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Research Article

Invasion of the little fire ant *Wasmannia auropunctata* (Roger, 1863) (Hymenoptera: Formicidae) in Taiwan

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Abstract

The little fire ant, *Wasmannia auropunctata* is an invasive species native to Central and South America. We report the establishment of this species in Taiwan, which is also the first confirmed population in the East Asian region. Little fire ants were found at two locations in central Taiwan, separated by approximately 30 km. Genetic and behavioral analyses indicated that the two populations both employ clonal reproduction and showed no aggression towards each other. Results of population genetic analyses indicate that the two populations are most likely derived from a common source population or genetically similar populations.

Key words: mitochondrial DNA, microsatellite, new record, clonal reproduction, unicoloniality

Introduction

Wasmannia auropunctata (Roger, 1863), also known as little fire ant, is a species native to Central and South America. Worldwide spread of this invasive ant is believed to be associated with human-mediated jump dispersal via transportation of nursery stock, plant parts, soil, food packaging materials, agricultural products, timber products, etc. (Lubin 1984; Roque-Albelo and Causton 1999; Wetterer and Porter 2003). Currently, the invasive population of this ant has been confirmed in Australia, West Africa, Galapagos Island, U.S. Mainland (e.g., California, Florida), Middle East, as well as many Caribbean and Pacific islands (Wetterer and Porter 2003; Vonshak et al. 2010).

The little fire ant is a generalist yet preferentially feeds on invertebrates and honeydew secreted by phloem-feeding hemipterans (Clark et al. 1982; Ulloa-Chacon and Cherix 1990; Delabie et al. 1994; Naumann 1994). The predaceous nature of *W. auropunctata*, coupled with the possession of a venomous sting that can be used for subduing prey (Holway et al. 2002), has led to significant reductions of native arthropod fauna in its introduced ranges (Clark et al. 1982; Lubin 1984; Jourdan 1997; Vonshak et al. 2010). By virtue of numerical advantage, this species also outcompetes and displaces native ant species through the combined effects of physical aggression and exploitative competition (Le Breton et al. 2003; Walker 2006). Furthermore, there have been numerous cases in which the *W. auropunctata* invasions are linked to incidences of blindness in both domestic and native mammals (Wetterer 1997; Wetterer et al. 1999).

Unlike most ant species, *W. auropunctata* possesses a unique reproductive system: while a part of its populations displays a haplodiploid reproductive system that is typical in hymenopterans, in most introduced populations queens and males are produced clonally and workers are produced sexually (Fournier et al. 2005a; Foucaud et al. 2007). This system makes offspring queens and males genetically identical to their mother and father, respectively, resulting in two separate gene pools almost without gene flow. The presence of two genetically divergent gene pools has been considered to be advantageous as it potentially facilitates a species to overcome genetic bottlenecks during the invasion process (Pearcy et al. 2011). *Wasmannia auropunctata* also shares traits in common with other global invasive ants. For instance, the expansion of *W. auropunctata* is primary through colony budding, where a mated queen accompanied by a group of workers establishes a new colony in close proximity to her natal colony (Hölldobler and Wilson 1977, 1990). This dispersal mode, despite relatively short distance, ensures the survival and successful establishment of a newly founded colony. Furthermore, *W. auropunctata* is unicolonial, forming an extensive supercolony where intraspecific aggression is nearly absent among physically separate nests (Le Breton et al. 2004).

The presence of *W. auropunctata* in Taiwan was first reported by a citizen myrmecologist who photographed an alate queen at Heping District, Taichung City in 2019 and uploaded it to a major social media platform. The photo was later made available to one of the authors (YCH) who confirmed the identity of this alate as *W. auropunctata*. Later in June 2021, another citizen myrmecologist photographed and reported both female alates and a few active ant nests in a secondary forest in Wuri District, Taichung City (approximately 30 km from the first location), further indicating the likelihood of the ant's establishment in Taiwan. As part of the early detection and rapid response (EDRR) action, we conducted a preliminary survey to determine the current distribution of *W. auropunctata* in Taiwan with an emphasis on the areas where the ant

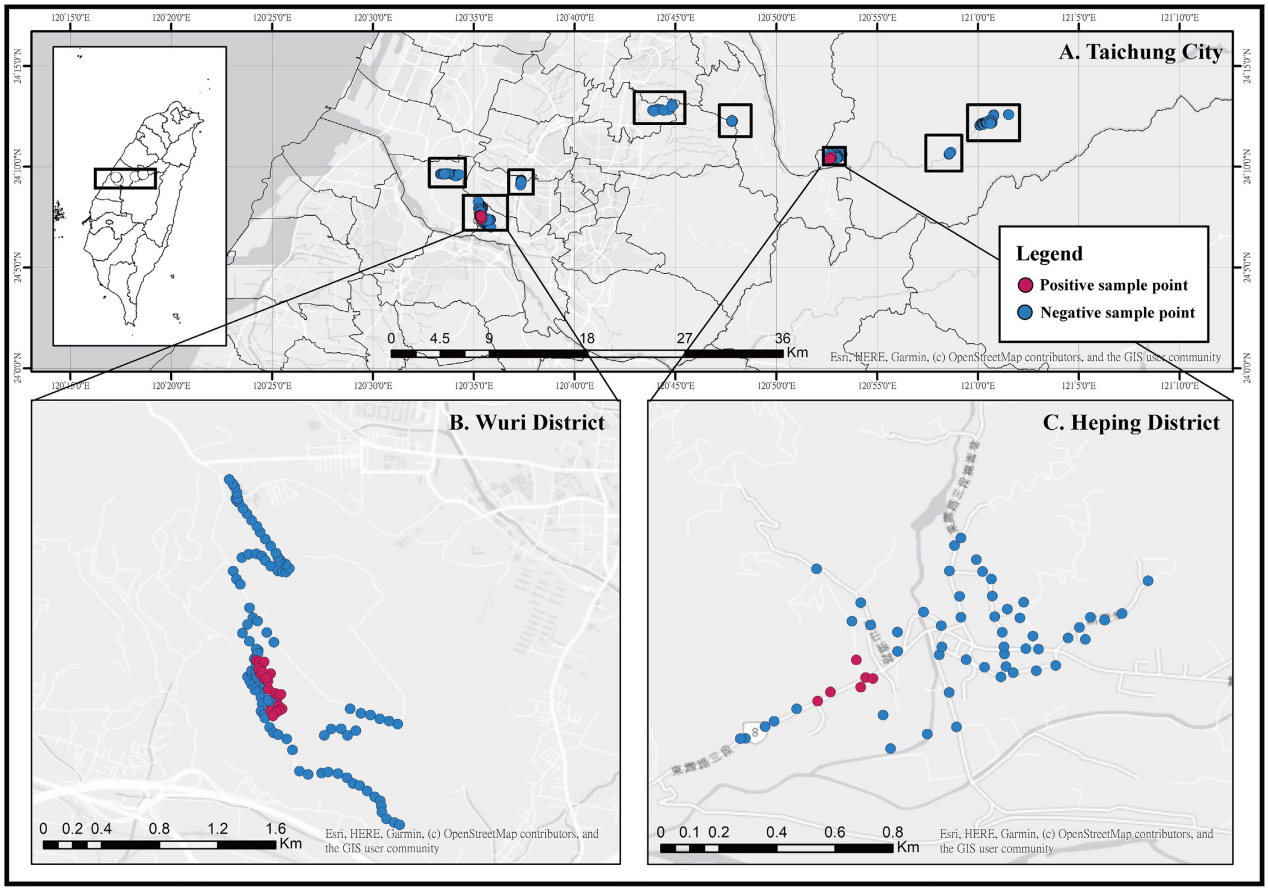

Figure 1. (A) Geographical locations surveyed for the infestation of the little fire ant, *Wasmannia auropunctata*. Two locations, Wuri (B) and Heping (C), were confirmed with established little fire ant colonies. Blue circles are lure stations without *W. auropunctata* and red circles are lure stations where *W. auropunctata* was present.

was initially reported. We also examined the social organization of this ant through intraspecific aggression assays. Genetic analyses were performed to assess the genetic structure of the species in Taiwan to infer its invasion history and reproduction mode. Given the high invasiveness and potential threats this species may pose to the native fauna in Taiwan, this study represents a critical first step to establish baseline information to facilitate the planning of an EDRR program against this invasive ant.

Materials and methods

Study sites and surveying methods

Little fire ant surveillance was carried out in eight locations (Figure 1, Supplementary material Table S1) from June to November in 2021. The eight locations, including two in which *W. auropunctata* alates were previously photographed, were selected based on the public reporting deriving from a search for little fire ant initiative advertised on the same social media platform. Active surveillance was conducted in areas of approximately 500–3,000 m in diameter. Tree trunks, dead woods, stones, and artificial disposals in the target locations were manually examined for the presence of *W. auropunctata*. Potato chips (Original flavor, Pringles,

USA) were used as lures in all surveyed locations to detect *W. auropunctata*, being placed every 10–50 m alongside the roads for at least 30 mins prior to assessment. Ants found on the lures were collected and identified to species level. Specimen was photographed using Leica M205C stereomicroscope (Wetzlar, Germany) mounted with a Nikon D500 camera (Tokyo, Japan), and were over-laid by the software Helicon Focus 6.7.

Genetic Analyses

Wasmannia auropunctata foragers or colony fragments, if found during the surveillance, were transferred to the lab for both behavioral assays and genetic analysis. In addition, one worker specimen from the Solomon Islands intercepted at the Taiwanese border (Lee et al. 2020) was also included. Genomic DNA of *W. auropunctata* was individually extracted using Gentra Puregene Tissue Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Two mitochondrial gene regions, including cytochrome *c* oxidase subunit I (*COI*, 658 base-pair) and *COI-tRNA-COII* (433 base-pair), were sequenced. Only a single queen in each of the two locations was sequenced. Polymerase chain reactions (PCR) were conducted with primer sets and respective thermal cycling conditions described in Folmer et al. (1994) and Mikheyev and Mueller (2007). Sequences were compared against the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST). All *W. auropunctata* sequences from NCBI and Barcode of Life Data Systems (BOLD) were later retrieved, sorted, and analyzed using R. 4.0 (R Core Team 2020) and MEGA 7 (Kumar et al. 2016). The sequences were aligned using MUSCLE with default settings, and the pairwise distances were calculated using the Kimura-2-parameter model with 95% partial deletion to search for the identical sequences. For microsatellite analysis, a total of 23 individuals including nine queens and 14 workers from a nest from each of the locations (Wuri: four queens and six workers; Heping: five queens and eight workers), as well as the border-intercepted worker specimen from the Solomon Islands were genotyped at eight previously published microsatellite loci. Multiplex PCR reactions were carried out using primer sets and thermal cycling conditions described in Fournier et al. (2005b) and Blacket et al. (2012). Microsatellite genotypes were utilized to infer the reproductive system and putative origin of *W. auropunctata* in Taiwan.

Intraspecific aggression assay

To determine the social organization (i.e., unicoloniality) of *W. auropunctata* in Taiwan, a total of three nest fragments (group of workers, brood and/or queens within rotten wood or tree bark) were collected from each of the two locations. Within each location, the collected nest was spaced at least 50 m (up to 355 m; mean ± SD: 193.7 ± 90.0 m) apart from each another.

The distance between the two locations was approximately 30 km (Figure 1). Both intra-location (i.e., between nests within the same location) and inter-location (i.e., between nests from different locations) aggression assays were carried out, resulting into six intra-location nest pairs and nine inter-location nest pairs. An individual worker from each of two different nests was gently introduced to a fluon-coated screw cap of a 15 × 45 mm 1 dram Shell Vial (inner diameter = 1.2 cm; DWK Life Sciences, Rockwood, United States) in which their interactions were observed. The highest level of aggression shown by the two workers during the first 5 minutes was recorded under a stereo microscope (Leica M205, Wetzlar, Germany). Interactions were scored according to the following aggression index (slightly modified from Suarez et al. 1999): 1 = physical contact and antennation without aggressive behavior; 2 = prolonged antennation; 3 = biting or pulling legs and antennae; 4 = prolonged aggression, often involving the use of sting. The aggression test was repeated 10 times for each nest pair, but each worker was used only once. The aggression index was calculated based on the mean value of the 10 trials. If the calculated aggression index was below 2.5, the respective two nests were considered as belonging to the same supercolony.

Statistical analysis

Given that the data were not normally distributed, aggression levels between different nest pairs were compared using Kruskal-Wallis tests followed by Dunn's post hoc tests. Statistical analysis was performed using SPSS version 16.0 (SPSS, Chicago, IL, USA).

Results

Distribution and nesting habitat of Wasmannia auropunctata *in Taiwan*

Wasmannia auropunctata was detected in two of the eight locations reported by the public, namely Wuri and Heping (Figures 1–3). The two locations were approximately 30 km away from each other and separated by highly urbanized areas. The landscape and vegetation are similar between the two locations (low-elevation, semi-disturbed montane with artificial planation). In both locations, *W. auropunctata* colonies can be found nesting in various microhabitats including pre-existing cavities under/within dead woods and logs, stones, leaf litters, or sometimes underneath living tree bark or hollow structure. *Wasmannia auropunctata* foragers were detected in 29.5% (43/146) and 10.2% (6/59) of the lure stations in Wuri and Heping, respectively, and were clearly localized with the maximum linear distance between positive stations being 190 m and 610 m respectively in the two locations. Multiple dealate queens and winged castes of both sexes were observed in the colonies collected from both locations (Figure 3E).

Figure 2. Macro- and microhabitats where *Wasmannia auropunctata* was discovered in Taiwan. (A) A semi-disturbed secondary forest in Wuri, Taichung. (B) A fruit tree orchard in Heping, Taichung. (C–G) *Wasmannia auropunctata* can be found nesting in various microhabitats, such as leaf litters (C–D), dead woods (E), and tree bark (F–G). Photos by Ching-Chen Lee and Yu-Chun Hsiao.

Genetic analysis

Queens from the two locations (i.e., Wuri and Heping) shared an identical sequence at the two mitochondrial gene regions (*COI*: ON222744–ON222745, and *COI-tRNA-COII*: ON237734–ON237735, 1,091 bp in total). The species was confirmed to be *W. auropunctata* based on the BLAST output. For *COI*, the haplotype was identical to 30 of the 322 sequences retrieved from the

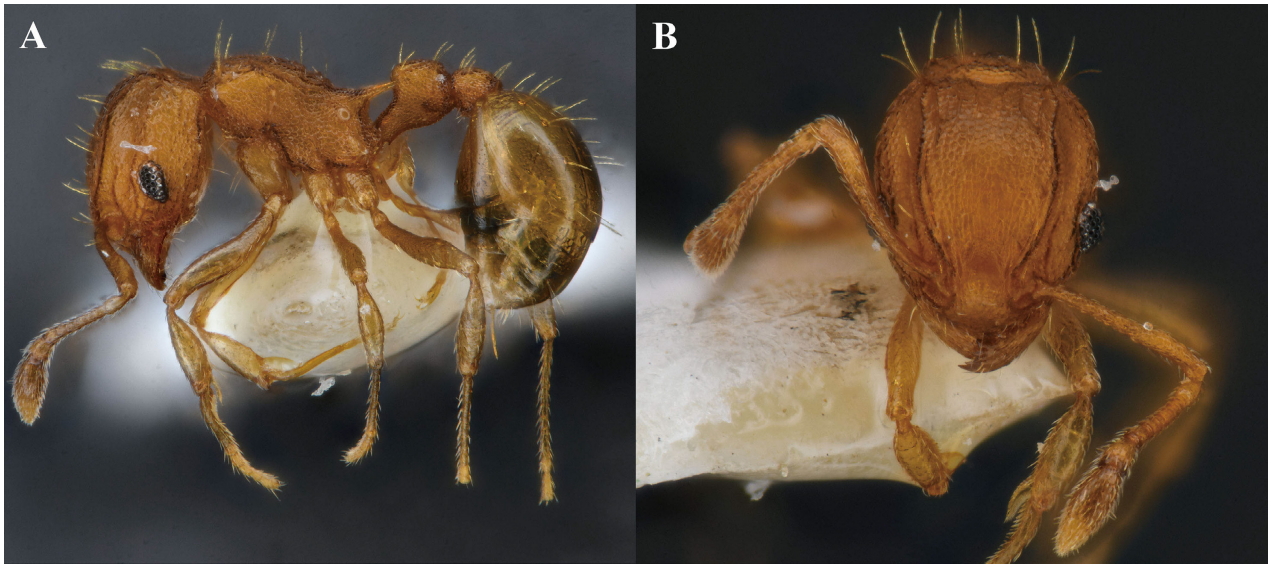

Figure 3. *Wasmannia auropunctata* worker collected in Wuri, Taichung, Taiwan, in lateral view (A) and frontal view (B). Photos by Ai-Shan Lu.

NCBI and BOLD, which can be consistently traced to New World populations including French Guiana and Dominica (Table 1). For *COI-tRNA-COII*, 14 of the 64 sequences retrieved from the NCBI were found to be identical to the haplotype in this study. The 14 sequences were derived from individuals in both species' native (Columbia and Panama) and introduced ranges (Cuba, Dominica and Guadeloupe [France], Ecuador, Florida and Hawaii [USA], Galapagos Islands, and Solomon Islands) (Table 1). However, the attempt to amplify sequences of the two mitochondrial regions from the intercepted worker specimen originating from the Solomon Islands was unsuccessful, which was most likely due to the sample being aged. Microsatellite genotype data indicated clonal reproduction of queens from both locations in Taiwan (Table 2). Within each location, queens shared identical genotypes across all loci except one (*Waur-566*). When comparing the genotypes of ants between the two locations, allelic variations were found at two loci (*Waur-566* and *Waur-2164*), with one of which (*Waur-2164*) being consistently different by one allele (318/322 vs 318/324, Table 2). These data suggest these queens were likely produced clonally and might belong to two or more independent lineages.

A total of five multilocus haplotypes were found from nine queens across the eight loci. All workers, on the other hand, were shown to be produced sexually as would be expected if the species was undergoing double clonal reproduction. As workers always carried one allele identical to that of the clonal queens from the same locality in each of the locus, identification of paternal allele became possible (marked as boldface in Table 2). Paternal genotypes of the two localities, as determined from worker genomes, were consistent with a single clonal patriline except one that carried a different allele (154) at *Waur-275* (Table 2), which could otherwise be explained by the occurrence of a single-step mutation from the original clone patriline. The single intercepted worker specimen from

Table 1. Detailed information of the mitochondrial sequences that are identical to those recovered from *Wasmannia auropunctata* in Taiwan. Sequences obtained from introduced populations are indicated by * (see Janicki et al. 2016).

Cytochrome *c* oxidase subunit I (*COI*)			
Accession number & Sequence identifier	Sample location	Reference	Note
EF459759.1_ADJJ_01_(CBGP) to EF459782.1_ADJJ_24_(CBGP)	French Guiana	Foucaud et al. 2007	Location A, K, P, or RN in reference
KR106375.1_WAS131_01_W1_Sulph	*Sulphur Springs, Dominica	Chifflet et al. 2016	Haplotype 31 in reference
KR106382.1_P2-1-Q1	Sinnamary, French Guiana	Chifflet et al. 2016	Haplotype 31 in reference
KR106383.1_P2-2-Q1	Sinnamary, French Guiana	Chifflet et al. 2016	Haplotype 31 in reference
KR106384.1_P3-W7	Sinnamary, French Guiana	Chifflet et al. 2016	Haplotype 31 in reference
KR106385.1_Pi41-Q2	Sinnamary, French Guiana	Chifflet et al. 2016	Haplotype 31 in reference
KX146469_GBMNA9989-19	Not provided	Duan et al. 2016	Uploaded by Shaanxi Normal University, China
Cytochrome *c* oxidase subunit I - tRNA-Leu gene - Cytochrome *c* oxidase subunit II (*COI-tRNA-COII*)			
Accession number	Sample location	Reference	Note
EF409392.1	*Guadeloupe, France	Mikheyev and Mueller 2007	
EF409394.1	*Dominica, France	Mikheyev and Mueller 2007	
EF409395.1	Panama	Mikheyev and Mueller 2007	
EF409396.1	*Solomon Islands	Mikheyev and Mueller 2007	
EF409397.1	*Florida, USA	Mikheyev and Mueller 2007	
EF409398.1	*Galapagos, Ecuador	Mikheyev and Mueller 2007	
EF409399.1	Colombia	Mikheyev and Mueller 2007	
EF409400.1	*Cuba	Mikheyev and Mueller 2007	
EF409402.1	*Hawaii, USA	Mikheyev and Mueller 2007	
EF409403.1	*Florida, USA	Mikheyev and Mueller 2007	
EF409404.1	*Hawaii, USA	Mikheyev and Mueller 2007	
KY433397.1	Cali,Colombia	Silva et al. 2018	
KY433402.1	Caloto, Colombia	Silva et al. 2018	
KY433416.1	Cali,Colombia	Silva et al. 2018	

the Solomon Islands showed a relatively high level of homozygosity, which was different to those workers in Taiwan despite having shared alleles at some loci.

Behavioral assay

No significant difference in the mean aggression index was observed among the three types of nest pairs (Kruskal-Wallis test: $H = 1.410$, df = 2, $P = 0.494$). The mean aggression index ranged from 1.29 ± 0.09 to 1.42 ± 0.06 (Table 3). *Wasmannia auropunctata* workers from all locations in Taiwan behaved amicably towards each other, never displaying level 3 aggression or above in all nest pair interactions.

Discussion

Our study confirms the establishment of *W. auropunctata* in Taiwan, which is also the first confirmed population of *W. auropunctata* in East Asia (Janicki et al. 2016). Our finding raises an immediate concern that other parts of this region (e.g., East Asia) may be at high risk of invasions by *W. auropunctata*, especially given the ant's hitchhiker nature, high adaptability and clonal reproductive system (Mikheyev et al. 2009). Indeed, previous studies have shown that the clonal reproduction mode is prevalent in most introduced populations of *W. auropunctata*, suggesting that clonality may have facilitated invasiveness/ecological dominance in this

Table 2. Genotypes of *Wasmannia auropunctata* at eight microsatellite loci. Data are based on nine dealate queens and 14 workers from a nest from each of the two locations in Taiwan, as well as an additional border-intercepted worker originating from the Solomon Islands. Paternal alleles inferred from the queen-worker data are in bold.

	Colony/Sample origin	Caste	Waur-1gam		Waur-3176		Waur-2164		Waur-566		Waur-1166		Waur-225		Waur-275		Waur-680	
1	Solomon Islands (intercepted)	Worker	300	300	235	235	322	330	284	300	100	108	238	238	154	154	185	185
2	Wuri, Taichung, Taiwan	Queen	300	306	235	235	318	322	298	300	100	112	234	236	128	142	191	191
3	Wuri, Taichung, Taiwan	Queen	300	306	235	235	318	322	296	300	100	112	234	236	128	142	191	191
4	Wuri, Taichung, Taiwan	Queen	300	306	235	235	318	322	298	300	100	112	234	236	128	142	191	191
5	Wuri, Taichung, Taiwan	Queen	300	306	235	235	318	322	298	300	100	112	234	236	128	142	191	191
6	Wuri, Taichung, Taiwan	Worker	300	**318**	235	**243**	318	**330**	**284**	300	**108**	112	236	**238**	128	**154**	**185**	191
7	Wuri, Taichung, Taiwan	Worker	300	**318**	235	**243**	318	**330**	**284**	298	**108**	112	236	**238**	128	**152**	**185**	191
8	Wuri, Taichung, Taiwan	Worker	300	**318**	235	**243**	322	**330**	**284**	298	**108**	112	234	**238**	142	**152**	**185**	191
9	Wuri, Taichung, Taiwan	Worker	306	**318**	235	**243**	318	**330**	**284**	300	100	**108**	236	**238**	128	**152**	**185**	191
10	Wuri, Taichung, Taiwan	Worker	300	**318**	235	**243**	322	**330**	**284**	300	**108**	112	236	**238**	128	**152**	**185**	191
11	Wuri, Taichung, Taiwan	Worker	300	**318**	235	**243**	318	**330**	**284**	300	100	**108**	236	**238**	128	**152**	**185**	191
12	Heping, Taichung, Taiwan	Queen	300	306	235	235	318	324	300	300	100	112	234	236	128	142	191	191
13	Heping, Taichung, Taiwan	Queen	300	306	235	235	318	324	298	300	100	112	234	236	128	142	191	191
14	Heping, Taichung, Taiwan	Queen	300	306	235	235	318	324	298	300	100	112	234	236	128	142	191	191
15	Heping, Taichung, Taiwan	Queen	300	306	235	235	318	324	300	300	100	112	234	236	128	142	191	191
16	Heping, Taichung, Taiwan	Queen	300	306	235	235	318	324	298	298	100	112	234	236	128	142	191	191
17	Heping, Taichung, Taiwan	Worker	300	**318**	235	**243**	324	**330**	**284**	298	**108**	112	236	**238**	128	**154**	**185**	191
18	Heping, Taichung, Taiwan	Worker	306	**318**	235	**243**	324	**330**	**284**	298	100	**108**	234	**238**	142	**154**	**185**	191
19	Heping, Taichung, Taiwan	Worker	300	**318**	235	**243**	318	**330**	**284**	298	100	**108**	234	**238**	142	**154**	**185**	191
20	Heping, Taichung, Taiwan	Worker	306	**318**	235	**243**	318	**330**	**284**	298	100	**108**	234	**238**	142	**154**	**185**	191
21	Heping, Taichung, Taiwan	Worker	306	**318**	235	**243**	318	**330**	**284**	300	**108**	112	234	**238**	142	**154**	**185**	191
22	Heping, Taichung, Taiwan	Worker	300	**318**	235	**243**	318	**330**	**284**	298	**108**	112	234	**238**	142	**154**	**185**	191
23	Heping, Taichung, Taiwan	Worker	306	**318**	235	**243**	324	**330**	**284**	300	100	**108**	236	**238**	128	**154**	**185**	191
24	Heping, Taichung, Taiwan	Worker	300	**318**	235	**243**	324	**330**	**284**	298	100	**108**	234	**238**	142	**154**	**185**	191

Table 3. Mean aggression index for interactions between two *Wasmannia auropunctata* workers from different nests at the same site or from different sites.

Nest combination	Aggression index (Mean ± SE)
Within site	
Wuri – Wuri	1.29 ± 0.09
Heping – Heping	1.33 ± 0.10
Between site	
Wuri – Heping	1.42 ± 0.06

ant. Our study is consistent to this hypothesis as our microsatellite analysis indicates that *W. auropunctata* in Taiwan also employs clonal reproduction.

Intriguingly, *W. auropunctata* has only been documented once in approximately 500 ant interception records at the Taiwanese borders since 2011. This particular intercepted sample was found in the goods that originated from the Solomon Islands, which is within the species' exotic range (Lee et al. 2020). However, the Solomon Islands *W. auropunctata* worker assessed here appears to be genetically different from *W. auropunctata* in Taiwan, suggesting that *W. auropunctata* in Taiwan arose from a different invasion event and probably a different source population. Although the current genetic dataset may not allow us to pinpoint the putative source of *W. auropunctata* in Taiwan, the incursion appears to have occurred via secondary introduction because the mitochondrial haplotype recovered from *W. auropunctata* in Taiwan can be mostly found in the known introduced populations (Table 1), including those from several islands in the Pacific.

Previous study has shown that this ant tends to disperse through budding in the introduced range (Hölldobler and Wilson 1977; Lubin 1984), and this is supported by the localized distribution of the ants in the two locations in Taiwan. However, the distance between the two locations indicated the possibility of human-mediated dispersal. In addition, analysis of the queens' genotypes indicated that queens from the two locations likely originated from two or more different clonal lineages. However, the high genetic resemblance between these lineages, as well as the low aggression level found in ants between the two locations, may reflect that *W. auropunctata* in the two locations has derived from a single population containing queens of independent clonal lineages with a closely related genetic and behavioral assembly. High similarity in the inferred paternal haplotypes of ants in the two locations (Table 2) supports this scenario.

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Authors contribution

CCSY and CCLin provided funding, designed the research methods and sampling strategies; CCLee, FCH, CHS, JWT, and YCH carried out the field survey and collected samples; CCLee carried out the behavioral experiments and the following statistics; PWH and SPT carried out the molecular experiments and genetic analyses; PWH wrote the original draft of manuscript; CCLee, FCH, SPT, CHS, JWT, and YCH reviewed and edited the manuscript; PWH, CCSY, and CCLin finalized the manuscript.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Geo-referenced information for the surveys conducted in this study.

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2022/Supplements/BIR_2022_Hsu_etal_SupplementaryMaterial.xlsx