Received: 4 August 2022 | Revised: 12 December 2022 | Accepted: 14 December 2022

DOI: 10.1111/mec.16827

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Genetic analysis reveals the putative native range and widespread double-clonal reproduction in the invasive longhorn crazy ant

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Funding information
Kyoto University Future Development Funding Program; Virginia Tech Faculty Start-up Research Fund

Handling Editor: Rosemary Gillespie

Abstract

Clonal reproduction can provide an advantage for invasive species to establish as it can circumvent inbreeding depression which often plagues introduced populations. The world's most widespread invasive ant, *Paratrechina longicornis*, was previously found to display a double-clonal reproduction system, whereby both males and queens are produced clonally, resulting in separate male and queen lineages, while workers are produced sexually. Under this unusual reproduction mode, inbreeding is avoided in workers as they carry hybrid interlineage genomes. Despite the ubiquitous distribution of *P. longicornis*, the significance of this reproductive system for the ant's remarkable success remains unclear, as its prevalence is still unknown. Further investigation into the controversial native origin of *P. longicornis* is also required to reconstruct the evolutionary histories of double-clonal lineages. Here, we examine genetic variation and characterize the reproduction mode of *P. longicornis* populations sampled worldwide using microsatellites and mitochondrial DNA sequences to infer the ant's putative native range and the distribution of the double-clonal reproductive system. Analyses of global genetic variations indicate that the Indian subcontinent is a genetic diversity hotspot of this species, suggesting that *P. longicornis* probably originates from this geographical area. Our analyses revealed that both the inferred native and introduced populations exhibit double-clonal reproduction, with queens and males around the globe belonging to two separate, nonrecombining clonal lineages. By contrast, workers are highly heterozygous because they are first-generation interlineage hybrids. Overall, these data indicate a worldwide prevalence of double clonality in *P. longicornis* and support the prediction that the unusual genetic system may have pre-adapted this ant for global colonization by maintaining heterozygosity in the worker force and alleviating genetic bottlenecks.

Shu-Ping Tseng and Hugo Darras contributed equally to this work.

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KEYWORDS
biological invasion, clonal reproduction, inbreeding, parthenogenesis, social insects

1 | INTRODUCTION

Reproduction represents one of the key factors for the successful establishment and spread of an invasive species (Barrett et al., 2008). Empirical and theoretical studies have shown that the reproductive mode of a species can determine the genetic and demographic structure of its invasive populations via shaping dispersal ability, the genetic composition of propagules and thus the evolutionary potential of introduced populations (Barrett et al., 2008; Gutekunst et al., 2018; Ness et al., 2010). As one example, clonal organisms are more than four times over-represented among invasive/pest invertebrates (Hoffmann et al., 2008), suggesting that clonality is probably an important mechanism underlying the invasion success of introduced populations (reviewed in Vallejo-Marín et al., 2010). Some forms of asexual reproduction may especially help mitigate negative effects associated with low population densities during colonization (Hagan & Gloag, 2021; Taylor & Hastings, 2005).

Social insects are among the most successful invaders and account for half of the worst invasive insect species listed by the IUCN's Global Invasive Species Database (www.iucngisd.org). The ecological success of some of these infamous invasive species appears to be partly attributed to their modes of reproduction, with clonal reproduction being one of the primary mechanisms (Eyer & Vargo, 2021). A unique form of reproduction involving clonality was reported in multiple invasive ant species including *Wasmannia auropunctata*, *Vollenhovia emeryi*, *Paratrechina longicornis* and *Cardiocondyla kagutsuchi*. Under this system, termed double-clonality, queens are clones of their mother, males are clones of their father and sterile workers arise sexually (Fournier et al., 2005; Kobayashi et al., 2008; Ohkawara et al., 2006; Okita & Tsuchida, 2016; Pearcy et al., 2011). This two-fold asexuality prevents inbreeding in the workers, which arise from sexual reproduction between divergent queen and male clones (Foucaud et al., 2007; Pearcy et al., 2011). Previous studies in *W. auropunctata* observed canonical sexual populations in native populations, but an overwhelming occurrence of male and female clonality in introduced populations, suggesting that double-clonality is under selection in human-modified habitats and potentially contributes to the invasion success of this invasive ant species (Foucaud et al., 2007, 2009, 2010). Genetic studies also revealed that the genomes of the clonal males and queens of *W. auropunctata* occasionally recombine, although the evolutionary significance of these introgression events is yet undetermined (Foucaud et al., 2007). To better understand the link between the reproductive systems of a species and its ability to become invasive, further research on species with such noncanonical reproductive systems is necessary. Such studies could offer valuable insights to aid in monitoring biological invasions.

The longhorn crazy ant, *Paratrechina longicornis* (Latreille, 1802), is a common household, garden, and agricultural pest which can disrupt ecosystems by enhancing populations of phloem-feeding hemipterans and displacing other invertebrates (Koch et al., 2011; Wetterer, 2008; Wetterer et al., 1999). This species thrives in anthropogenically modified environments (LaPolla et al., 2013) and is frequently transported by cargo and other human-associated articles (Bertelsmeier et al., 2018; Lester, 2005; Weber, 1939). Consequently, it has spread across most subtropical and tropical areas of the world for over a century and has become arguably the most widely distributed ant today (Figure 1; Wetterer, 2008). Double-clonal reproduction has been previously reported in this species in one locality in Thailand (Figure 2a), but whether this system has contributed to its global invasion success and whether the gene pools of the queens and males regularly recombine, as in *W. auropunctata*, or remain divergent over time are unclear. Evaluating the prevalence of double-clonality and comparing genetic variation across both native and invasive populations of *P. longicornis* is necessary to understand how double-clonality evolved (e.g., pre-adaptation in native range) and may have aided the invasion success in this species. However, despite the long establishment history of *P. longicornis*, attempts to deduce its geographical origin failed to reach consensus

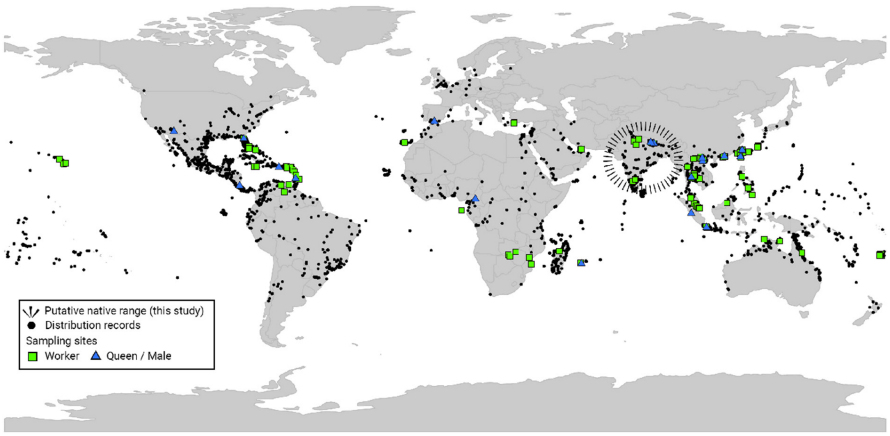

FIGURE 1 Distribution and sampling sites of *Paratrechina longicornis*. The 256 collection sites studied are indicated by squares and sites in which males or queens were collected are indicated by triangles ($N = 22$ localities). The global distribution of the species was obtained from Wetterer (2008), Antmaps.org and iNaturalist

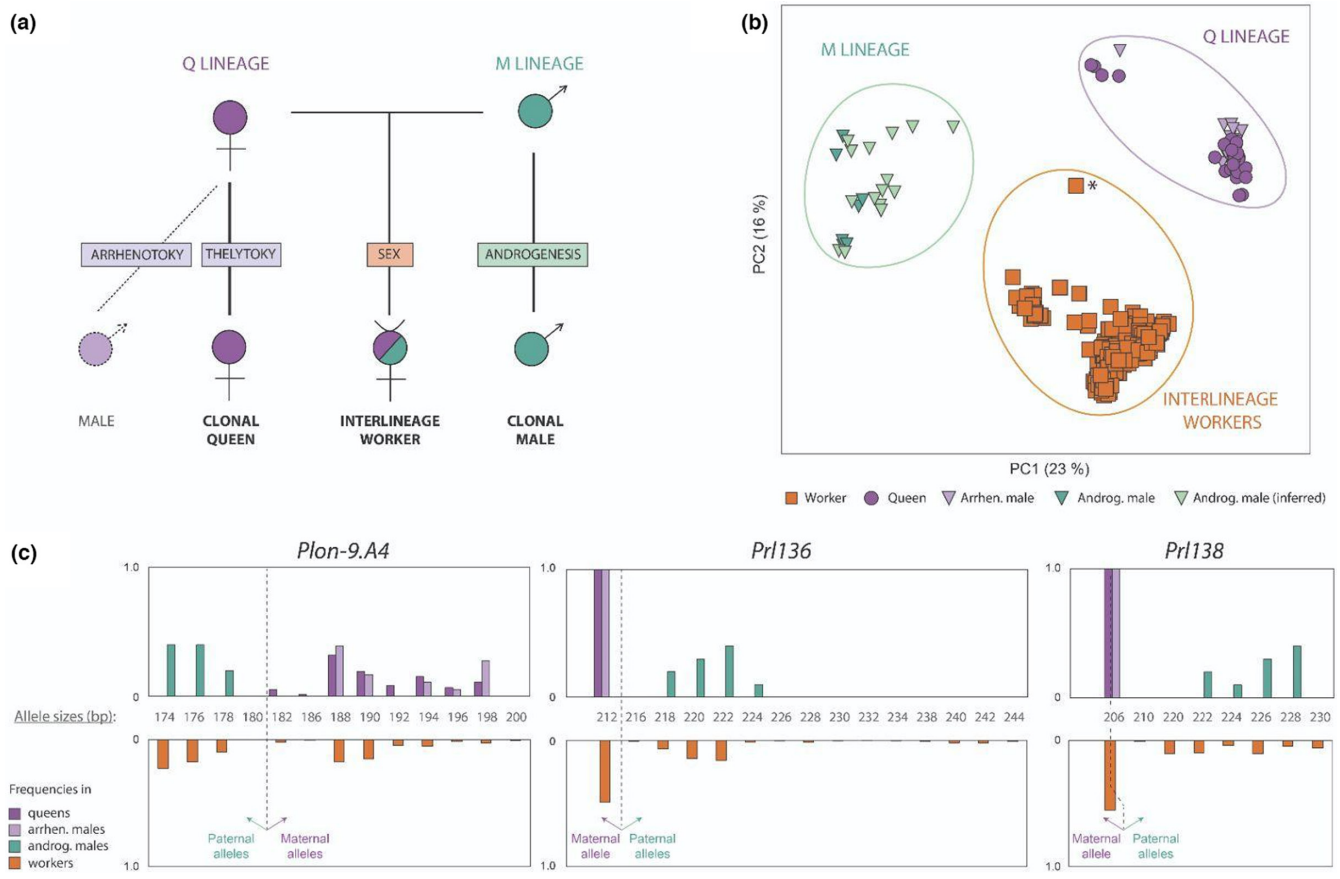

FIGURE 2 Two interdependent lineages (Q and M) coexist and interbreed for the production of workers across the range of *Paratrechina longicornis*. (a) Schematic representation of the double-clonal system of *P. longicornis*. New queens belong to the Q lineage and are produced by parthenogenesis (thelytoky). Two main types of males coexist. Clonal male reproductives belong to M lineages and develop from male gametes with no maternal nuclear genome contribution (androgenesis). Arrhenotokous males (dashed lines) carry alleles from the Q lineage and develop from unfertilized eggs. Queens are virtually always found mated with males from the M lineage and produce interlineage workers by sexual reproduction. The significance of arrhenotokous males is undetermined. (b) Distribution of genetic diversity in queens, males and workers sampled in different localities across the world (see Figure 1). The first two axes from a principal coordinates analysis (PCoA) based on microsatellite Bruvo's distance are shown with the percentage of total variation explained by the axes. In localities with both queen and worker samples, male genotypes were inferred from workers. The worker indicated with a star was collected from the putative native range in Nepal and carried rare alleles. (c) Examples of how workers' parental alleles are inferred. Three of the 19 microsatellite loci at which Q and M lineages had nonoverlapping allele size ranges in the 22 localities where reproductive individuals were sampled are shown (other markers are shown in Figures S1 and S2). Top panels: Allele frequencies of the Q lineage ($N = 37$ queens and $N = 17$ arrhenotokous males) and the M lineage ($N = 10$ androgenetic males) at each of the three loci. Bottom panels: Allele frequencies of workers ($N = 341$ individuals from 252 localities). The dashed lines represent the boundaries between the maternal and paternal alleles of workers, corresponding to the Q and M lineages, respectively. Boundaries were inferred from the comparison of reproductive individuals' genotypes and used to infer the parental origin of workers' alleles in localities in which reproductive individuals were not collected. The allele size ranges of the Q and M lineages overlap at the 206-bp allele for marker Prl138

(LaPolla et al., 2010; LaPolla & Fisher, 2014; Wetterer, 2008). The species has been recorded in undisturbed habitats in Southeast Asia (Wetterer, 2008) and is related to the Southeast Asian genera *Pseudolasius* and *Euprenolepis* (LaPolla et al., 2010), but congeneric species have recently been found in Africa (LaPolla & Fisher, 2014). With the ongoing disagreement regarding the ant's geographical origin, the understanding of invasive processes of *P. longicornis* and the significance of this reproductive system in the ant's outstanding invasion success is currently impeded.

Here, we investigate the population genetics, phylogeography and breeding system of *P. longicornis* worldwide using both microsatellite and mitochondrial DNA (mtDNA) data for 256 localities sampled across most of the species' range (22 of which included reproductive individual samples, Table 1). These analyses revealed that the species probably originates from the Indian subcontinent; and that queens and males from all invasive populations around the globe belong to two nonrecombining clonal lineages which interbreed to produce workers.

TABLE 1 Information about localities and sample sizes genotyped for *Paratrechina longicornis*

Region	Subregion	Sampled localities	Genotyped individuals			Inferred genotypes	
			Male	Queen	Worker	Male	Queen
Arabia	Arabia	3	–	–	4	3	3
Caribbean	Caribbean	19	2	–	30	17	30
Central America	Costa Rica	1	2	1	4	0	4
East Asia	China	19	0	1	27	19	18
	Taiwan	17	17	15	28	15	14
Indian subcontinent	India	14	–	–	18	14	14
	Nepal	21	4	6	32	21	27
North America	Mainland USA	8	1	2	14	7	6
Northeast Asia	Japan	22	–	–	22	22	22
Oceania	Fiji	9	–	–	10	9	10
	USA-Hawaii	9	–	–	9	9	9
	Australia	7	–	–	7	7	7
South America	Venezuela	4	–	–	4	4	4
Southeast Africa	Reunion	2	–	1	9	2	4
	Madagascar	1	–	–	2	1	1
	Zambia	1	–	–	2	1	1
	Zimbabwe	2	–	–	3	2	2
	Mozambique	3	–	–	6	3	3
Southeast Asia	Indonesia	7	4	3	13	9	9
	Vietnam	10	–	3	16	10	13
	Thailand	32	1	3	34	31	31
	Philippines	3	–	–	4	3	3
	Singapore	7	–	–	7	7	7
	Laos	1	–	–	1	1	1
	Malaysia	26	–	–	26	26	26
	Cambodia	2	–	–	2	2	2
Southern Europe	Spain	2	1	1	1	1	1
	Greece	2	–	–	2	2	2
West Africa	Cameroon	1	1	1	–	–	–
	São Tomé and Príncipe	1	–	–	4	1	1
Total		256	33	37	341	249	275

Note: Numbers of longhorn crazy ant localities, numbers of genotyped males, queens and workers, and inferred male and queen haplotypes/genotypes for each surveyed geographical region.

2 | MATERIALS AND METHODS

2.1 | Sample collection

We collected *Paratrechina longicornis* in 67 localities and performed additional genetic analyses on previously collected samples from 189 localities (Tseng, Darras, et al., 2019; Tseng, Wetterer, et al., 2019). In total, we obtained samples from 256 different localities across the range of the species (32 from America, 11 from Africa, four from Europe, 184 from Asia and 25 from Oceania; Figure 1). To study the distribution of queen and male clonality, reproductive individuals were sampled in 22 representative localities, including the original locality in Thailand where double-clonality was first discovered (Pearcy et al., 2011; Table 1).

Our sampling revealed three different types of males (see Section 3). To investigate the evolutionary significance of these males, two colony fragments consisting of ~50 reproductive queens and a few thousand workers were collected in two neighbouring Spanish populations (Malaga) and maintained in the laboratory. Some of the emerging males were dissected to assess whether they produced sperm and were preserved for genetic analyses. The remaining males were left to mate with new queens within the nests. After a year, the spermathecae of the new generation of queens

were collected to determine the mating success of each male type (see Section 2.4 for the detailed methodology).

2.2 | Microsatellite genotyping

Queens, males and a sample of workers (mean ± SD = 4.1 ± 3.0 per locality) were genotyped in 22 localities. We also analysed one or two workers for each of the remaining 234 localities. A total of 411 selected individuals were genotyped at 40 microsatellite loci using published primer sequences (MERPDC et al., 2011; Tseng, Darras, et al., 2019; Data S1). DNA extraction and genotyping were carried out as described in Tseng, Darras, et al. (2019). For some localities, partial genotypes were released earlier to answer other research questions (Tseng, Darras, et al., 2019; Tseng, Wetterer, et al., 2019).

Queen, male and sperm samples collected from the two laboratory colonies were genotyped at 13 microsatellite loci (see Data S1). Microsatellite data derived from these colonies were analysed separately from the main data set. All genotypes are available as Data S2 and Data S3.

2.3 | Sequencing and processing of mtDNA

To compare patterns of nuclear and mitochondrial variation, a portion of the mitochondrial genome containing the *cytochrome oxidase subunit I*, *cytochrome oxidase subunit II* and *tRNA–Leu* genes, and an intergenic spacer was sequenced and analysed following methods described by Tseng, Wetterer, et al. (2019). A female from each of the 67 new localities sampled was sequenced (63 workers and four queens). Additional castes were sequenced in nine localities (both sexes were sequenced in four localities and one individual of each caste was sequenced in each of the remaining five localities). These sequences were aligned with published sequences of *P. longicornis* (GenBank accession numbers: KY769964–KY770017). The region containing the intergenic spacer and the *tRNA–Leu* gene showed a low level of conservation and was removed from the alignment. For further analyses, the sequences were collapsed into haplotypes using DNASP version 5.10 (Librado & Rozas, 2009). The new haplotype sequences identified in this study were deposited in GenBank (MZ398110–MZ398127). We constructed a median-joining mtDNA haplotype network using POPART (Leigh & Bryant, 2015) to visualize the geographical distribution of mtDNA haplotypes.

2.4 | Distribution and characteristics of double-clonality

The double-clonal mode of reproduction previously described in Thailand resulted in different genetic makeups for each caste: queens and males belong to different genetic groups, while workers are highly heterozygous individuals carrying alleles from both groups at each marker. To investigate the global distribution of double-clonality, we compared the microsatellite genotypes of reproductive individuals and workers (22 and 252 localities, respectively). Workers' paternal genotypes were reconstructed based on the genotypes of mothers when the latter was available (Pearcy et al., 2011). Five out of 33 males carried more than one allele at one or multiple markers. PCRs (polymeracse chain reactions) were replicated to confirm that these unusual genotypes did not stem from contaminations, and these were subsequently excluded from the main phylogeographical analyses. The genotypes of the remaining males were encoded as diploids by doubling their alleles to enable simultaneous analysis of male and female genotypes with the population genetic programs GENALEX version 6.502 (Peakall & Smouse, 2012), SPAGEDI version 1.5 (Hardy & Vekemans, 2002) and STRUCTURE version 2.3.3 (Pritchard et al., 2000). This approach has no effect on pairwise distance calculation but gives more weight to males when allelic frequencies are estimated. Allelic frequencies and individual heterozygosity (i.e., number of heterozygous loci divided by the total number of loci analysed per individual) were estimated using GENALEX version 6.502 (Peakall & Smouse, 2012). F_{ij} kinship coefficients were estimated according to Loiselle et al. (1995) using SPAGEDI version 1.5 (Hardy & Vekemans, 2002). Genetic variation was investigated using principal-coordinate analyses (PCoAs). Genetic distances were estimated with the R package "poppr v2.9.1" (Kamvar et al., 2014) using the method of Bruvo, which considers mutational distances between microsatellite alleles (Bruvo et al., 2004). PCoAs were then performed on raw distances using GENALEX. The Bayesian clustering method implemented in STRUCTURE version 2.3.3 was used to determine the number of genetic lineages (K) among reproductive individuals. Only one queen and one male per locality were included in this analysis to avoid unbalanced sampling. The program was run 10 times for each value of K ranging from 1 to 10, with 200,000 Markov chain Monte Carlo iterations and a burn-in period of 50,000. Analyses were performed under the admixture model with correlated allele frequencies and without prior population information. The most likely genetic lineage numbers were determined using the ad-hoc ΔK method (Evanno et al., 2005). The occurrence of clonal reproduction was assessed by comparing the genotypes of nestmate reproductive individuals when multiple queens or males were sampled within a locality. To determine whether androgenetic *P. longicornis* males still inherit mtDNA from their mothers, we compared the mtDNA sequences of male and female nestmates in seven localities.

2.5 | Clonal diversity

Our results indicate that queens and males of *P. longicornis* belong to two widespread, nonrecombining clonal lineages, which interbred to produce workers (see Section 3). To analyse the distribution of genetic diversity within each of these two interdependent lineages, we complemented the genotypes of reproductive individuals sampled in the field (22 localities) with parental haplotypes inferred from workers (252 localities, including 18 of the 22 localities

mentioned before). Sixteen loci showed no overlap between the allele size ranges of the two lineages in our queen and male samples (Table S1). This allowed us to assign the parental origin of a worker's allele solely based on its size when reproductive individuals were not available for deductive inferences (see Appendix S1). The 24 remaining loci had overlapping allele size ranges between the two lineages and were not considered for this analysis. When multiple workers were sampled in a locality, redundant inferred parental genotypes, if any, were discarded.

To study genetic variation in clonal (androgenetic) males, we constructed a median-joining haplotype network of microsatellite genotypes using NETWORK FLUXUS version 10.2 with the epsilon parameter set to 0 (Bandelt et al., 1999). This approach requires that microsatellite loci are linked, which we assumed to be the case as haploid male genomes are transmitted as complete linkage groups during androgenesis. Male haplotypes were clustered into multilocus clonal lineages (MLLs) using a Bruvo's distance threshold of 0.2 with the *mlg.filter* function of the R package "poppr version 2.9.1." To study queen genetic variation, we performed PCoAs instead as queens have diploid genomes that may recombine during parthenogenesis (Pearcy et al., 2006).

The queen and male data sets were reduced to one genotype per locality per lineage to compare the diversity of the two lineages. Genetic differentiation, as expressed by Wright's F_{ST} (F_{ST}), Hedrick's standardized G_{ST} (G''_{ST}) and Jost's estimate of differentiation (*Dest*) was estimated using GENALEX (Peakall & Smouse, 2012). Analyses of molecular variance (AMOVAs) were performed with GENALEX using Bruvo's distances.

2.6 | Inference of the native range

To infer the native range of *P. longicornis*, we examined whether some parts of the world displayed higher microsatellite and mtDNA richness than the rest of the species range. We assumed that genetic diversity would be lower in invasive populations due to genetic bottlenecks during colonization (Caron et al., 2014). We only used one mtDNA sequence per locality and the first worker genotyped of each locality to avoid biases. In addition, indexes were corrected for differences in sample sizes among geographical regions using rarefaction methods. Microsatellite allelic richness was estimated with the rarefaction algorithm implemented in ADZE version 1.0 (Szpiech et al., 2008), while mtDNA haplotype richness was estimated using the rarefaction method of PAST version 2.17c (Hammer et al., 2001). We also used the presence of the private, divergent alleles as an indication of the native range in addition to the genetic diversity approach, which is not always a reliable indicator of a species origin (Estoup et al., 2016). We estimated the mean numbers of private microsatellite alleles (e.g., alleles restricted to one geographical region) using ADZE version 1.0 (Szpiech et al., 2008). We examined the geographical distribution of individuals carrying a larger number of rare microsatellite alleles (i.e., alleles that occur at frequencies <0.01) or divergent haplotypes/genotypes, assuming these individuals are more likely to be found in native areas (Dlugosch & Parker, 2008; Fitzpatrick et al., 2012).

3 | RESULTS

3.1 | Distribution of double-clonality

To investigate the distribution of double-clonality in *Paratrechina longicornis*, we analysed the genotypes of workers, queens and males from 22 localities across Africa, America, Asia and Europe (Table 1). The genotypes of reproductive individuals from multiple localities clustered into two main groups (Figure 2b). The first group contained all queen genotypes including the queen clone originally described in Thailand, as well as 40% of the male genotypes (Morgan Pearcy et al., 2011). The similarity of these male genotypes with those of queens suggested that these were arrhenotokous males developing from unfertilized eggs. The second group consisted of all the remaining males and included all the male genotypes inferred from queen–worker comparisons and the male clone identified by Pearcy et al. (2011) in Thailand. The two groups were distinguishable by their first PCoA coordinate and had diagnostic, nonoverlapping allele size ranges at 19 of the 40 microsatellite loci genotyped (Figure 2c; Figures S1 and S2). The observed high divergence between these two groups (corresponding to the queen and male clonal genetic groups originally described in Thailand) suggests that queens and males worldwide belong to two distinct clonal lineages. Clustering analyses with STRUCTURE (Pritchard et al., 2000) confirmed these results. The second-order rate of change of the likelihood (ΔK) was the highest for a predefined number of groups set to 2, and all runs performed with this number showed identical results with all individuals assigned to the same two respective lineages mentioned above with virtually no admixture (<0.2% per individual; Figure S3). The two lineages are hereafter referred to as Q and M lineages.

All workers from the 22 localities were almost completely heterozygous (mean individual heterozygosity ±SD = 91 ± 3%, range: 83%–97%, N = 91 individuals) and carried one allele from each lineage at each of the 19 diagnostic loci. This indicates that workers were all Q/M hybrids produced from interlineage mating. To determine whether Q and M lineages identified in these 22 localities coexisted in all other populations, we investigated the allelic patterns of workers in the 234 localities where no reproductive individual was available. Workers were always extremely heterozygous (mean individual heterozygosity ± SD = 89 ± 4%, range: 75%–98%; N = 250 workers; Figure S4) with one allele typically corresponding to the size range of the Q lineage and the other to the size range of the M lineage at each of the 19 diagnostic loci (Figure 2c and Figure S1). Accordingly, in all but one Nepalese locality (NP01), individual inbreeding coefficients across the 19 loci were lower than expected under random mating (mean F_{ij} = −0.378; max = −0.073; N = 251 workers, 95% confidence interval based on 1000 permutations of gene copies across individuals: [−0.016 to 0.014]). In line with these

results, the PCoA coordinates of workers were all intermediate between those of the Q and M lineage groups (Figure 2b). These results further support the view that workers of *P. longicornis* are always first-generation hybrids of Q and M lineages.

Queen genotypes were consistent with clonal reproduction through apomixis or central fusion automixis, as previously suggested (Pearcy et al., 2011). These two modes of parthenogenesis result in a partial decrease of heterozygosity at each generation due to mitotic or meiotic recombination events (Rabeling & Kronauer, 2013). Accordingly, queens were all highly homozygous (mean individual heterozygosity ± SD = 16 ± 8%, range: 0%–30%; $N = 37$ queens from 19 localities; Figure S1). Genotype variation among matrilines could be assessed within 17 localities (Data S4). In 13 localities, maternal genotypes were either completely identical or displayed minor allelic differences which could be explained by the occurrence of recombination events during parthenogenesis (Pearcy et al., 2006). In four localities, female genotypes were not consistent with a single clonal matriline, suggesting that different clonal matrilines coexisted locally or that some queens were produced by intralineage sexual reproduction.

Two main types of male genotypes were observed (Figure 2c). (i) Twenty-five males, including all the males inferred from queen–worker comparisons, carried one allele from the M lineage at each diagnostic locus, suggesting that they were produced clonally by androgenetic reproduction ($N = 20$ localities). (ii) Eighteen males carried one allele from the Q lineage at each locus ($N = 5$ localities). These males had variable genotypes at loci where the queens were heterozygous ($N = 12$ males from two localities), suggesting that they developed from unfertilized eggs with random segregation of maternal alleles as is typical for most hymenopteran males (arrhenotokous parthenogenesis). In addition, five males with more than one allele per marker were found in another three localities. It is unclear whether these males were diploid individuals as sometimes occurs in ants (Cournault & Aron, 2009; Pamilo et al., 1994; Ross & Fletcher, 1986) or chimeras resulting from cytological incidents (Aamidor et al., 2018; Michez et al., 2009). No detectable morphological differences were found among males with different genotypes.

The production of each type of male was not confined to particular localities as multiple male types could be found within several localities (Data S2). To gain insights into the relative frequency of each type, we genotyped males produced in two laboratory colonies. Out of 223 males, 80 were inferred to be androgenetic, 127 were inferred to be arrhenotokous and 16 had more than one allele per locus, suggesting that these were diploids or chimeras. Note that arrhenotokous males have never been described before in *P. longicornis*. To investigate whether these males are functional, we inspected their reproductive systems and genotyped the spermathecal contents of queens to search for sperm of arrhenotokous origin. Live sperm was observed in seminal vesicles of arrhenotokous males ($N = 20$), but all dissected queens carried sperm with a single allele from the M lineage at each locus, suggesting that they had mated with androgenetic males only ($N = 86$).

To summarize, workers sampled in 252 localities across four continents were first-generation hybrids of the same two nuclear lineages. The two lineages appear to have remained genetically distinct over generations through clonal reproduction of each sex: the genome of the M lineage is transmitted through androgenesis from males to males. In contrast, the genome of the Q lineage is parthenogenetically transmitted from queens to queens. Although males carrying Q alleles occur regularly, we found no evidence that they reproduce. Together, our results suggest that double-clonality is not a local phenomenon in *P. longicornis*.

3.2 | Clonal diversity

PCoAs of the queen and male genotypes from the 22 localities with reproductive individuals suggested the existence of multiple subgroups within the queen and androgenetic male lineages (Figure S5a,b). To further investigate the two interbreeding lineages' variability, we reconstructed the maternal and paternal haplotypes of all workers from the 234 localities where reproductive individuals were not available (Table S1 and Appendix S1).

Analysis of the 257 queen and 256 male genotypes across the 256 localities surveyed revealed that the allelic diversity of the M lineage was 2.7 times greater than that of the Q lineage ($N = 253$ genotypes per lineage, after reduction to one genotype per locality per lineage; Table S2).

PCoA (Figure 3b) separated queens into two main groups (referred to as Clone A and Clone B), which differed at seven of the 16 loci analysed (Figure S6) and appeared widespread in invasive populations (Figure 3a). Most queens belonged to Clone A (233 of 257 genotypes). These queens carried identical homozygous genotypes at 14 of the 16 loci analysed, with only rare, seemingly recent, mutations in some individuals (genotypes with one or two stepwise mutation differences from the dominant genotype). The two remaining loci were more variable and occasionally heterozygous (*Plon-9.A4* was heterozygous in 30 of 32 queens sampled, while *Plon-8.F2* was heterozygous in one of 32 queens sampled and potentially heterozygous in queens of two localities, where nestmate workers carried different maternal alleles). Queens of Clone B were relatively less common (20 of 257 genotypes). Most variations within this second clone appeared to stem from recent mutations (one repeat mutation present in a small group of individuals). The two queen clones were genetically differentiated as all differentiation estimates were significant ($F_{ST} = 0.511$; $G''_{ST} = 0.829$; $Dest = 0.485$; all p-values $< .001$). Finally, four queens from Nepal had rare alleles, had distinct PCoA coordinates and could not be assigned to a particular clone.

In total, 138 different haploid genotypes were observed among the 256 androgenetic males analysed. A median-joining network of the genotypes revealed a star-like structure with lower-frequency genotypes at the tips of the branches (Figure 3d), consistent with clonal radiation (Weetman et al., 2002). Alternative genotypes differing by one or a few repeats were collapsed into 15 main MLLs

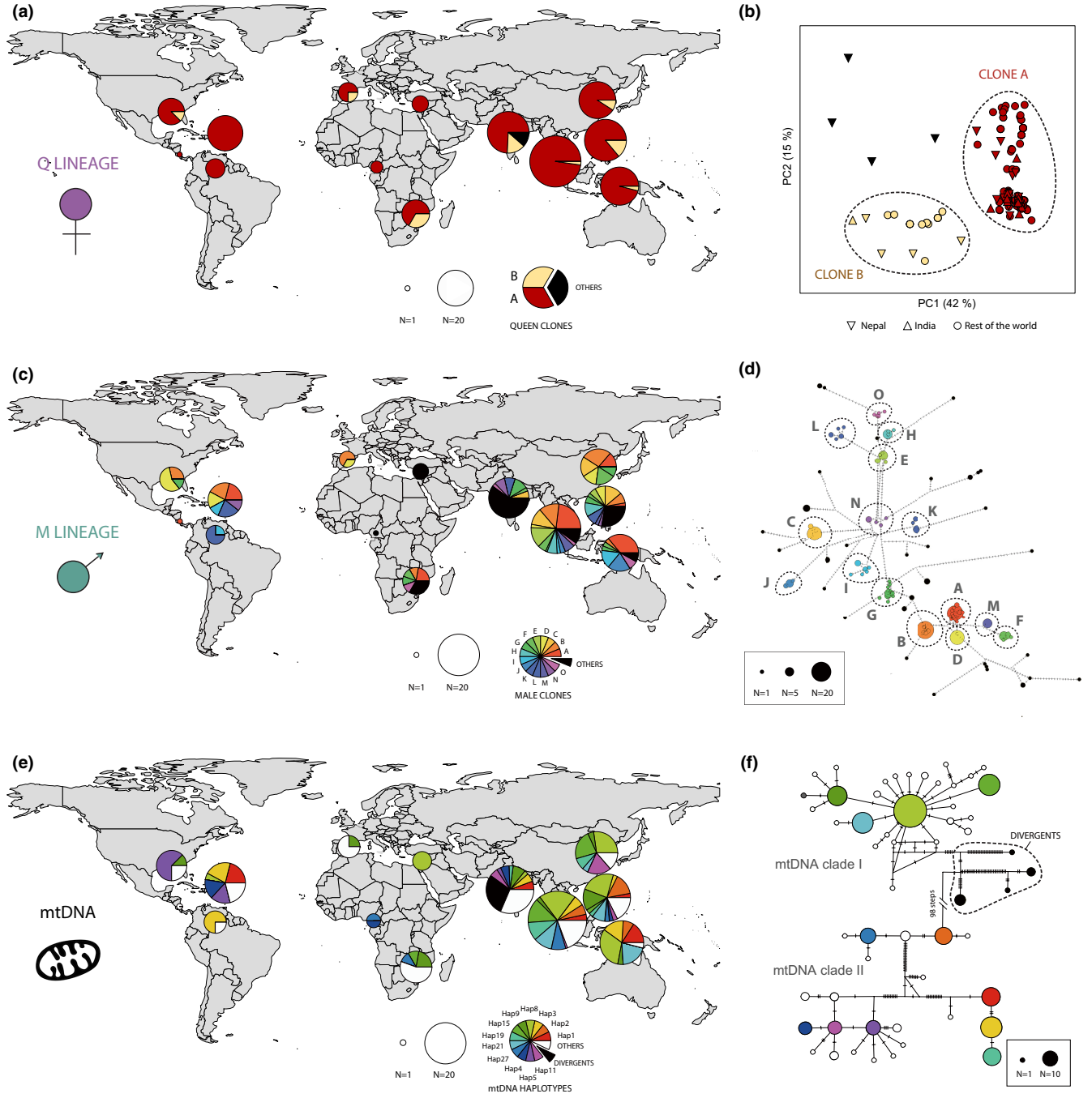

FIGURE 3 Phylogeography of *Paratrechina longicornis*: microsatellite variation of the Q lineage (a, b) and M lineage (c, d), and mitochondrial DNA variation (e, f). For each panel pair, colours correspond to divergent genetic groups or haplotypes that are commonly encountered. Rare genotypes/haplotypes are depicted in black. The size of pie charts on the maps are functions of the sample sizes (ln(*N*)+0.5) (a, c, e). (a, b) Diversity of the Q lineage based on 257 microsatellite genotypes of queens (256 localities). (a) Frequencies of the two main clonal lineages identified through principal coordinate analyses. (b) First two axes from a principal coordinate analysis based on microsatellite Bruvo's distance with the percentage of total variation explained by the axes. Genotypes from the putative native range (India and Nepal) are shown with different symbols to highlight their outlier positions. (c, d) Diversity of the M lineage based on 256 microsatellite genotypes of androgenetic males (253 localities). (c) Frequencies of the 15 main clonal lineages identified by POPPR across the 13 geographical regions surveyed. (d) Median-joining network of multilocus male haplotypes. Each haplotype is represented by a disc, and tick marks on the lines joining haplotypes represent the number of mutated positions. Disc sizes are proportional to sample sizes. Disc colours and letters correspond to the multilocus clonal lineages in A. (e, f) mtDNA variation based on 55 haplotype sequences of a mitochondrial region of 1726 bp containing the COI and COII genes (254 localities). (e) Frequencies of the 12 most common mtDNA haplotypes, as well as of the four "divergent" haplotypes observed in the phylogenetic analyses (see f). (f) Median-joining mtDNA haplotype network. Disc sizes are proportional to the number of sequences sampled for each haplotype. Single nucleotide substitutions are shown as ticks on the connecting lines between haplotypes. The haplotype network was divided into two divergent clades as discussed in Tseng, Wetterer, et al. (2019) (see also Figure S7)

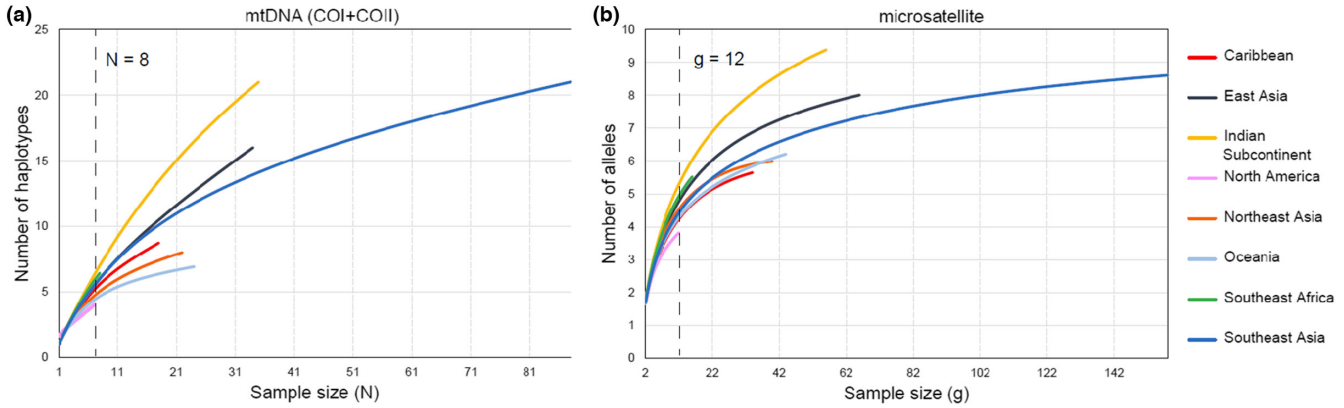

FIGURE 4 Rarefaction curves of (a) mtDNA haplotype richness (number of distinct mtDNA haplotypes) and (b) microsatellite allele richness (mean number of distinct alleles per locus) of *Paratrechina longicornis* workers for each geographical region. Sample sizes correspond to the number of mtDNA sequences sampled (*N*) and the number of microsatellite alleles sampled per locus (*g*; two per individual). South America, Arabia and Southern Europe were not included in these analyses due to small sample sizes ($N < 5$; $g < 10$). The vertical dashed lines indicate the lowest sample size in any of the remaining regions

accounting for 83% of the sampling. These MLLs appeared randomly distributed across the world (Figure 3c). Accordingly, only 10% of the androgenetic male genetic variation could be explained by differences among geographical regions (AMOVA based on Bruvo's distances, $p < .001$, 9999 permutations; 215 males from 11 regions).

In most invasive ant species, reproduction is assumed to occur among nestmates with dispersion primarily via colony budding (Passera, 1994). In line with this, sib-mating is common in laboratory colonies of *P. longicornis* (Pearcy et al., 2011). In addition, we noted that reproductives virtually never fly out of the nest under laboratory conditions. If no dispersion occurs among colonies, the queen and male genomes should evolve in parallel, leading to an association between queen clones, maternal mtDNA and male MLLs. No strong correlation between the queen mitochondrial and male nuclear variations was observed and no one-to-one association between the 15 most common males' MLLs and the 12 most common maternal mtDNA haplotypes was found. Yet, some male MLLs appeared to mate more frequently with one queen clone than the other (Table S3). For example, < 1% of the queens of Clone A (two out of 233) were mated with one of the four clones always associated with queens of Clone B ($N = 19$ queens). Similarly, 19% of male genetic variation could be explained by the difference among maternal mtDNA haplotypes (AMOVA based on Bruvo's distances, $p < .001$, 9999 permutations; 217 males carrying 22 haplotypes). Together, these results suggest that the queen and male genotypes are not randomly assorted: movements of reproductive individuals among colonies may erase patterns of parallel clonal evolution. Gene flow between colonies could result from the occasional fusion of genetically distinct colonies following multiple introduction events as seems to have occurred in one of our sampled localities where four different androgenetic clones were probably brought together (locality pIID04 in Indonesia). Alternatively, it is possible that reproductive individuals occasionally disperse by flight between colonies (Ito et al., 2016).

3.3 | Variation in mtDNA

We identified 55 mtDNA haplotypes. Of these, 12 were common haplotypes found in more than two geographical regions and appeared to be randomly distributed (Figure 3e,f; Figure S7). The two queen clones did not share any mtDNA haplotypes (46 haplotypes were observed in localities comprising queens of Clone A, while six haplotypes were observed in localities with queens of Clone B). Despite the haplotype segregation following the clone origin (Figure 3f), the patterns of queens' nuclear and mitochondrial variations were not congruent. For example, identical queen genotypes were regularly associated with mtDNA haplotypes from two divergent mtDNA clades. The average genetic distance between these two mtDNA clades was 5.7%, translating into 1.6–2.5 million years of divergence using standard mtDNA evolutionary rates of 2.3%–3.54% per million years (Papadopoulou et al., 2010). If queens' mitochondrial and nuclear genomes had linked histories, such large divergence times would have resulted in significant microsatellite genotypic differences given that microsatellites have a mutation rate of about 10^{-4} mutations per locus per generation (Crozier et al., 1999; Weber & Wong, 1993). The incongruence of nuclear and mtDNA variation suggests that the nuclear and mitochondrial genomes of queens might have undergone different evolutionary histories.

The androgenetic males had the same mtDNA haplotypes as queens and workers in each of the nine localities studied (Data S5), suggesting that the mtDNA is also maternally inherited also for these males.

3.4 | Inference of the native range

Rarefaction analyses indicated that the Indian subcontinent is a genetic diversity hotspot for *P. longicornis*. This region harboured the highest haplotype/allele richness for both mtDNA (Figure 4a)

and microsatellites (Figure 4b) of all regions surveyed. To explore further whether the Indian subcontinent could be the native range of the species, we evaluated whether ants there harbour more private alleles (i.e., alleles restricted to one geographical region), rare alleles (i.e., alleles that occur in frequencies <0.01) and divergent mitochondrial haplotypes than other regions of the world. All the divergent queen clonal genotypes were collected from the Himalayas, which is part of the Indian subcontinent (see Figure 3a,c; Table S4). Similarly, most of the 101 private alleles identified in worker samples (N = 252 individuals genotyped at 40 markers) originated from the Indian subcontinent (65% of the private alleles) even though this region accounted for only 14% of the sampling effort (Table 2). Furthermore, among the 22 workers with five or more rare microsatellite alleles, 18 (82%) were collected from the Indian subcontinent and the three workers carrying the highest number of rare alleles were found in Nepal and India (number of rare alleles = 27, 11 and nine, respectively; Table S5).

Analysis of mtDNA diversity showed that 73% of haplotypes were private to one geographical region (Figure S7). Southern Europe had the highest frequency of private haplotypes (75%; three of four sequences), followed by the Indian subcontinent (60%; 21 of 35 sequences) and Southeast Africa (56%; five of nine sequences; Table S6). However, the private mtDNA haplotypes from Southern Europe and Southeast Africa were all closely related to widespread haplotypes (divergence of 25,190–50,380 years using a conservative low divergence rate of 2.3% per million years; Papadopoulou et al., 2010), suggesting that they result from recent mutations of common haplotypes. By contrast, sequences with no close relationship to any widespread haplotypes were found in the Indian subcontinent. More specifically, four Himalayan haplotypes (Hap31, Hap37, Hap46 and Hap48) were unrelated to any other haplotypes sampled in our study (minimum divergence of 378,261–1,008,696 years; Figure 3f). Together, our results suggest that the Indian subcontinent and more precisely the Himalayas is a richness and rarity hotspot for *P. longicornis* and probably constitutes the native range of the species.

4 | DISCUSSION

Our analyses of genetic variation across the range of the invasive ant *Paratrechina longicornis* reveal that this cosmopolitan species probably originates from the Indian subcontinent, and that most, if not all, populations of this species display a double-clonal system with the presence of two divergent, yet interdependent queen and male clonal lineages. Double-clonality was initially reported in a single Thailand population of *P. longicornis* (Pearcy et al., 2011). This study extends this finding and shows that this genetic system is widespread across the species range. We found direct evidence for double-clonality in 22 localities. At these localities, queens appear to be typically produced by thelytokous parthenogenesis and inherit maternal DNA only, while most males are androgenetic cytonuclear hybrids carrying divergent paternal nuclear DNA and maternal mtDNA. Although queen and male samples were only obtained in 22 populations, the highly heterozygous genotypes of workers in

TABLE 2 Distribution of private microsatellite alleles across the range of longhorn crazy ant

Region	Workers	N_A	N_{PA}	Mean PA (SE)	Significance groups				
Arabia	3	82	1	0.225 (0.061)	A	B			
Caribbean	19	230	2	0.135 (0.023)	A		C		
Central America	1	78	0	NA	–	–	–	–	–
East Asia	36	325	16	0.262 (0.028)				D	
Indian subcontinent	35	396	66	0.504 (0.046)					E
North America	7	160	0	0.078 (0.015)	A	B			
Northeast Asia	22	218	0	0.105 (0.019)	A	B			
Oceania	25	255	1	0.207 (0.031)			C	D	
South America	4	117	1	0.244 (0.049)	A	B	C	D	
Southeast Africa	9	230	3	0.246 (0.03)				D	
Southeast Asia	87	349	9	0.19 (0.025)			C	D	
Southern Europe	3	115	2	0.144 (0.05)		B			
West Africa	1	71	0	NA	–	–	–	–	–

Note: The number of worker genotypes, number of alleles (N_A), total number of private alleles (N_{PA}), and mean number of private alleles per locus (Mean PA) across the 40 microsatellite loci surveyed are indicated for each geographical region. The mean number of private alleles per locus was calculated using the rarefaction approach with a standardized sample size of four. Differences in the mean number of private alleles among regions were analysed using Kruskal–Wallis tests followed by post hoc Wilcoxon signed-rank tests with a false discovery rate (FDR) correction. Regions that differ significantly ($p < .05$) in their mean private allele richness are indicated by different sets of letters.

all 256 localities surveyed indicate that their parents always belong to divergent lineages as expected for double-clonality. Our analyses therefore suggest that invasive populations of *P. longicornis* are probably all double-clonal.

Remarkably, our phylogeographic analyses revealed that all clonal queens and males of *P. longicornis* in the invasive range belong to two respective, nonrecombining Q and M lineages that have remained divergent across time and space. This result was supported by the segregation of queen and male samples from different continents in two divergent groups (22 localities) and the finding that workers always carry F_1 hybrid combinations of these two parental groups (256 localities). Interestingly, the M lineage appears far more variable than the Q lineage. This could be partially explained by different mutation rates among the two *P. longicornis* lineages that result from differences in ploidy: the Q lineage is carried by diploid individuals which undergo a modified meiosis, while the M lineage is only found in the haploid stage and never goes through meiosis (Sharp et al., 2018). Alternatively, selection may be much stronger in queens than males, leading to stronger clonal sweeps in the former. Ant males are short-lived, and their only task is to mate, which is expected to limit the opportunity for selection (Feldhaar et al., 2008).

In contrast to *P. longicornis*, recombination among queen and male clones occurs regularly in populations of the ant *Wasmannia auropunctata*. In this latter species, gene flow among the queen and male gene pools results from the occasional production of functional hybrid queens by sexual reproduction (Foucaud et al., 2007). It is possible that such sexual reproduction events also exist in native populations of *P. longicornis*, but were not captured in our dataset. Our sampling in the Himalayas suggests that native local genotypes are being outnumbered by dominant, invasive genotypes, which makes them increasingly more difficult to collect.

The complete segregation of *P. longicornis* parental genomes in the two lineages is reminiscent of social hybridogenesis, another genetic system which has been reported in the ant genera *Pogonomyrmex*, *Solenopsis*, *Messor* and *Cataglyphis*. Under this system, two lineages consisting of both queens and males coexist across populations and interbreed regularly for the production of workers (Lacy et al., 2019; Leniaud et al., 2012; Norman et al., 2016; Romiguier et al., 2017; Sirviö et al., 2011). Social hybridogenesis has been shown to be maintained by genetic caste determination systems, whereby the fate of a female egg is determined by its genotype, rather than by environmental factors as is usually the case in social hymenopterans (Cahan et al., 2004; Darras et al., 2014; Schwander et al., 2010). A similar mechanism probably underlies the maintenance of double-clonality in *P. longicornis*.

One unexpected finding of our study was the frequent occurrence of nonclonal, arrhenotokous males developing from unfertilized eggs. Arrhenotokous males were collected in five localities and were also regularly produced in laboratory colonies. The presence of live sperm in seminal vesicles of arrhenotokous males suggested that these arrhenotokous males could produce sperm, but we did not find evidence supporting mating between arrhenotokous males and queens in the laboratory. Although these males appear to have low fitness, we argue that occasional reproduction by arrhenotokous males may have played a key role in the spread and maintenance of the double-clonal system. The first evidence for a role of arrhenotokous males derives from the cytonuclear incongruences observed in queens, which suggests that their nuclear genome has introgressed into diverse mitochondrial backgrounds. The most parsimonious candidate mechanism for such introgression is reproduction by arrhenotokous males. Following the emergence of clonality, clonality-inducing mutation(s) may have spread contagiously to neighbouring populations through arrhenotokous males carrying the mutation(s) (Simon et al., 2003). Second, that fertilization by the Q lineage sperm of an arrhenotokous male could potentially lead to queen development is also of high evolutionary significance for maintenance of the system. As mentioned above, the perfect association between the genotype of females and their caste indeed suggests the existence of a hardwired genetic caste determination system, whereby diploid eggs carrying Q genotypes only develop into new queens. Fertilization of a Q lineage egg by the Q lineage sperm of an arrhenotokous male should therefore lead to queen development. Such sexual production of new queens, albeit rare, may allow the purging of deleterious mutations and the generation of new allelic combinations through recombination. Such occasional recombination events have been reported in other clonal ants such as *Mycocepurus smithii* (Rabeling et al., 2011), *Oocerea biroi* (Trible et al., 2020) or desert ants of the genus *Cataglyphis* (Darras et al., 2014; Doums et al., 2013).

The patterns of both nuclear and mitochondrial variations placed the genetic diversity hotspot of *P. longicornis* in the Indian subcontinent. This result, together with the fact that rare and divergent mitochondrial haplotypes/clonal lineages were only identified in the Indian subcontinent, and that workers with the highest number of rare microsatellite alleles were found in this region, suggests that it is the native range of the species. Previous authors listed *P. longicornis* as originating either in tropical Africa or Asia (LaPolla & Fisher, 2014; Wetterer, 2008). Although our sampling was limited both in number and in geographical range for the African continent, two additional lines of evidence suggest an Asian origin. First, relatively few records of *P. longicornis* and virtually no record of its associated symbionts are found in continental Africa (Wetterer, 2008). Second, the distinctive genetic pattern found in the Indian subcontinent renders the African origin hypothesis improbable: considering the high propagule pressure of the species, rare/divergent alleles of African origin would be expected to be distributed over the entire invasive range rather than concentrated in the Indian subcontinent. Surprisingly, the three other species of the genus *Paratrechina* (*P. ankarana*, *P. antsingy* and *P. zanjensis*) are all endemic to Africa (Boudinot et al., 2021; LaPolla & Fisher, 2014). The incongruence between the inferred South Asian origin of *P. longicornis* and the African distribution of its sister species may stem from an intercontinental dispersal event which resulted in a disjunctive Afro-Asian distribution of *Paratrechina* species, similar to other ant genera such as *Bothroponera* and *Parasyscia* (Borowiec, 2016; Joma & Mackay, 2020).

The global spread of ant species has been greatly facilitated by major events along recent human history, especially during the last two waves of globalization (1850–1914 and 1960–today) (Bertelsmeier et al., 2017). The distribution of *P. longicornis* rapidly expanding after 1860 coincides with the first wave of globalization (Bertelsmeier et al., 2017). In the late 19th century, the domestic and foreign trade of India grew exponentially (Kuehlwein, 2021), and by 1900, India had become the largest exporter in Asia and ranked the ninth largest in the world (Hanson, 1980; Kuehlwein, 2021). Such India-outbound commerce activities may have fuelled the early transcontinent spread of *P. longicornis* (i.e., from its native range to regions across the world). Furthermore, our findings of multiple, randomly distributed clonal sublineages and mtDNA haplotypes within each region of the species' invasive range suggest that multiple introduction events involving genetically distinct propagules are common. Border inspection records worldwide are consistent with our interpretation as *P. longicornis* is one of the most frequently intercepted species (Bertelsmeier et al., 2018; Lee et al., 2020; Ward et al., 2006).

Our findings support the idea that reproductive characteristics are a key determinant for the success of invasive species. More importantly, our study suggests that double-clonal reproduction occurs in the inferred native range of *P. longicornis*, and may have pre-adapted this species to be a successful invader. Indeed, double-clonality functionally eliminates the risk of inbreeding depression in the sexually produced workers, which constitute the majority of the colony and face adverse environments, while queens and males remain within the safety of the nest. As queens and males worldwide belong to two nonrecombining lineages, mating always produces outbred first-generation hybrid workers even when it takes place between siblings (Pearcy et al., 2011; Trager, 1984). This mechanism, potentially circumventing the consequences of genetic bottlenecks and depletion which usually hampers the viability of founding populations during biological invasion, may well explain the worldwide establishment success of *P. longicornis*. These results are a steppingstone towards understanding the evolutionary advantages of double-clonal reproduction in social Hymenoptera, and raise the necessity of additional studies on invasive ants to gain insight into the patterns and processes of adaptation to new environments and transition in reproductive modes.

AUTHOR CONTRIBUTIONS

C.C.S.Y., S.-P.T. and H.D. originally conceived the ideas. J.W., L.K. and C.-Y.L. helped develop an overarching research programme and collect the samples. C.C.S.Y., L.K. and T.Y. applied for the grant. S.-P.T., H.D. and P.-W.H. performed the experiments. S.-P.T. and H.D. carried out the analyses and drafted the manuscript. All authors contributed substantially to editing the manuscript and approved the submitted version.

ACKNOWLEDGEMENTS

Morgan Pearcy performed preliminary phylogeographical analyses. We thank Amy Low, Ni-Chen Chang, Chung-Wei You, Chùn-Têng Coody Chiu, Chun-Yi Lin, Ching-Chen Lee, En-Cheng Yang, Han-Chih Ho, Hui-Siang Tee, Hung-Wei Hsu, Jia-Wei Tay, Kean Teik Koay, Li Yan Gan, Mark Ooi, Nellie Wong, Peter G. Hawkes, Ping-Chih Lin, Singham Veera, Su-Chart Lee, Sylvain Hugel, Yi-Ming Weng, Yueh-Hua Wu, Yu-Fang Tseng, Andy Suarez, Jennifer Lii, Shih-Chia Yeh, Chih-Chi Lee and Zhengwei Jong for assistance in field collection and Qiaowei M. Pan for comments on the manuscript. This research was funded by the Future Development Funding Program of the Kyoto University Research Coordination Alliance and the Virginia Tech Faculty Start-up Research Fund. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The new mtDNA haplotype sequences identified in this study have been deposited in GenBank (MZ398110–MZ398127). The data associated with this paper have been archived in the Dryad Digital Repository (https://doi.org/10.5061/dryad.gmsbcc2rq).

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How to cite this article: Tseng, S.-P., Darras, H., Hsu, P.-W., Yoshimura, T., Lee, C.-Y., Wetterer, J. K., Keller, L., & Yang, C.-C. (2023). Genetic analysis reveals the putative native range and widespread double-clonal reproduction in the invasive longhorn crazy ant. *Molecular Ecology*, *32*, 1020–1033. https://doi.org/10.1111/mec.16827