國立臺灣大學生物資源暨農學院昆蟲學系

碩士論文

Department of Entomology

College of Bioresources and Agriculture

National Taiwan University

Master's Thesis

# 穴居鋸針蟻的超級群落與微衛星標幟 － 台灣新興引入針蟻亞科鋸針蟻之初探

# Exploring supercoloniality and novel microsatellite markers in *Odontomachus troglodytes*: insights into a recently introduced Ponerinae ant in Taiwan

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中華民國 113 年 7 月

July 2024

doi:10.6342/NTU202403329

# 致謝

這本碩論的成果，是經過身邊所有人的不吝相助，才得以完成。三年前一頭熱地從法律斜槓進昆研所，憑藉著的，只有從小對節肢動物的熱情。然而現實告訴了我除非天時地利人和，光有熱情是無法在研所生存的，因此我這趟讀研的旅程得能順利走完，實在是有太多貴人需要感謝。首先，我要感謝我的指導老師 – 書萍老師對我的栽培，願意接納我這樣一個理科實驗零基礎、對實驗室生態一無所知的外系生。無論是在研究方面抑或是在培養身為研究生的自覺的過程中，想必我都給老師您帶來超出預期的麻煩，真的很感謝您在我到處闖禍的時候對我的包容與不離不棄。再來是這兩年陪伴我做實驗與撰寫論文的三位實驗室助理：子嘉、怡婷與宗翰。很慶幸我做分子實驗的啟蒙是子嘉，聰明的她總是能簡化複雜的問題，讓我這個分生初學者可以更快融會貫通；統計分析、R軟體的操做還有論文的校對，要是沒有細心的怡婷手把手指導，我可能連畢業門檻都勾不著；口試報告與圖表的規劃最後還需宗翰來救火，抱歉讓你剛入職就得為我文稿上的粗心多費心。碩班生涯能有這幾位給力的助理們我真是感激涕零，一定是上輩子燒了好香。同時要感謝溫泉大學長，不只是採集時的螞蟻百科，作為實驗室的暖男擔當，任何不順在經你開導後都是小事一樁，你與世無爭的態度，總是讓人心神嚮往。感謝彰師大的伯誠學長、品誌學長，補足我採集的經驗又送我螞蟻還幫我割壓克力板，螞蟻的大小事有你們的協助真的太幸福。針蟻實驗的野外採集要感謝柏磊，為銓與凱文，要不是有你們的協助，實驗採集不會如此順利。謝謝世祥、Kiwi、亮得、克軒、昇鴻等同袍，沒想到碩班生涯能遇到合拍的一群人，為我帶來好多歡笑的回憶，相信大家畢業後都能在各自的崗位上發光發熱。另外要感謝世祥在我口試前提供許多報告上的建言，謝謝你願意在忙碌與迷惘之際仍待我的報告如此認真。謝謝我的老鄉 – 張作曲家與賴建築師，高中、大學再到出社會，能遇到一起成長的好友實屬難得，不同領域打拼的酸甜苦辣，讓我們再續寫下一個十、二十年。除此之外，我很感激父母對我考昆研的容忍，謝謝你們在知道我從法律斜槓到昆蟲後只有支持，更在我迷惘休學時讓我得能以自己的步調來決定下一步。

最後，謝謝妳粲蓉，我的愛。休學期間的焦慮與掙扎，搖擺於回歸國考或返校復讀之時，沒有比妳更支持我的人。是妳一路自始至終伴在我身邊，並總是鼓勵我勇敢去追夢。期待下一階段的妳跟我，在有妳和我一步一腳印的時間裡，所有努力後都是甘甜。

“I will finish what I started.”是我下定決心復讀時我對我自己的諾言。現在，我已達成當初的承諾，並準備好邁入下一篇章。

2024/07/31 朱孝洋 於 台大學新館

doi:10.6342/NTU202403329

# 摘要

隨著人類活動擴張，許多物種被帶離其原生地，對全球生態和農業造成嚴重威脅。非洲穴居鋸針蟻（*Odontomachus troglodytes*）原生於非洲，近年來族群爆發於台灣西南部果樹園，對於台灣生態及農業經濟造成潛在危害。與此同時，穴居鋸針蟻也展現出超級群落的潛力，加深此物種潛在的侵略性。目前有關這種鋸針蟻的族群結構侵略特性及基因層面的研究十分有限，這將使得制定相對防治方針時缺乏參考資料。本研究旨在填補有關穴居鋸針蟻的知識缺口，了解其行為模式、生殖策略和族群結構。

本研究收集了嘉義和高雄的穴居鋸針蟻族群，進行巢間攻擊行為實驗，以探討此物種是否存在超級群落。實驗結果顯示，不同巢的穴居鋸針蟻並未展現攻擊性，表示入侵族群間具有相容性，可能形成高密度蟻群壟斷周圍資源。由於目前非洲穴居鋸針蟻沒有可用的微衛星遺傳標記，本研究中利用次世代定序技術篩選微衛星基因座，測試 20 組標記後，篩選出 11 個具有多型性的遺傳標記進行遺傳分析。遺傳分析結果顯示穴居鋸針蟻只使用有性生殖模式，並且在等位基因上缺乏多樣性。族群分化分析顯示嘉義族群與高雄族群可分為兩個不同的族群，代表台灣的入侵族群有可能是多次入侵的。然而，嘉義的族群卻同時具有較低的等位基因數，並且缺乏族群獨有的等位基因，使得嘉義的族群也有可能源自等位基因數更高並且具有獨有的等位基因的高雄族群。

本研究不僅提供台灣的穴居鋸針蟻入侵族群在行為、生殖模式及族群結構上的資訊，也是鋸針蟻族內首次記錄到超級群落的特性。我們的研究有助於解析新



doi:10.6342/NTU202403329

入侵螞蟻族群的結構和特性, 並為該物種的生物防治管理和策略制定提供生態習性與族群遺傳分化上的貢獻。

關鍵字：入侵物種、微衛星、巢間攻擊行為、穴居鋸針蟻、針蟻亞科、超級群落



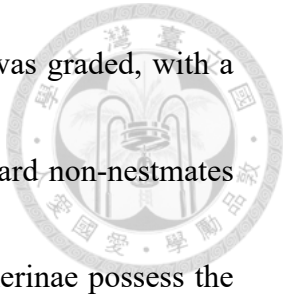
doi:10.6342/NTU202403329

# Abstract

Human activities have facilitated the global spread of numerous invasive species. Among invasive insects, ants are particularly successful due to reproductive flexibility, including mechanisms like polygyny and asexual reproduction, which likely facilitate their establishment and expansion in new environments. Besides reproductive flexibility, some ant species develop “Supercoloniality”, where individuals from geographically disparate colonies recognize each other as colony members. Supercoloniality diminishes intraspecific competition and strengthens resource monopolization, allowing these ants to dominate native competitors and disrupt the entire ecosystem. *Odontomachus troglodytes*, a recently-invaded African Ponerinae that took root in the southern part of Taiwan, is suspected to possess traits similar to other successful invasive ants. Clarifying the social structure and reproductive mechanism of this alien species can contribute to its scarce studies and the development of effective prevention measures that may mitigate its impact on the native fauna. Twelve colonies were collected followed by ant-to-ant aggression assays that were conducted to test the hostility of the colonies within and between the two invaded



doi:10.6342/NTU202403329



regions: Chiayi and Kaohsiung. The aggressiveness of every pair was graded, with a total of 107 bioassay pairs exhibiting no aggressive behaviour toward non-nestmates up to 120 kilometers apart, suggesting that this newly invaded Ponerinae possess the fundament trait of supercoloniality, which would be the first record in the Odontomachini tribe and in the Ponerinae subgamily. Rapid antennation among different pairing groups were analyzed and the duration of rapid antennation over time was met with a steep decline from the second bout. Microsatellite genotyping with the assist of field and laboratory observations were used to clarify the breeding structure and reproductive mechanism of the invaded *O. troglodytes* populations. Twenty potential microsatellite loci were selected, of which 12 were successfully amplified and 11 were found to be polymorphic. The collected colonies exhibited a high percentage of polygyny, with none of the genotyping results of the colonies indicate a reproduction method outside of reproducing sexually. Additionally, eight out of the nine genotyped colonies produced diploid males and/or extracted diploid sperm, indicating the introduced populations are under inbreeding situation typical to newly invaded ant species. The population differentiation analysis suggests two distinct genetic clusters exist between the two regions, supporting the hypothesis of multiple invasions by two



doi:10.6342/NTU202403329

distinct populations, yet the allelic summary shows that the Chiayi region has less abundant allele composition and lacks regional private alleles, arguing the case of post-colonization genetic mutation due to geographical barriers after inland bridgehead expansion. Our study fills the knowledge gaps about this newly invaded ant species on both behavioral and molecular perspectives and serve as the fundamental base for future mitigation measurements to minimize the impact of this alien species on the native ecosystems of Taiwan.

**Key words**: aggression assay, invasive species, microsatellite marker, *Odontomachus troglodytes*, Ponerinae, polygynous, supercolony



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# 1. Introduction

Ever since the transportation revolution of the Anthropocene, civilisation activities re-shaping the environments and connecting the globe has led to the global explosion of invasive species. These species took advantage of these transportation channels and thrived in ecosystems hundreds of thousands of miles away from their origins. They outcompete their native competitors either by out-producing or out-resourcing, destroying the stable formation of the native ecosystem, leading to the extinctions of crucial components species and corrupting the balance of the food chain. Out of the top 100 world's worst invasive alien species organized by the International Union for Conservation of Nature, invertebrates are the most prevalent, with almost 60% being composed of insects (Luque *et al*. 2013). Due to their smaller sizes, insects are more prone to hitchhike along human transportations, hence leading to an astonishing amount of case reports of invasive insects. More than 2,800 insect species were reported to have environmental impact outside of their native distribution range, above 500 with literature evidence of negative environmental impacts and beyond 240 species have multiple references (McGeoch *et al*. 2020). Among the most prevalent pathways



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utilized by invasive insects, Hymenoptera, especially ants, had the most versatile introductions, accounting for more than one in eight invasive species assessed, three times as many as any other insects (McGeoch *et al*. 2020). This is no surprise as ants are easily portable due to the petite body sizes even in comparison to other insects, yet the midgets possess the advantage to outnumber other species owing to their prolific breeding systems and cooperative social structures, which aids them in establishing and colonizing foreign continents or islands (Bertelsmeier *et al.* 2017, Abril and Gómez 2020, Bertelsmeier 2021). The influence of invasive ants to the ecosystems may seem trivial, but the outcome to the fauna as a whole could be devastating. Ants can alter plantation fauna (Brener and Ghermandi 2008, Green and O'Dowd 2009, Lach *et al.* 2010), displace niche-overlapping invertebrates (Giraud *et al*. 2002, Goodman and Warren 2019, Jourdan *et al*. 2022), endanger native vertebrates (Lubin 1984, Ligon *et al.* 2011, Long 2015), nurture invasive plant pests (Helms and Vinson 2002, Holway *et al.* 2002, Lach 2013) and induce plantation demolition (Mikheyev 2008).

Behind all the colonizing records, invasive ants that are the most successful are especially competent in out-numbering and out-resourcing native ant competitors, with their triumph aided by their unique colony structures and/or breeding systems (Holway



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*et al.* 2002, Tsutsui *et al.* 2003, Eyer *et al*. 2018, Lacy *et al*. 2019). Dr. Eyer and Dr. Vargo (2021) analyzed various ant genera and identified three key factors that contributed to the success of some invasive species: (1) supercoloniality, (2) polygyny, and (3) asexuality.

Commonly, an ant colony would shape a certain range of area as the nest territory and act agonistically towards those who intruded, including those of the same species (Adam 2016). Some colonies would become polydomous, with their nests growing exceptionally large and may expand satellite nests that spread over large areas with linked inter-nest networks in-between (Robinson 2014). But the extreme of these social organization could even exceed the limitation of polydomus nests by excluding actual connections between nests, with workers being separated by large geographical distance or barriers (*sensu* Hoffmann *et al*. 1999, Krushelnycky *et al.* 2005, Hoffmann and Saul 2010, Thomas *et al.* 2010) and yet still displayed no aggression toward each other with the potential of workers and resources exchange between different nests (Giraud 2002, Hoffmann 2014). This phenomenon, called supercolony, ceased inter-nest hostility and strengthened resource monopolization of a species. Supercolony was initially defined as a conceptual idea of three factors at its introduction into



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myrmecology: little to no aggression towards non-nestmates between geographical separated nests, dispersal of resource within the supercolony, and a constant worker exchange between nests (Wilson 1971, Bourke and Franks 1995). However, its definition turned out to be hard to categorize, as no uniformity in the definition exist even within the same species (Le Breton *et al*. 2004, Pedersen *et al*. 2006, Buczkowski 2010, Hoffmann 2014). Although the concept is yet to be solidified, it is disproportionately common among invasive ants (Eyer and Vargo 2021) such as the likes of *Linepthima humile* (Heller 2004), *Wasmannia auropunctata* (Foucaud *et al*. 2009) or *Anoplolepis gracilipes* (Hoffmann 2014), which are being defined as supercolonial due to sharing the fundamental trait of supercoloniality: Individual populations that are geographically, and thus reproductively, isolated from others, yet display little or no aggression between workers from different nests (Hoffmann 2014). This makes any ant species that showcases such a trait a potentially agonistic invader that could very possibly devastate other native faunas if it was being brought in.

Aside from supercoloniality, having multiple egg-laying queens is also a pivotal trait that promotes the invasive success of ant species (Seifert 2010). Most ant colonies are composed of a reproductive female (queen) and a great number of infertile females



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(workers). However, some species are polygynous, which increases not only the rate of colony growth (Vargo and Fletcher 1989), but also the chance for a reproductive to be included in transported propagules (Tsutsui and Suarez 2003), making invasive ants that feature polygyny notoriously hard to eradicate. Introduced fragments of a nest could consist of multiple fertilized queens that could start reproducing once settled in the new fauna; reproduction rate that doubled or even tripled in comparison to the native ant colonies ensured resource monopolization by outnumbering it competitors, dominating the original food chain and more often replace the native ants. (Greenberg *et al.* 1985, Ross *et al*. 1985, Espadaler and Rey 2001, Ingram 2002, Buczkowski 2010).

Lastly, clonal and asexuality are both prevalent breeding system characters in some infamous invasive species such as double lineages clonal of which new queens are clones of their mothers while sons are clones of their fathers in *Wasmannia auropunctata* (Fournier *et al*. 2005), *Paratrechina longicornis* (Pearcy *et al*, 2011, Tseng *et al*. 2019, Tseng *et al*. 2023) or unmated queens can produce both new queens and workers asexually in *Mycocepurus smithii* (Rabeling *et al*. 2009, Rabeling *et al*. 2011). These unorthodox traits can promote a single queen colonization, which greatly raise the severity of a species' invasiveness. To summarize, all the above-mentioned



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features are essential and functioned as great indicators in determining the potential invasive severity a newly invasive ant could induce when recently introduced into a foreign land or island. Potential alien species that consist of one or multiple characters mentioned holds the potential to be extremely devastating to other faunas and the invasion could result in irreversible outcomes in the likes of reduced biodiversity, species extinctions or the collapse of a whole ecosystem (Abbott 2005, Parker *et al*. 2021).

An outbreak by a recently invaded Ponerinae: *Odontomachus sp*., had been reported in the southwestern part of Taiwan. Collected and reported by amateurs in 2017 and later became more populated, the *Odontomachus* sp. was classified to be *Odontomachus troglodytes* and the origin of its populations in Taiwan confirmed via phylogenetic relationship and haplotype COI network building by Lin *et al.* in 2023. This species is native to sub-Saharan African, with the population of Taiwan possibly originated from Cameron due to sharing the same haplotype (Lin *et al*. 2023), and has become the second invaded population after the Madagascar population (Fisher and Smith 2008, Lin *et al*. 2023). *O. troglodytes* displays high aggression to disturbances (Lin *et al*. 2023) and the species is characterized by its stout mandibles and short blunt



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subapical teeth, strongly striated body sculpture and 4, 3 palp formula (Brown Jr 1976, Sorger and Zettel 2011, Lin *et al*. 2023). It possesses formidable mandibles that can easily break the chitin surfaces of other invertebrates and potent stings that can paralyze prey or give out extremely painful strikes. Unlike the colony population of most other Ponerinae species that are usually comprised of no more than a few hundreds of workers (Wheeler 1900, Paiva and Brandão 1995, Ito *et al*. 1996), the average colony size of *O. troglodytes* often exceeds thousands (Colombel 1970, Brown Jr 1976, Schmidt and Shattuck 2014, Lin *et al*. 2023, personal observation 2023), with previous reports of being polygynous and their workers potentially fertile (Ledoux 1952, Colombel 1970, Colombel 1972, Brown Jr 1976, Schmidt and Shattuck 2014, Lin *et al*. 2023). In addition to being ground dwelling, the sighting of workers tending Homopterans and occupying tree trunks suggested that *O. troglodytes* can fully exploit resources in a three-dimensional latitude (Evans and Leston 1971, Colombel 1972, personal observation 2023), with field study (Evans and Leston 1971) and personal observations revealing the ant's preference in habituating secondary forest and agricultural farmland while displaying the potential to thrive in habitats different to its native environment (Lin *et al*. 2023). Most importantly, the species also exhibited the potential of



doi:10.6342/NTU202403329

supercoloniality via being low to none aggressive toward non-nestmates in preliminary tests (Lee unpublished). Aside from the above-mentioned traits, members from the same genus such as *O. haematodus*, *O. bauri*, *O. ruginodis* and *O. simillimus* had all be reported to establish populations outside of their native origins (Brown Jr 1976, Fisher and Smith 2008, Framenau and Thomas 2008, Herrera *et al*. 2014, MacGown *et al*. 2014, Deyrup 2016, Wetterer 2020, Lin *et al*. 2023), we therefore speculate that this newly introduced alien species could be preadapted to being invasive and the introduced population of *O. troglodytes* could impose destructive consequences to the environment of Taiwan similar to other notorious invasive ants.

Currently, the behavioural studies of the species are mostly confined to basic biology (Colombel 1970, 1972, 1978, Evans and Leston 1971, Dejean and Lachaud 1991) and lacks empirical studies on its invaded populations and its supercoloniality. With no previous studies discussing its potential to possess this particular behavioural trait as other infamous ant invaders, our study aims to validate its supercoloniality via different nest combinations of ant-to-ant aggression trials. This would not only enrich the behaviour study of this species by bringing its invasive nature to light, the present study would also potentially be the first official record for a member of the Ponerinae



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to possess supercoloniality if this non-aggressive behavioural trait among non-nestmates is true.

Aside from the behavioural aspect, little molecular studies had been done besides taxonomy and phylogeny level classification (Schmidt 2013, Schmidt and Shattuck 2014) and gut content examination (Fayle *et al.* 2015). In the same aspect, not many studies focused on examining the breeding systems of Ponerinae via molecular analysis, with the majority emphasizing on specialized ergatogyne, gamergates or oligogyny reproductions (Haskins *et al.* 1971, Peeters 1991, Dejean and Fénéron 1996). Since the extensive molecular analysis of this species is almost non-existence, it may hinder us on clarifying its breeding system and its colony structure, which could prosopon the invasiveness assessment of this highly aggressive Ponerinae. Hence, we developed novel species-specific microsatellite markers and performed population level genotyping in order to shed light on the ambiguity of the breeding system of the invaded populations. This would help us unveil the breeding dynamics behind the abnormally high amount of worker number observed in the colonized sites in comparison to most other Ponerinae, which could have derived from unorthodox systems such as production via clonality. Furthermore, the genetic analysis of molecular data would



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help us understand more about the population differentiation among populations in Taiwan, providing insights into its population origins and contributing to reconstructing its invasion pathway and the number of invasion events. The goal of the present study is to provide an outset for subsequent ecology and molecular research of the invaded *O. troglodytes* populations in Taiwan, and offers insights for future mitigations through assessing its invasiveness and reproduction dynamics.



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# 2. Materials and Methods

## 2.1 Sampling

A total of 12 nests were collected between August 14th and 15th in 2023 (Fig. 1A). Four nests were excavated from the pseudostems and rhizomes of banana plantations and one from the base of a tree trunk alongside the pineapple farmland in the north-east of East District, Chiayi (Fig. 1B); three nests collected at the base of bushes and tree trunks along the hiking trail of north-east Fongshan, Kaohsiung (Fig. 1C); two nests from the pseudostems of a banana plant and the base of a tree trunk uphill of the Fongshan cemetery in the south-east of Fongshan, Kaohsiung (Fig. 1D) and two nests within two separate rotten wood at Dapingding Tropical Botanical Garden in the east of Siaogang District, Kaohsiung (Fig. 1E). Every nest was housed either in a 13 x 6 cm or a 20.5 x 8 cm plastic pie-shape enclosure regarding to the collected colony size (small: 50~100 worker; large: 100~) with Petri-dish as nest cover and moist plaster as the nest substrate base. Nests were provided with water tube before the aggression assay and kept at an average room temperature of 25 °C. For the aggression trial, 10 nests with more abundant workers: CY02, CY03, CY04, CY05, KH02, KH03, KH04, KH05,



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KH06 and KH07 (Fig1B – Fig.1E), were selected to go under trial; for population genotyping, nine nests: CY01, CY02, CY04, CY05, KH01, KH03, KH04, KH05, KH06 (Fig1B – Fig.1E), either with male samples retrieved or multiple queens collected, were chosen for colony structure genotyping in order to obtain a more comprehensive genomic data. Maximum of three queens, all collected males and four workers of each chosen colony were sampled and used for subsequent population genetic analysis. Additionally, sperm samples were also genotyped via the spermatheca dissection of all genotyped queens.

**2.2 Aggression assays**

2.2.1 Number differences of worker-worker in aggression assays

Prior to the aggression assay, preliminary tests were carried out to test the outcome of aggressiveness with different ant number combinations. According to the conclusion of Roulston *et al.* (2003), a worker grouping of 5 vs. 5 induces a higher and more stable aggression result than 1 vs. 1. However, in order to conserve workers for the subsequent aggression assay, we added a 3 vs. 3 group as an inter-grading to assess the minimum number needed for a single trial. The pre-test followed the protocol of Roulston *et al.*



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(2003), of which consisted ant-to-ant bioassay combinations of (1) 1 vs. 1, (2) 3 vs. 3 and (3) 5 vs. 5 of Within-Chiayi and Chiayi vs. Kaohsiung. A total of five repetitions for combination (1) and one repetition for (2) and (3), each trial lasted 5 minutes. The minimum-worker-required preliminary test resulted in no aggression differences in all categories, with all three combinations displaying no hostility despite the number differences. The nonchalant reaction suggests that the non-agonistic interaction between *O. troglodytes* workers from distinct nests/colonies may not be affected by the number of workers encountered.

2.2.2 Assessment of worker introduction methods in aggression assays

Ant transferring in aggression assay is a factor often overlooked in the process of the bioassay. Excessive tension could be induced to contestants if not maneuvered carefully before being introduced to non-nestmates, which would result in false outcomes. This affects highly irritable species such as the likes of *O. troglodytes* to the extent that hostility could even be induced within nestmates if being introduced abruptly. Only few studies stated their method of ant transfer (Roulston et al 2003, Vásquez and Silverman 2008), others emphasized little to none on a less stress-inducing



doi:10.6342/NTU202403329

method of worker introducing (Suarez *et al.* 1999, Giraud *et al.* 2002, Tanner and Adler 2009, Gruber *et al.* 2012, Sorger *et al.* 2017), or lack the subsequent acclimatization period before a trial to avoid conducting an assay at a high-tension situation (Suarez *et al.* 1999, Vásquez and Silverman 2008, Tanner and Adler 2009). The workers of *O. troglodytes* were found to be less disturbed after transfer if they are maneuvered at their appendages. In contrast, if seized at the thorax or abdomen, the struggle would be fierce as the ant would immediately curl in reflex and try to attack or escape, this would leave the ant in a hyper condition that is far from a natural non-nestmate introduction scenario and could very possibly induce misleading results. As for ant acclimatation, comparing to previous studies that did employ this procedure before ant-to-ant introduction (Roulston *et al.* 2003, Gruber *et al.* 2012), we extended the acclimatization period to 10 minutes in the present study to prolonged the time for workers to acclimatize, ensuring the tensions caused by being transported into the arena were minimized and the interaction more neutral before both sides were being introduced.

2.2.3 Colour marking

To recognize the representatives from different colonies during the aggression assay, colonies that were assigned for trials were given a specific colour combination



doi:10.6342/NTU202403329

of their own. Randomly selected workers were placed in a 7ml plastic tube and put into an ice bucket for 3 minute to induce chill-coma (Roeder *et al.* 2021), the workers were then transported to a “Colour Petri-dish” for their thorax and abdomen to be marked with a specific colour combination using SNOWMAN-CP paint markers. After being marked, the workers were then moved to a “Rest Petri-dish” for 10 minutes of recovery, allowing the workers to regain from the coma stage before placing them back to their respective colonies. At least 20 to 25 workers for each nest were marked to meet the minimum requirement of the trial setting for the aggression assay. The marked workers remained in their respective nests for over 24 hours before the assay took part to volatilize the scent of the markers in case of unwanted aggressiveness that was induced due to the odor of freshly painted markers.

2.2.4 Aggression assays

According to previous preliminary aggression test (Lee unpublished), the aggression between colonies escalated after being kept in lab for over two weeks, suggesting that a relatively more realistic aggression assay in-lab could only be achieved by performing the bioassay as soon as the ants were being collected to at least within a week after collecting. Hence, the aggression trials of the present study were all



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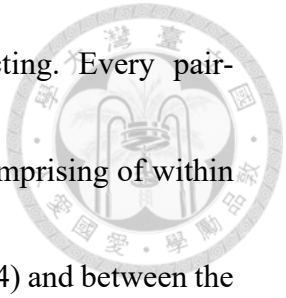

performed on the same day within the first week after collecting. Every pair-combination of the 10 marked nests led to a total of 107 pairings comprising of within nest (e.g., CY02_1 vs. CY02_2), between nests (e.g., CY02 vs. CY04) and between the two regions (e.g., CY02 vs. KH02). Following the aggression assay protocol of Giraud *et al.* (2002) with a slight material modification, the marked workers were randomly selected and placed in a plastic cup of 8 x 8 cm diameter that served as the arena with an equal sized circular sheet as exchangeable base. For ant contestant transport, we used forceps to grasp the worker at the hind appendage and transfer the workers into the arena steadily and precisely. Randomly selected individuals out of each pairing nest would be isolated by a smaller Petri-dish of 4 x 4 cm diameter inside of the plastic cup arena. The separation by the small Petri-dish would last 10 minutes, this functioned as the acclimatization period for each pairing before the barrier in between the two individuals was removed. The interactions were recorded using a Keystone FHD Webcam (AVA001) for 10 minutes starting from the initial contact between the two workers. The aggression scale used in the current study to assess the hostility referred to the likes of Suarez *et al*. (1999), Giraud *et al*. (2002) and Roulston *et al*. (2003) and Murata *et al*. (2017), but also with modifications of level specific behaviours that suited



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more to the nature of *O. troglodytes*. The interaction scores were as follows: (0): Touch and go, where the two workers ignore each other right after initial contact; (1): Mild antennation, workers stop and exchange antennation slowly with all four antennae clearly visible; (2): Rapid antennation (RA), the exchange of antennae strikes too rapid to be capture either by eye or camera. RA, also referred to as: intense antennation, antennal strikes, antennal boxing, antennal dueling or antennal drumming, is differentiated with other antennal movements by being fast enough to induce blurring and often occurred when ants initiate dominant or aggressive context (O'Fallon *et al.* 2016). In the present study, we observed that RA may escalate into aggression or demote to being non-aggressive, hence we deemed RA as a pre-aggression behaviour.; (3): Bite and pull, the act of nipping the other's leg or antenna while trying to maneuver its direction; and (4): Snap and sting, snapping of the trap-jaw and aggressive stinging when the jaw latched on to the other. The highest grading that occurred would be the result of a pairing; if a record of grade 3 or above was recorded, the interaction of the pair would be deemed as aggressive. In contrast, as long as the interactions were graded 2 or below, the result would be deemed as "non-aggressive." All workers and circular sheet-bases were used in no more than one trial; individuals were proceeded to be



doi:10.6342/NTU202403329

preserved in 95% ETOH after a trial had ended. The classification of gradings and the inspection of ant-to-ant interactions of each level were analyzed via the built-in camera app of Microsoft Windows 10.

2.2.5 Analysis of RA and encounter occurrence

Due to the lack of agonistic expressions under the aggression assay, we turned the focus on to assessing the duration of RA of each bout among all recorded colony pairs (O'Fallon *et al*. 2016, Murata *et al*. 2017). We analyzed the aggression trial recordings and calculated the significancy of RA duration between the initial and the secondary bouts of (1) between nests of the two regions, (2) within nests of the two regions and (3) between regions using Generalized Estimating Equations (GEE). Encounter occurrence was compared between within-nest and between-nests of the two regions to see if the bout frequency is affected by being tested with nestmates and non-nestmates. The significancy was calculated using the Mann-Whitney U test.

**2.3 Development of microsatellite markers**

DNA libraries were prepared from two genomic DNA samples of a queen and a worker from Kaohsiung and Chiayi, respectively, by applying the Celero EZ DNA-Seq



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Library Preparation Kit (TECAN). Owing to the genome size record of six *Odontomachus* species being around 400 to 500 Mb (Tsutsui *et al.* 2008.), the sequencing size was set to be a total of 12 Gb from the two samples each to begin with. Whole genome sequencing and the following *de novo* assembly were performed via Next Generation Sequencing (NGS) using NovaSeq (NovaSeq 6000 Sequencing System). The above-mentioned procedures were carried out by Tri-I Biotech Inc. (Taipei, Taiwan) with the application of the universal library structure adaptor of Illumina for library construction, adaptors and low-quality bases trimming (QV $\geq$ 25) using the CLC Genomic Workbench v 10 and *De Novo* assembly employing the default settings of SPAdes v3.15.3. The draft contigs were then screened for potential microsatellite loci following the methods of Tseng *et al.* (2019) with slight modification. The contigs were screened for dinucleotide repeats greater than 10 but lesser than 17 using Krait (Lianming, 2018). A total of 20 potential microsatellite loci were selected for further analysis, half from the library of the queen collected in Kaohsiung and the other from the library of the worker collected in Chiayi to have a more comprehensive sampling. Corresponding primer pairs were designed using the Primer3 program (Rozen and Skaletsky, 1999) embedded in Krait with the range of the product size set



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from 100 bp to 250 bp, the primer pairs were then synthesized by Genomics BioSci and Tech Corp. (Taipei, Taiwan). The 20 potential microsatellite loci were screened for positive PCR amplification using agarose gel electrophoresis. PCR reactions contained a total volume of 20 μl, composed of 10 μl of AMPLIQON 2x Master Mix RED (Ampliqon, Denmark), 1 μl of 1 μM primer pairs, 8 μl of nuclease-free water and 1 μl of genomic DNA from one adult male (Kaohsiung). The PCR conditions were as follows: initial denaturation at 95°C (5 min) followed by 35 cycles of 95°C (30 s), 58°C (30 s) and 72°C (30 s), with a final extension phase at 72°C (10 min) and at last held at 4°C (infinite).

All of the products obtained from the PCR process underwent gel electrophoresis; in order to genotype in a cost-effective manner, those that yield a single band within the expected size corresponding to the design followed the strategy of multiplex PCR reactions using fluorescently labeled universal primers described in Blacket *et al.* (2012) with a slight modification. Four fluorescent labeled universal primers (FAM, VIC, NED and ROX) and modified locus-specific primers with a 5' universal primer sequence tail were used. Each microsatellite locus was amplified independently to ensure maximal procedure quality. PCR reactions contained a total volume of 20 μl,



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composed of 10 μl of AMPLIQON 2x Master Mix RED (Ampliqon, Denmark), 1 μl of 1 μM primer pairs, 8 μl of nuclease-free water and 1 μl of 1:50 first PCR product. The PCR conditions were as follows: initial denaturation at 95°C (5 min) followed by 35 cycles of 95°C (30 s), 58°C (30 s) and 72°C (25 s), with a final extension phase at 72°C (10 min) and at last held at 4°C (infinite). The products of the second PCR procedure that, again, yielded a single band at the expected base pair size were analyzed on an ABI-3730XL DNA Analyzer with GeneMapper v4.0 by Genomics BioSci and Tech Co., Ltd (Taipei, Taiwan). GeneMarker program (version V3.0.1, SoftGenetics LLC) was used to visualize and score alleles, loci that produced the most distinguishable peaks would be applied later in the whole colony structure genotyping.

**2.4 DNA extraction and microsatellite genotyping**

Genomic DNA extraction was performed following the manual instruction of Gentra Puregene Tissue kit (Qiagen). The eluted DNA samples were stored at -20 ºC.

To genotype more efficiently, the PCR processes were completed on 96 wells PCR plates. The PCR procedure, as mentioned before, followed the strategy of Blacket *et al.* (2012) with a slight modification of amplifying each locus independently. The resulting PCR products were analyzed on ABI-3730XL DNA Analyzer by Genomics BioSci and



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Tech Co., Ltd (Taipei, Taiwan) and the alleles were visualized and scored using GeneMarker program (version V3.0.1, SoftGenetics LLC).

**2.5 Characterization of microsatellite loci**

Statistical summary of novel microsatellite markers including the number of alleles (Na), Shannon's information index (*I*), observed heterozygosity (*Ho*) and private allele summary (PAS) were calculated using the GenAlEx 6.503 software (Peakall and Smouse 2006).

**2.6 Colony affiliation**

Determination of the colony affiliation from samples collected at different sites were estimated using FSTAT (Goudet 1995). We followed the method of Vargo (2003) by treating each colony as a population to validate neighboring colony boundaries of all closely sampled nests from Chiayi and Kaohsiung with the application of default settings. Pairwise nest groups include: (1) CY01/CY02/CY04/CY05, (2) KH01/KH03, (3) KH04/KH05, South sampling site was not tested because only one nest was genotyped. Due to this unique interpretation of $F_{ST}$, we only focus on the *p*-value significance of the $F_{ST}$ result between paired nests.



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## 2.7 Genetic variance

The analysis of molecular variance (AMOVA) was calculated using the GenAlEx 6.503 software to evaluate genetic variances at three hierarchical levels: variance among the 2 regions of Chiayi and Kaohsiung, variance among colonies and variance within colonies. The variance among the two regions indicates the differentiation between the Chiayi population and the Kaohsiung population; the percentage of variance among colonies and within colonies would help explain the genetic variance composition among the two levels. The fixation indices for pairwise comparisons were determined using 999 per mutations rate (Nei. 1973, Liu *et al.* 2022).

## 2.8 Breeding structure analysis

With the intent of assigning parentage and estimating the possible number of queens and their mates, we applied the COLONY v.2.0 software (Jones & Wang 2010) that implemented a maximum likelihood method among sampled nests. The two populations from Chiayi and Kaohsiung were run separately. Females were set to be polygamous due to the observation of multiple queens both in the present and in the previous studies (Colombel 1970, Colombel 1978). Males were also set to be polygamous as the allele peak of sperm genotypes suggested the possible existence of



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polyandry. Furthermore, inbreeding was considered because of the high percentage of diploid sons genotyped. The Species option was set to "HaploDiploid" and the length of run was set to "very long." Marker type error rate was acquired using the CREATE v.1.37 software (Coombs *et al.* 2008) with the input of the whole colony. Questionable sperm samples that could either be diploid sperm or due to multiple mating were excluded. We estimated the number of queens (maternity), their corresponding mates (paternity) and assigned workers to their reconstructed parentages among populations (parent-pair, half-siblings and full-siblings).

**2.9 Population differentiation**

Regional genetic differentiation expressed by Wright's $F_{ST}$ ($F_{ST}$), Jost's estimate of differentiation (*Dest)*, and Hedrick's standardized $G_{ST}$ for small number of populations ($G''_{ST}$) were obtained and summarized using GenAlEx 6.503. Estimation was done by selecting one worker each from the nine genotyped colonies and performing the same calculation with non-repeat workers for four repetitions.



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Population level principal coordinate analysis (PCoA) was performed via the GenAlEx 6.503 software and visualized using the ggplot2 package of R (R Development Core Team 2010).

Allele frequency was exported to Structure format via the GenAlEx software and genetic structure analysis implementing the Bayesian clustering algorithm was performed via STRUCTURE v.2.3.1 (Pritchard *et al.* 2000) with both the length of burn-in period and the number of MCMC reps after burn-in set to 500,000 (Cordonnier *et al.* 2020). Iteration was set to 10 with a number of K = nine to compute for the most likely number of clusters (K) according to the allele frequency of the populations in Taiwan. The optimal K was selected by StructureSelector (Li and Liu 2018) applying the Evanno's method and Puechmaille's method (Evanno *et al.* 2005, Puechmaille 2016), and was visualized by CLUMPAK (Kopelman *et al.* 2015) and R.



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# 3. Results

### 3.1 Aggression assays and rapid antennation analysis

All 107 aggression assay trials resulted in a final scoring of level 2 (Fig. 3). Every trial from both the experiment group (between nests / colonies) and the controls (within nests) had shown at most the behaviour of RA with no interactions that could be deemed as hostile (level 3 - Bite and pull or level 4 - Snap and sting) observed being expressed toward each other.

The common routine of a bout consists of (1) RA, (2) Mild antennation, and lastly (3) Part-away. The RA of initial contacts between every trialed pair were usually the most intense as the average RA duration was the highest in the first bout between two workers either from the experiment groups or the controls (Appendix Fig. 1). The whole process of a bout was observed to gradually shorten during the 10 minutes trial routine due to the time span of RA becoming shorter as more bouts occurred. The result of GEE revealed that the duration of RA was significantly different among different bouts of contacts during the trials ($p < 0.05$). However, the durations showed no significant difference after the first bout (Appendix Fig. 2), so we removed bouts higher than two and continued the comparison between the initial and the second contact.



doi:10.6342/NTU202403329

The RA duration was significantly higher in the first contacts than the second contacts in Kaohsiung (between-nests) and Chiayi vs. Kaohsiung ($p < 0.001$ and $p = 0.005$, respectively), while showing no differences in the Chiayi (between-nests), the Chiayi (within-nest) and the Kaohsiung (within-nest) groups ($p = 0.295$, $p = 0.693$, and $p = 1$, respectively. Fig. 4A). When comparing the initial contacts among different groups, the RA duration of Kaohsiung (between-nests) was significantly higher than Kaohsiung (within-nest) ($p = 0.032$), but non-significant in the Chiayi comparison ($p = 0.212$, Fig. 4B). The encounter occurrence of within-nest was significantly higher than between-nests in both regions ($p < 0.001$ and $p = 0.001$, respectively. Fig. 5).

### 3.2 Microsatellite loci result analysis

Of the 20 primer sets tested, 12 loci succeeded in amplification and 11 showed polymorphisms (Table 1). A total of 27 alleles were amplified from the 12 loci based on 36 genotyped worker samples (Table 1). The number of alleles per locus ranged from one to three, averaging 2.042 alleles per locus for the worker dataset (Table 1). The 12 loci were also successfully amplified from queens, males and sperm samples with the number of alleles per locus also ranging from one to three (Table 1). Of the 27



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alleles observed, five private alleles were found only among the Kaohsiung population on locus OT1, OT4, OT15, OT16 and OT17, with the frequency of 0.406, 0.311, 0.123 0.387 and 0.302, respectively (Table 3). The number of alleles (*Na*), Shannon's information index (*I*), observed heterozygosity (*Ho*) for the two regions are displayed in Table 2. The microsatellite polymorphism and genetic diversity, indicated by the average number of alleles per locus and average Shannon's information index, were both higher in the *O. troglodytes* population in Kaohsiung (Table 2). The observed heterozygosity among the two regions are generally low, with the average values being 0.198, 0.308 for ants in Chiayi and Kaohsiung.

### 3.3 Observed breeding and social structure

Via the summary table of allele genotypes, we observed the introduced population in Taiwan exhibiting only sexual breeding with no sign of parthenogenesis or clonality found. On the other hand, the *O. troglodytes* sampled displayed a high percentage of diploidy among males genotyped, with 2/6 and 7/9 male samples from Chiayi and Kaohsiung respectively expressed clear double peaks during allele screening (Appendix Table 20). As for sperm samples dissected from the spermathecae of queens,



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2/5 and 4/12 from Chiayi and Kaohsiung respectively exhibited double peaks, which could either be diploid sperms or the extraction of multiple sperm types due to possible polyandry (Appendix Table 20). Colony social structure-wise, six nests out of the 12 collected were polygynous, with the maximum queen number being nine. The other six consisted of five monogyny nests and one lacking of reproductive caste. No sign of worker reproducing on their own (noted by Colombel 1972) was proven under in-lab rearing condition. However, a newborn male was sampled from one of the queen-less nests. It is uncertain whether it was reproduced by the remaining workers or it was from the remaining cocoon produced by previous queens.

### 3.4 Colony affiliation of the two invaded regions

Colony affiliation calculated by FSTAT helped determine colony boundaries of neighboring nests. The pairwise $F_{ST}$ values of the four colonies from Chiayi were all non-significant, suggesting that the collected neighboring nests in Chiayi to be of the same colony (Appendix Table 12). The same application was applied to nests sampled within the same sampling site in Kaohsiung, which could be separated to three sites of North Kaohsiung (KH01 and KH03), Mid Kaohsiung (KH04 and KH05) and South



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Kaohsiung (only KH06). The pairwise $F_{ST}$ values within each group (except South Kaohsiung, which has only one colony) were all non-significant (Appendix Table 13 and 14), suggesting that nests collected within the same sampling site are all affiliated to the same colony.

### 3.5 Genetic variance percentage between the invaded populations, among colonies and within colonies of Taiwan

The analysis of molecular variance (AMOVA) revealed a 28% of genetic variance among regions ($F_{RT}$ = 0.283*)*, 9% among colonies ($F_{SR}$ = 0.129) and 62% within colony ($F_{ST}$ = 0.375) (Table 4). The *F* value of genetic variance among the two regions, among colonies and within colony were all significant. The percentage of variance within colony (62%) expressed a much higher ratio than among colonies (9%).

### 3.6 Breeding structure of inferred parentages

The inferred sibship and parentage analysis of *O. troglodytes* suggested a total of six genotypes of maternal lines detected among the four sampled nests of Chiayi region and 11 types among the five nests of Kaohsiung. Paternity-wise, a total of eight male mates each were estimated from the genotyped workers of Chiayi and Kaohsiung



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(Appendix Table 16 and 17). The mean number of estimated queens in Chiayi was 3 with the ±SD of 0, whereas in Kaohsiung it was 3 with the ±SD of 1.41; the mean number of estimated male mates in Chiayi was 3.5 with the ±SD of 0.7 whereas in Kaohsiung it was 3 with the ±SD of 0 (Appendix Table 16 and 17). The number of estimated maternities were estimated to be three in all the genotyped Chiayi nests and ranged from two to four in Kaohsiung; estimated paternity number ranged from three to four in Chiayi and all three in Kaohsiung (Fig. 8). An average of 2.89 paternal lines and 2.69 maternal lines were sampled from the two regions.

Sibship reconstruction-wise, workers of the same region displayed a high variation of parentage even within nests (Appendix Table 18 and 19), with the inferred parentage of multiple workers deriving from neighboring nests and a higher percentage of half-siblings than full-siblings (Appendix Table 18 and 19), suggesting the exchange and intermixing of workers among nests within reasonable distance. The probability of parentage pair for most Kaohsiung workers were higher among their collected local distribution of North, Mid and South Kaohsiung, yet some workers still displayed a high offspring probability for parent pairs of more distanced localities, suggesting either alate sexual among the Kaohsiung region had overcome the geographical barriers



doi:10.6342/NTU202403329

within region or the genetic variances between the Kaohsiung colonies are still low enough to induce high relatability between distanced nests.

### 3.7 Population differentiation

Population genetic differentiation obtained by calculating the $F_{ST}$, *Dest* and $G''_{ST}$ between the two regions of Chiayi and Kaohsiung with four repetitions resulted in a mean $F_{ST}$ of 0.222 (± SD 0.055), a mean *Dest* of 0.213 (± SD 0.017) and a mean $G''_{ST}$ of 0.420 (± SD 0.067). All three values were revealed to be significant, if not highly significant between Chiayi and Kaohsiung among all four repetitions (Appendix Table 15).

Population level principal coordinate analysis (PCoA) showed two partially overlapped clusters of genetic distribution between the Chiayi and Kaohsiung regions (Fig. 7A). This indicates a certain level of genetic variance between the Chiayi population and the Kaohsiung population, which could either be interpretated as the result of multiple invasions by two different lineages or a bridgehead expansion by a one-time invasion of a single lineage.



doi:10.6342/NTU202403329

The number of clusters estimated using the Evanno's method by StructureSelector suggested a maximum Delta K value at K = 2 (Fig. 7B) and the highest Mean LnP(K) value at K = 2 (Appendix Fig. 3A). Estimation by the Puechmaille's method that calculated the 'MedMeaK' (median of means), 'MaxMeaK' (maximum of means), 'MedMedK' (median of medians) and 'MaxMedK' (maximum of medians) also suggested an optimal K value at K = 2 (Appendix Fig. 3C). The most likely clustering of K = 2 was visualized by R with each nest contribution listed below (Fig. 7C).



doi:10.6342/NTU202403329

# 4. Discussion

## 4.1 Supercoloniality and its potential in Poneroids

Official documentations of supercoloniality have always incorporated a certain degree of non-nestmate aggression assay aside from field observations, and complementary molecular analysis were sometimes incorporated (Giraud *et al*. 2002, Foucaud *et al*. 2009). Those explicitly deemed as supercolonial or fulfilled all the criteria are disproportionately documented among the Formicoids, with most recorded from the Myrmicinae and the Formicinae (e.g., *W. auropunctata*, *Pheidole megacephala, A. gracilipes, Nylanderia fulva*) and some sporadic documentations from Dolichoderinae (e.g., *L. humile*) and Pseudomyrmicinae (e.g., *Pseudomyrmex peperi*) (Helanterä 2022, Appendix Table 21). In contrast, no official record of supercolony were found among Poneroids to the knowledge of the present study and only a few species from the Ponerinae subfamily had been reported of supercolonial potentials (Taheri *et al*. 2016, Murata *et al*. 2017). The invaded *O. troglodytes* expressed not only the supercolonial trait of low aggression between non-nestmates and the overwhelming worker number, its populations also consisted of a polygyne breeding system similar to



doi:10.6342/NTU202403329

many supercolonial invasive predecessors. With its supercoloniality being validated, this newly invaded Ponerinae represent a mixture of individual ferocity of a Poneroid and strength in number of a Formicoid with high potential of resoruce monopolization within invaded faunas.

The rarity of *O. troglodytes*, a Ponerinae species fulfilling the criteria of being supercolonial is unprecedented to our knowledge and could be the pioneer for future discovery of other supercolonial species among the Poneroids, as *Brachyponera chinensis, Odontomachus chelifer* and a few other Ponerinae species that were reported to express supercolonial potentials such as low aggressiveness among conspecies could be factually validated as supecolonial if further behavioural and extensive molecular examinations were carried out.

**4.2 RA and bout frequency**

The display of rapid/intense antennation between workers of *O. troglodytes* seemed to be a common act of communication in the genus *Odontomachus* (O'Fallon *et al*. 2016). Nest recording revealed that this behaviour exists even among nestmates in nest norm (Dejean and Lachaud 1991, Powell and Tschinkel 1999, Smith *et al*. 2013,



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O'Fallon *et al*. 2016, personal observation 2023). The attribution of RA was indicated to be none quantitatively different towards nestmates or non-nestmates (O'Fallon *et al*. 2016). However, in the present study, the RA duration differentiation shown significance between the initial and the second contact from the Kaohsiung (between-nests) group and the Chiayi vs. Kaohsiung group via GEE analysis (Fig. 4A). Furthermore, the RA duration of the initial contact was statistically significant between the Kaohsiung (between-nests) group and the Kaohsiung (with-nest) group. We propose that the duration differentiation of RA between the first and the second contact corresponds to nest-to-nest familiarity, and that its contribution to possibly providing another point of view to the gradual familiarization of non-nestmates of different colonies may be left unnoticed. The first bout is the most intense due to the initial exposure of unfamiliar cuticular hydrocarbon (Hölldobler and Wilson, 1990; Tsutsui, 2004), but the unfamiliarity could be within the threshold of the supercolonial recognition plasticity of *O. troglodytes* due to genetic basis such as the case of *L. humile* (Tsutsui and Suarez 2003, Thomas *et al.* 2006, Thomas *et al.* 2007) or due to non-genetic factors (Tsutsui and Case 2001, Pedersen *et al.* 2006, Drescher *et al.* 2007, Kùmmerli and Keller 2007, van Zweden *et al.* 2007, Holzer *et al.* 2009), and overtime,



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the low-aggression workers may accommodate to the scent of the distanced colonies counterparts (Murata *et al.* 2017) and potentially leads to subsequent nest merging. Furthermore, the significance of RA result distributed correlate to the geographical distribution and the genomic result, with the relatively indifferentiable Chiayi nests that nested closer expressing non-significant RA interaction, whereas the more distanced Kaohsiung nests that consisted of more abundant genomic variations displayed significant RA interactions between the "within-nest" and the "between-nests," suggesting the geographical separation and the variance in genomic may had its effect on the result of the initial RA between workers.

Additionally, we also analyzed the encounter occurrence of between-nestmates and between-non-nestmates (Fig. 5). Contradictory to the personal observation of O'Fallon et al (2016), which suggested that bouts were more frequent between non-nestmates than nestmates for all four *Odontomachus* species in the study (Smith and O'Fallon's personal observations), the finding of the present study indicated a opposite result of more frequent bouts between nestmates than non-nestmates. This may be due to nestmate pairings having less confusion and tension between workers from the same nest. Unlike non-nestmate pairings that spent more time on RA, nestmate pairs



doi:10.6342/NTU202403329

accommodate each other in lesser time, which accelerate the bout process and ultimately increased the frequency of encounter occurrence.

### 4.3 Additional behavioral observations in *O. troglodytes*: nestmate carrying and queen domination

A carrying behaviour was observed to be initiated by *O. troglodytes* workers once in the preliminary bioassay test and twice in the final aggression assay. One worker would show a higher RA tendency towards the other, with the other worker expressing a more or less submissive pose with lesser RA. The more dominant individual would then proceed to loom over the other worker and try to hold it on the back of the thorax using the mandibles in a manner similar to maneuvering particles. The initiator handled the submissive individual in a rather gentle way unlike the agonistic behaviours of level 3 or level 4 aggressions. The submissive individual would straighten the abdomen and fold the head and appendages close to the torso, reincarnating a pupal posture that allowed the initiator to lift with ease. Previous studies have recorded this phenomenon within the same colony (Dejean and Lachaud 1991, Medeiros *et al.* 1992), stating that matured foragers would carry premature individuals that wandered into the foraging



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area back to the nest. This suggests the trial pairs that exhibited nestmates transfer phenomenon may be paired combinations of a matured forager and a premature pre-forager. A change of duty overtime called polyethism is prevalent among Ponerinae (Fresneau and Dupuy 1988, Dejean and Lachaud 1991, Schatz *et al*. 1996, Pie 2002, Melo and Giannotti 2012), where premature workers in the Ponerinae subfamily usually take the role of nest care and brood nursing while matured workers mainly focus on foraging for resources. This may correspond to the act of matured foragers carrying premature individuals back to the nest to maintain the order of labor division. The estimated range of age of premature and matured workers differed greatly among Ponerinae species (Dejean and Lachaud 1991, Melo and Giannotti 2012), with Dr. Dejean and Dr. Lachaud (1991) observing the transition of premature duties to matured duties of *O. troglodytes* workers happening after two weeks since eclosion. The fact that all the carrying behaviour occurred between non-nestmates in the aggression assay of the present study may further strengthen the hypothesis of *O. troglodytes* being supercolonial as the phenomenon observed was simply the act of nestmate carrying within an immense supercolony cluster.



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Queen-to-queen behavioural domination was observed inside multiple nests in captivity. The observed behaviours consisted of agonistic actions where the alpha queens pulled the antennae and appendages of the beta queens with its mandibles while employing RA to continuously strike the betas until they lowered themselves into a submissive posture identical to the pupal form or the alphas lost interests. Previous study documented this behviour and concluded it as a dominance hierarchy contest between queens of *Odontomachus* for reproductive rights and hence leads to variation in reproduction rate (Medeiros *et al.* 1992). If this is also true for closely related *O. troglodytes*, it should hinder the population growth of the introduced population. Hence, we propose that this phenomenon may have occurred more frequently in captivity due to the artificial nests only provided one to two chambers in comparison to the natural environment, where multiple queens of a large colony could minimize reproduction inhibition induced by queen-to-queen aggression via establishing in separated chambers within the territory of a colony. However, future studies on the composition of the chambers and the distribution of the queens in a nest would be needed to validate this hypothesis.



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### 4.4 Diploidy and breeding structure of *O. troglodytes*

In Hymenoptera, sex is determined by a single sex-determining locus where unfertilized haploid eggs develop into males, and diploid eggs develop into females if heterozygous at the sex-determining locus (Pearcy et al, 2011). Under the haplodiploidy system, the phenomenon of inbreeding often depletes the worker force needed to perform the core task of a colony due to producing diploid males that are instead inviable, infertile or sire offspring that are infertile themselves (Whiting 1939, Whiting 1943, Cook and Crozier 1995, Butcher *et al.* 2000, Beye *et al.* 2003, van Wilgenburg *et al.* 2006, Pearcy et al, 2011), and thus induce fitness costs to the whole colony (Hagan and Gloag 2021). High diploidy consequently increase colony mortality and decrease colony growth rate (Ross and Fletcher 1986, Whitehorn *et al.* 2009, Hagan and Gloag 2021), and it is often related to invasive species due to the founder effect. In a native population, species maintain a dozen of distinct alleles at the sex locus (Yokoyama and Nei 1979, Ding *et al.* 2020, Hagan and Gloag 2021), this minimizes the chance of costly diploidy male being reproduced. However, when a smaller group of the population is separated from the main population and thus its sex-determining allele exchange is being constrained, the frequency of overlapping alleles on the sex-determining locus



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would become more frequent, leading to the outcome of increased diploidy (Tsutsui and Suarez 2003, Hagan and Gloag 2021). The introduced *O. troglodytes* population in Taiwan expressed high frequency of diploidy, with 60% of the sampled males expressing double allele peaks on microsatellite loci. The diploid sperm dissected from queen spermathecae may have resulted from the insemination of diploid male, the occurrence of multiple mating, or both. It would be no surprise that queens encountered and copulated with diploid males more frequently as recently invaded species often produce a higher percentage of diploid males due to excessive inbreeding (Ross and Fletcher 1985, Ross and Fletcher 1986, Ross *et al*. 1993, Lenancker *et al*. 2019). On the other hand, queens may reduce the risk of mating with a diploid male by being polyandry, which is mating with multiple males (Fernández-Escudero *et al*. 2002, Hagan and Gloag 2021). Nonetheless, the vast majority of queens mate only once and the effective mate number is close to one should still be considered (Strassmann 2001). We propose that *O. troglodyte* may belong to the minority of being polyandry and the implementation of multi-mating may be valid in the introduced population. The preference of mating more than once may be the key to how the *O. troglodyte* population in Taiwan overcame its founder effect and contribute to its explosion in



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expansion as being polyandry may have increased the chance for introduced queens to receive viable sperm by mating with multiple males (Ding *et al*. 2017). Future analysis of spermathecae content combination via extensive sperm-typing of more sample data and isolation culturing of queens would be required to factually validate the polyandry status of *O. troglodytes* in Taiwan. Furthermore, all matured nests could be polygynous. Half of the sampled nests displayed polygyny, and the genotyping result of workers from those collected as monogyny exhibit alleles inherited from other maternal lineages. Breeding system analysis via COLONY v.2.0 estimated an average of three queens per nest (Fig. 8), yet queen number of above five had been sampled numerously from the two regions, which suggest an underestimation on the number of queens per nest in the field. Additionally, the average paternal lines per nest (2.89) being higher in comparison to the average maternal lines (2.67) may be evidence that supports the existence of polyandry (Fig. 8). We propose that the *O. troglodytes* populations in Taiwan are either polygynous or at least facultatively polygynous like some other *Odontomachus* species (Medeiros *et al*. 1992), which would help explain the growth explosion and high density of workers (Vargo and Fletcher 1989, Seifert 2007) from colonies sampled in Taiwan.



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### 4.5 The influence of the founder effect

A low number of alleles ($N_A$) was observed among the *O. troglodytes* populations in Taiwan. The mean number of alleles over each locus was 2.042, with the $N_A$ ranging from 1 to 3 (Fig. 6). Taking the invasive fire ant as a comparative, the mean number of alleles between the native and invasive fire ant populations expressed significant differences. The two native sources from South America (Argentina) consisted of a mean number of alleles of 15.8, 15.8 and a maximum $N_A$ of 196, 95; the invasive populations in America (Mississippi and western Louisiana) displayed a mean number of alleles of 5.7, 5.8 and a maximum $N_A$ of 34, 35, respectively. The invasive population of the invasive fire ant in Taiwan (Taoyuan County and Chiayi County) expressed a mean number of alleles of 3.8, 4.8 and a maximum $N_A$ of 23, 26. (Shoemaker *et al.* 2006, Ross *et al.* 2007, Yang 2008). The introduced population of *O. troglodytes* expressed an even lower mean number of alleles and $N_A$, indicating yet another possible outcome of the founder effect on its genomic diversity. However, a study on the microsatellite marker development of another Ponerinae from the same genus – *Odontomachus chelifer*, where the mean rarefied allelic richness was discovered to be 4.27 alleles per locus (Lemos *et al.* 2020). This study applied *O. chelifer* samples collected from its



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native distribution, nevertheless, a considerably low allelic richness was obtained. This may provide another explanation to the seemingly extremely low mean number of alleles and $N_A$ of the introduced *O. troglodytes* population, where the species could have possessed a low allelic richness and the introduced populations did not suffer that significantly a drop in mean number of alleles and $N_A$ comparing to its native population. Further validation would require the samples of the native populations from Africa for extensive genotyping comparative analysis.

### 4.6 Population differentiation and its possible introduction pathways

The invasion history of *O. troglodytes* populations in Taiwan remained obscure, as no decisive evidence from the two scenarios of one-time invasion or multiple times of invasions were established. Population differentiation analysis, first via the GenAlEx software, revealed the pairwise genetic differentiation of the $F_{ST}$, *Dest* and $G''_{ST}$ values among the two sampled *O. troglodytes* populations to be all significant between the two regions. Genomic structure analysis using the STRUCTURE v.2.3.1 software resulted in a most likely cluster group of two ancestral populations – Chiayi and Kaohsiung (Fig. 7B & 7C), which corresponded to the result of two separated populations carried out by FSTAT. Likewise, the result of the principal coordinates analysis (PCoA) suggested



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a double cluster of Chiayi and Kaohsiung with only slight overlapping (Fig. 7A), which could be interpreted as two separate population clustering, this also correlate with the aforementioned surmise of introductions of two different lineages. These results indicate that two distinct populations exist in Taiwan, which may be explained as the consequence of multiple invasions events that introduced two differentiable populations. However, the debate of the number of times *O. troglodytes* had been introduced does not stop here. Allele table summary applying the GenAlEx software suggested a higher abundance of allele composition in the Kaohsiung population. Moreover, private allele distribution analysis summary resulted in the distribution of private alleles only in Kaohsiung and no private allele detected within the Chiayi region (Table 1). This argued that the Chiayi population, having a lower abundance of alleles and possessing no private allele unlike the Kaohsiung population, could be a bridgehead scenario resulting from an inland expansion of a single invaded population, which would be Kaohsiung. $F_{ST}$, STRUCTURE and PCoA analysis that suggested the two populations to be differentiable could have their outcomes deriving from differentiations induced due to genetic geographical barrier constraint between the two separate regions after the bridgehead event. Addition to the perspective of molecular



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analysis, continuing the suspicion of Lin *et al.* 2023, where they speculate the port-neighboring Kaohsiung to be the introduced origin, we discovered that the collecting sites of the central Kaohsiung colonies (KH04 and KH05, under plantations located uphill of the Kaohsiung ossuary) situated next to the cargo storage of OCEAN NICE CONTAINER SERIVCE INC. (Fig. 1D and Fig. 2), which seemed to be a highly possible introduction pathway for invasive species. Although the one-time invasion hypothesis may fit both the genetic and field observations, multiple times of invasions cannot be completely excluded. More conclusive evidence would be needed to factually determine how many times had the species invasion event occurred.

The invasion event could either be long distanced, stemming from its Cameroon origin, or via multiple trade routes, even though commercial trades between Taiwan and Cameroon has not been frequent, and no literature had recorded the invasion of the species in neighboring countries around Taiwan (Lin *et al.* 2023). Vector-wise, due to the import prohibition that forbid the import of oil to Taiwan (https://www.moa.gov.tw/ws.php?id=6108), hitchhiking inside logs may be more plausible, as logs are also the choice of nesting sites for *O. troglodytes*. Additionally, the possibility of introduction via smuggling is always not to be neglected (Gippet and



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Bertelsmeier 2021, Lin *et al.* 2023), as the ant is also for sale among multiple online traders on various social platforms pet trade communities, which has always been a major pathway in invasive species introduction.



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# 5 Conclusion

In conclusion, the recently introduced *O. troglodytes* population in Taiwan displays no sign of aggression towards either nestmates or non-nestmates. Neither group of within-regions nor between-regions expressed any physical hostility that would be deemed as aggressive. This result fulfilled the most crucial component of supercoloniality, which is the lack of aggression towards non-nestmates from different nests that are geographically separated (Hoffmann 2014). This made *O. troglodytes* a new record of supercolonial invasive ant species that may be on par with the likes of *W. auropunctata*, *A. gracilipes*, or *L. humile*, and it is also the very first official record of supercoloniality in the Ponerinae subfamily. The analysis of the RA among nests revealed a corresponding result to the genetical analyses, as the less differentiable Chiayi population exhibited no RA significance, while the more distanced Kaohsiung nests, also expressing higher genetic variance, were relatively more affected by the geographically separation, inducing significantly longer initial RA between different colonies within the Kaohsiung region.



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Aside from developing and establishing novel microsatellite markers for future potential molecular studies on this Ponerinae, the genetical analyses of the species helped clarified the breeding system of the invaded population and elucidated its colony structure, suggesting the existence of both polygynous and possible polyandrous within colony hierarchy and a solely sexual reproduction that excluded coloniality behind its significant worker number. Extensive genomic analysis revealed that both multiple invasions and one-time invasion to be plausible, with multiple population differentiation analysis suggesting the existence of two distinct populations in Taiwan, yet the possibility of a one-time introduction cannot be excluded, as the allele summary of sampled colonies suggested a more abundant allele composition in Kaohsiung, and that the Chiayi population lacks its own private alleles, supporting a single invasion that originated from Kaohsiung. In addition, the sampled site in mid Kaohsiung neighbored a cargo container entrepot, providing a highly possible origin of the introduction pathway for the colonization of the Taiwan population. Still more research is required on both the genomic and field level to truly unveil its actual introduction history. Our finding is exciting not only due to the discovery of a new supercolonial ant is rare, which is found in less than 1% of all ants (Sorger *et al.* 2017), but that



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supercolonialty is disproportionally displayed by invasive ants (Hoffmann 2014), and that we unveiled a new invasive species with high potential in both quality and quantity to induce severe invasion due to its similar nature to other highly invasive ants. The breeding system of only sexual could provide reference for potential mitigation protocols, accelerating the procedure of both invasiveness assessment and subsequent practical field eradication. The present study strives to contribute as a fundament for future studies of this newly invaded Ponerinae in southwestern Taiwan, and hopes to provide both behavioural and genetical research knowledge on the invasiveness of this aggressive Ponerinae for future eradication measures with the aim to mitigate the impact of this alien species on the native biosystem of Taiwan.



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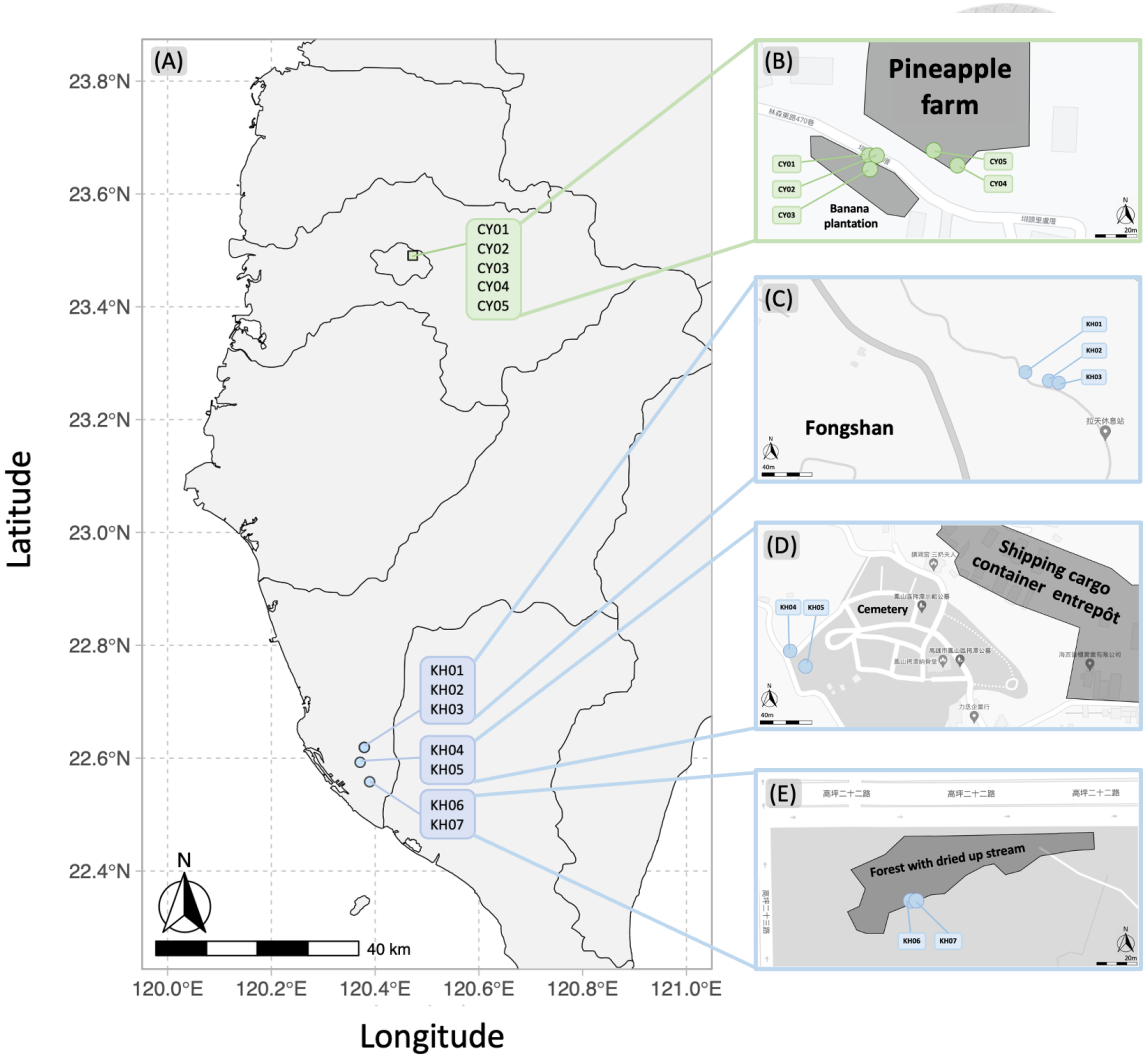


**Figure 1.** Collection sites in Taiwan. (A) Five nests were collected at the same site in Chiayi; seven nests were collected at three different sites in Kaohsiung, including northern Fongshan (three nests), southern Fongshan (two nests) and northern Siaogang (two nests). (B) Five nests were collected from plantations around a pineapple farm in East District, Chiayi. CY01, CY02, CY03 and CY05 were collected from the pseudostems and rhizomes at the base of banana trees; CY04 was excavated from the trunk-base of a small fruit tree at the edge of the pineapple farm. (C) Three nests were collected from a hiking trail at the North of Fongshan District, Kaohsiung, right next to the R.O.C Military Academy. All were excavated from the dirt nest covering the base of three trunks. (D) KH04 was collected from the rhizome of a banana plantation and KH05 from the base of a tree trunk uphill of the Fongshan cemetery at the south of Fongshan District, Kaohsiung. A shipping cargo container entrepôt company situates to the east of the cemetery. (E) KH06 was collected from the base of a dead tree trunk and KH07 from a fallen rotten wood at the Dapingding Tropical Botanical Garden in Siaogang District, Kaohsiung.



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**Figure 2.** Cargo container entrepôt. Close-up entrance of the entrepôt of Ocean Nice Container Service Inc. that situated to the east of the Fongshan cemetery.



doi:10.6342/NTU202403329

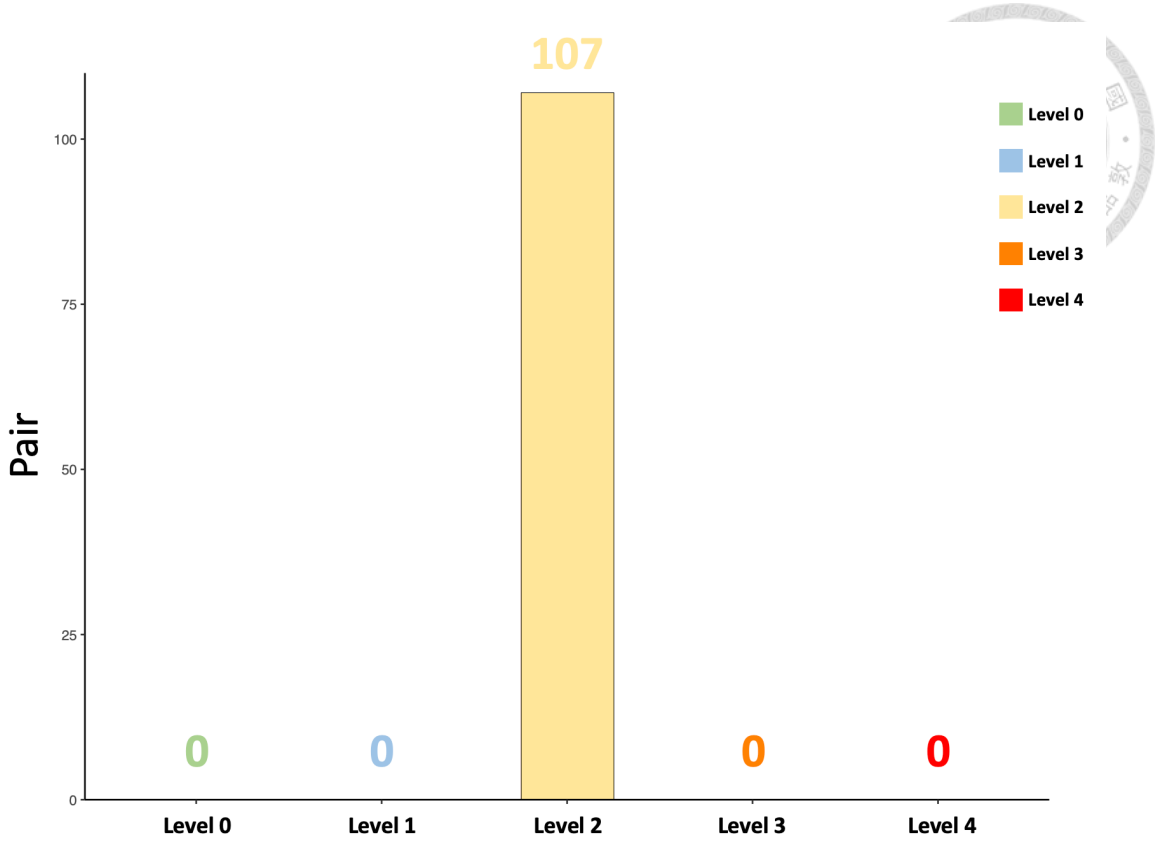


**Figure 3.** The aggression assay result of all 107 pairs. Level 0: Touch and go. Level 1: Mild antennation. Level 2: Rapid antennation. Level 3: Bite and pull. Level 4: Snap and sting.



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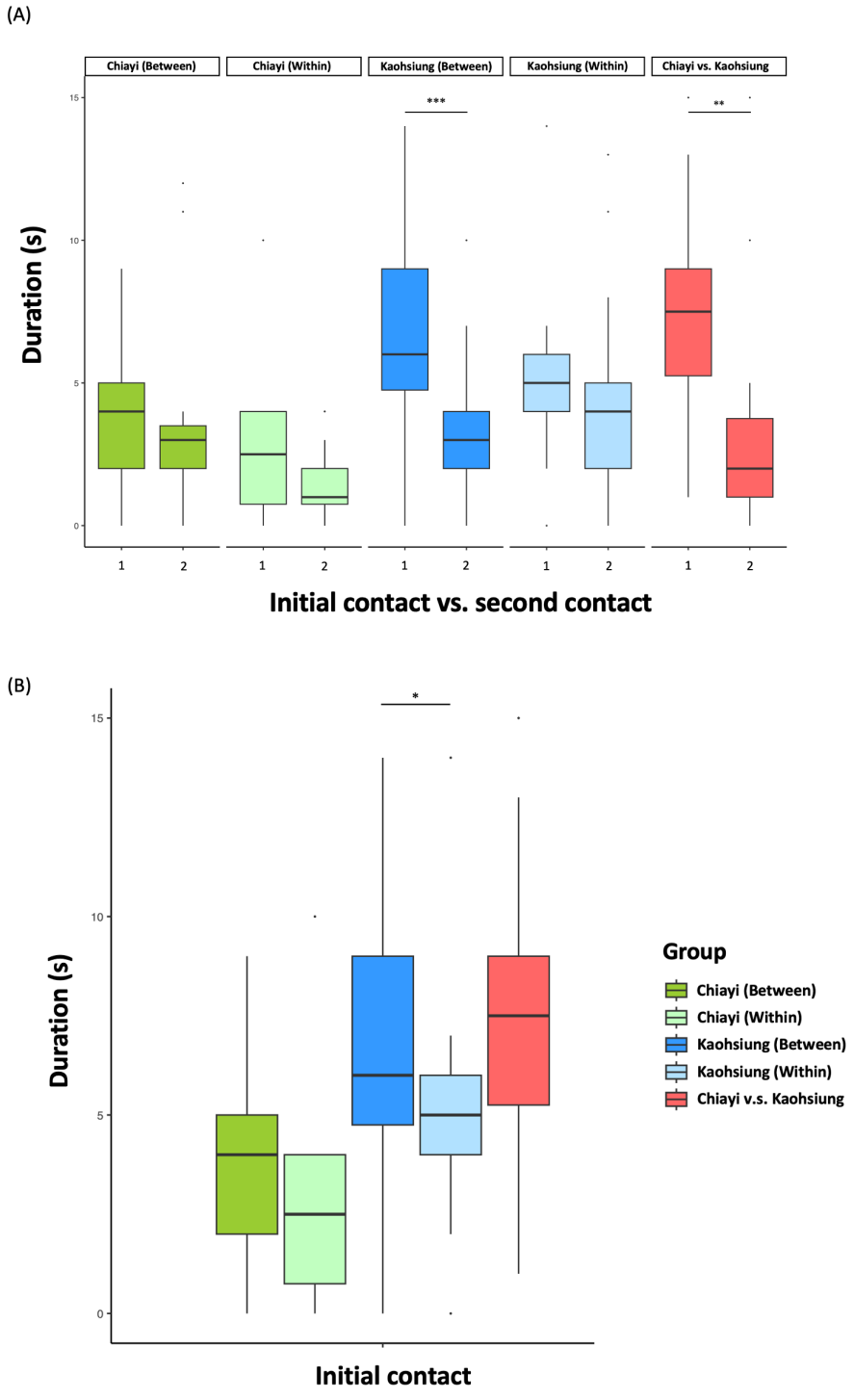


**Figure 4.** Rapid antennation duration analysis. (A) Duration between the initial contact and the second contact among the five groups. (B) Duration of the initial contact among the five groups. Dark green = between-nests of Chiayi; dark blue = between-nests of Kaohsiung; light green = within-nests of Chiayi; light blue = within-nests of Kaohsiung; red = Chiayi vs. Kaohsiung. (GEE: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)



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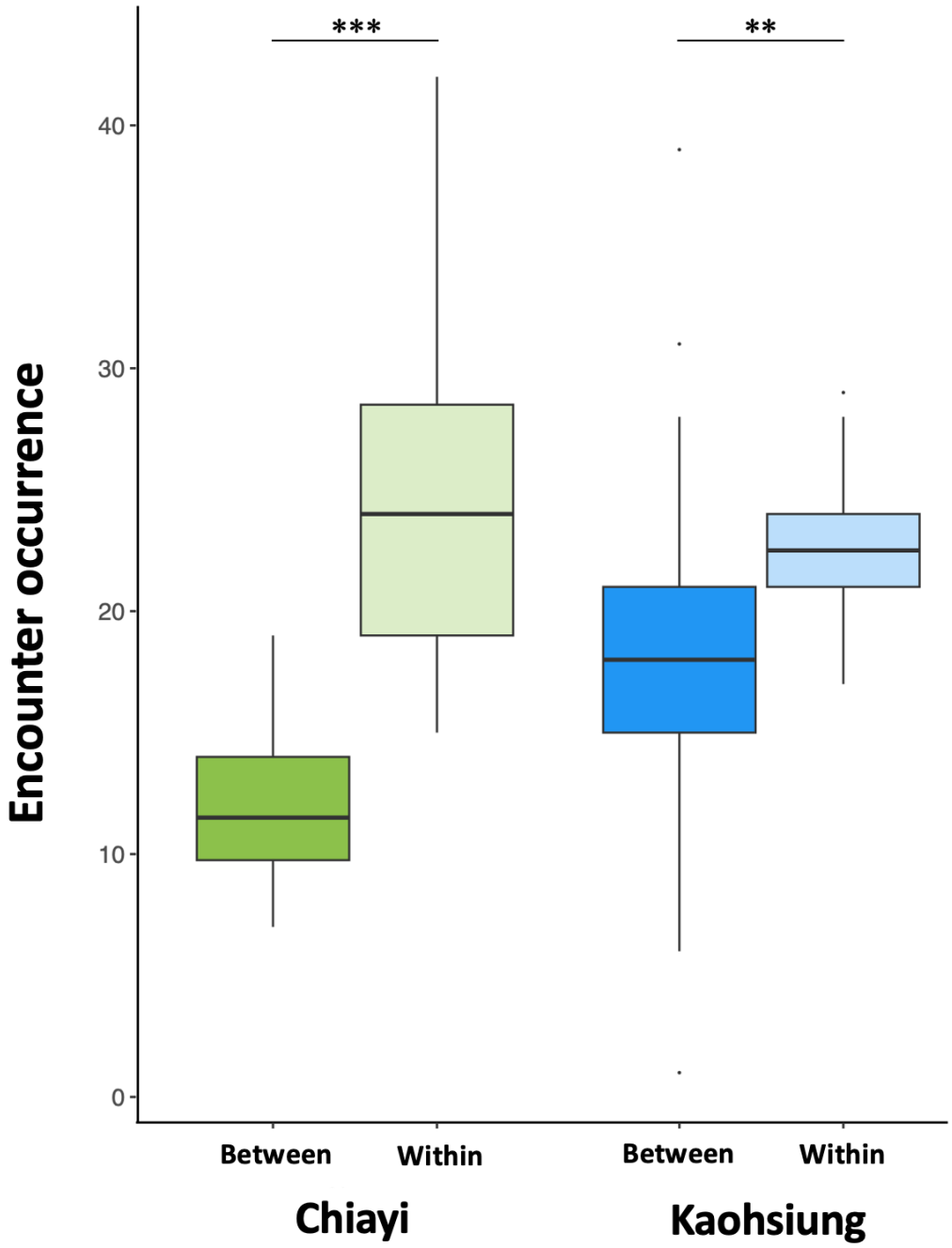


**Figure 5.** Encounter occurrence of within-nests and between-nests from the two populations. (Mann-Whitney U test: $^{**}p < 0.01$, $^{***}p < 0.001$)



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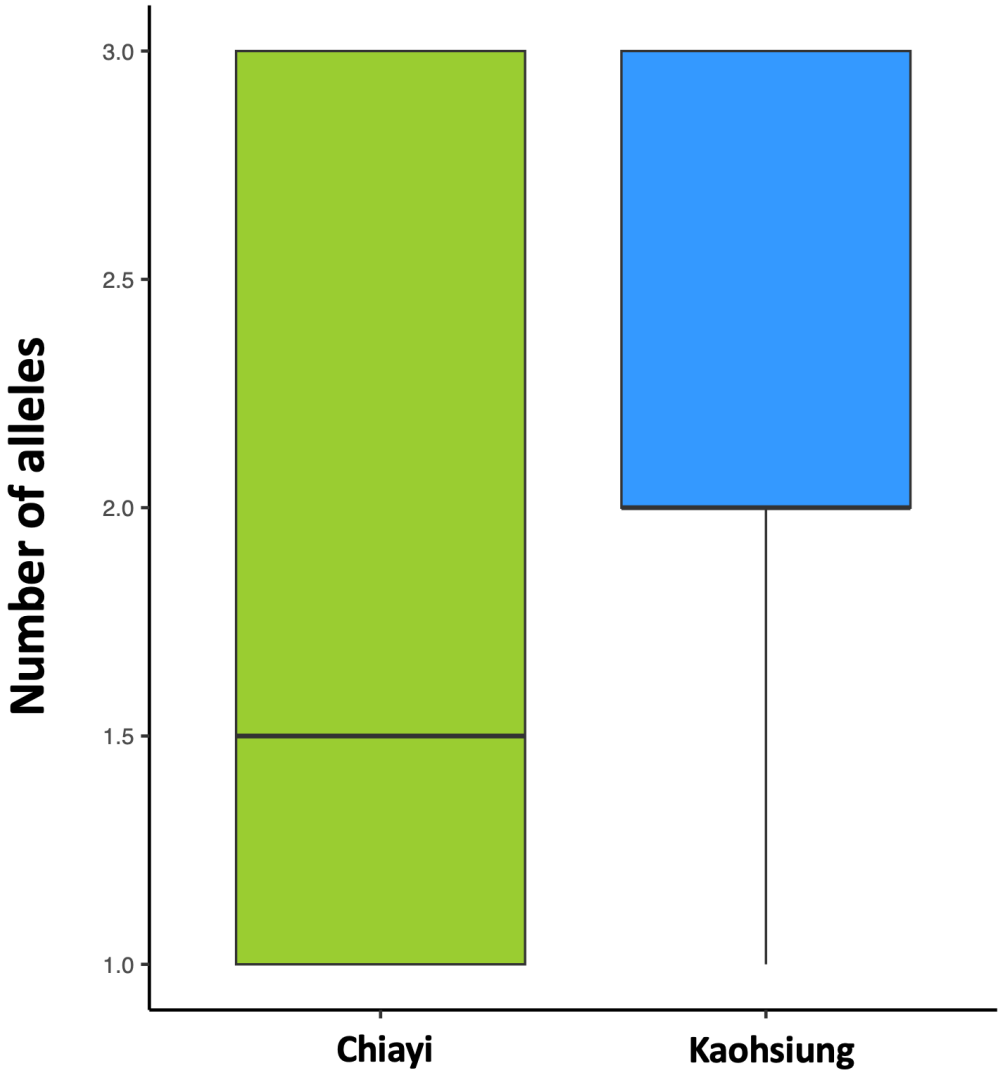


**Figure 6.** Average number of alleles (*Na*) at each locus of the two populations.



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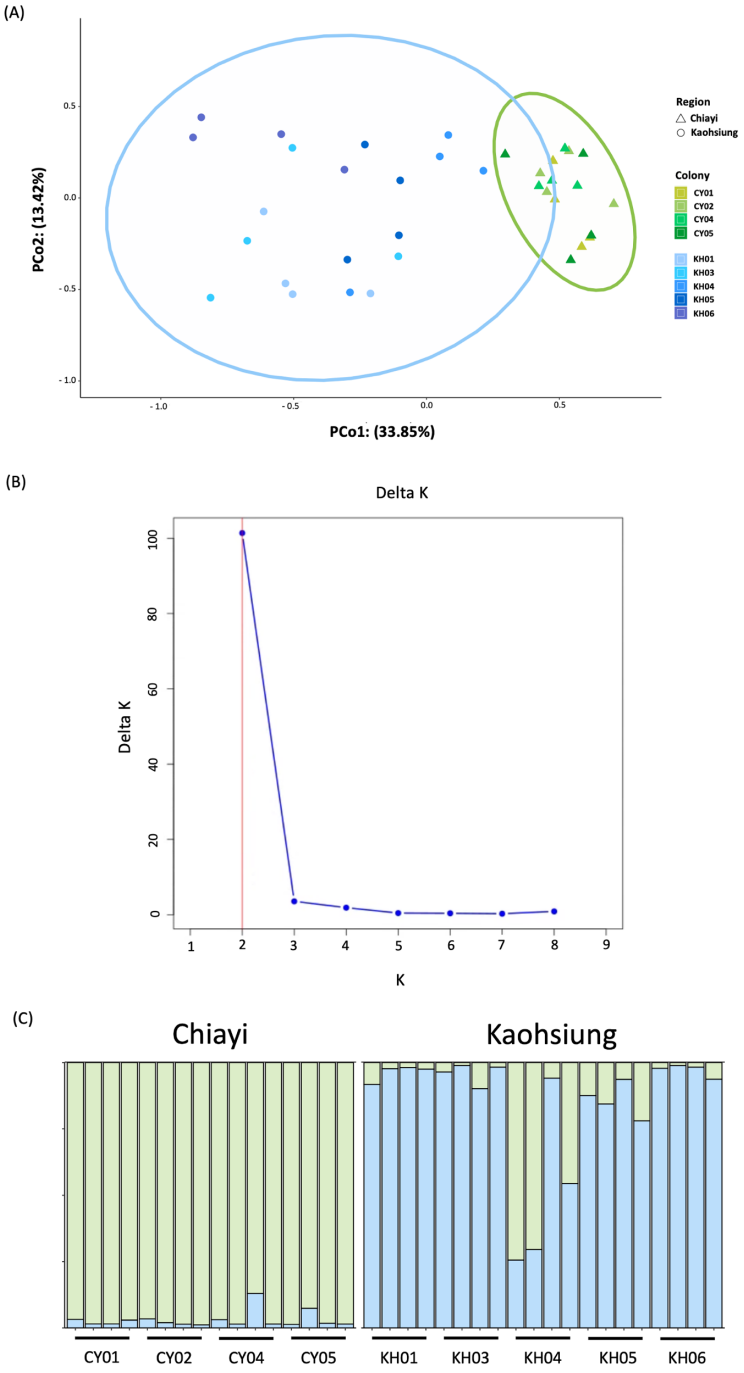


**Figure 7.** Genetic differentiation of the two populations based on 36 worker genotypes among nine nests. (A) Principal coordinate analysis (PCoA) of the genetic distance with 95% confidence ellipse for Chiayi and Kaohsiung population. (B) Summary of the Delta K value among the hypothesized range of K (1-9) generated by StructureSelector. (C) Barplot obtained from STRUCTURE clustering when K = 2. Chiayi nests and Kaohsiung nests are listed below.



doi:10.6342/NTU202403329

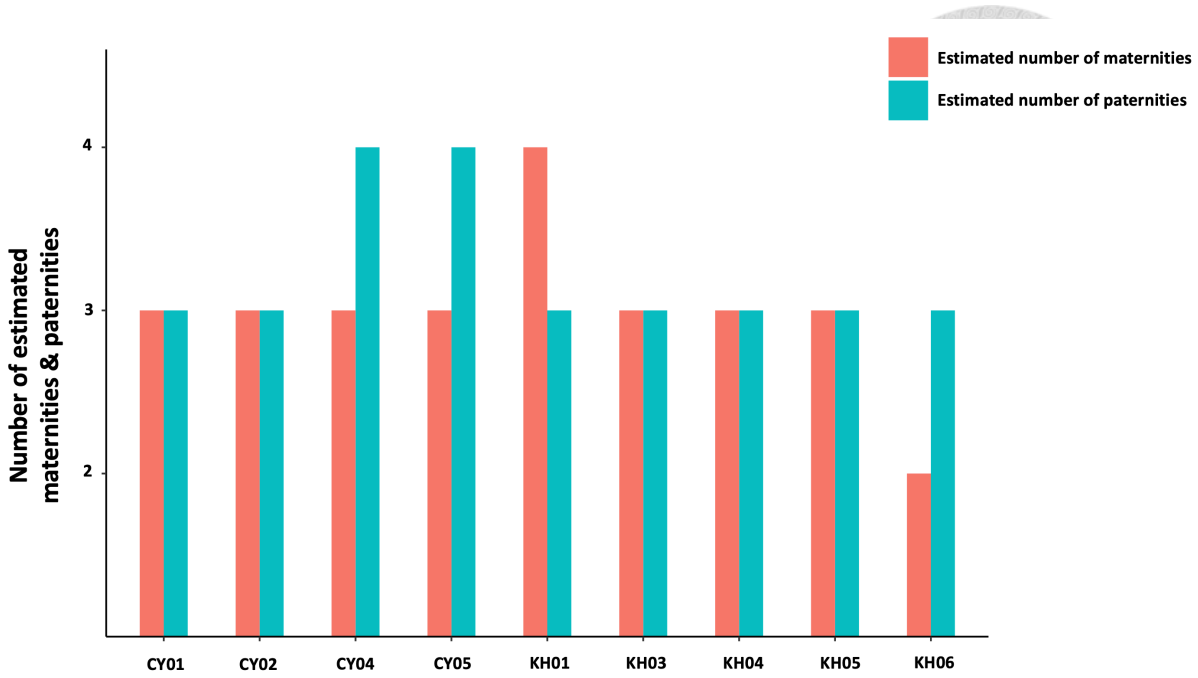


**Figure 8.** Number of the estimated maternal lines and paternal lines. Estimated parentage of genotyped nests computed by COLONY v.2.0.



doi:10.6342/NTU202403329

**Table 1.** Summary of general information of the 12 successfully amplified microsatellite loci isolated from *Odontomachus troglodytes*. (Na-W: number of alleles in workers; Na-Q: number of alleles in queens; Na-M: number of alleles in males; Ta: annealing temperature; Size range: product size of allele.)

| Locus | Repeat motif | Primer sequence (5'-3') | Na-W | Na-Q | Na-M | Ta (°C) | Size range (bp) |
|---|---|---|---|---|---|---|---|
| **OT1** | (CT)^16 | F: AACAGGGTGCGTTCTCTCTC | 2 | 2 | 2 | 58 | 9054-9085 |
| | | R: TAATTTTCGTGGCACGGCAC | | | | | |
| **OT2** | (GTC)^12 | F: CGTAATTAACGCCTCGACGC | 2 | 2 | 2 | 58 | 10235-10270 |
| | | R: AATCCGTCCGCTTATGGTGC | | | | | |
| **OT4** | (GA)^11 | F: ACGAGTCGGTATGTGCCTTC | 2 | 2 | 2 | 58 | 4497-4518 |
| | | R: AACGACACGAAAACAAGCGG | | | | | |
| **OT5** | (CA)^11 | F: ACCGTCACACTGTCGTTACG | 3 | 3 | 3 | 58 | 11607-11628 |
| | | R: GATTCAACGAACCGTCTCGC | | | | | |
| **OT8** | (CT)^11 | F: AGAAAACGCGCCTCTCTCTC | 3 | 3 | 3 | 58 | 54328-54349 |
| | | R: GAGTCGCCGCATTCGAAATG | | | | | |
| **OT11** | (CT)^11 | F: TCTGCGTGTCAGACAGGTTC | 1 | 1 | 1 | 58 | 4220-4241 |
| | | R: GATGAATCAACGCGGCAGAC | | | | | |
| **OT12** | (CA)^14 | F: TTTCCCGTTGGAGTATGGCC | 3 | 3 | 3 | 58 | 6394-6421 |
| | | R: AAGGCCGGAGACTTTTGGAG | | | | | |
| **OT15** | (TG)^11 | F: AGCAGCGTCCCTTAAGTACG | 2 | 2 | 2 | 58 | 12173-12194 |
| | | R: TCCAAGCGCGGTACTAACAG | | | | | |
| **OT16** | (CA)^12 | F: ACACGCACACACACTCTCTC | 2 | 2 | 2 | 58 | 23199-23222 |
| | | R: ATTACGAGCGACGAGAGCTG | | | | | |
| **OT17** | (CT)^12 | F: TTTCGGGCAATGCGTTTCTG | 2 | 2 | 2 | 58 | 13577-13600 |
| | | R: GCTAATACCGACAGGGGAGC | | | | | |
| **OT18** | (CT)^13 | F: CCACGCTCGCTTTCGATAAC | 3 | 3 | 3 | 58 | 10581-10606 |
| | | R: TACAACGACCGCGCTAATGG | | | | | |
| **OT20** | (AG)^15 | F: GGCTGGAGAGGACATGATGG | 2 | 2 | 2 | 58 | 47936-47965 |
| | | R: GGTCTCACCTCCTCTTTCGC | | | | | |



doi:10.6342/NTU202403329

**Table 2.** Genetic diversity across the 12 microsatellite loci in *Odontomacus troglodytes* workers from Chiayi and Kaohsiung. (N: number of samples; *Na*: number of alleles; *I*: Shannon index; *Ho*: Observed heterozygosity; *: non-polymorphic marker.)

| Locus | Chiayi population | | | | Kaohsiung population | | | |
|---|---|---|---|---|---|---|---|---|
| | N | *Na* | *I* | *Ho* | N | *Na* | *I* | *Ho* |
| **OT1** | 16 | 1.000 | 0.000 | 0.000 | 20 | 2.000 | 0.688 | 0.400 |
| **OT2** | 16 | 2.000 | 0.311 | 0.188 | 20 | 2.000 | 0.688 | 0.400 |
| **OT4** | 16 | 1.000 | 0.000 | 0.000 | 20 | 2.000 | 0.682 | 0.150 |
| **OT5** | 16 | 3.000 | 0.875 | 0.313 | 20 | 3.000 | 1.067 | 0.600 |
| **OT8** | 16 | 3.000 | 1.092 | 0.625 | 20 | 3.000 | 0.999 | 0.350 |
| **OT11*** | 16 | 1.000 | 0.000 | 0.000 | 20 | 1.000 | 0.000 | 0.000 |
| **OT12** | 16 | 3.000 | 0.692 | 0.438 | 20 | 3.000 | 0.976 | 0.650 |
| **OT15** | 16 | 1.000 | 0.000 | 0.000 | 20 | 2.000 | 0.377 | 0.150 |
| **OT16** | 16 | 1.000 | 0.000 | 0.000 | 20 | 2.000 | 0.673 | 0.300 |
| **OT17** | 16 | 1.000 | 0.000 | 0.000 | 20 | 2.000 | 0.631 | 0.150 |
| **OT18** | 16 | 3.000 | 0.99 | 0.688 | 20 | 3.000 | 0.633 | 0.400 |
| **OT20** | 16 | 2.000 | 0.234 | 0.125 | 20 | 2.000 | 0.377 | 0.150 |
| **Mean (± SD)** | 16 (0) | 1.833 (0.271) | 0.349 (0.126) | 0.198 (0.075) | 20 (0) | 2.250 (0.179) | 0.649 (0.086) | 0.308 (0.057) |



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**Table 3.** Private alleles summary of the Kaohsiung population.

| Locus | Allele | Frequency |
|---|---|---|
| **OT1** | 114 | 0.406 |
| **OT4** | 115 | 0.311 |
| **OT15** | 221 | 0.123 |
| **OT16** | 212 | 0.387 |
| **OT17** | 219 | 0.302 |



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**Table 4.** Summary table of Analysis of Molecular Variance (AMOVA). (Df: Degree of freedom; MS: Mean square. *: $p < 0.001$)

| **Source** | **Df** | **MS** | **Percentage of variation (%)** | ***F* value** |
|---|---|---|---|---|
| **Among Regions** | 1 | 32.787 | 28.31% | $F_{RT} = 0.283$* |
| **Among Colonies** | 7 | 3.913 | 9.24% | $F_{SR} = 0.129$* |
| **Within Colonies** | 63 | 1.792 | 62.45% | $F_{ST} = 0.375$* |
| **Total** | 71 | | 100% | |



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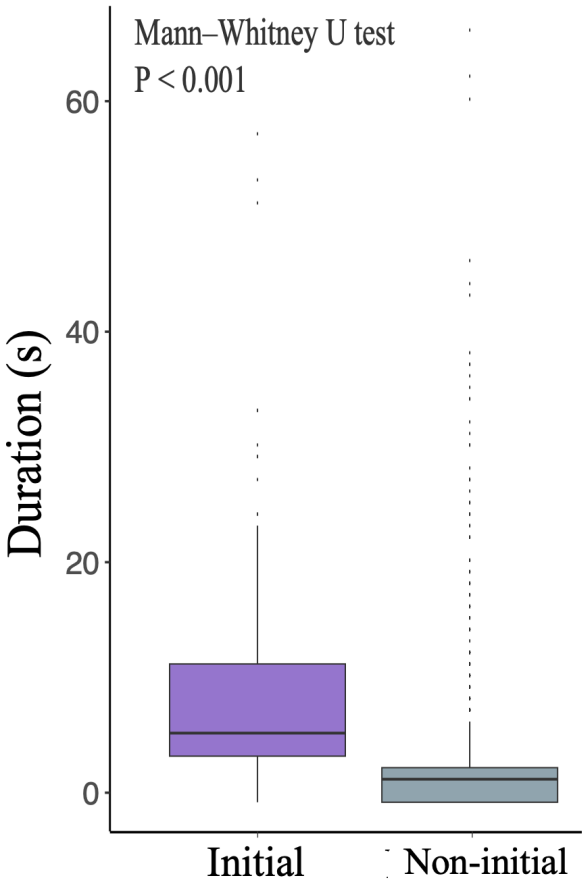
doi:10.6342/NTU202403329

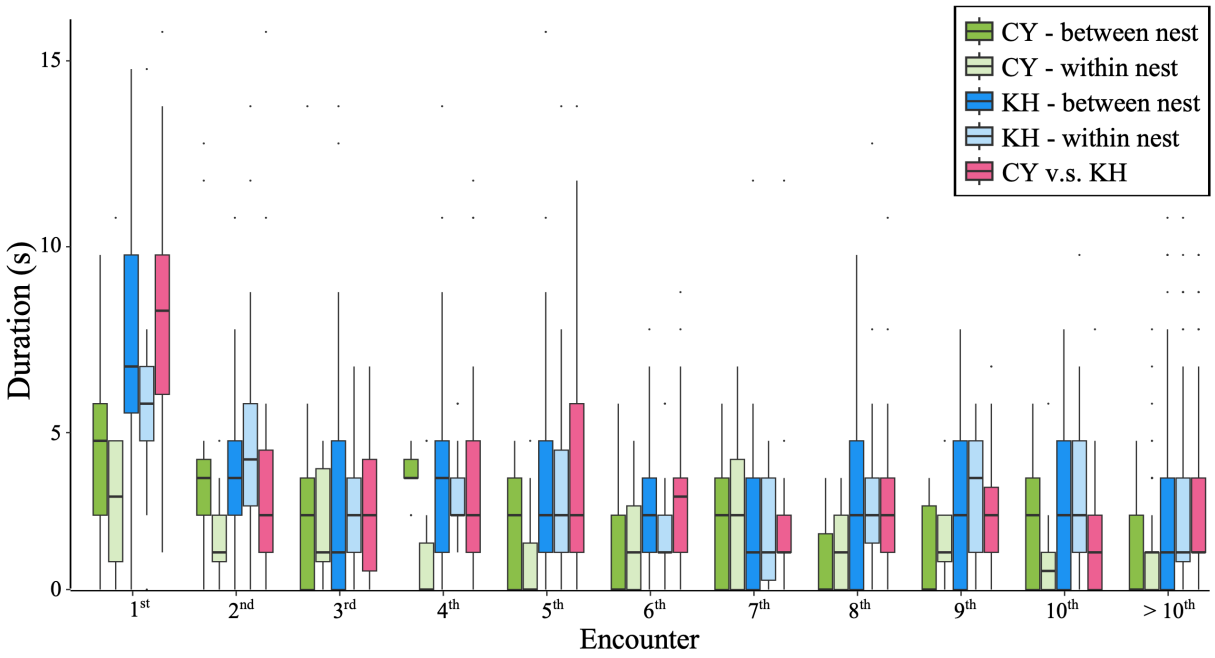
# Appendix




**Appendix Figure 1.** Initial rapid antennation vs. Non-initial rapid antennation box chart. The RA duration data was separated into Initial and non-initial. Comparison between the two was plotted using R with the significancy of duration differentiation calculated applying the Mann-Whitney U test.



doi:10.6342/NTU202403329

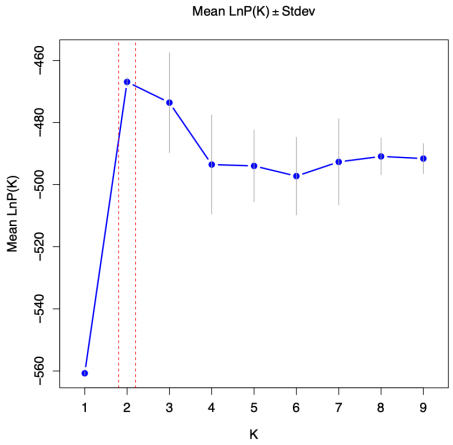



**Appendix Figure 2.** RA duration box chart of each bout (bouts over 10 are grouped together) plotted using R to demonstrate overtime bout duration pattern among all five groups. Dark green: Duration of nests-to-nest in Chiayi; Light green: Duration of within nests in Chiayi; Dark blue: Duration of nests-to-nest in Kaohsiung; Light blue: Duration of within nests in Kaohsiung; Red: Chiayi vs. Kaohsiung.



doi:10.6342/NTU202403329

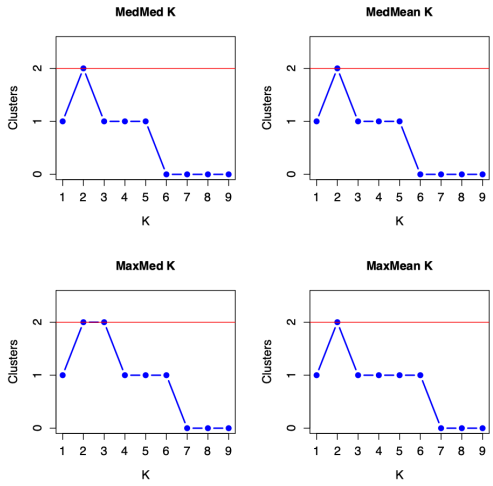
(A)




(B)

| K | Reps | Mean LnP (K) | Stdev LnP (K) | Ln'(K) | \| Ln"(K) \| | Delta K |
|---|---|---|---|---|---|---|
| 1 | 10 | -560.73 | 0.54985 | NA | NA | NA |
| 2 | 10 | -466.92 | 1.33566 | 93.81 | 100.48 | 75.22847 |
| 3 | 10 | -473.59 | 16.06905 | -6.67 | 13.27 | 0.82581 |
| 4 | 10 | -493.53 | 15.91701 | -19.94 | 19.5 | 1.2251 |
| 5 | 10 | -493.97 | 11.56076 | -0.44 | 2.84 | 0.24566 |
| 6 | 10 | -497.25 | 12.50211 | -3.28 | 7.86 | 0.62869 |
| 7 | 10 | -492.67 | 13.85312 | 4.58 | 2.82 | 0.20356 |
| 8 | 10 | -490.91 | 5.86164 | 1.76 | 2.45 | 0.41797 |
| 9 | 10 | -491.6 | 4.81572 | -0.69 | NA | NA |

(C)



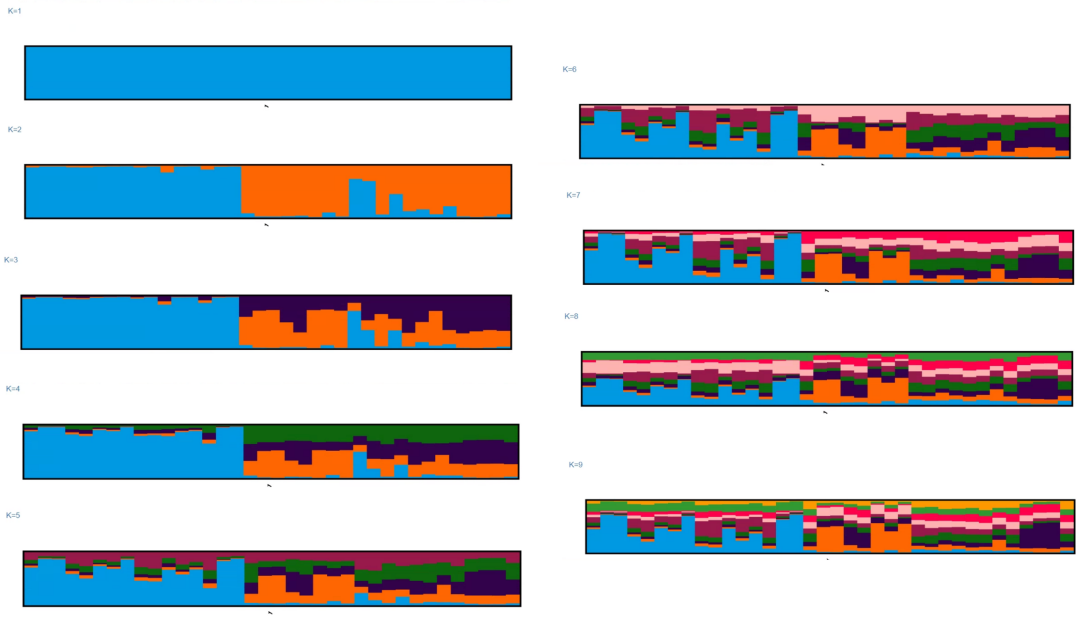

(D)

| K | MedMed | MedMean | MaxMed | MaxMean | Reps |
|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 10 |
| 2 | 2 | 2 | 2 | 2 | 10 |
| 3 | 1 | 1 | 2 | 1 | 10 |
| 4 | 1 | 1 | 1 | 1 | 10 |
| 5 | 1 | 1 | 1 | 1 | 10 |
| 6 | 0 | 0 | 1 | 1 | 10 |
| 7 | 0 | 0 | 0 | 0 | 10 |
| 8 | 0 | 0 | 0 | 0 | 10 |
| 9 | 0 | 0 | 0 | 0 | 10 |
| | MedMedK | MedMeaK | MaxMedK | MaxMeaK | |
| ALL | 2 | 2 | 2 | 2 | |

**Appendix Figure 3.** Summaries of Evanno's and Puechmaille's method on the optimal K. (A) Visualized Mean LnP(K). (B) Summary of Evanno's method. (C) Visualized MedMedK, MedMeanK, MaxMedK and MaxMeanK. (D) Summary of Puechmaille's method.



doi:10.6342/NTU202403329




**Appendix Figure 4.** STRUCTURE clustering from K = 1 to K = 9.



doi:10.6342/NTU202403329

**Appendix Table 1.** Colony IDs, collected counties, latitude and longitude of collected nests. Latitude and longitude data are represented in WGS84 (World Geodetic System 1984).

| Nest | County | Latitude | Longitude |
|---|---|---|---|
| **CY01** | Chiayi | 23.49076 | 120.47226 |
| **CY02** | Chiayi | 23.49076 | 120.4723 |
| **CY03** | Chiayi | 23.49069 | 120.47228 |
| **CY04** | Chiayi | 23.49069 | 120.47276 |
| **CY05** | Chiayi | 23.49076 | 120.47264 |
| **KH01** | Kaohsiung | 22.61907 | 120.37898 |
| **KH02** | Kaohsiung | 22.61907 | 120.37921 |
| **KH03** | Kaohsiung | 22.61898 | 120.37931 |
| **KH04** | Kaohsiung | 22.59272 | 120.37104 |
| **KH05** | Kaohsiung | 22.59255 | 120.37126 |
| **KH06** | Kaohsiung | 22.55818 | 120.38945 |
| **KH07** | Kaohsiung | 22.55820 | 120.38947 |



doi:10.6342/NTU202403329

**Appendix Table 2.** Genotypes of the queens, males and workers from CY01 collected in Chiayi. Q: Queen. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY01Q_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 114 | 114 | 210 | 210 |
| **CY01M_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **CY01M_2** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 114 | 210 | 210 |
| **CY01M_3** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 114 | 210 | 210 |
| **CY01_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 134 | 114 | 116 | 210 | 210 |
| **CY01_2** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 114 | 210 | 210 |
| **CY01_3** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 116 | 210 | 210 |
| **CY01_4** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 114 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY01Q_1** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 214 | 214 |
| **CY01M_1** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 214 |
| **CY01M_2** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 214 | 214 |
| **CY01M_3** | CY | 209 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 214 | 214 |
| **CY01_1** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY01_2** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY01_3** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY01_4** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 212 | 214 |



doi:10.6342/NTU202403329

**Appendix Table 3.** Genotypes of the queens, males and workers from CY02 collected in Chiayi. Q: Queen. s: Sperm extraction of spermatheca. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY02Q_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 114 | 114 | 210 | 210 |
| **CY02Q_1s** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 114 | 210 | 210 |
| **CY02M_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 116 | 116 | 210 | 210 |
| **CY02M_2** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 116 | 116 | 210 | 210 |
| **CY02_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 134 | 112 | 116 | 210 | 210 |
| **CY02_2** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 114 | 116 | 210 | 210 |
| **CY02_3** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 114 | 210 | 210 |
| **CY02_4** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 114 | 114 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY02Q_1** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY02Q_1s** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 214 | 214 |
| **CY02M_1** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 214 | 214 |
| **CY02M_2** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 214 | 214 |
| **CY02_1** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY02_2** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 214 | 214 |
| **CY02_3** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY02_4** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 214 | 214 |



doi:10.6342/NTU202403329

**Appendix Table 4.** Genotypes of the queens, males and workers from CY04 collected in Chiayi. Q: Queen. s: Sperm extraction of spermatheca.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY04Q_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **CY04Q_2** | CY | 112 | 112 | 112 | 115 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |
| **CY04Q_3** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 112 | 112 | 210 | 210 |
| **CY04Q_1s** | CY | 112 | 112 | 112 | 115 | 113 | 113 | 111 | 134 | 114 | 116 | 210 | 210 |
| **CY04Q_2s** | CY | 112 | 112 | 112 | 115 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |
| **CY04Q_3s** | CY | 112 | 112 | 115 | 115 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **CY04_1** | CY | 112 | 112 | 112 | 115 | 113 | 113 | 134 | 134 | 116 | 116 | 210 | 210 |
| **CY04_2** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |
| **CY04_3** | CY | 112 | 112 | 112 | 115 | 113 | 113 | 109 | 109 | 112 | 114 | 210 | 210 |
| **CY04_4** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 112 | 116 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY04Q_1** | CY | 207 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 226 | 226 | 214 | 214 |
| **CY04Q_2** | CY | 207 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY04Q_3** | CY | 209 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 226 | 226 | 212 | 214 |
| **CY04Q_1s** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 226 | 214 | 214 |
| **CY04Q_2s** | CY | 207 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY04Q_3s** | CY | 207 | 207 | 215 | 215 | 214 | 214 | 216 | 216 | 226 | 226 | 214 | 214 |
| **CY04_1** | CY | 209 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 226 | 214 | 214 |
| **CY04_2** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 226 | 226 | 214 | 214 |
| **CY04_3** | CY | 207 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY04_4** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 226 | 214 | 214 |



doi:10.6342/NTU202403329

**Appendix Table 5.** Genotypes of the queens, males and workers from CY05 collected in Chiayi. Q: Queen. s: Sperm extraction of spermatheca. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY05Q_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 116 | 210 | 210 |
| **CY05Q_1s** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 111 | 116 | 116 | 210 | 210 |
| **CY05M_1** | CY | 112 | 112 | 115 | 115 | 113 | 113 | 109 | 109 | 116 | 116 | 210 | 210 |
| **CY05_1** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 134 | 112 | 112 | 210 | 210 |
| **CY05_2** | CY | 112 | 112 | 112 | 115 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |
| **CY05_3** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 116 | 116 | 210 | 210 |
| **CY05_4** | CY | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 114 | 114 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CY05Q_1** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY05Q_1s** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 214 | 214 |
| **CY05M_1** | CY | 209 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 212 | 212 |
| **CY05_1** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 218 | 214 | 214 |
| **CY05_2** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 226 | 212 | 214 |
| **CY05_3** | CY | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |
| **CY05_4** | CY | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 214 | 214 |



doi:10.6342/NTU202403329

**Appendix Table 6.** Genotypes of the queens, males and workers from KH01 collected in Kaohsiung. Q: Queen. s: Sperm extraction of spermatheca. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH01Q_1** | KH | 114 | 114 | 112 | 115 | 115 | 115 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH01Q_1s** | KH | 114 | 114 | 115 | 115 | 113 | 113 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH01M_1** | KH | 112 | 114 | 115 | 115 | 113 | 115 | 111 | 111 | 112 | 116 | 210 | 210 |
| **KH01M_2** | KH | 114 | 114 | 115 | 115 | 115 | 115 | 134 | 134 | 116 | 116 | 210 | 210 |
| **KH01_1** | KH | 114 | 114 | 115 | 115 | 113 | 113 | 109 | 109 | 116 | 116 | 210 | 210 |
| **KH01_2** | KH | 112 | 114 | 112 | 112 | 115 | 115 | 109 | 111 | 116 | 116 | 210 | 210 |
| **KH01_3** | KH | 112 | 114 | 112 | 115 | 115 | 115 | 109 | 109 | 114 | 116 | 210 | 210 |
| **KH01_4** | KH | 114 | 114 | 112 | 115 | 115 | 115 | 111 | 111 | 112 | 114 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH01Q_1** | KH | 211 | 211 | 215 | 215 | 212 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH01Q_1s** | KH | 211 | 211 | 215 | 215 | 212 | 212 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH01M_1** | KH | 207 | 211 | 215 | 215 | 212 | 214 | 216 | 219 | 224 | 226 | 212 | 214 |
| **KH01M_2** | KH | 209 | 211 | 215 | 221 | 212 | 214 | 216 | 219 | 224 | 224 | 212 | 212 |
| **KH01_1** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |
| **KH01_2** | KH | 209 | 211 | 215 | 215 | 212 | 214 | 216 | 219 | 224 | 224 | 212 | 212 |
| **KH01_3** | KH | 207 | 209 | 215 | 215 | 212 | 214 | 219 | 219 | 224 | 226 | 212 | 212 |
| **KH01_4** | KH | 211 | 211 | 215 | 215 | 212 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |



doi:10.6342/NTU202403329

**Appendix Table 7.** Genotypes of the queens, males and workers from KH03 collected in Kaohsiung. Q: Queen. s: Sperm extraction of spermatheca.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH03Q_1** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 111 | 111 | 114 | 116 | 210 | 210 |
| **KH03Q_2** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 109 | 134 | 116 | 116 | 210 | 210 |
| **KH03Q_1s** | KH | 112 | 112 | 112 | 112 | 115 | 115 | 109 | 109 | 116 | 116 | 210 | 210 |
| **KH03Q_2s** | KH | 114 | 114 | 115 | 115 | 113 | 113 | 134 | 134 | 116 | 116 | 210 | 210 |
| **KH03_1** | KH | 112 | 114 | 115 | 115 | 113 | 113 | 111 | 134 | 112 | 116 | 210 | 210 |
| **KH03_2** | KH | 114 | 114 | 112 | 112 | 115 | 115 | 109 | 111 | 116 | 116 | 210 | 210 |
| **KH03_3** | KH | 112 | 112 | 112 | 112 | 113 | 115 | 109 | 134 | 114 | 114 | 210 | 210 |
| **KH03_4** | KH | 112 | 114 | 112 | 112 | 115 | 115 | 111 | 134 | 114 | 114 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH03Q_1** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 111 | 111 | 114 | 116 | 210 | 210 |
| **KH03Q_2** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 109 | 134 | 116 | 116 | 210 | 210 |
| **KH03Q_1s** | KH | 112 | 112 | 112 | 112 | 115 | 115 | 109 | 109 | 116 | 116 | 210 | 210 |
| **KH03Q_2s** | KH | 114 | 114 | 115 | 115 | 113 | 113 | 134 | 134 | 116 | 116 | 210 | 210 |
| **KH03_1** | KH | 112 | 114 | 115 | 115 | 113 | 113 | 111 | 134 | 112 | 116 | 210 | 210 |
| **KH03_2** | KH | 114 | 114 | 112 | 112 | 115 | 115 | 109 | 111 | 116 | 116 | 210 | 210 |
| **KH03_3** | KH | 112 | 112 | 112 | 112 | 113 | 115 | 109 | 134 | 114 | 114 | 210 | 210 |
| **KH03_4** | KH | 112 | 114 | 112 | 112 | 115 | 115 | 111 | 134 | 114 | 114 | 210 | 210 |



doi:10.6342/NTU202403329

**Appendix Table 8.** Genotypes of the queens, males and workers from KH04 collected in Kaohsiung. Q: Queen. s: Sperm extraction of spermatheca. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH04Q_1** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 111 | 111 | 112 | 116 | 210 | 210 |
| **KH04Q_2** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |
| **KH04Q_3** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **KH04Q_1s** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 116 | 116 | 210 | 210 |
| **KH04Q_2s** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 134 | 112 | 116 | 210 | 210 |
| **KH04Q_3s** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **KH04M_1** | KH | 112 | 114 | 112 | 112 | 113 | 113 | 111 | 111 | 112 | 116 | 210 | 210 |
| **KH04M_2** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |
| **KH04_1** | KH | 112 | 112 | 112 | 115 | 113 | 113 | 111 | 134 | 112 | 116 | 210 | 210 |
| **KH04_2** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **KH04_3** | KH | 114 | 114 | 112 | 115 | 113 | 113 | 109 | 134 | 116 | 116 | 210 | 210 |
| **KH04_4** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 134 | 112 | 112 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH04Q_1** | KH | 209 | 209 | 215 | 221 | 214 | 214 | 216 | 219 | 218 | 226 | 212 | 214 |
| **KH04Q_2** | KH | 207 | 211 | 215 | 221 | 212 | 214 | 216 | 219 | 224 | 226 | 212 | 214 |
| **KH04Q_3** | KH | 211 | 211 | 215 | 215 | 212 | 214 | 216 | 216 | 224 | 224 | 212 | 214 |
| **KH04Q_1s** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 219 | 219 | 224 | 224 | 212 | 212 |
| **KH04Q_2s** | KH | 207 | 209 | 215 | 221 | 214 | 214 | 216 | 216 | 218 | 218 | 212 | 214 |
| **KH04Q_3s** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH04M_1** | KH | 209 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |
| **KH04M_2** | KH | 207 | 211 | 215 | 221 | 214 | 214 | 216 | 219 | 224 | 226 | 212 | 212 |
| **KH04_1** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 219 | 224 | 226 | 214 | 214 |
| **KH04_2** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH04_3** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 219 | 224 | 224 | 212 | 212 |
| **KH04_4** | KH | 211 | 211 | 215 | 221 | 212 | 214 | 216 | 216 | 218 | 224 | 212 | 214 |



doi:10.6342/NTU202403329

**Appendix Table 9.** Genotypes of the queens, males and workers from KH05 collected in Kaohsiung. Q: Queen. s: Sperm extraction of spermatheca. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH05Q_1** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 111 | 134 | 112 | 116 | 210 | 210 |
| **KH05Q_2** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 109 | 134 | 112 | 116 | 210 | 210 |
| **KH05Q_3** | KH | 112 | 114 | 112 | 112 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **KH05Q_1s** | KH | 114 | 114 | 112 | 112 | 113 | 113 | 109 | 109 | 116 | 116 | 210 | 210 |
| **KH05Q_2s** | KH | 114 | 114 | 112 | 112 | 113 | 113 | 109 | 134 | 116 | 116 | 210 | 210 |
| **KH05Q_3s** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 109 | 109 | 112 | 112 | 210 | 210 |
| **KH05M_1** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 109 | 111 | 112 | 116 | 210 | 210 |
| **KH05_1** | KH | 112 | 112 | 115 | 115 | 113 | 113 | 111 | 134 | 112 | 112 | 210 | 210 |
| **KH05_2** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 109 | 111 | 112 | 116 | 210 | 210 |
| **KH05_3** | KH | 112 | 114 | 112 | 115 | 113 | 115 | 109 | 111 | 112 | 116 | 210 | 210 |
| **KH05_4** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 111 | 112 | 112 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH05Q_1** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 226 | 212 | 212 |
| **KH05Q_2** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |
| **KH05Q_3** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |
| **KH05Q_1s** | KH | 209 | 209 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH05Q_2s** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |
| **KH05Q_3s** | KH | 211 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH05M_1** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 219 | 224 | 224 | 212 | 212 |
| **KH05_1** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH05_2** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 218 | 224 | 212 | 212 |
| **KH05_3** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 219 | 219 | 224 | 224 | 212 | 214 |
| **KH05_4** | KH | 209 | 211 | 215 | 215 | 214 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |



doi:10.6342/NTU202403329

**Appendix Table 10.** Genotypes of the queens, males and workers from KH06 collected in Kaohsiung. Q: Queen. s: Sperm extraction of spermatheca. M: Male.

| Sample | Pop | OT1 | | OT2 | | OT4 | | OT5 | | OT8 | | OT11 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH06Q_1** | KH | 114 | 114 | 115 | 115 | 113 | 113 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH06Q_2** | KH | 112 | 112 | 115 | 115 | 113 | 113 | 111 | 134 | 112 | 112 | 210 | 210 |
| **KH06Q_3** | KH | 112 | 112 | 112 | 115 | 113 | 115 | 111 | 134 | 116 | 116 | 210 | 210 |
| **KH06Q_1s** | KH | 112 | 112 | 112 | 112 | 115 | 115 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH06Q_2s** | KH | 112 | 114 | 112 | 115 | 115 | 115 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH06Q_3s** | KH | 112 | 112 | 112 | 115 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **KH06M_1** | KH | 112 | 112 | 115 | 115 | 115 | 115 | 111 | 134 | 112 | 116 | 210 | 210 |
| **KH06M_2** | KH | 112 | 112 | 112 | 112 | 113 | 113 | 111 | 111 | 116 | 116 | 210 | 210 |
| **KH06M_3** | KH | 112 | 112 | 112 | 112 | 115 | 115 | 134 | 134 | 114 | 114 | 210 | 210 |
| **KH06M_4** | KH | 112 | 112 | 115 | 115 | 113 | 113 | 134 | 134 | 112 | 112 | 210 | 210 |
| **KH06_1** | KH | 112 | 114 | 112 | 115 | 113 | 115 | 111 | 134 | 112 | 112 | 210 | 210 |
| **KH06_2** | KH | 112 | 112 | 115 | 115 | 115 | 115 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH06_3** | KH | 114 | 114 | 115 | 115 | 115 | 115 | 111 | 111 | 112 | 112 | 210 | 210 |
| **KH06_4** | KH | 112 | 114 | 112 | 115 | 113 | 113 | 134 | 134 | 112 | 116 | 210 | 210 |

| Sample | Pop | OT12 | | OT15 | | OT16 | | OT17 | | OT18 | | OT20 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **KH06Q_1** | KH | 211 | 211 | 215 | 215 | 212 | 212 | 216 | 219 | 224 | 224 | 212 | 214 |
| **KH06Q_2** | KH | 207 | 209 | 215 | 215 | 212 | 212 | 216 | 219 | 218 | 224 | 212 | 212 |
| **KH06Q_3** | KH | 207 | 211 | 215 | 221 | 212 | 214 | 216 | 216 | 224 | 226 | 212 | 214 |
| **KH06Q_1s** | KH | 207 | 207 | 221 | 221 | 212 | 212 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH06Q_2s** | KH | 207 | 207 | 215 | 215 | 212 | 214 | 216 | 219 | 224 | 224 | 212 | 212 |
| **KH06Q_3s** | KH | 207 | 207 | 215 | 215 | 212 | 212 | 216 | 219 | 224 | 224 | 212 | 214 |
| **KH06M_1** | KH | 211 | 211 | 215 | 215 | 212 | 212 | 216 | 216 | 224 | 224 | 212 | 214 |
| **KH06M_2** | KH | 209 | 209 | 215 | 215 | 214 | 214 | 219 | 219 | 224 | 224 | 212 | 212 |
| **KH06M_3** | KH | 211 | 211 | 215 | 215 | 212 | 212 | 216 | 216 | 226 | 226 | 212 | 212 |
| **KH06M_4** | KH | 207 | 209 | 215 | 215 | 212 | 212 | 216 | 219 | 224 | 224 | 212 | 214 |
| **KH06_1** | KH | 207 | 207 | 215 | 215 | 212 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH06_2** | KH | 207 | 211 | 215 | 221 | 212 | 212 | 216 | 216 | 224 | 224 | 212 | 212 |
| **KH06_3** | KH | 207 | 211 | 215 | 215 | 212 | 212 | 216 | 216 | 224 | 224 | 212 | 214 |
| **KH06_4** | KH | 207 | 209 | 215 | 215 | 212 | 214 | 216 | 216 | 224 | 224 | 212 | 212 |



doi:10.6342/NTU202403329

**Appendix Table 11.** Information on microsatellite markers developed. Empty slots in the *He* and *Ho* columns are NA values of markers that were not successfully amplified and were consequently not used in later analysis. (*He*: Expected heterozygosity; *Ho*: Observed heterozygosity.)

| Marker | Repeat motif | Primer sequence (5'-3') | *He* | *Ho* |
|---|---|---|---|---|
| **OT1** | (CT)^16 | F: AACAGGGTGCGTTCTCTCTC | 0.241 | 0.179 |
| | | R: TAATTTTCGTGGCACGGCAC | | |
| **OT2** | (GTC)^12 | F: CGTAATTAACGCCTCGACGC | 0.373 | 0.270 |
| | | R: AATCCGTCCGCTTATGGTGC | | |
| **OT3** | (AG)^13 | F: CTTTGTAACCCCGACGAAGC | — | — |
| | | R: TCTCCCTCTCTCCCTCTCAC | | |
| **OT4** | (GA)^11 | F: ACGAGTCGGTATGTGCCTTC | 0.214 | 0.047 |
| | | R: AACGACACGAAAACAAGCGG | | |
| **OT5** | (CA)^11 | F: ACCGTCACACTGTCGTTACG | 0.603 | 0.365 |
| | | R: GATTCAACGAACCGTCTCGC | | |
| **OT6** | (AG)^14 | F: GAGAGAAACGGGAAAGGGGG | — | — |
| | | R: TACCGACTGCTACGTTGCTC | | |
| **OT7** | (CT)^14 | F: AACCTGCAGGGAGACGTTAC | — | — |
| | | R: GAGAGAGAGCGAGAGAGAGAAG | | |
| **OT8** | (CT)^11 | F: AGAAAACGCGCCTCTCTCTC | 0.616 | 0.382 |
| | | R: GAGTCGCCGCATTCGAAATG | | |
| **OT9** | (AG)^12 | F: AACGAGACAAAGTAGGGAGTGG | — | — |
| | | R: TCTCTCTCTTTCTCCCTCCCC | | |
| **OT10** | (AGTG)^11 | F: ACGTGAGTGAGTGAGTGAGTG | — | — |
| | | R: ACACACACACACACACACAC | | |
| **OT11** | (CT)^11 | F: TCTGCGTGTCAGACAGGTTC | 0.000 | 0.000 |
| | | R: GATGAATCAACGCGGCAGAC | | |
| **OT12** | (CA)^14 | F: TTTCCCGTTGGAGTATGGCC | 0.549 | 0.418 |
| | | R: AAGGCCGGAGACTTTTGGAG | | |
| **OT13** | (AT)^12 | F: GTGAAACTGCCGTCGTTCTG | — | — |
| | | R: GTGAAACTGCCGTCGTTCTG | | |
| **OT14** | (TC)^13 | F: TACGTCACACCAGCTGGAAC | — | — |
| | | R: GAATCGATTCGACCTCCGGG | | |



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| Marker | Repeat motif | Primer sequence (5'-3') | *He* | *Ho* |
|---|---|---|---|---|
| **OT15** | (TG)^11 | F: AGCAGCGTCCCTTAAGTACG | 0.085 | 0.108 |
| | | R: TCCAAGCGCGGTACTAACAG | | |
| **OT16** | (CA)^12 | F: ACACGCACACACACTCTCTC | 0.237 | 0.142 |
| | | R: ATTACGAGCGACGAGAGCTG | | |
| **OT17** | (CT)^12 | F: TTTCGGGCAATGCGTTTCTG | 0.211 | 0.151 |
| | | R: GCTAATACCGACAGGGGAGC | | |
| **OT18** | (CT)^13 | F: CCACGCTCGCTTTCGATAAC | 0.502 | 0.437 |
| | | R: TACAACGACCGCGCTAATGG | | |
| **OT19** | (TA)^12 | F: AATTTCCTCCCGGATTTCTCTG | — | — |
| | | R: TGACTGACAGCTCGTACGAC | | |
| **OT20** | (AG)^15 | F: GGCTGGAGAGGACATGATGG | 0.204 | 0.183 |
| | | R: GGTCTCACCTCCTCTTTCGC | | |



doi:10.6342/NTU202403329

**Appendix Table 12.** Colony affiliation result among colonies collected in Chiayi. *P*-values obtained after 1000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is: 0.008333. Upper-right: $F_{ST}$ value; Lower-left: *P*-value.

| | **CY01** | **CY02** | **CY04** | **CY05** |
|---|---|---|---|---|
| **CY01** | 0 | -0.0443 | 0.0617 | -0.0839 |
| **CY02** | 0.822 | 0 | 0.0476 | -0.0021 |
| **CY04** | 0.114 | 0.158 | 0 | -0.0354 |
| **CY05** | 0.913 | 0.61 | 0.736 | 0 |



doi:10.6342/NTU202403329

**Appendix Table 13.** Colony affiliation result among nests genotyped in northern Fongshan, Kaohsiung. *P*-values obtained after 1000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is: 0.050000. Upper-right: $F_{ST}$ value; Lower-left: *P*-value.

| | **KH01** | **KH03** |
|---|---|---|
| **KH01** | 0 | -0.0068 |
| **KH03** | 0.413 | 0 |



doi:10.6342/NTU202403329

**Appendix Table 14.** Colony affiliation result among nests genotyped in southern Fongshan, Kaohsiung. *P*-values obtained after 1000 permutations. Indicative adjusted nominal level (5%) for multiple comparisons is: 0.050000. Upper-right: $F_{ST}$ value; Lower-left: *P*-value.

| | **KH04** | **KH05** |
|---|---|---|
| **KH04** | 0 | 0.0336 |
| **KH05** | 0.296 | 0 |



doi:10.6342/NTU202403329

**Appendix Table 15.** Pairwise genetic differentiation between the two studied populations of *Odontomachus troglodytes*. Significancy was tested using 999 permutations for pairwise $F_{ST}$, *Dest*, and $G''_{ST}$ (*: $p < 0.05$).

| Population pair | Mean $F_{ST}$ | ± SD | Mean *Dest* | ± SD | Mean $G''_{ST}$ | ± SD |
|---|---|---|---|---|---|---|
| **Chiayi - Kaohsiung** | 0.222* | 0.055 | 0.213* | 0.017 | 0.420* | 0.067 |



doi:10.6342/NTU202403329

**Appendix Table 16.** Estimated paternal / maternal line and contribution of the Chiayi population. Data order – Section 1: Nest ID - Est. number of parentage line - Parentage ID; Section 2: Parentage ID - Est. number of nest contributed - Nest ID; *: Potential paternal line; #: Potential maternal line)

**Section 1**

| **Estimate "paternal" line / colony：8** | **Estimate "maternal" line / colony：6** |
|---|---|
| CY01: 3 (CY05Q_1s/CY02Q_1s/*1) | CY01: 3 (CY02Q_1/CY05Q_1/#1) |
| CY02: 3 (CY05Q_1s/*2/*1) | CY02: 3 (CY02Q_1/CY04Q_2/#2) |
| CY04: 4 (*3/*4/*5/*6) | CY04: 3 (CY04Q_2/#2/#3) |
| CY05: 4 (CY02Q_1s/*4/*5/*6) | CY05: 3 (CY05Q_1/#1/#2) |

**Section 2**

| **Paternal line distribution** | | **Maternal line distribution** | |
|---|---|---|---|
| CY02Q_1s: | 2 (CY01/CY05) | CY02Q_1: | 2 (CY01/CY02) |
| CY05Q_1s: | 2 (CY01/CY02) | CY04Q_2: | 2 (CY02/CY04) |
| *1: | 2 (CY01/CY02) | CY05Q_1: | 2 (CY01/CY05) |
| *2: | 1 (CY02) | #1: | 2 (CY01/CY05) |
| *3: | 1 (CY04) | #2: | 3 (CY02/CY04/CY05) |
| *4: | 2 (CY04/CY05) | #3: | 1 (CY04) |
| *5: | 2 (CY04/CY05) | | |
| *6: | 2 (CY04/CY05) | | |
| **Mean (±SD)** | | | |
| 3.5 (0.7) | | 3 (0) | |



doi:10.6342/NTU202403329

**Appendix Table 17.** Estimated paternal / maternal line and contribution of the Kaohsiung population. Data order – Section 1: Nest ID - Est. number of parentage line - Parentage ID; Section 2: Parentage ID - Est. number of nest contributed - Nest ID; *: Potential paternal line; #: Potential maternal line)

**Section 1**

| **Estimate "paternal" line / colony：8** | **Estimate "maternal" line / colony：11** |
|---|---|
| KH01: 3 (KH01Q_1s/KH03Q_1s/KH05Q_1s) | KH01: 4 (KH05Q_2/#1/#2/#3) |
| KH03: 3 (KH01Q_1s/KH03Q_1s/KH04Q_1s) | KH03: 3 (#2/#4/#5) |
| KH04: 3 (KH03Q_2s/KH04Q_3s/KH05Q_3s) | KH04: 3 (KH04Q_2/KH05Q_2/#6) |
| KH05: 3 (KH04Q_1s/KH04Q_3s/KH05Q_3s) | KH05: 3 (KH05Q_2/#7/#8) |
| KH06: 3 (KH01Q_1s/KH04Q_3s/KH06Q_1s) | KH06: 2 (#4/#9) |

**Section 2**

| **Paternal line distribution** | **Maternal line distribution** |
|---|---|
| KH01Q_1s: 3 (KH01/KH03/KH06) | KH04Q_2: 1 (KH04) |
| KH03Q_1s: 2 (KH01/KH03) | KH05Q_2: 3 (KH01/KH04/KH05) |
| KH03Q_2s: 1 (KH04) | #1: 1 (KH01) |
| KH04Q_1s: 2 (KH03/KH05) | #2: 2 (KH01/KH03) |
| KH04Q_3s: 3 (KH04/KH05/KH06) | #3: 1 (KH01) |
| KH05Q_1s: 1 (KH01) | #4: 2 (KH03/KH06) |
| KH05Q_3s: 2 (KH04/KH05) | #5: 1 (KH03) |
| KH06Q_1s: 1 (KH06) | #6: 1 (KH04) |
| | #7: 1 (KH05) |
| | #8: 1 (KH05) |
| | #9: 1 (KH06) |
| **Mean (±SD)** | |
| 3 (0) | 3 (1.41) |



doi:10.6342/NTU202403329

**Appendix Table 18.** Pairwise Half-siblings and full-siblings probability summary of the Chiayi population. Data order – Half-sibling: Worker ID1 - Worker ID2 - Paternal/Maternal - Probability; Full-siblings: Worker ID1 - Worker ID2 - Probability.

| Half-siblings | Full-siblings |
|---|---|
| CY01_1, CY01_2, MaternalSib, 0.993 | CY01_1, CY02_2, 0.004 |
| CY01_1, CY02_1, PaternalSib, 1.000 | CY01_2, CY02_4, 0.006 |
| CY01_1, CY02_2, MaternalSib, 0.132 | CY01_2, CY05_4, 0.174 |
| CY01_1, CY02_3, MaternalSib, 0.048 | CY01_3, CY05_4, 0.784 |
| CY01_1, CY02_4, MaternalSib, 0.947 | CY01_4, CY02_3, 0.008 |
| CY01_1, CY05_4, MaternalSib, 0.004 | CY04_2, CY05_2, 0.003 |
| CY01_2, CY01_3, PaternalSib, 0.993 | |
| CY01_2, CY01_3, MaternalSib, 0.005 | |
| CY01_2, CY02_2, MaternalSib, 0.003 | |
| CY01_2, CY02_3, MaternalSib, 0.001 | |
| CY01_2, CY02_4, PaternalSib, 0.005 | |
| CY01_2, CY02_4, MaternalSib, 0.943 | |
| CY01_2, CY04_3, PaternalSib, 0.227 | |
| CY01_2, CY05_3, MaternalSib, 0.005 | |
| CY01_2, CY05_4, PaternalSib, 0.819 | |
| CY01_2, CY05_4, MaternalSib, 0.001 | |
| CY01_3, CY02_2, PaternalSib, 0.001 | |
| CY01_3, CY02_4, PaternalSib, 0.004 | |
| CY01_3, CY02_4, MaternalSib, 0.007 | |
| CY01_3, CY04_3, PaternalSib, 0.225 | |
| CY01_3, CY05_3, MaternalSib, 1.000 | |
| CY01_3, CY05_4, PaternalSib, 0.216 | |
| CY01_4, CY02_1, MaternalSib, 0.216 | |
| CY01_4, CY02_3, PaternalSib, 0.120 | |
| CY01_4, CY02_3, MaternalSib, 0.024 | |
| CY01_4, CY02_4, PaternalSib, 0.307 | |
| CY01_4, CY02_4, MaternalSib, 0.009 | |
| CY01_4, CY04_2, MaternalSib, 0.012 | |
| CY01_4, CY04_4, MaternalSib, 0.014 | |



doi:10.6342/NTU202403329

| Half-siblings | Full-siblings |
|---|---|
| CY01_4, CY05_1, PaternalSib, 0.026 | |
| CY01_4, CY05_1, MaternalSib, 0.223 | |
| CY01_4, CY05_2, PaternalSib, 0.004 | |
| CY01_4, CY05_2, MaternalSib, 0.515 | |
| CY01_4, CY05_4, MaternalSib, 0.015 | |
| CY02_1, CY02_2, PaternalSib, 0.013 | |
| CY02_1, CY02_3, MaternalSib, 0.110 | |
| CY02_1, CY04_2, MaternalSib, 0.005 | |
| CY02_1, CY04_3, MaternalSib, 0.042 | |
| CY02_1, CY04_4, MaternalSib, 0.104 | |
| CY02_1, CY05_1, MaternalSib, 0.422 | |
| CY02_1, CY05_2, MaternalSib, 0.161 | |
| CY02_2, CY02_3, PaternalSib, 0.088 | |
| CY02_2, CY02_3, MaternalSib, 0.045 | |
| CY02_2, CY02_4, MaternalSib, 0.004 | |
| CY02_2, CY04_1, PaternalSib, 0.013 | |
| CY02_2, CY04_1, MaternalSib, 0.293 | |
| CY02_2, CY04_2, PaternalSib, 0.004 | |
| CY02_2, CY04_2, MaternalSib, 0.006 | |
| CY02_2, CY04_3, MaternalSib, 0.007 | |
| CY02_2, CY04_4, MaternalSib, 0.022 | |
| CY02_2, CY05_2, PaternalSib, 0.004 | |
| CY02_2, CY05_3, PaternalSib, 0.002 | |
| CY02_2, CY05_4, PaternalSib, 0.001 | |
| CY02_2, CY05_4, MaternalSib, 0.067 | |
| CY02_3, CY02_4, PaternalSib, 0.183 | |
| CY02_3, CY02_4, MaternalSib, 0.054 | |
| CY02_3, CY04_2, MaternalSib, 0.010 | |
| CY02_3, CY04_4, MaternalSib, 0.161 | |
| CY02_3, CY05_1, PaternalSib, 0.040 | |
| CY02_3, CY05_1, MaternalSib, 0.169 | |
| CY02_3, CY05_2, MaternalSib, 0.086 | |
| CY02_3, CY05_4, MaternalSib, 0.016 | |



doi:10.6342/NTU202403329

| Half-siblings | Full-siblings |
|---|---|
| CY02_4, CY04_3, PaternalSib, 0.004 | |
| CY02_4, CY05_1, PaternalSib, 0.003 | |
| CY02_4, CY05_3, MaternalSib, 0.007 | |
| CY02_4, CY05_4, PaternalSib, 0.001 | |
| CY02_4, CY05_4, MaternalSib, 0.113 | |
| CY04_1, CY04_2, PaternalSib, 0.078 | |
| CY04_1, CY04_2, MaternalSib, 0.001 | |
| CY04_1, CY04_3, MaternalSib, 0.024 | |
| CY04_1, CY04_4, PaternalSib, 0.053 | |
| CY04_1, CY04_4, MaternalSib, 0.017 | |
| CY04_1, CY05_2, PaternalSib, 0.076 | |
| CY04_2, CY04_4, PaternalSib, 0.005 | |
| CY04_2, CY04_4, MaternalSib, 0.355 | |
| CY04_2, CY05_1, MaternalSib, 0.006 | |
| CY04_2, CY05_2, PaternalSib, 0.275 | |
| CY04_2, CY05_2, MaternalSib, 0.003 | |
| CY04_3, CY04_4, PaternalSib, 0.046 | |
| CY04_3, CY04_4, MaternalSib, 0.035 | |
| CY04_3, CY05_1, PaternalSib, 0.104 | |
| CY04_3, CY05_1, MaternalSib, 0.042 | |
| CY04_3, CY05_4, PaternalSib, 0.202 | |
| CY04_4, CY05_1, PaternalSib, 0.021 | |
| CY04_4, CY05_1, MaternalSib, 0.179 | |
| CY04_4, CY05_2, PaternalSib, 0.019 | |
| CY04_4, CY05_2, MaternalSib, 0.014 | |
| CY04_4, CY05_3, PaternalSib, 0.223 | |
| CY05_1, CY05_2, PaternalSib, 0.001 | |
| CY05_1, CY05_2, MaternalSib, 0.195 | |
| CY05_3, CY05_4, MaternalSib, 0.784 | |



doi:10.6342/NTU202403329

**Appendix Table 19.** Pairwise Half-siblings and full-siblings probability summary of the Kaohsiung population. Data order – Half-sibling: Worker ID1 - Worker ID2 - Paternal/Maternal - Probability; Full-siblings: Worker ID1 - Worker ID2 - Probability.

| **Half-siblings** | **Full-siblings** |
|---|---|
| KH01_1, KH04_2, MaternalSib, 0.434 | KH01_2, KH01_3,0.045 |
| KH01_1, KH04_3, MaternalSib, 0.999 | KH01_2, KH03_2, 0.072 |
| KH01_1, KH05_2, PaternalSib, 0.001 | KH01_2, KH03_4, 0.072 |
| KH01_1, KH05_2, MaternalSib, 0.999 | KH01_3, KH03_2, 0.076 |
| KH01_1, KH06_4, PaternalSib, 0.091 | KH01_3, KH03_4, 0.106 |
| KH01_2, KH01_3, PaternalSib, 0.929 | KH03_2, KH03_4, 0.793 |
| KH01_2, KH01_3, MaternalSib, 0.013 | KH04_2, KH05_1, 0.002 |
| KH01_2, KH01_4, MaternalSib, 0.056 | KH05_1, KH05_2, 0.017 |
| KH01_2, KH03_2, PaternalSib, 0.811 | KH05_1, KH05_4, 0.002 |
| KH01_2, KH03_2, MaternalSib, 0.039 | KH06_2, KH06_3, 0.035 |
| KH01_2, KH03_3, MaternalSib, 0.008 | |
| KH01_2, KH03_4, PaternalSib, 0.810 | |
| KH01_2, KH03_4, MaternalSib, 0.009 | |
| KH01_2, KH05_1, MaternalSib, 0.021 | |
| KH01_2, KH05_3, PaternalSib, 0.048 | |
| KH01_2, KH05_3, MaternalSib, 0.051 | |
| KH01_3, KH03_2, PaternalSib, 0.807 | |
| KH01_3, KH03_2, MaternalSib, 0.117 | |
| KH01_3, KH03_3, PaternalSib, 0.006 | |
| KH01_3, KH03_3, MaternalSib, 0.512 | |
| KH01_3, KH03_4, PaternalSib, 0.777 | |
| KH01_3, KH05_3, PaternalSib, 0.074 | |
| KH01_3, KH05_3, MaternalSib, 0.008 | |
| KH01_4, KH03_1, PaternalSib, 1.000 | |
| KH01_4, KH06_2, PaternalSib, 0.073 | |
| KH01_4, KH06_3, PaternalSib, 0.993 | |
| KH03_1, KH06_1, MaternalSib, 0.010 | |
| KH03_1, KH06_2, PaternalSib, 0.070 | |
| KH03_1, KH06_3, PaternalSib, 0.993 | |



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| Half-siblings | Full-siblings |
|---|---|
| KH03_1, KH06_4, MaternalSib, 0.533 | |
| KH03_2, KH03_4, PaternalSib, 0.207 | |
| KH03_3, KH03_4, MaternalSib, 0.007 | |
| KH03_3, KH05_3, PaternalSib, 0.952 | |
| KH04_1, KH04_2, PaternalSib, 0.096 | |
| KH04_1, KH04_4, PaternalSib, 0.189 | |
| KH04_1, KH05_1, PaternalSib, 0.261 | |
| KH04_1, KH05_2, PaternalSib, 0.829 | |
| KH04_1, KH05_3, MaternalSib, 0.028 | |
| KH04_1, KH05_4, PaternalSib, 0.830 | |
| KH04_1, KH06_1, PaternalSib, 0.017 | |
| KH04_1, KH06_4, PaternalSib, 0.024 | |
| KH04_2, KH04_3, MaternalSib, 0.433 | |
| KH04_2, KH04_4, PaternalSib, 0.600 | |
| KH04_2, KH05_1, PaternalSib, 0.424 | |
| KH04_2, KH05_1, MaternalSib, 0.152 | |
| KH04_2, KH05_2, MaternalSib, 0.434 | |
| KH04_2, KH05_4, MaternalSib, 0.011 | |
| KH04_2, KH06_1, PaternalSib, 0.952 | |
| KH04_2, KH06_4, PaternalSib, 0.780 | |
| KH04_3, KH05_2, MaternalSib, 0.998 | |
| KH04_4, KH05_1, PaternalSib, 0.532 | |
| KH04_4, KH05_2, PaternalSib, 0.132 | |
| KH04_4, KH05_4, PaternalSib, 0.146 | |
| KH04_4, KH06_1, PaternalSib, 0.569 | |
| KH04_4, KH06_2, MaternalSib, 0.013 | |
| KH04_4, KH06_3, MaternalSib, 0.007 | |
| KH04_4, KH06_4, PaternalSib, 0.464 | |
| KH05_1, KH05_2, PaternalSib, 0.006 | |
| KH05_1, KH05_4, PaternalSib, 0.358 | |
| KH05_1, KH05_4, MaternalSib, 0.024 | |
| KH05_1, KH06_1, PaternalSib, 0.412 | |
| KH05_1, KH06_4, PaternalSib, 0.304 | |



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| Half-siblings | Full-siblings |
| --- | --- |
| KH05_2, KH05_4, PaternalSib, 0.999 | |
| KH06_1, KH06_2, PaternalSib, 0.013 | |
| KH06_1, KH06_2, MaternalSib, 0.501 | |
| KH06_1, KH06_3, PaternalSib, 0.002 | |
| KH06_1, KH06_3, MaternalSib, 0.664 | |
| KH06_1, KH06_4, PaternalSib, 0.736 | |
| KH06_1, KH06_4, MaternalSib, 0.010 | |
| KH06_2, KH06_3, MaternalSib, 0.034 | |



doi:10.6342/NTU202403329

**Appendix Table 20.** Male offspring and sperm summary of the nine genotyped nests.

| Colony | Son | Sperm | Diploid son | Double alleles sperm |
|---|---|---|---|---|
| **CY01** | 3 | NA | 1 | NA |
| **CY02** | 2 | 1 | 1 | 0 |
| **CY04** | NA | 3 | NA | 2 |
| **CY05** | 1 | 1 | 0 | 0 |
| **KH01** | 2 | 1 | 2 | 0 |
| **KH03** | NA | 2 | NA | 0 |
| **KH04** | 2 | 3 | 2 | 1 |
| **KH05** | 1 | 3 | 1 | 1 |
| **KH06** | 4 | 3 | 2 | 2 |
| **Sum** | 15 | 17 | 9 | 6 |



doi:10.6342/NTU202403329

**Appendix Table 21.** Summary list of supercolony documentations of ant species.

| Species | Subfamily | Citation | Supercolony description |
|---|---|---|---|
| ***Myrmica rubra*** | Myrmicinae | Naumann *et al*. 2018 | Both supercolonial and multi-colonial populations are known in native and invasive ranges. |
| ***Wasmannia auropunctata*** | Myrmicinae | Breton *et al*. 2004; Foucaud *et al*. 2009 | A tropical supercolonial invader with small supercolonies in native range and large supercolonies in invaded East Asia countries. |
| ***Solenopsis invicta*** | Myrmicinae | Morel *et al*. 1990 | Listed to be supercolonial and fulfills all supercoloniality criteria including mutual tolerance of polygyne nests |
| ***Solenopsis saevissima*** | Myrmicinae | Lenoir *et al.* 2016 | French Guiana. Forms native supercolonies that holds high invasive potential. |
| ***Monomorium pharaonis*** | Myrmicinae | Schmidt *et al*. 2010 | Low levels of non-nestmate discrimination despite having high genetic differentiation. |
| ***Pheidole megacephala*** | Myrmicinae | Fournier *et al.* 2009 | None aggressive towards conspecifics from different nests, even at large geographical scales. Forms a large supercolonial population across northern/eastern Australia. |
| ***Tetramorium bicarinatum*** | Myrmicinae | Astruc *et al*. 2001 | Workers do not discriminate against conspecific non-nestmate individuals. |
| ***Tetramorium caespitum*** | Myrmicinae | Steiner *et al.* 2003 | Nearly total absence of aggression between workers from different nests suggests supercoloniality. |
| ***Anoplolepis gracilipes*** | Formicinae | Abbott. 2005; Drescher *et al*. 2010 | Supercolonial and highly invasive among East Asia and Pacific islands |
| ***Lepisiota canescens*** | Formicinae | Sorger *et al*. 2017 | A recently discovered supercolonial species within native range (Ethiopia) with very high invasive potential |



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| | | | |
|---|---|---|---|
| ***Cataglyphis niger*** | Formicinae | Leniaud *et al*. 2011 | Found in the desert of Israel. Forms small supercolonies with workers from different nests over hundreds of square meters being none aggressive to each other. |
| ***Polyrhachis robsoni*** | Formicinae | Van Zweden *et al*. 2007 | An Australian weaver ant. Aggression tests showed that hostility between ants from different nests was minimal. |
| ***Nylanderia fulva*** | Formicinae | Eyer *et al*. 2018 | Lack of aggressive behaviors towards conspecifics from different nests and displaying a supercolonial system with no clear boundaries among nests in its invasive range. |
| ***Formica paralugubris*** | Formicinae | Holzer *et al*. 2006 | Ants within populations showed no aggressive behaviour, but recognized non-nestmates as shown by longer antennation bouts. |
| ***Linepithema humile*** | Dolichoderinae | Giraud *et al*. 2002; Pedersen *et al*. 2006; Van Wilgenburg *et al.* 2010 | Invasive supercolonies are the largest described, spanning several continents. In the native range supercolonies are relatively smaller. |
| ***Tapinoma sessile*** | Dolichoderinae | Buczkowski and Bennett 2008 | Exhibits supercoloniality and becomes a dominant invasive pest in invaded urban areas. |
| ***Technomyrmex albipes*** | Dolichoderinae | Dejean *et al*. 2010 | Arboreal. Supercolonies exist along the coast of Madagascar. |
| ***Pseudomyrmex veneficus*** | Pseudomyrmicinae | Janzen 1973 | One of the earliest described supercolonies among ants. |
| ***Pseudomyrmex peperi*** | Pseudomyrmicinae | Kautz *et al*. 2009 | Establishes distinct, but highly polygynous supercolonies. |



doi:10.6342/NTU202403329

**Appendix Table 22.** Pairwise population $F_{ST}$ values. $F_{ST}$ Values below diagonal and *p*-values above diagonal.

| | CY01 | CY02 | CY04 | CY05 | KH01 | KH03 | KH04 | KH05 | KH06 | |
|---|---|---|---|---|---|---|---|---|---|---|
| **CY01** | 0.000 | 0.398 | 0.128 | 0.375 | 0.001 | 0.001 | 0.007 | 0.002 | 0.001 | **CY01** |
| **CY02** | 0.000 | 0.000 | 0.197 | 0.348 | 0.001 | 0.001 | 0.002 | 0.001 | 0.001 | **CY02** |
| **CY04** | 0.069 | 0.053 | 0.000 | 0.468 | 0.001 | 0.001 | 0.003 | 0.001 | 0.001 | **CY04** |
| **CY05** | 0.000 | 0.017 | 0.000 | 0.000 | 0.001 | 0.001 | 0.030 | 0.001 | 0.001 | **CY05** |
| **KH01** | 0.389 | 0.445 | 0.389 | 0.377 | 0.000 | 0.199 | 0.004 | 0.014 | 0.015 | **KH01** |
| **KH03** | 0.393 | 0.422 | 0.411 | 0.401 | 0.034 | 0.000 | 0.008 | 0.010 | 0.011 | **KH03** |
| **KH04** | 0.193 | 0.168 | 0.173 | 0.138 | 0.226 | 0.193 | 0.000 | 0.213 | 0.001 | **KH04** |
| **KH05** | 0.316 | 0.344 | 0.297 | 0.291 | 0.145 | 0.218 | 0.043 | 0.000 | 0.010 | **KH05** |
| **KH06** | 0.517 | 0.521 | 0.473 | 0.498 | 0.176 | 0.177 | 0.269 | 0.195 | 0.000 | **KH06** |
| | **CY01** | **CY02** | **CY04** | **CY05** | **KH01** | **KH03** | **KH04** | **KH05** | **KH06** | |



doi:10.6342/NTU202403329