Genetic Diversity and *Wolbachia* Infection Patterns in a Globally Distributed Invasive Ant

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Understanding the phylogeographic history of an invasive species may facilitate reconstructing the history and routes of its invasion. The longhorn crazy ant, *Paratrechina longicornis*, is a ubiquitous agricultural and household pest throughout much of the tropics and subtropics, but little is known about the history of its spread. Here, we examine worldwide genetic variation in *P. longicornis* and its associated *Wolbachia* bacterial symbionts. Analyses of mtDNA sequences of 248 *P. longicornis* workers (one per colony) from 13 geographic regions reveal two highly diverged mtDNA clades that co-occur in most of the geographic regions. These two mtDNA clades are associated with different *Wolbachia* infection patterns, but are not congruent with patterns of nDNA (microsatellite) variation. Multilocus sequence typing reveals two distinct *Wolbachia* strains in *P. longicornis*, namely, *wLonA* and *wLonF*. The evolutionary histories of these two strains differ; *wLonA* appears to be primarily transmitted maternally, and patterns of mtDNA and nDNA variation and *wLonA* infection status are consistent with a relatively recent *Wolbachia*-induced selective sweep. In contrast, the observed patterns of mtDNA variation and *wLonF* infections suggest frequent horizontal transfer and losses of *wLonF* infections. The lack of nDNA structure among sampled geographic regions coupled with the finding that numerous mtDNA haplotypes are shared among regions implies that inadvertent long-distance movement through human commerce is common in *P. longicornis* and has shaped the genetic structure of this invasive ant worldwide.

**INTRODUCTION**

Globalized human commerce has facilitated and intensified the spread of alien species, and the number of invasive species threatening native biodiversity, natural resources, and the economy continues to increase (Pimentel et al., 2000; Leppc et al., 2002; Occhipinti-Ambrogi and Savini, 2003; Meyerson and Mooney, 2007). Knowledge of the invasion histories, routes, and subsequent spread of invasive species provides important information for developing practical management strategies (Estoup and Guillemaud, 2010). Population genetic analyses on invasive species may provide insights into the introduction pathways and help us understand the mechanisms underlying...
the invasion success. Such analyses also may help define management objectives and assist policy makers in developing management, prevention, and restoration strategies (Abdelkrim et al., 2005; Le Roux and Wieczorek, 2009; Chadès et al., 2011; Cristescu, 2015).

The longhorn crazy ant, *Paratrechina longicornis* (Latreille, 1802) (Hymenoptera: Formicidae), is a widespread agricultural and household pest found throughout much of the tropics and subtropics in both the Old World and New World (Wetterer, 2008). A previous study demonstrated the occurrence of an extraordinary, double-clonal reproduction system in a population of *P. longicornis* from Thailand. In this population, queens are produced clonally from their mother, males are produced clonally from their fathers, and workers are produced sexually and characterized by an excess of heterozygosity (Peary et al., 2011). High heterozygosity of workers, close association with humans, and high adaptability in disturbed environments of this species may help explain to some extent how this ant spread quickly in the world even prior to the 20th century (Weber, 1939; Harris and Berry, 2005; Lester, 2005; Wetterer, 2008; Peary et al., 2011). While the precise native range of this ant has been a source of debate and remains uncertain, distributional records of *P. longicornis* and its closest relatives suggest either a Southeast Asian or African origin (Wetterer, 2008; LaPolla et al., 2010; LaPolla et al., 2013; LaPolla and Fisher, 2014). A comprehensive phylogeographic study of *P. longicornis* is needed to help identify more precisely where the species originated as well as its subsequent dispersal routes around the globe.

Researchers routinely analyze both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) data to address questions in molecular ecology and invasion biology. Typically, low genetic variation within a focal population is interpreted as resulting from one or more population bottlenecks after colonization. However, low mtDNA variation also can result from a recent “selective sweep” of a single, highly successful mtDNA variant, a process which may have no discernable effect on nDNA variation (Nei et al., 1975; Aquadro, 1997). This pattern also can stem from indirect selection associated with a selectively-favored, maternally-inherited symbiont (Hurst and Jiggins, 2005). Such symbionts are common in many insect populations and play a major role in shaping host mtDNA evolutionary history (Hurst and Jiggins, 2005; Moran et al., 2008; Charlat et al., 2009; Feldhaa, 2011; Richardson et al., 2012; Bennett and Moran, 2015; Schuler et al., 2016; Schuler et al., 2018). If a maternally-inherited symbiont confers a sufficient selective advantage to spread within and among host populations, the mtDNA variant originally associated with this symbiont may spread with it, and result in a skewed frequency distribution of mtDNA alleles during the process (Caspari and Watson, 1959; Kambhampati et al., 1992; Turelli et al., 1992; Narita et al., 2006; Atayme et al., 2011; Schuler et al., 2016). Several genera of such bacterial symbionts are found in insects, including *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Arsenophonus* (Duron et al., 2008; Engelstädter and Hurst, 2009). Among these, *Wolbachia* appears to be the most widespread maternally-transmitted symbiont in insects (Zug and Hammerstein, 2012; Weinert et al., 2015). *Wolbachia* variants typically spread within host species by increasing the relative fitness of infected females, either by conferring direct fitness benefits, such as increased fecundity (Vavre et al., 1999; Weeks et al., 2007; Zélé et al., 2018) or providing nutrients (Hosokawa et al., 2010; Nikoh et al., 2014), or by manipulating host reproduction via cytoplasmic incompatibility (CI), male-killing, feminization of genetic males, or thalytokous parthenogenesis (Werren et al., 2008; Saridaki and Bourtzis, 2010; Ma and Schwander, 2017). A relatively high proportion of ant species harbor Wolbachia infections (34%; Russell, 2012; Russell et al., 2012). Thus, possible symbiont effects on mtDNA variation in ants cannot be ignored. Incorporation of data from nuclear genes is essential to verify results obtained for mtDNA data because Wolbachia selective sweeps often, but not always, have little to no effects on nuclear variation (Rokas et al., 2001).

In this study we attempt to understand worldwide genetic variation and prevalence of Wolbachia in *P. longicornis*. We also assessed the geographic patterns of mtDNA variation in *P. longicornis*, to see if phylogeographic structure can help track the routes of dispersal of this invasive ant species. Our combined results allow us to test whether Wolbachia have exerted some selective pressure on mtDNA variation in *P. longicornis*. Also, patterns of mtDNA and nDNA variation were compared for congruence, which would be predicted if mtDNA variation has been affected by co-evolving reproductive parasite. Lastly, because mtDNA genomes and endosymbionts are maternally co-inherited, analyses of mtDNA structure and variation can shed light on historical transmission patterns (e.g., potential source and spread) of endosymbionts in *P. longicornis*.

**MATERIALS AND METHODS**

**mtDNA Sequencing and Phylogenetic Analyses**

We obtained *P. longicornis* workers from field collections and from other researchers (Table S1). A total of 248 ant colonies were sampled across the current geographic distribution of *P. longicornis*, including 22 colonies from Northeast Asia, 81 colonies from East Asia, 71 colonies from South Asia, 9 colonies from Indian Subcontinent, 17 colonies from Oceania, 9 colonies from Polynesia, 9 colonies from North America, 2 colonies from South America, 19 colonies from Caribbean, 2 colonies from Arabia, 2 colonies from Southeastern Europe, 4 colonies from West Africa, and 1 colony from South Africa. To generate statistically unbiased samples, only a single worker ant was used from each colony for subsequent genetic analyses.

DNA was extracted from individual *P. longicornis* using the Gentra Puregene cell and tissue kit (Qiagen, USA) following the manufacturer’s instructions, and stored at −20°C. Portions of the cytochrome oxidase subunit I (COI, 1,203 bp), the cytochrome oxidase subunit II (COII, 547 bp) genes were amplified following the PCR conditions described below (Degnan et al., 2004; Table S2). PCR mixtures contained 1–2 μL of template DNA and 0.2 μM of each primer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 U of Taq polymerase in a final volume of 25 μL. PCRs were performed using the thermocycler (Table S2) with the following PCR conditions: an initial denaturation at 94°C for 2 min, followed by 25–35 cycles of denaturation at 94°C for 40 s, annealing at 45°C for 40 s, and extension at 72°C for 90 s, with a final extension at 72°C for 5 min.

The sequences were compared and aligned using ClustalX 1.83 (Huang et al., 2001) and MEGA 6.06 (Tamura et al., 2013). Sequences of *P. longicornis* were compared with those obtained from GenBank and Entrez Genetic Library. mtDNA haplotypes were identified using DnaSP 6.0 (Librado and Rozas, 2009) and framed haplotypes were tested for significant statistical differences using Fisher’s exact test in DnaSP 6.0 (Librado and Rozas, 2009).

**mtDNA Variations and Wolbachia Infections**

To test whether Wolbachia infection has an effect on mtDNA variation, the proportion of 248 mtDNA sequences from *P. longicornis* was compared with the proportion of *P. longicornis* infected with Wolbachia (34%; Russell, 2012; Russell et al., 2012). The identity of Wolbachia infections was determined following the methods described by Week et al. (2007) (Table S2) using the Wolbachia-specific primers RCOI-F and RCOI-R (LaPolla and Fisher, 2014). Wolbachia infections were detected in 34% of the samples.

The frequency distribution of mtDNA haplotypes in *P. longicornis* was calculated using DnaSP 6.0 (Librado and Rozas, 2009). A “selective sweep” of a single, highly successful mtDNA variant often, but not always, could result in a skewed frequency distribution of mtDNA haplotypes. Wolbachia infection frequencies were plotted in relation to the frequency distribution of mtDNA haplotypes to determine whether mtDNA variation was significantly correlated with Wolbachia infection frequencies.
DNA, 0.2 μM of each primer, Takara EmeraldAmp Max PCR Master Mix (Takara, Japan) and water (20 μL reactions). PCR conditions included an initial denaturation step at 98°C (3 min) followed by 35 cycles of 94°C (30 s), 52°C (30 s), 72°C (2 min), and a final extension phase at 72°C (7 min). All PCR products were sequenced in both directions by Genomics BioSci and Tech Corp. (Taipei, Taiwan) using an ABI3730 sequencer. Sequence data were assembled using Sequencher 4.9 (GeneCodes).

Sequences were aligned using MUSCLE as implemented in MEGA 6 with default settings (Tamura et al., 2013). The intergenic spacer and tRNA-Leu region were excluded from phylogenetic analyses due to its ambiguous alignment. We performed phylogenetic analyses using two mtDNA datasets, one including all 248 P. longicornis workers and a second containing only a single representative sequence for each of the 43 mitochondrial haplotypes and plus two outgroup taxa, P. zanjensis and P. ankarana (45 OTUs). PartitionFinder 1.0.1 software (Lanfear et al., 2012) was used to determine the best fit substitution model and partitioning scheme based on Akaike information criterion (AIC) scores. PartitionFinder for our full dataset indicated the best scheme had four partitions: first position of COI and COII, second position of COI, third position of COI and COII, and second position of COI. The preferred evolutionary model for these four partitions were GTR + G, HKY + I, GTR + G, and F81, respectively (GTR = General Time Reversible; G = gamma distribution; HKY = Hasegawa-Kishino-Yano; I = proportion of invariable sites; F81 = Felsenstein 1981). For the singleton haplotype dataset (45 OTU), PartitionFinder suggested the best scheme had five partitions: 1) first position of COI, 2) second position of COI, 3) third position of COI and COII, 4) first position of COII, and 5) second position of COII. The preferred evolutionary model for these five partitions was GTR + G, HKY + I, GTR + G, HKY + G, and F81, respectively. These best schemes were used as priors for Bayesian phylogeny inference. A Bayesian phylogeny was reconstructed using MrBayes 3.2.1 (Ronquist et al., 2012). Two independent runs of 107 generations with 4 MCMC (Markov Chain Monte Carlo) chains were conducted simultaneously, starting from random trees and resampling each tree every 1,000 generations. Posterior probabilities were obtained from the 50% majority-rule consensus of trees sampled after discarding the first 25% of sampled trees.

Network Analysis and Neutrality Tests
A median joining mtDNA haplotype network was constructed using POPART (Leigh and Bryant, 2015; software available at: www.popart.otago.ac.nz) to infer relationships among haplotypes. Net genetic divergence between and within groups (p-distance) was estimated using MEGA 6 (Tamura et al., 2013). Population genetic parameters, including number of segregating sites S (Watterson, 1975), number of haplotypes h, haplotype diversity Hd (Nei, 1987), and nucleotide diversity π/bp (Nei, 1987), were estimated using DNASP v5.10 (Librado and Rozas, 2009). This software also was used to perform neutrality tests including Tajima’s D (Tajima, 1989), Fu and Li’s D* and F* tests (Fu and Li, 1993) and McDonald and Kreitman test (McDonald and Kreitman, 1991). A mtDNA sequence from P. zanjensis was used as the outgroup for neutrality tests. Negative values of Tajima’s D, Fu and Li’s D* and F* may reflect a recent population expansion, purifying selection, or genetic hitchhiking, whereas positive values generally reflect a population bottleneck, genetic structure and/or balancing selection. The McDonald and Kreitman test (M–K test) compares the ratio of fixed and polymorphic synonymous and nonsynonymous changes (McDonald and Kreitman, 1991). Additionally, the DHEW test (Zeng et al., 2007b) was performed to detect the signatures of positive selection and hitchhiking on host mtDNA as implemented in the DH program (Zeng et al., 2007a; Zeng et al., 2007b). The DHEW test we used was a compound test of Tajima’s D (Tajima, 1989), Fay and Wu’s Hn (Fay and Wu, 2000), and Eyens–Watterson test (Watterson, 1978), and is thought to be more powerful in detecting positive selection and more robust to historical demographic changes. P-values of the DHEW test were estimated using 100,000 replications of coalescent simulation using DH package (available online: http://zeng-lab.group.shef.ac.uk/wordpress/?page_id=28). Normalized Fay and Wu’s Hn (Fay and Wu, 2000; Zeng et al., 2006) was also calculated using the same package.

Screening for Wolbachia Infection and MLST Sequencing
We screened the DNA samples for Wolbachia using three primer pairs that amplified part of the Wolbachia surface protein gene (wsp), 16S rRNA gene, and cell division protein (ftsZ) (Table S3). PCR primers are published elsewhere and listed in Table S3. PCRs were carried out for each of the three genes, and at least one Wolbachia-positive sample and deionized/distilled H2O were included as positive control and blank, respectively. Three workers per colony were used to determine the infection status for each colony. Our preliminary results indicated a high intra-colony infection rate of Wolbachia in both workers and queens (approximately 0.96–0.97, Tseng et al., unpublished data). All PCR amplicons that yielded a single band on agarose gels were sequenced by Genomics BioSci and Tech Corp. (Taipei, Taiwan) using an ABI3730 sequencer. Some workers appeared to be infected with multiple Wolbachia (see Results section for more details), and, in these cases, sequence data for individual Wolbachia was obtained by PCR using group- or strain-specific primers (Table S4).

Although Wolbachia surface protein (wsp) gene sequence data have been used for phylogenetic analyses in numerous studies, the phylogenetic relationships inferred using data based on a single gene may not be robust due to a high level of recombination among Wolbachia strains (Baldo and Werren, 2007). Therefore, we employed a multilocus sequence typing (MLST) approach developed by Baldo et al. (2006) in which a total of five MLST genes (gatB, coxA, hcpA, ftsZ, and fbpA) were sequenced following the methods of Baldo et al. (2006). Wolbachia strains were characterized by comparisons with other sequences in the Wolbachia MLST database (http://pubmlst.org/Wolbachia/) and NCBI Genbank database (https://www.ncbi.nlm.nih.gov/genbank/). ClonalFrame version 1.1 was used to construct a Wolbachia MLST genealogy (Didelot and Falush, 2007).
ClonalFrame accounts for both substitutions and recombination events, providing more reliable clonal relationships based on multilocus data (Didelot and Falush, 2007). Two independent runs were performed, each with 1,000,000 MCMC burn-in iterations and 1,000,000 as sampling period and a sampling frequency of 1,000. A 50% majority rule consensus tree was built from combined data from the two independent runs.

Reconstruction of Ancestral States of Wolbachia Infection Status
The program BayesTraits was utilized to reconstruct the ancestral states of Wolbachia infections in P. longicornis mtDNA lineages (Pagel et al., 2004; Mark and Andrew, 2006). This software considers uncertainties in estimating a tree and its branch lengths when inferring ancestral states, and thus may provide different inference results from the parsimonious expectation. Two Wolbachia strains, wLonA and wLonF, were found in some of our P. longicornis samples (see Results section for details). We tested for a correlation between the occurrence/absence of wLonA and the occurrence/absence of wLonF by performing BayesTrait analyses using both dependent (i.e., the infection history of wLonA was correlated with wLonF) and independent models. The difference between the two models was assessed by Bayes Factor (BF) based on the final harmonic mean of the likelihoods model. A log BF value greater than two was interpreted as supporting the dependent model (i.e., correlated patterns of infections). Prior to the MCMC runs, maximum likelihood analyses were performed using the consensus tree obtained from MrBayes, and the derived results were used to set the priors for MCMC analyses. Considering the results of the likelihood analysis, all MCMC priors were set as uniform distribution for all rates, with different ranges used for each parameter. A total of 7,500 trees were generated by MrBayes (full dataset with 248 OTUs, discarded first 25% trees as burn-in) and used in the MCMC inferences to account for phylogenetic uncertainty. These input trees did not include an outgroup species because we focused only on infection histories of the two Wolbachia strains in P. longicornis. Terminal taxa were coded for presence (1) or absence (0) of Wolbachia infection. The rate deviation parameter was tuned automatically to achieve an average acceptance rate between 20% and 40% and ancestral states were reconstructed using the command “addnode”. The MCMC chains were run for $10^4$ iterations, sampled every $10^3$ iterations with a burn-in of $10^4$ iterations.

We tested for associations between mitochondrial lineages and Wolbachia infection status by using the BaTS program (Bayesian tip-association significance testing) to compute the parsimony score statistic of clustering strength (PS), the association index statistic (AI), and the exclusive single-state clade size statistic (MC) (Parker et al., 2008). PS represents the most parsimonious number of character changes in the phylogeny. AI is an estimate of the frequency of the most common branch tip subtree subtended by internal nodes. MC measures the size of the maximum monophyletic clade in which all tips share the same trait. Thus, a significantly lower value of PS, lower AI and higher MC would indicate a strong phylogeny–trait association. The association between mitochondrial lineage and Wolbachia infection is predicted to be strong if Wolbachia infections are transmitted vertically only, while frequent horizontal transfers of Wolbachia infections would erode this association. In BaTS analyses, 7,500 trees were generated by MrBayes as input and tested each parameter by generating a null distribution from 1,000 replicates.

nDNA Analyses
We genotyped a subset of P. longicornis workers from three well-sampled regions (41 colonies from East Asia, 21 colonies from Northeast Asia, and 71 colonies from South Asia) at 20 microsatellite loci (Tseng et al., 2019) (Table S5) to test for congruence (or incongruence) of mtDNA and nuclear DNA variation patterns. DNA of the same worker (one worker per colony) was used for both mtDNA and microsatellite analyses. Microsatellite loci were amplified by using a multiplex PCR method following procedures described by Blacket et al. (2012). The purified PCR products were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) by Genomics BioSci and Tech Co., Ltd (Taipei, Taiwan). GeneMarker (version 2.4.0, Softgenetics LLC) was employed to visualize and score alleles. Genetic variation at each microsatellite locus was characterized in terms of number of alleles (Na), effective number of alleles (Ne), observed (Ho) and expected (He) heterozygosity, Shannon’s information index (I), fixation index (F), and Hedrick’s standardized Gs for small number of populations (Gst), using the program GENALEX 6.502 (Peakall and Smouse, 2006).

Genetic structure was assessed using the Bayesian model-based clustering software STRUCTURE 2.3.4 (Pritchard et al., 2000; Hubisz et al., 2009). Five independent STRUCTURE runs were executed for each of K = 1–10 (K, the number of assumed genetic clusters) under the admixture model and allele frequencies correlated with 1,000,000 MCMC iterations and an initial burn-in of 100,000 generations. The optimal number of genetic clusters within the data was estimated by Evanno et al. (2005) in STRUCTURE HARVESTER v. 0.9.94 (Earl and vonHoldt, 2012) (available online: http://taylor0.biology.ucla.edu/structureHarvester/). STRUCTURE results were visualized using CLUMPAK server (Jakobsson and Rosenberg, 2007) (available online: http://clumpak.tau.ac.il/). Genetic relationships among populations were examined by applying a discriminant analysis of principal components (DAPC) (Jombart et al., 2010) available in the R (R Core Team, 2014) package adegenet (Jombart, 2008) on all microsatellite data. Population labels were input as the prior cluster information in DAPC. The first 20 principal components (PCs) accounted for 80% of the total microsatellite genetic variation and were retained from the analysis.

RESULTS
mtDNA Analyses
We sequenced mtDNA of 248 P. longicornis workers (one per colony) from 13 geographic regions. A total of 43 different mtDNA haplotypes were found with 172 polymorphic sites present over the entire 1,750bp COI-COII region (Table S6;
collection site information in Table S1). Nucleotide diversity was highest in samples from the Indian Subcontinent (0.041) (Figure 1; Table S6) genetic diversity values from Arabia, Southeastern Europe, West Africa, and South America are likely biased due to low sample size). Nevertheless, the populations across Old World regions exhibit similar levels of genetic diversity. Bayesian phylogenetic analyses indicated the presence of two mtDNA clades (Clade I and II), one of which (Clade II) was divided into three subclades (Clade II-1, -2 and -3) (Figure 2). The average genetic distance between Clades I and II was 0.057, suggesting deep divergence between the two clades. The average pairwise genetic distance among haplotypes was higher within Clade II (0.010) compared with Clade I (0.002). Average genetic distances among workers within the three subclades each had a mean value of 0.001. MtDNA variation was not strongly correlated with geographic location (Figure 3). Workers belonging to Clades I and II were found at 11 of the 13 sampled geographic regions (all except South and West Africa; Figure 3). Workers with haplotypes belonging to subclade II-1 were found in the Old World, but not in the New World (Figure 3). The median-joining network constructed for all 43 unique mtDNA haplotypes further revealed no clear spatial clustering of haplotypes from Clade I (Figure 4). In particular, haplotype Hap08 (Clade I) was common across the sampled ranges and was connected to several tip haplotypes with low frequency, implying that this haplotype may represent a putative ancestral haplotype within Clade I from which the latter are derived. Similar to Clade I, the haplotype network revealed negligible spatial clustering in Clade II (Figure 4), with approximately half of all haplotypes in this clade present in more than one geographic region.

**Wolbachia Infections in P. longicornis**

Both sequence data and phylogenetic analyses of concatenated MLST data suggest that two Wolbachia strains, wLonA and wLonF, occur in *P. longicornis*, with the former belonging to supergroup A and the latter to supergroup F (Figure S1). Forty-two of the 248 *P. longicornis* workers were infected with wLonA only (17%), 55 workers were infected with wLonF only (22%), 56 workers were co-infected with wLonA and wLonF (23%), and 95 workers were uninfected (38%) (Table 1). Wolbachia infection status was strongly associated with mtDNA variation. Specifically, the majority of ants belonging to Clade I were either infected with wLonA only (33%) or co-infected with wLonA and wLonF (44%), whereas none of workers belonging to Clade II was infected with wLonA (Table 1). We did not observe a significant association between Wolbachia infection status and host geographic range (Figure S2).

The MLST allelic profile for wLonA was identical to a sequence type in the Wolbachia MLST database, whereas
FIGURE 2 | The 50% majority rule consensus tree for all sampled Paratrechina longicornis, inferred by Bayesian analysis. Numbers above branches indicate Bayesian posterior probability calculated by MrBayes. Refer to Table S1 for respective geographic information of each haplotype.

FIGURE 3 | Distribution of all mitochondrial haplogroups in the study regions. Haplogroups are denoted by colors: Clade I (blue), Clade II-1 (orange), Clade II-2 (red), and Clade II-3 (pink).
LonF represented a new sequence type that has not been reported in the database. LonA allelic profiles for gatB, coxA, hcpA, ftsZ, fbpA and wsp were 7, 6, 7, 3, 8, and 18, respectively (Table S7). LonA belongs to sequence type 19 (ST-19), and is similar to Wolbachia variants detected in a moth (Ephestia kuehniella), several ants (Technomyrmex albipes, Leptomyrmex sp., Pheidole plagiara, Ph. sauberi, and Leptogenys sp.) and two butterflies (Ornipholidotos peucetia and Aricia artaxerxes).

**TABLE 1 | Prevalence of Wolbachia wLonA and wLonF infections in Paratrechina longicornis.**

| No. nests (percentage) | wLonA | wLonAF | wLonF | Uninfected |
|------------------------|--------|---------|--------|------------|
| Clade I                | 42 (33%) | 56 (44%) | 8 (6%) | 21 (17%) |
| Clade II               | 0 (0%)  | 0 (0%)  | 47 (39%) | 74 (61%) |
| Total                  | 42 (17%) | 56 (23%) | 55 (22%) | 95 (38%) |

†Three workers from each nest were used to screen for Wolbachia infections.

**FIGURE 4 | Haplotype networks of the mitochondrial genes. Circle sizes are proportional to the number of sequences per haplotype. Colors correspond to geographic regions.**
wLonA shared an identical sequence type with Wolbachia Ekue_A (ID 13) detected from Ephesia kuehniella, a transinfected A group Wolbachia from Cadra cautella (wCauA) to E. kuehniella. Wolbachia wCauA has been reported to cause cytoplasmic incompatibility (CI) in C. cautella, and male killing in E. kuehniella (Sasaki et al., 2002; Sasaki et al., 2005). wLonF allelic profiles for gatB, coxA, hcpA, fisZ, fbpA and wsp were 168, 147, 262, 132, 226, and 708, respectively (Table S7), and were unique to consider wLonF a new sequence type (denoted as ST-471). Similar sequence types included ST-239, ST-242, and ST-243, all of which were detected from two dragonflies (Brachythemis contaminata and Orthetrum sabina). The most similar wsp sequence to wLonF in GenBank was from a Wolbachia variant infecting bat flies Cyclopodia dubia (KT751165; 99% similarity) (Wilkinson et al., 2016).

Wolbachia Infection History in P. longicornis

The BayesTraits analyses indicated the dependent model was not significantly better than the independent model (log Bayes factors = 0.707), suggesting no correlation between wLonA infection status and wLonF infection status. Therefore, infection history of wLonA and wLonF was inferred separately (Figure 5). BayesTraits analyses suggested a single ancestral wLonA infection (on the common ancestor of node 2 or node 3, Figure 5) occurred in P. longicornis that subsequently has been characterized by vertical Wolbachia transmission in the populations of Clade I with only occasional losses of infections (Figure 5). On the other hand, the history wLonF infections in P. longicornis appears to be characterized by frequent gains of wLonF through horizontal transmission as well as frequent...
losses of wLonF over time. Although the association between wLonF infection status and host mtDNA phylogeny was weaker than that of wLonA (both AI and PS values of wLonF were higher than those of wLonA), the BaTS results indicated that both wLonA and wLonF are significantly associated with the host mtDNA phylogeny (Table 2). These results suggest that wLonF infection within P. longicornis has been shaped by both horizontal and vertical transmission. For example, individuals bearing haplotype 2 (Hap 2) likely obtained wLonF via horizontal transmissions whereas the high prevalence of wLonF in Clade II-3 is consistent with vertical transmission of wLonF over time (Figure 5).

**Patterns of mtDNA Variation Within Wolbachia-Infected and -Uninfected Ants**

wLonA and wLonF may have undergone independent invasion histories in P. longicornis (Figure 5), therefore wLonA (or F)-positive individuals (including individuals with co-infection) are grouped together when mtDNA variation was analyzed to show the effect of a single strain on the mtDNA evolution. Analyses of mtDNA variation revealed that nucleotide diversity and numbers of segregating sites are much lower in wLonA-infected workers than those in wLonA-uninfected workers within all sampled regions, except North America (Figure 1; Table S6). Estimates of nucleotide diversity were more than 8-fold lower for wLonA-infected workers than in wLonA-uninfected workers despite limited differences in mtDNA variation between wLonF-infected workers and wLonF-uninfected workers.

Statistical tests of departures from neutral expectations are presented in Table S8 and Figure 6. Estimates of Tajima’s D, Fu and Li’s D*, Fs and Fay’s F* statistics of all groups harboring wLonF were all positive, but only five of these estimates were significant (Table S8; Figure 6). The estimates of Fay and Wu’s H* in wLonA-uninfected and wLonF-uninfected workers were negative and significantly less than zero. However, the results of DHEW tests failed to support the presence of positive selection of these two groups. The results of M-K tests indicated that the NI of these three groups were not significantly different from one.

Similar trends were found for estimates of Tajima’s D, Fu and Li’s D*, and Fu and Li’s F* statistic for all groups harboring wLonA (i.e., generally less than zero). However, the normalized H* statistics of Fay and Wu’s H* test and results of DHEW varied among regions (Table S8). We obtained a negative yet significant estimate of H* only for wLonA-infected workers from East Asia, and the results of DHEW tests for wLonA-infected groups from East Asia and South Asia regions were significant. The NI estimated from M-K test were larger than one for all groups harboring wLonA, and were significant in groups from Northeast and East Asia.

Tajima’s D, Fu and Li’s D*, Fu and Li’s F* generally were positive for wLonA-uninfected, wLonF-infected and wLonF-uninfected groups for each region with few exceptions. The significant negative estimates of D*, D*, F*, H* and significant results of DHEW test were observed in three groups, wLonA-uninfected workers from Oceania, wLonF-infected workers from North America, and wLonF-uninfected workers from Caribbean.

**Nuclear DNA Variation and Population Genetic Structure**

We compared the extent of mtDNA and nuclear (microsatellite) differentiation in the three selected Asia regions. A total of 191 alleles were observed across all loci for the 134 sampled workers. The average number of alleles in each sampled region ranged from 4.85 to 8.10 (Table S9). Shannon’s information index was used to assess gene diversity (Figure 7; Table S9), and, when incorporating infection status into analysis, genetic diversities among Wolbachia-infected workers are similar to those for uninfected workers across the three selected regions.

Bayesian cluster analysis performed using STRUCTURE revealed six distinct genetic clusters for the entire data set (K = 3 based on ΔK statistic; Table S10). Most workers were admixed (i.e., had membership in more than one cluster; Figure S3), and

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**TABLE 2** Significance of correlations between Paratrechina longicornis mtDNA phylogeny and Wolbachia infection status as identified by BaTS. Association index statistic (AI) and parsimony score (PS) statistic of clustering strength, and exclusive single-state clade size (MC) statistic.

| Statistics | Observed mean (95% CI) | Null mean (95% CI) | P-value |
|------------|------------------------|--------------------|---------|
| wLonA      |                        |                    |         |
| AI         | 3.10 (2.03, 4.19)      | 10.46 (9.82, 11.06) | <0.0001 |
| PS         | 24.29 (22.00, 27.00)   | 77.69 (74.31, 80.41)| <0.0001 |
| MC (uninfected) | 121.00 (121.00, 121.00) | 5.63 (5.03, 6.50) | 0.001 |
| MC (infected) | 19.89 (14.00, 24.00)  | 3.46 (3.17, 4.26)  | 0.001  |
| wLonF      |                        |                    |         |
| AI         | 6.37 (4.87, 7.89)      | 10.82 (10.17, 11.47)| <0.0001 |
| PS         | 49.67 (45.00, 54.00)   | 81.54 (77.87, 84.61)| <0.0001 |
| MC (uninfected) | 33.40 (30.00, 42.00)  | 4.97 (4.45, 5.71)  | 0.001  |
| MC (infected) | 10.56 (5.00, 18.00)   | 3.90 (3.55, 4.57)  | 0.001  |
genetic differentiation among geographic regions or mtDNA clades was not observed. DAPC analysis indicated the lack of differentiation among groups in each of the three selected regions as well as between the two mtDNA clades (Figure S4). The estimate of $G''_{ST}$ between the two mtDNA clades was 0.049 ($P = 0.001$), suggesting a low level of nuclear differentiation between workers from the two mtDNA clades.

DIscussion

Our results showed that global patterns of mtDNA and nDNA variation among populations of *P. longicornis* were discordant, characterized by two highly divergent mtDNA clades with no parallel pattern of nuclear genetic divergence (based on microsatellite loci) between workers from the two mtDNA clades.
clades. Several evolutionary scenarios possibly explaining such mitochondrial-nuclear discordance include sex-biased dispersal, local adaptation, historical demography, incomplete lineage sorting, and endosymbiont-driven hitchhiking effects (reviewed in Toews and Brelsford, 2012). Male-biased dispersal (López-UrIBE et al., 2014) and local adaptation of mtDNA haplotypes (Cheviron and Brunfeld, 2009; Ribeiro et al., 2011; Spottiswoode et al., 2011) are unlikely for at least two reasons: 1) the geographical distributions of ants from the two mtDNA clades overlap considerably and coexist in virtually all geographic regions we sampled and 2) all ant samples were collected from human-modified habitats (e.g., roadsides, parks or near buildings), suggesting negligible habitat preference between ants from the two clades. The strong association between Wolbachia infection status and host mtDNA lineage, as well as reduced mtDNA diversity associated with wLon in P. longicornis, are consistent with Wolbachia influencing patterns of host mtDNA structure and variation. Levels of nuclear variation were nearly identical for ants from the wLonA-infected workers (mtDNA Clade I) and uninfected groups (mtDNA Clade II), which is consistent with the prediction that Wolbachia endosymbionts have minimal or no effects on nuclear genetic variation and divergence (assuming host reproduces sexually) due to biparental inheritance (Rokas et al., 2001).

Results from additional analyses also were largely consistent with the predicted effects of Wolbachia on mtDNA variation. Tajima’s D, Fu and Li’s D*, and Fu and Li’s F* tests for departures from neutral evolution were negative for all groups harboring wLonA and for global datasets. Moreover, the results of Fay and Wu’s Hn and DHWEW were also consistent with expected patterns for a relatively recent Wolbachia-driven selective sweep occurring in some, but not all, geographic regions, such as East and South Asia, despite the fact that mtDNA variation is low in almost all populations harboring wLonA in every geographic region. One potential explanation for these inconsistencies is that selective sweeps of Wolbachia in some of these populations may have occurred far enough in the distant past such that any signature of selection on the mtDNA may have been eroded. The results of M–K tests also imply mtDNA substitution patterns may have been influenced by wLonA, but surprisingly that a signature of purifying selection is registered. The NI values of M–K test for groups harboring wLonA ranged between 5,074 to 12,888, and were significantly larger than values for groups from Northeast and East Asia. One possible explanation is that hitchhiking events associated with wLonA resulted in accumulation of slightly deleterious mutations (Shoemaker et al., 2000; Fay and Wu, 2000) followed by negative selection as Wolbachia-driven haplotype replacements cease (Bazikin and Kondrashov, 2011).

The virtual absence of genetic structure across a large geographic area in P. longicornis and the co-occurrence of divergent mtDNA haplotypes in almost every geographic region suggest that human-mediated, long-distance movement of this species is common. The time of divergence between any random pair of wLonA-uninfected groups (i.e., mtDNA subclades II-1, 2, and 3) is roughly 34,000 years ago (estimated based on average of pairwise genetic distances assuming a substitution rate of 1.455% per site per million years, the estimated rate for the COI gene of ants; Resende et al., 2010), which apparently predates potential human dispersal. However, this divergence, along with the deep divergence between Clades I and II, may stem from accelerated mtDNA substitution rates due to recurrent Wolbachia sweeps (Shoemaker et al., 2000), with the assumption that the former had infected ancestor at some point in time in the past. A likely explanation for the specific genetic patterns observed in P. longicornis is the ant has experienced multiple human-mediated dispersal events from genetically distinct source populations followed by global dispersal (i.e., high propagule pressure as a result of rampant migration/movement). The genetic patterns we describe are similar to those found in several other globally distributed insects, especially those that are common in human-modified landscapes [e.g., German cockroach (Blattella germanica), American cockroach (Periplaneta americana); Vargo et al., 2014; von Beeren et al., 2015], further highlighting the role of human-mediated dispersal in shaping population structure of insect species closely associated with humans.

Reduction in genetic variation as a result of a population bottleneck is a common feature observed in the introduced ranges of numerous invasive species (Nei et al., 1975; Allendorf and Lundquist, 2003; Dlugosch and Parker, 2008). The general consensus is that P. longicornis originated in the Old World tropics, but to narrow this down further to a specific sub-region has been somewhat controversial (Wetterer, 2008). We did not find evidence for reduced mtDNA diversity in any sampled subregions of the Old World. Also, mtDNA structure within Clade II appears to be less associated with Wolbachia infections, and inferring the origin of this ant using mtDNA patterns of variation in Clade II remains challenging primarily due to insufficient sampling in certain areas (Table S11). Identification of the native range of P. longicornis on a finer geographic scale is further obscured by presumed frequent human-mediated dispersals, a multi-century old invasion history, and the potential effects of Wolbachia infections on mtDNA variation. However, it is interesting to note that haplotypes from the northern part of India and Nepal (Himalayan region) (Hap31 and Hap37) are divergent from other haplotypes in Clade I and form a clade sister to all other haplotypes in this clade (Figure 2), implying the populations in Himalayan region might be the source of invasive populations of Clade I. More comprehensive sampling and additional nuclear data from queens and males may help efforts to identify the likely origin and to reconstruct with more confidence the invasion history of this ant.

The loss of Wolbachia in invasive ranges is common, but not universal, in invasive insects possibly due to founder effects or altered selection pressures in the new habitats (Shoemaker et al., 2000; Tsutsui et al., 2003; Reuter et al., 2004; Yang et al., 2010; Nguyen et al., 2016). For example, both the red imported fire ant, Solenopsis invicta, and the Argentine ant, Linepithema humile, had higher Wolbachia infection prevalences in their native populations compared with introduced populations where the symbionts are nearly absent (Shoemaker et al., 2000; Tsutsui et al., 2003; Reuter et al., 2004; Yang et al., 2010). However, we did not observe a similar phenomenon in P. longicornis. Both wLonA and wLonF are found throughout geographic range of P. longicornis, including known invasive areas such as North America and Caribbean (Figure S2). Nevertheless, a few individuals in clade I (wLonA lost) and
subclade II-3 (wLonF lost) have lost Wolbachia and this loss appears to be stochastic. The loss of Wolbachia likely is attributable to imperfect maternal transmission or natural curing events (Stevens and Wicklow, 1992; Hoffmann and Turelli, 1997; Clancy and Hoffmann, 1998).

Our simulation results suggest wLonA was acquired by the common ancestor of Clade I, and the fitness advantage associated with harboring wLonA infections compared with uninfected ants may have facilitated the spread of Wolbachia and the associated mtDNA haplotype. One possible fitness advantage of harboring wLonA infections could be Wolbachia-induced cytoplasmic incompatibility (CI). Although spread of Wolbachia inducing CI in haplodiploid species appears to be less efficient than in diploid species (Vavra et al., 2000), limited movement of P. lonigornis (Trager, 1984; Harris and Berry, 2005) could have enabled Wolbachia to increase in frequency within small local populations through genetic drift, allowing the bacterium to exceed the threshold frequency for spread in a host population (Vavra et al., 2003). While this possibility remains to be tested, a survey of Wolbachia prevalence across numerous ant species appears supportive that Wolbachia infections generally are more prevalent in ant species that have limited mobility (i.e. reproducing by budding or fusion) (Wenseleers et al., 1998).

In contrast, wLonF appears to have been gained and lost multiple times in P. longicornis over evolutionary time and has had little or no significant effect on host mtDNA variation. One possible explanation for this pattern is that wLonF is simply a passive passenger in longhorn crazy ant (e.g., having negligible fitness effects), and persists because the rate of wLonF loss occurs roughly at the same rate as horizontal transmission (Hoffmann et al., 1996; Charlat et al., 2004; Bouwma and Shoemaker, 2011). Invasive species may acquire new Wolbachia in their new environments (Rocha et al., 2005; Himler et al., 2011; Schuler et al., 2013). For example, the North American fruit fly, Rhagoletis cingulata (Diptera: Tephritidae) acquired a new Wolbachia strain through interspecific horizontal transmission from the Eurasian endemic R. cerasi (Schuler et al., 2013). However, supergroup F Wolbachia, to the best of our knowledge, has only been discovered in a single ant species, Ocmynrmex picardi (Russell et al., 2009), suggesting that the prevalence of this variant is very low in ants, and that acquisition of this variant from other sympatric ant species in the introduced range of P. longicornis appears unlikely. Effective and efficient horizontal transmission of Wolbachia depends on intimate ecological associations that provide opportunities to bring Wolbachia into close contact with novel hosts (Sintupachee et al., 2006; Stahlhut et al., 2010; Boivin et al., 2014). Host-parasitoid associations have been commonly suggested as a route of the Wolbachia horizontal transmission, and evidence this has occurred includes between host and parasitoid (Heath et al., 1999), between hosts (Ahmed et al., 2015), and between parasitoids sharing the same host (Huigens et al., 2000; Huigens et al., 2004). Other putative ecological associations for successful Wolbachia horizontal transmission are prey-predator and parasite-host associations (Le Clech et al., 2013; Brown and Lloyd, 2015). One well-known case for the latter involves an inquiline social parasite (Solenopsis daguerrei) and its ant host (S. invicta) (Dedeine et al., 2005). While no social parasites have been reported in P. longicornis to date, colonies of this ant often host a variety of arthropods, such as the ant cricket Myrmecophilus americanus, the beetle Colhocera maderae and the ant mite Macrocyclus multispinosus (Wollaston, 1854; Wetterer, 2008; Werren et al., 2008; Lachaud et al., 2016). These arthropods, termed “myrmecophiles,” represent good candidates for Wolbachia transfer because they all have intimate ecological associations and interactions with their ant hosts (Kronauer and Pierce, 2011). A future study of Wolbachia in the organisms ecologically associated with P. longicornis may uncover the routes and mechanisms underlying Wolbachia horizontal transmission in this ant.

**DATA AVAILABILITY**

MtDNA sequences (accession numbers KY769964-KY770018) are deposited at GenBank. The sequences of the five MLST genes and wsp gene of two Wolbachia strains are deposited at PubMedMLST database (ID:1827 and 1828).

**ETHICS STATEMENT**

We thank Future Development Funding Program of the Kyoto University Research Coordination Alliance for financial support.

**AUTHOR CONTRIBUTIONS**

CCSY, S-PT, and DS originally conceived the ideas. JW, AS, and C-YL helped develop an overarching research programme and collect the samples. TY and CCGS applied the grant. SPT performed the experiments. S-PT, DS, and CCGS carried out the analyses and drafted the manuscript. All authors contributed substantially to editing the manuscript.

**FUNDING**

We thank Future Development Funding Program of the Kyoto University Research Coordination Alliance for financial support.

**ACKNOWLEDGMENTS**

We thank Amy Low, Ni-Chen Chang, Chung-Wei You, Chun-Teng Coody Chiu, Chun-Yi Lin, Ching-Chen Lee, En-Cheng Yang, Han-Chih Ho, Hui-Siang Tee, Hugo Darras, Hung-Wei Hsu, Jia-Wei Tay, Kean Teik Koay, Li Yan Gan, Nellie Wong, Peter G. Hawkés, Ping-Chih Lin, Po-Wei Hsu, Singham Veera, Su-Chart Lee, Sylvain Hugel, Yi-Ming Weng, Yueh-Hua Wu, Yu-Fang Tseng, and Zhengwei Jongs, for assistance in field collection. We also thank the Future Development Funding Program of the Kyoto University Research Coordination Alliance for financial support.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00838/full#supplementary-material
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