exp het) and expected homozygosity (Exp hom) for each population – Pennsylvania (PA) and Michigan (MI)

Pop	FIS	Pi	Private alleles	Obs het	Obs hom	Exp het	Exp hom
PA	0.083	0.15	474	0.13	0.87	0.14	0.86
MI	0.037	0.14	228	0.13	0.87	0.13	0.87

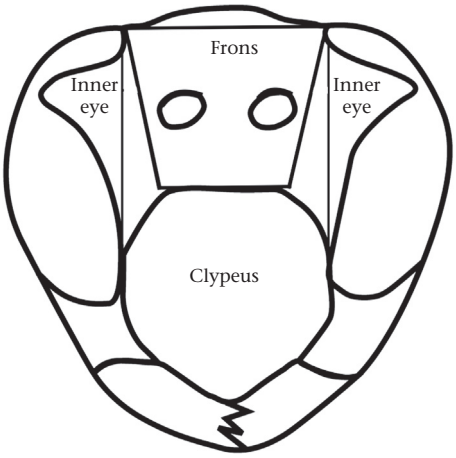

Figure A1. Portrait of *P. fuscatus* face, illustrating the three areas measured for analysis of individual identity signals (frons, inner eye (left and right), clypeus).

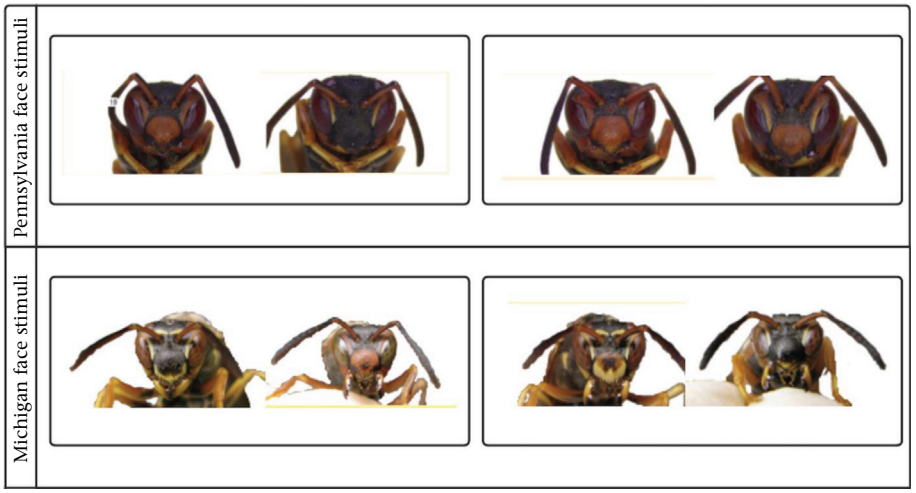

Figure A2. Examples of pairs of face stimuli used to train wasps. Pictures of *P. fuscatus* collected from Ann Arbor, Michigan (lower) and Rothrock, Pennsylvania (upper), U.S.A.

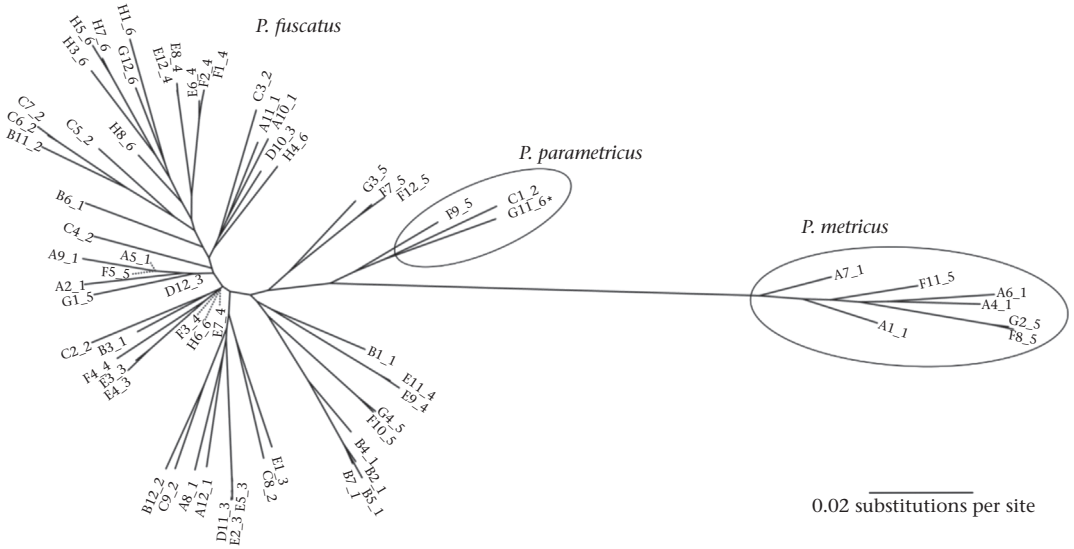

Figure A3. Estimated phylogenetic relationships among *Polistes* individuals based on a more geographically comprehensive data set (i.e. containing individuals not otherwise used in this study for comparative purposes), in addition to the focal Michigan and Pennsylvania populations of this study. Of these 67 individuals, seven individuals were of the nontarget species *P. metricus* (circled; species identification confirmed via photographs), and three individuals putatively corresponded to another nontarget species, *P. parametricus* (circled). We excluded individuals collected in our focal populations of nontarget species (four *P. metricus and one P. parametricus*) from behavioural and genomic data.

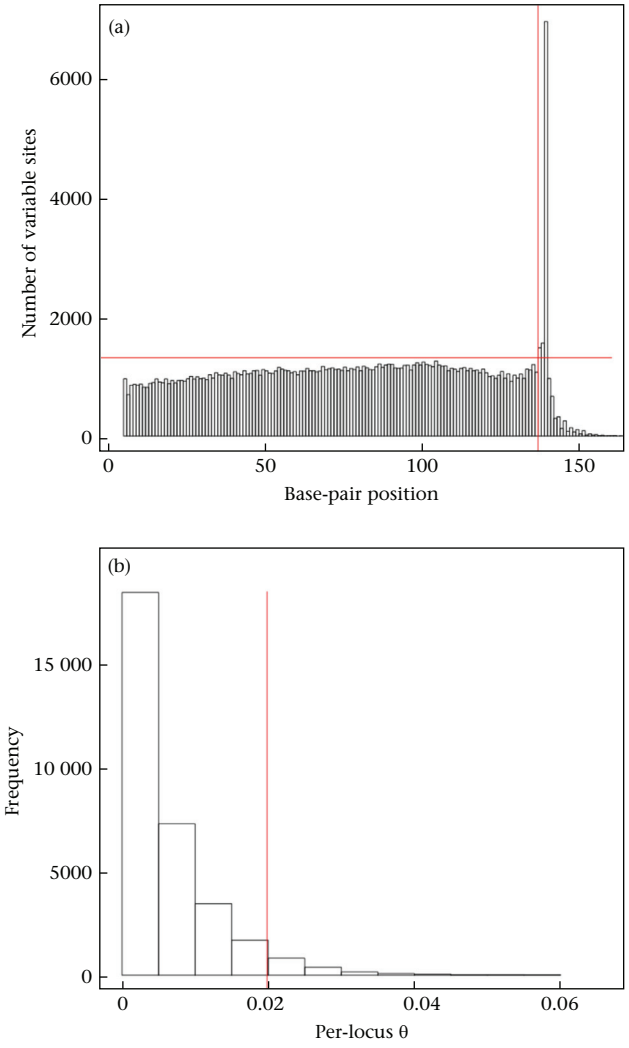

Figure A4. Sites were trimmed to exclude (a) the last three positions (to the left of the vertical red line), which exceeded typical levels of variation (horizontal red line summarized across loci), and loci excluded based on (b) per-locus levels of variation, as measured by θ, with a 95% threshold (vertical red line).

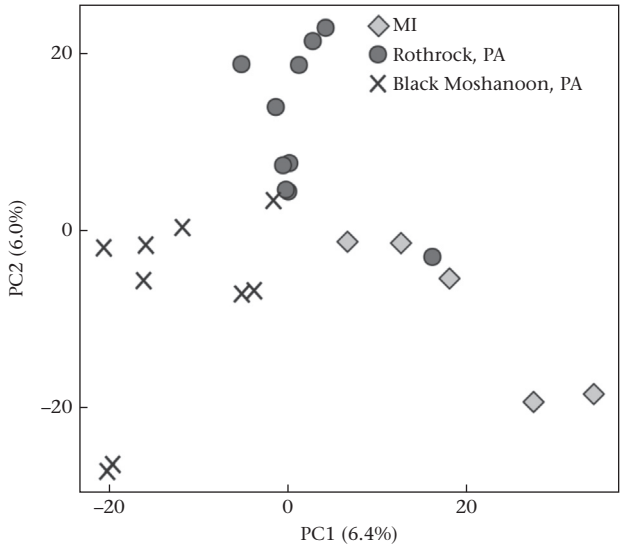

Figure A6. PCA of genomic data with an additional population from Black Moshanoon, PA, U.S.A. (marked by Xs), but for which we lacked behavioural data. Inclusion of another population highlights that genomic variation differs geographically (i.e. not due to differences in individual recognition per se; circles versus diamonds for the absence versus presence of individual recognition for the focal population).

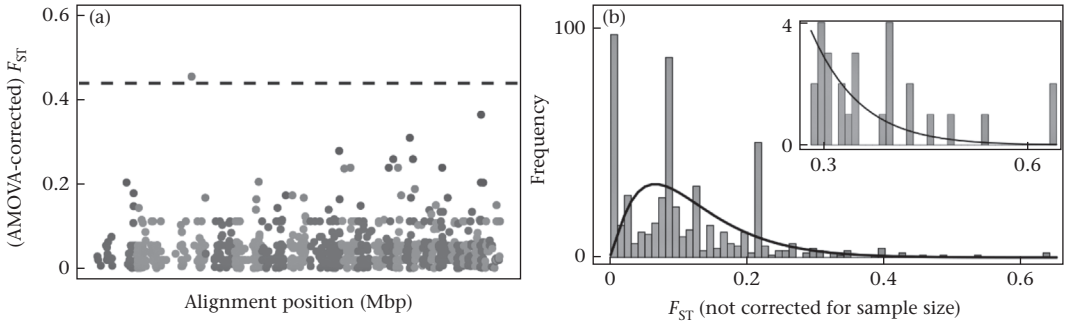

Figure A5. No locus-specific differentiation suggestive of selectively driven divergence was consistently identified across outlier tests using a threshold based on 9 SDs of the AMOVA-corrected F_{ST} values (on left) and the analysis based on OutFlank (on right).