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In Thailand, stingless beekeeping (meliponiculture) of *Heterotrigona itama* has expanded significantly, particularly in rural southern communities, where the Ministry of Agriculture and Cooperatives promotes it as a secondary occupation. However, the increasing interest in meliponiculture has led to the relocation of colonies beyond their native ranges, disrupting local bee populations and altering gene flow across large geographic areas. Additionally, large-scale artificial propagation using a limited number of colonies has contributed to increased inbreeding within managed populations and genetic introgression into wild populations. Colony expansion is also constrained by a shortage of queens. To address these challenges, investigating local genetic variation, mass-rearing virgin queens using in vitro method, and improving honey production practices could strengthen commercial stingless beekeeping. Our findings indicate slightly lower nuclear genetic variability but higher mitochondrial genetic variability, likely due to gene flow, colony transport, and nest division. Expanding colonies through nest division does not appear to negatively impact genetic diversity, as small-scale male dispersal and human-mediated transport help maintain it. For in vitro rearing, we observed that *H. itama* larvae developed into queens when fed 120–150 μL of larval food, producing in vitro queens with body sizes comparable to naturally produced ones. Microscopic analysis revealed that in vitro-reared queens had well-developed ovaries and spermathecae, whereas workers had smaller ovaries and lacked spermathecae. The acceptance of in vitro-reared queens was independent of worker age, and although their mating frequency was low, it did not significantly differ from that of naturally produced queens. Regarding honey production, heat treatment at 37 °C and 45 °C had minimal effects on physicochemical properties, total phenolic and flavonoid contents, and antioxidant activity ($p > 0.05$). Since heat treatment helps preserve the physicochemical and bioactive properties of dehydrated honey, the proposed method could serve as an alternative approach for preserving stingless bee honey. These findings support sustainable stingless beekeeping, conservation efforts, and enhanced honey production for native stingless bee species.

Enhancing Stingless Beekeeping in Thailand

*Genetic Diversity, Artificial Queen
Rearing, and Honey Processing*

Presented by

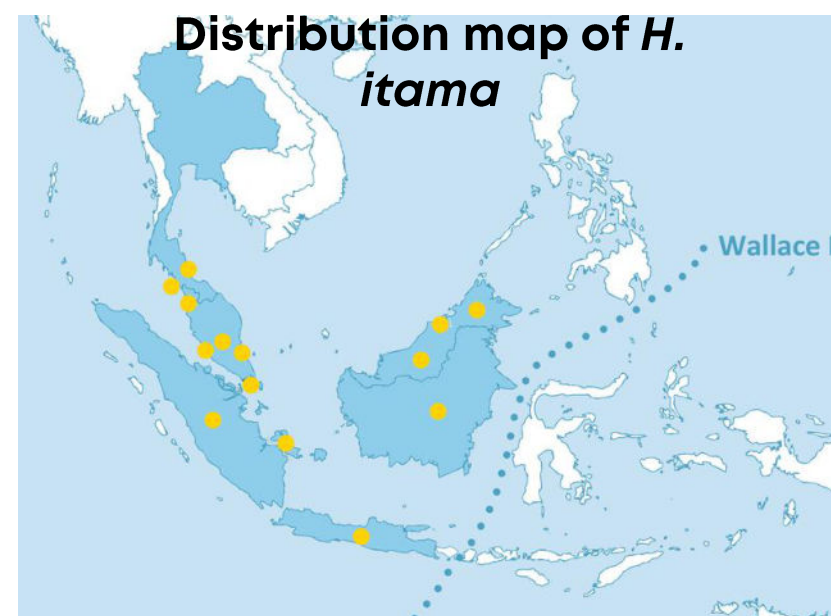
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Overview of *Heterotrigona itama*

- The species successfully managed in standard wooden hive boxes for commercial pollination, honey and propolis productions, and the commercial sale of colonies.
- This species is a medium-sized, with a **worker body length of 5.5–5.6 mm**.
- Perennial colonies comprise of approximately 8,000–10,000 worker bees and a **single physogastric queen**.
- *H. itama* is a limited distribution in **southern Thailand, Malaysia, Singapore, and Indonesia**





Importance of *Heterotrigona itama*

- The species is commonly used in meliponiculture in Thailand, where it is propagated in apiaries for the purpose of selling colonies, producing honey products, and pollinating crops.
- The selling price of *H. itama* products
 - honey is approximately **32–40 USD/kilogram**
 - colonies of *H. itama* are typically priced between **80–134 USD/colony**

Wongsa et al (2024)



Traditional
hive



Traditional
hive



Modern
hive



Importance of *Heterotrigona itama*

- With the growing demand for stingless bee honey, stingless bee keepers are expanding their apiaries by acquiring colonies, from other commercial apiaries and natural colonies.
- Thus, mass artificial rearing of virgin queens could address for commercially colony propagation.
- Additionally, Trade in colonies enable their geographic displacement **both within and outside their natural range**, might effect on local genetic structure.





Objectives

01

Genetic structure of the population

- We determined the genetic structure of managed and wild populations of *H. itama*, in Thailand.

02

Artificial queen rearing

- We explored the efficacy of *in vitro* queen rearing for *H. itama*, assessing the queen's **body size**, reproductive organ size (**ovary and spermatheca**), **acceptance rate into new**, and **mating frequency**.

03

impact of heat treatment on stingless bee honey

- We examine thermal treatment on the physical characteristics, phenolic and flavonoid contents, and antioxidant capacity of *H. itama* honey.

01 Genetic structure of the population

01

Workers sample collection

1 bee/hive ; 70 hive total

02

- DNA extraction
- PCR: COI, 16s and 28s
- DNA Seq. alignments

03

- diversity indices analyses
- Genetic differentiation
- gene flow

04

- Phylogenetic
- Haplotype network



RESEARCH ARTICLE

Genetic structure of the commercial stingless bee *Heterotrigona itama* (Apidae: Meliponini) in Thailand

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Wild colony



Managed colony

01 Results

1.1 DNA sequence variation

- Based on mitochondrial and nuclear sequences, the mean value of ***hd*** and ***Pi*** of all colony samples were high.
- Sample collected from managed apiaries exhibited higher haplotype diversity compared to wild colonies
- Higher number of haplotypes in the managed than in the wild.
- Both mtDNAs and nDNA diversity was high in both the managed and the wild.

Molecular diversity indices and population expansion test statistics of mitochondrial and nuclear gene sequences data of commercial and wild *H. itama* in Thailand

Gene		<i>N</i>	<i>No</i>	<i>S</i>	<i>k</i>	<i>hd</i> (± <i>SD</i>)	<i>P_i</i> (± <i>SD</i>)
<i>COI</i>	Province						
	Krabi	13	10	72	28.128	0.962(0.041)	0.0426(0.0036)
	Nakhon Si Thammarat	22	17	67	17.169	0.965(0.028)	0.0260(0.0049)
	Narathiwat	35	15	85	26.239	0.881(0.042)	0.0398(0.0049)
	Colony origin						
	Managed	39	30	116	34.663	0.985(0.009)	0.0525(0.0033)
	Wild	31	15	80	31.267	0.860(0.053)	0.0474(0.0032)
	All samples	70	42	121	36.718	0.966(0.012)	0.0556(0.0010)
<i>16s rRNA</i>	Province						
	Krabi	13	6	21	2.179	0.859(0.063)	0.00495(0.0023)
	Nakhon Si Thammarat	21	4	4	0.943	0.543(0.166)	0.00214(0.0007)
	Narathiwat	35	5	9	2.867	0.687(0.055)	0.00652(0.0009)
	Colony origin						
	Managed	39	9	24	3.828	0.808 (0.041)	0.0063(0.0011)
	Wild	30	4	9	3.386	0.646(0.058)	0.0077(0.0008)
	All samples	69	10	19	3.557	0.822(0.021)	0.0081(0.0005)
<i>28s rRNA</i>	Province						
	Krabi	13	4	6	1.051	0.526(0.153)	0.0017(0.0007)
	Nakhon Si Thammarat	21	3	2	0.505	0.467(0.113)	0.0008(0.0002)
	Narathiwat	35	3	2	0.387	0.375(0.086)	0.0006(0.0002)
	Colony origin						
	Managed	39	5	6	0.686	0.487(0.082)	0.0011(0.0003)
	Wild	30	3	2	0.354	0.343(0.097)	0.0006(0.0002)
	All samples	69	5	6	0.537	0.422(0.064)	0.0009(0.0002)

Wongsa et al. (2024)

<https://doi.org/10.1371/journal.pone.0312386.t00>

01 Results

1.2 Genetic differentiation and gene flow

- Based on sequence data, low to moderately high genetic differentiation and not significant ($p > 0.05$), suggesting that several population pairs of *H. itama* established a unique genetic group.
- Pairwise **Nm** values, most population pairs had values greater than one, implying that more than one queen per generation was estimated to exchange between all pair of populations.

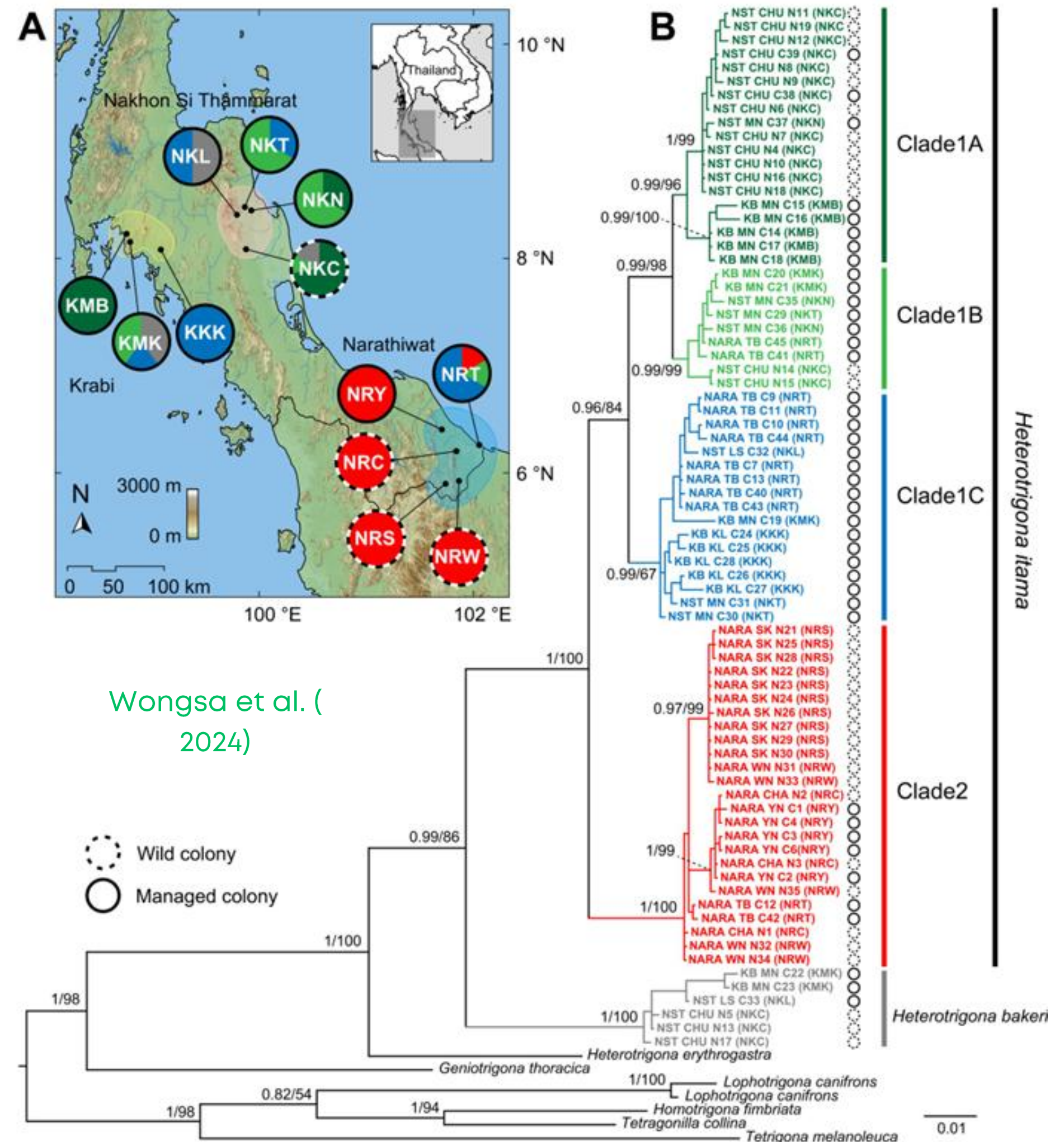
Wongsa et al. (
2021)



01 Results

1.3 Phylogenetic relationship

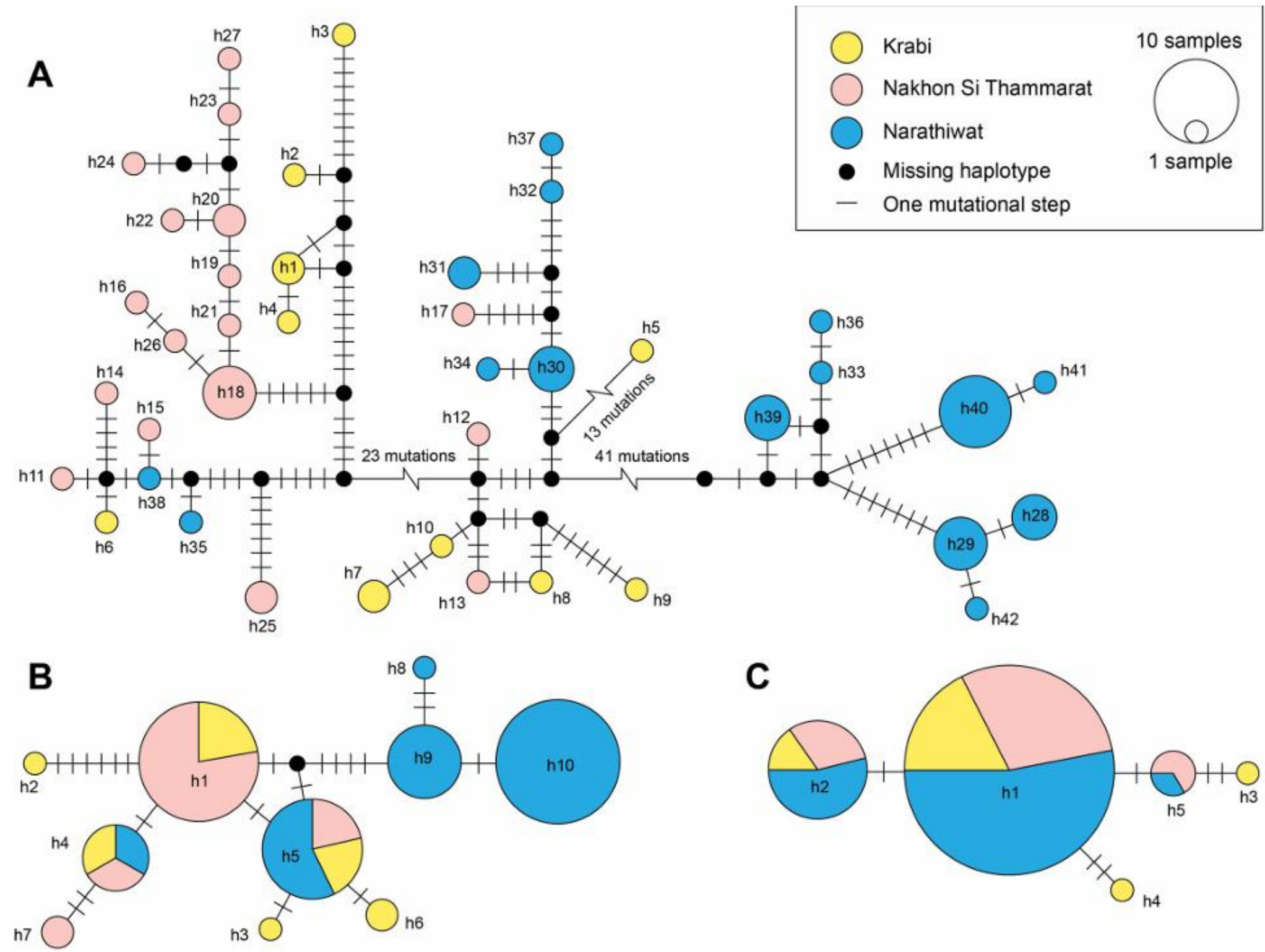
- The phylogenetic tree clearly illustrates the **monophyly** of *H. itama*.
- Two major clades were identified: Clade1 and Clade2.
- There was **no strong evidence of differentiation between wild and managed colonies** within these two clades.



01 Results

1.4 Haplotype network

- COI shows highly structuring network, whereas, 16s and 28s are lower-resolution structure.
- This study showed that wild and managed of *H. itama* exhibit high genetic variability, suggesting that the colony management practices of Thai beekeepers do not significantly affect mitochondrial or nuclear variability in the *H. itama* populations.



Wongsa et al. (2024)

02 Artificial queen rearing

01

Effect of larval food amount and incubation conditions on larva development in *in-vitro* condition

larval food amount

- 100 uL
- 120 uL
- 150 uL

larval food and queen-laid egg collection

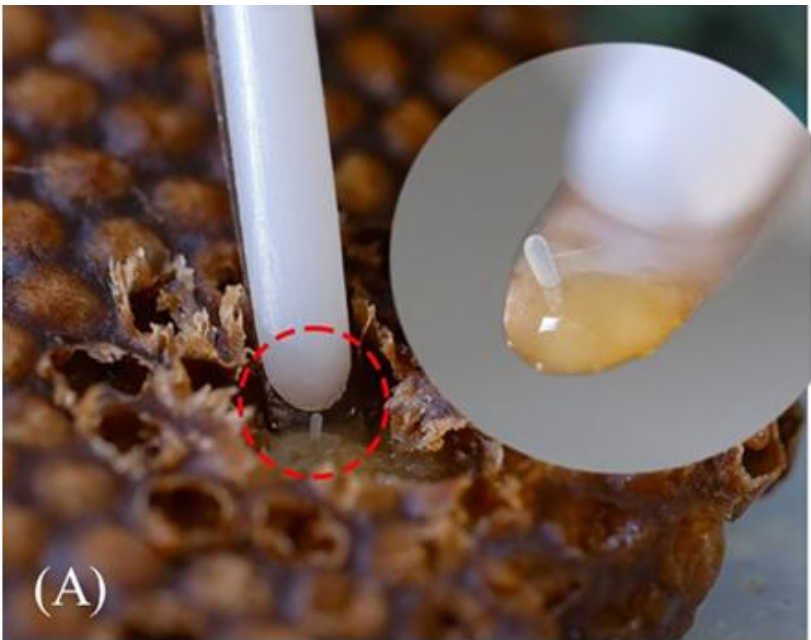
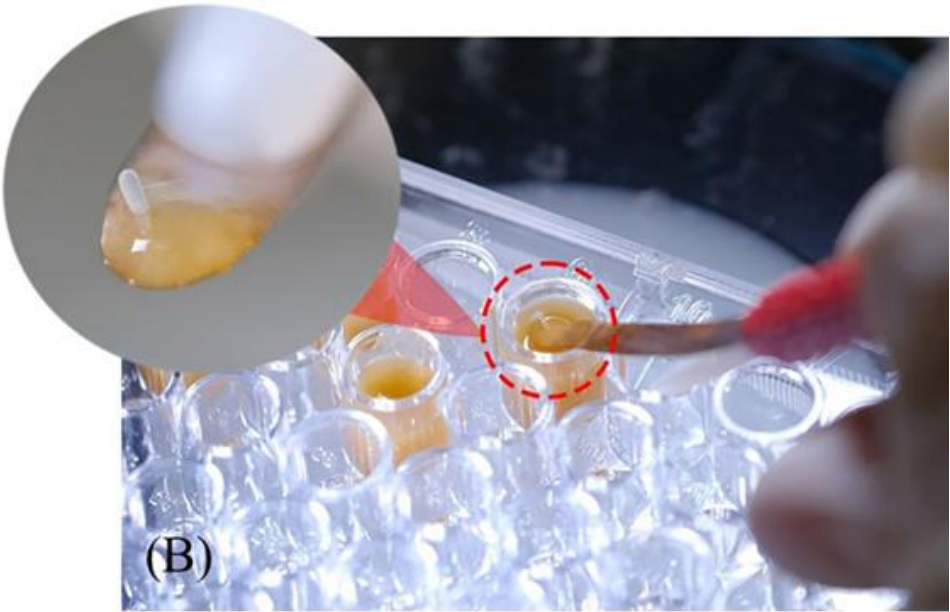


Table 1. Overall features of the queen-rearing protocol of *Heterotrigona itama*.

Development Stages	Saturated solution	Temperature (°C)	Humidity (%)	Days
Egg	Distilled water	30.0 ± 0.5	98	0 → 5
Feeding larvae	Distilled water+NaCl	30.0 ± 0.5	90	5 → 10
Defection larvae	Distilled water+NaCl	30.0 ± 0.5	90	10 → 17
Pupae	Distilled water+NaCl	30.0 ± 0.5	90	17 → 40
Pigmented pupae	No	30.0 ± 0.5	75	40 → 54

<https://doi.org/10.1371/journal.pone.0306085.t001>

02 Artificial queen rearing

01

Effect of larval food amount and incubation conditions on larva development in *in-vitro* condition

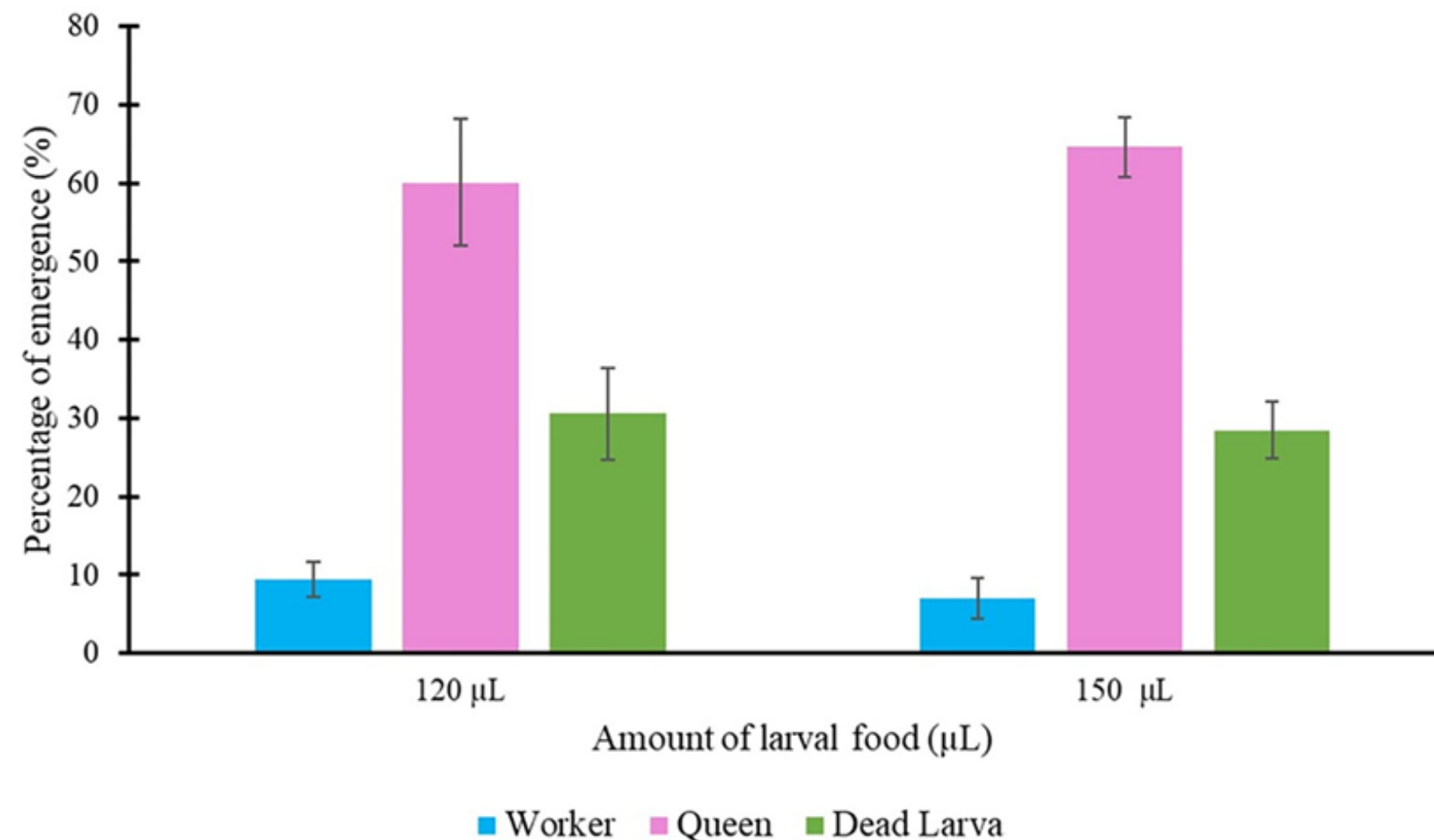
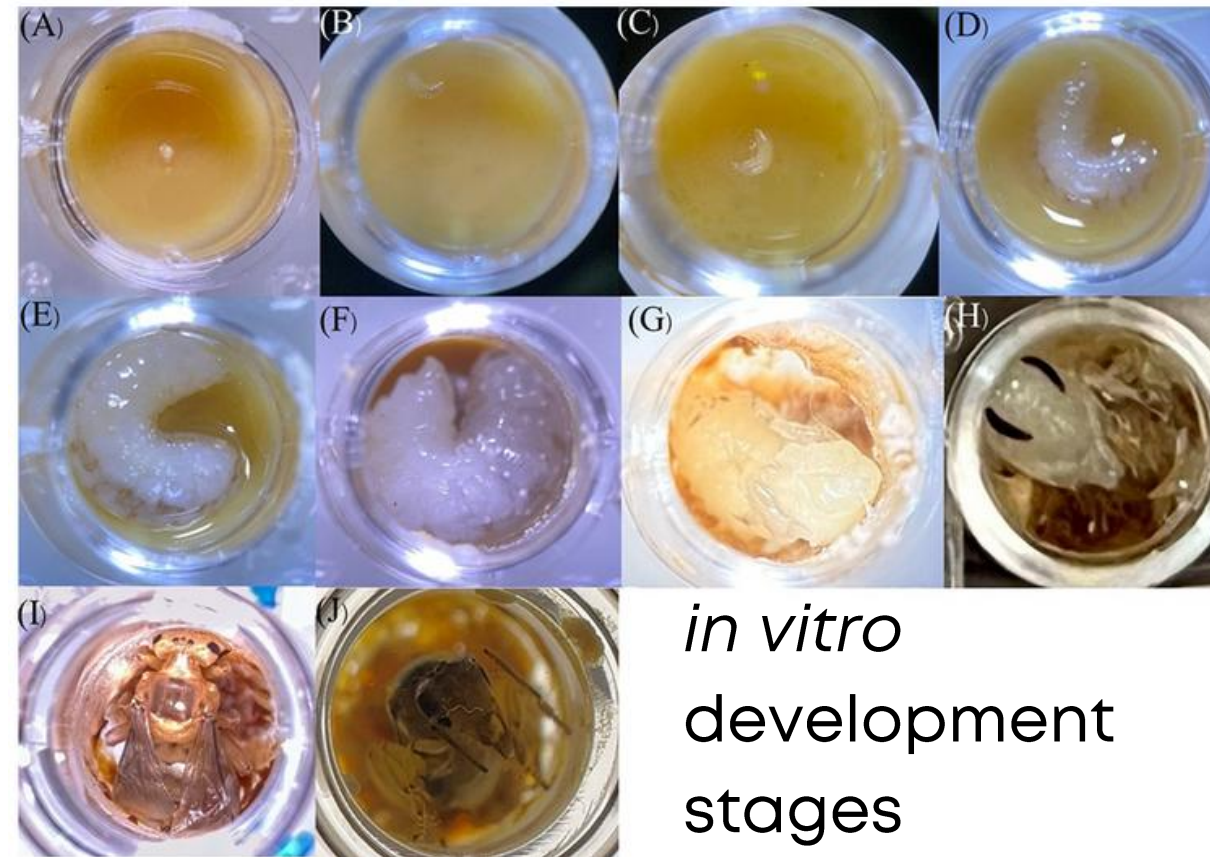


Fig 4. The percentage of worker and queen emergence of *Heterotrigona itama* in response to larval food amount of 120 µL (n = 173, 60.07 ± 5.92%) and 150 µL (n = 186, 64.58 ± 3.76%) from the *in-vitro* rearing experiment.

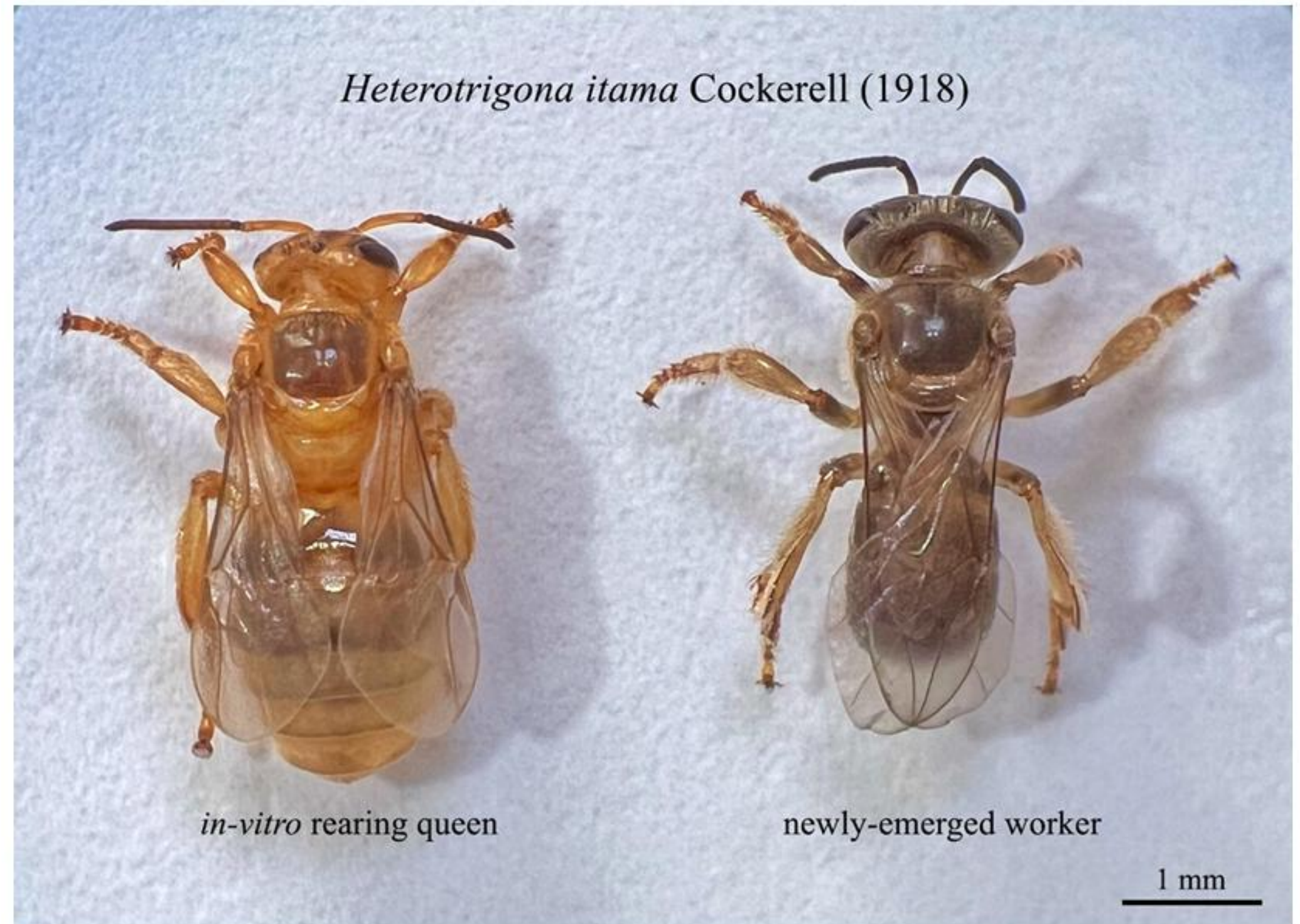
- No significant difference was found between the two larval food levels provided in response to queen emergence ($t = 1.115$, $df = 4$, $P = 0.327$)

02 Artificial queen rearing

02

Microscopic analysis of the reproductive system and External morphometry

- We conducted a comparative analysis of the ovaries and body size
 - *in vitro* queens
 - newly emerged workers obtained from natural colonies



02 Artificial queen rearing

02

Microscopic analysis of the reproductive system and External morphometry

- *In vitro* queens from the 120 and 150 μ L treatments were larger than workers, with a considerable abdomen and well-developed reproductive system.
- Discriminant analysis showed no significant differences between the *in vitro* and virgin queens produced under natural conditions.

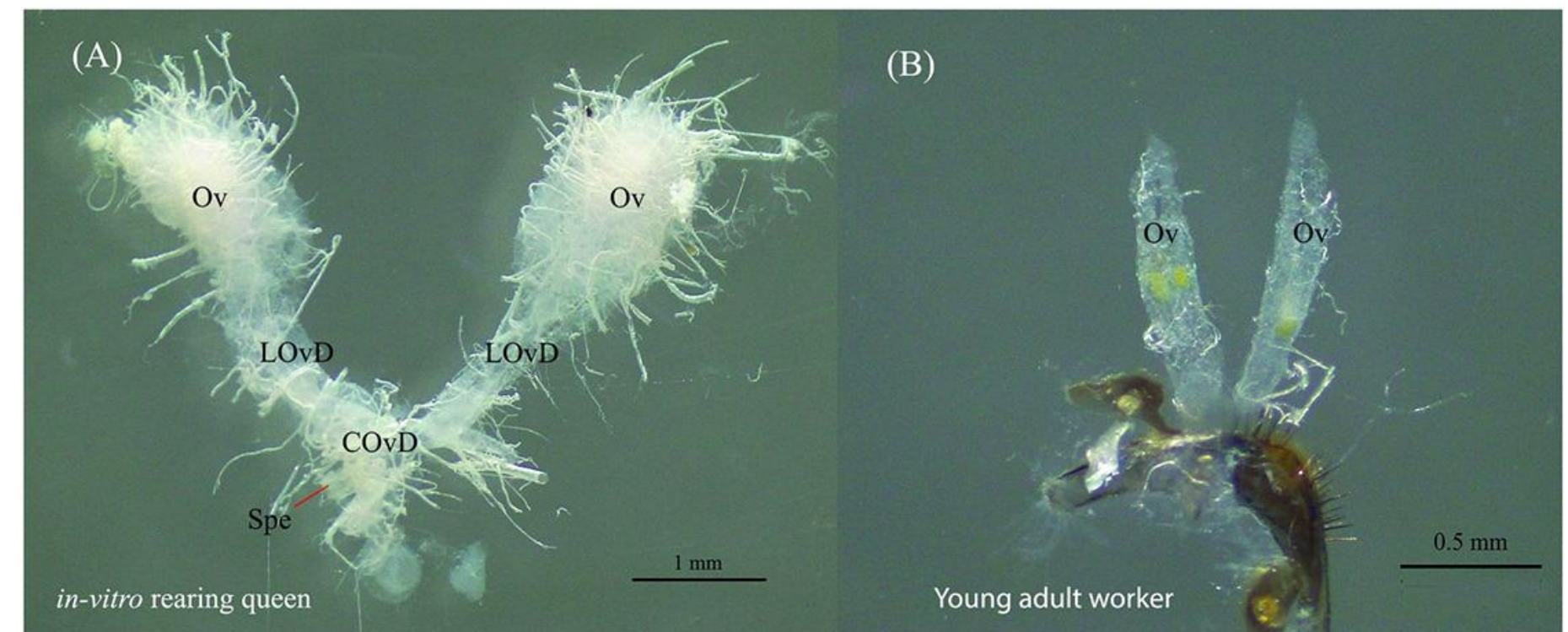


Fig 6. Microscopy images of gonadal development in *Heterotrigona itama*, displaying (A) the freshly dissected reproductive system of an *in vitro* queen with a well-developed ovarioles structure, and (B) a dissection of female workers, highlighting significant differences in ovaries and ovariole size compared to the *in vitro* queen. The abbreviations are ovary (Ov), lateral oviduct (LOvD), common oviduct (COvD), and spermatheca (Spe).

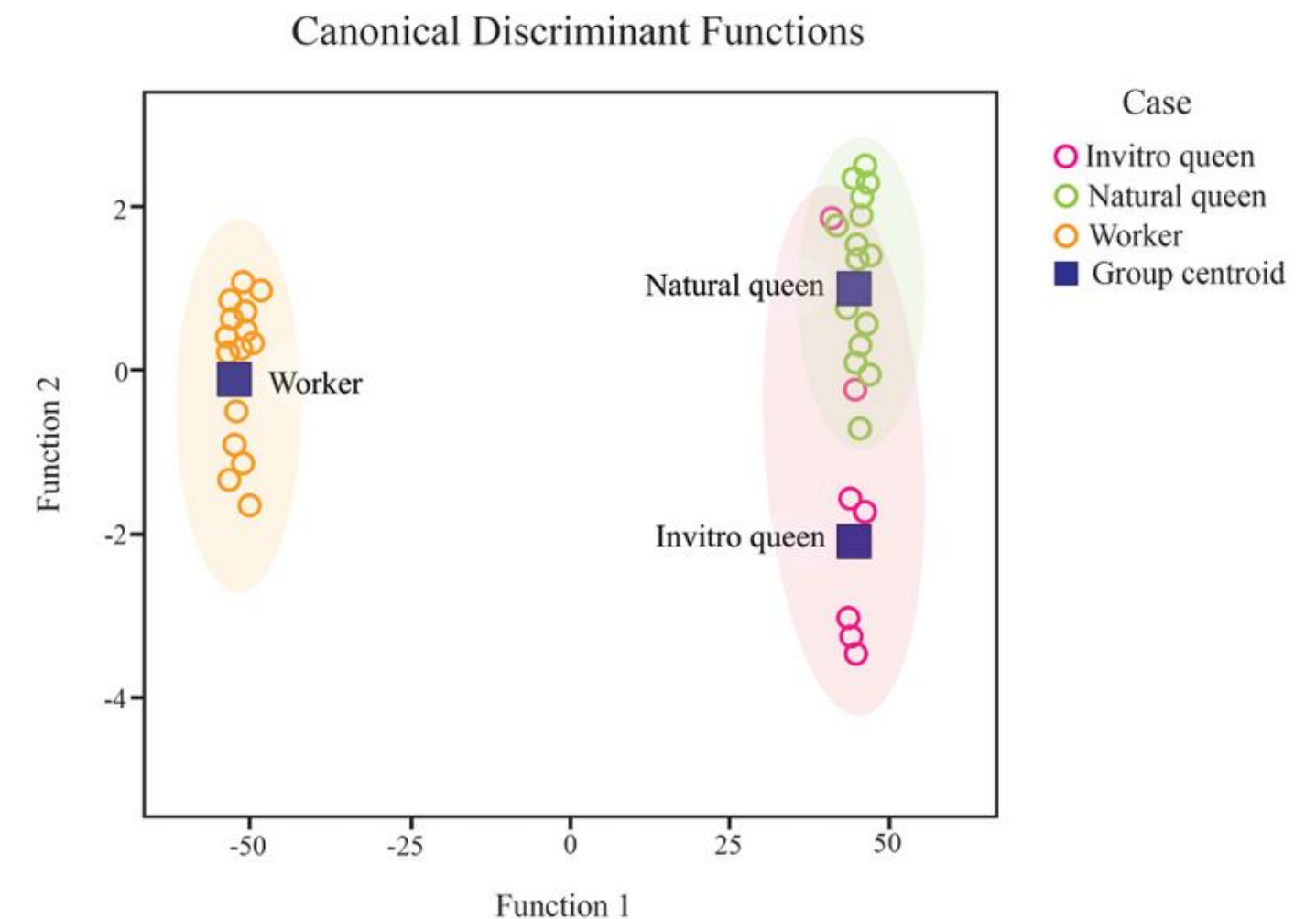


Fig 7. Linear discriminant analysis was conducted on the 34 morphometric characters measured in *in vitro* reared queens ($n = 11$), natural virgin queens ($n = 22$), and workers ($n = 30$) of *Heterotrigona itama*. The shaded areas represent the 95% confidence ellipse.

<https://doi.org/10.1371/journal.pone.0306085.g007>

02 Artificial queen rearing

03

Acceptance rate of the in vitro-reared virgin queens

(a) Introducing the *in vitro* queens into the new artificial queenless colonies

- **AQCa**: newly emergent workers only
- **AQCb**: newly emergent and old workers

(b) Analyzing the acceptance of in vitro queens

-The acceptance rate of the *in vitro* queens was compared between **AQCa** and **AQCb** using the Pearson's chi-square.



1-week-old queens reared in vitro



in-vitro queen was introduced

02 Artificial queen rearing

03

Acceptance rate of the *in vitro*-reared virgin queens

- Over 70% of the *in vitro*-reared queens were accepted after being introduced **AQCa**
- A lower acceptance rate (approximately 46%) was found in **AQCb**
- There was insignificant ($\chi^2 = 1.784$, $df = 1$, $P = 0.182$)

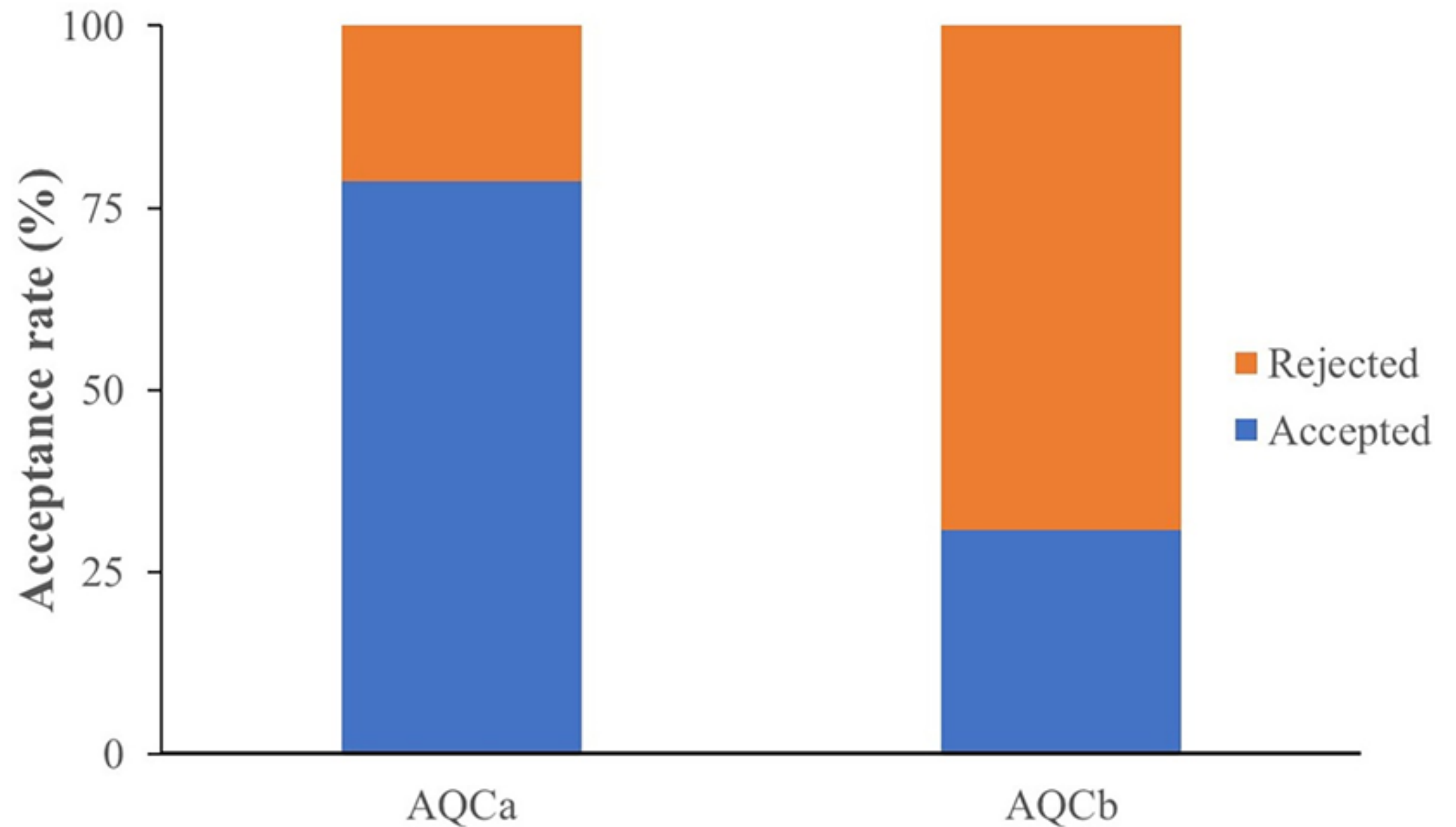


Fig 8. Acceptance rate of *Heterotrigona itama* queens reared *in vitro* inside artificial queenless colonies containing young workers only (AQCa, $n = 14$) or young and old workers (AQCb, $n = 13$).

<https://doi.org/10.1371/journal.pone.0306085.g008>

02 Artificial queen rearing

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04

Mating frequency

- No significant difference was found in observed mating frequency between the natural and *in vitro* reared queens.

Table 2. Paternity frequency in *Heterotrigona itama* colonies from two commercial apiaries in Thailand.

Apiary	Type of mother queen	<i>n</i>	<i>k</i>	<i>k</i> ₉	<i>m</i> _e	<i>r</i>
Nakorn Si Thammarat	Natural queen 1	10	2	0.48	1.839	0.522
	Natural queen 2	9	3	3.00	3.370	0.397
	Natural queen 3	10	2	0.40	2.189	0.478
	Mean (±SD)		2.3 (±0.6)	1.29 (±1.48)	2.466 (±0.802)	0.466 (±0.063)
	<i>in-vitro</i> rearing queen 1	10	2	0.48	1.839	0.522
	<i>in-vitro</i> rearing queen 2	10	3	0.51	2.419	0.457
	<i>in-vitro</i> rearing queen 3	10	1	1	1.000	0.750
	<i>in-vitro</i> rearing queen 4	10	1	1	1.000	0.750
	<i>in-vitro</i> rearing queen 5	15	3	1.69	2.795	0.429
	Mean (±SD)		2.0 (±1.0)	0.94 (±0.49)	1.811 (±0.814)	0.582 (±0.157)
Narathiwat	Natural queen 1	10	2	1.11	1.243	0.652
	Natural queen 2	10	1	1.00	1.000	0.750
	Natural queen 3	9	3	3.00	2.640	0.439
	Natural queen 4	10	3	0.48	2.704	0.435
	Mean (±SD)		2.3 (±1.0)	1.39(±1.10)	1.897 (±0.901)	0.569 (±0.157)
	<i>in-vitro</i> rearing queen 1	15	2	1.67	2.340	0.463
	<i>in-vitro</i> rearing queen 2	10	2	0.48	1.839	0.522
	<i>in-vitro</i> rearing queen 3	11	3	0.94	2.333	0.464
	Mean (±SD)		2.3 (±0.6)	1.03(±0.60)	2.171 (±0.287)	0.483 (±0.034)
Mean (±SE)			2.23 (±0.16)	1.164 (±0.217)	2.086 (±0.296)	0.525 (±0.059)

Number of workers analyzed per colony (*n*), observed paternity frequency (*k*), paternity frequency corrected to a standard sample size of 9 (*k*₉), effective paternity frequency (*m*_e), and average worker relatedness (*r*) are listed for each colony within each apiary. Bracketed numbers are the standard deviation (SD) and standard errors (SE).

03 Impact of heat treatment on stingless bee honey

01

Three different forest types honey (mangrove forest, swamp forest, and mixed

02

- The samples were heated to 37 °C and 45 °C for 24 and 48 h

03

- physicochemical properties
- total phenolic content
- the flavonoid content, and antioxidant activity



Thermal treatment on *H. itama* honey samples

03 Impact of heat treatment on stingless bee honey

- Raw honey from mixed forest had the highest radical scavenging activity with IC50 of 43.996 ± 0.377 mg/ml.
- After incubation at 45 °C for 48 hours, the moisture content decreased 27.93 ± 0.17 to 20.14 ± 0.34 g/100 g.
- Interestingly, heat treatment at 37 °C and 45 °C did not affect the total phenolic, flavonoid contents, and antioxidant activities ($p > 0.05$) in the honey samples.
- Heat treatment can be employed as an alternate method for preserving stingless bee honey from stingless bees





Conclusion

01 Genetic structure of the population

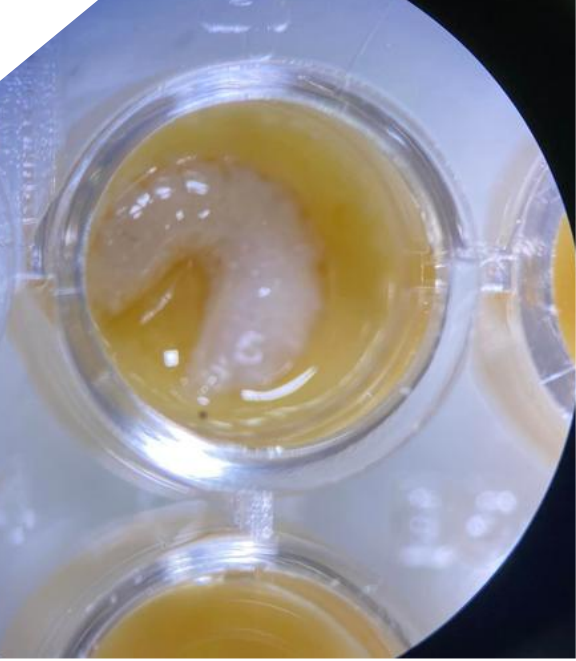
- We found no evidence that the trade of stingless bees affected population structure of *H. itama* populations in this region.

02 Artificial queen rearing

- The *in-vitro* rearing technique potentially increases the number of new colonies and the economic value for stingless beekeepers.

03 impact of heat treatment on stingless bee honey

- heat treatment at 37 °C and 45 °C for 24 and 48 h had no effects on total phenolic, flavonoid contents, and antioxidant activity all *H. itama* honey samples



Thank You
For Your Attention

