




# Genetic diversity of *Apis mellifera* complementary sex determiner (*csd*) in four Australian breeding populations

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**Abstract** – Honey bee (*Apis mellifera*) breeding has intensified as populations experience increasing stress and pollination demand increases. Breeding programmes risk genetic diversity losses as mating is focused on a small group of individuals. Loss of diversity at the *complementary sex determiner* (*csd*) locus results in decreased viability and reduced honey production. Bees that are homozygous at *csd* become inviable males rather than workers. We examined *csd* diversity in four Australian breeding populations: a queen bee breeder from New South Wales, another from Queensland, a Western Australian breeding programme involving 11 bee breeders, and a research population at the NSW Department of Primary Industries. We found 82 unique *csd* alleles across the four populations, 16 of which have not been previously reported. This study provides a snapshot of *csd* diversity in Australia which will be useful for the national honey bee genetic improvement programme (Plan Bee).

**honey bee / sex determination / breeding management / closed mating / inbreeding**

## 1. INTRODUCTION

Honey bees (*Apis mellifera*) are important pollinators of many crops (Klein et al. 2007; Gallai et al. 2009; Potts et al. 2010; Breeze et al. 2011; Hung et al. 2018). While the total number of honey bee colonies has increased worldwide (Phiri et al. 2022), pressures from pesticides, pests, diseases, heavy metal pollution, extreme weather, and lack of forage (Smith et al. 2013;

Goulson et al. 2015; Steinhauer et al. 2018; Insolia et al. 2023; Yang et al. 2023) have led to widespread annual colony losses (Gray et al. 2022). Increases in land devoted to pollination-dependent crops mean that honey bee populations are unlikely to meet demand for their services (Aizen and Harder 2009; Aizen et al. 2019; Mashilingi et al. 2022). To mitigate these factors, many countries have implemented modern animal breeding programmes (Bienefeld et al. 2008; Büchler et al. 2013; Zakour and Bienefeld 2014; Tahmasbi et al. 2015; Bixby et al. 2017; Costa-Maia et al. 2018; Guichard et al. 2020; Hoppe et al. 2020; Petersen et al. 2020; Maucourt et al. 2021).

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To support honey bee breeding programmes, evaluation of genetic diversity at the *complementary sex determiner* (*csd*) locus is crucial (Page and Laidlaw 1985). Honey bees have a haplodiploid sex determination system. Sex is determined by zygosity at *csd* (Beye et al. 2003). Bees that are heterozygous at the *csd* locus become females, hemizygous (unfertilised) individuals become males (drones), and those that are homozygous become abnormal diploid drones that are killed during development (Mackensen 1951; Woyke 1963, 1980). If a queen mates with a single male who carries the same *csd* allele as herself, 50% of her diploid offspring will be killed (Page 1980).

Colonies producing high rates of homozygous diploid males—as would occur if a queen has a genotype comprised of common alleles—often fail to thrive or be productive due to poor population growth (Page 1980; Woyke 1980), and are often inviable (Woyke 1963; Kaskinova et al. 2019). High allelic diversity at *csd* is therefore important for avoiding colony loss. Past studies have shown that increased *csd* diversity will result in higher expected brood viability in a population (Page and Laidlaw 1985).

Honey bee queens are polyandrous, typically mating with more than ten drones (Neumann et al. 1999; Tarpy et al. 2004, 2010, 2015; Simone-Finstrom and Tarpy 2018; Chapman et al. 2019a), at congregation areas that can attract drones from a distance of over 3.5 km (Utaipanon et al. 2019). As monandry is extremely rare (Neumann et al. 1999; Tarpy et al. 2004, 2010, 2015; Simone-Finstrom and Tarpy 2018; Chapman et al. 2019a) and the number of *csd* alleles is high (Bilodeau and Elsie 2021), *csd* homozygosity in diploid progeny is rare in nature (Page 1980). In closed breeding systems, there is potential for loss of *csd* alleles, as queens are produced from a small number of colonies. Over multiple generations, and without external genetic supplementation, there is a chance of decreased diversity of *csd* alleles in the population (Page and Laidlaw 1985). Another factor affecting *csd* diversity is the rapid collapse of colony numbers, for instance a pest or disease outbreak resulting in

widespread colony death, which can put honey bees at greater risk of inbreeding (Büchler et al. 2010; Hristov et al. 2020).

To assist with monitoring breeding programmes, investigation of the frequency and diversity of *csd* alleles can inform which colonies to produce queens and drones from (Hyink et al. 2013). Data on population-wide *csd* diversity can be used as a comparison point to measure changes over time, alerting breeders to decreased genetic diversity. Investigation of *csd* alleles has been performed in some overseas breeding programmes (Table I). All programmes found high *csd* diversity (Hyink et al. 2013; Zareba et al. 2017; Kaskinova et al. 2019; Bilodeau et al. 2020; Paolillo et al. 2022).

A national honey bee genetic improvement programme (Plan Bee) was launched in Australia in 2020 (Chapman and Frost 2021a; Chapman et al. 2022). Plan Bee provides a platform to implement modern animal breeding techniques in the Australian beekeeping industry. It consists of a research population managed by the New South Wales (NSW) Department of Primary Industries and a network of bee breeders that submit data. Plan Bee aims to drive improvement in traits important to the beekeeping and honey bee pollination-dependent industries, such as disease resistance, honey production, colony strength, and good temperament (Frost et al. 2021, 2022; Chapman and Frost 2021a). Successful implementation will allow commercial beekeeping to remain profitable and maintain strong healthy bee populations (Banks et al. 2021; Chapman and Frost 2021b, 2022), ensuring pollination services for industries that depend upon honey bees. In Australia, more than 35 industries rely on paid honey bee pollination services, estimated to be worth AU\$14.2 billion (Clarke and Le Feuvre 2021).

In this study, we investigate *csd* diversity at the hypervariable region (HVR) for the first time in four Australian breeding populations. The HVR is within the potential specifying domain (PSD) located on exons 6–8 of the *csd* gene (Zareba et al. 2017) and is the driving force of high allele variation seen in *csd* (Beye et al. 2013; Lechner et al. 2014; Zareba et al. 2017).

**Table I** Number of *complementary sex determiner (csd)* alleles found in the literature. Proportion is the number of alleles divided by the potential number of alleles found (number of colonies times two)

Location	Colonies	Alleles	Proportion	Source
New Zealand	42	16	19.0%	Hyink et al. (2013)
Wroclaw, Poland	99	85	42.9%	Zareba et al. (2017)
Ladek-Zdroj, Poland	94	74	39.4%	Zareba et al. (2017)
Republic of Bashkortostan, Russia	15	20	66.5%	Kaskinova et al. (2019)
Louisiana, USA (Pol-line)	47	60 <sup>a</sup>		Bilodeau et al. (2020)
Hawaii, USA (Hilo-line)	42			Bilodeau et al. (2020)
Italy	125	88	35.2%	Paolillo et al. (2022)
New South Wales, Australia	37	34	45.9%	This study
Western Australia, Australia	55	30	27.3%	This study
New South Wales, Australia	97	58	29.9%	This study
Queensland, Australia	20	19	47.5%	This study

<sup>a</sup>This study reported the number of *csd* alleles found across both countries; 11 of the 60 alleles were found in both population

The aim of the study was to determine if *csd* diversity was similar to that elsewhere in the world. If it were low, this would indicate that further sampling was required. If further sampling again showed low diversity, this would indicate that Australian populations could require careful management to both maintain diversity and avoid lethal *csd* allele combinations.

The first population was from a bee breeder in NSW who has been operating for approximately 8 years with four breeding lines sourced from commercial beekeepers and breeders around Australia with ~350 colonies. Mating is controlled mostly through drone flooding (providing many drones from the selected population such that queens mate predominantly with selected stock rather than drones from other colonies in the area) at isolated mating stations (> 4 km from other beekeepers) but may not be completely isolated due to the potential presence of feral (unmanaged) bees. There is also some use of artificial insemination.

The second population is from Better Bees WA Inc. The programme was initiated in 1979–1980 to provide Western Australia (WA) with quality stock following the closure of the borders to prevent European foulbrood from entering the state (Kühnert et al. 1989). For the first few years, an isolated mating station on Rottneest Island was used. From 1983, the

programme was maintained by artificial insemination with line breeding, queens being inseminated with homogenised semen collected from drones of all lines (Kühnert et al. 1989). When it was taken over by beekeepers in 1991, isolated mating was again utilised (Chapman et al. 2008). Annually, the beekeepers (11 at the time of this study) in the consortium take 50–70 of the top performing colonies to provide drones for hundreds of queens to mate with on Rottneest Island, where there are no feral bees (Chapman et al. 2008). These breeder queens are then returned to the mainland and used to produce daughter queens that are typically open-mated and distributed predominantly within WA. The number of lines in the programme has varied over the years, starting with 20 lines (Kühnert et al. 1989), having 24 in 2005 (Chapman et al. 2008), and 31 at the time of this study.

The third population is a research and development stock run by the New South Wales Department of Primary Industries. The population was established in March 2020 with stock from Western Australia, Queensland, and NSW bee breeders, with new genetics introduced in 2021 from South Australia and Tasmania. This population consists of 250 colonies made of two subpopulations—black and yellow. Stock is maintained using a mix of open mating and artificial insemination.

The last population is from a bee breeder located in Queensland who has been commercially breeding bees since 2013 with ~450 colonies. There are eight breeding lines, originating from several sources including queen bee breeders from NSW and Kangaroo Island, South Australia. Breeding stock is maintained by AI and open mating with drone flooding.

## 2. MATERIAL AND METHODS

### 2.1. Sample collection and DNA extraction

Up to six drone pupae (range 1–6) were collected per colony. As drones are unfertilised, sampling six provides a 96% probability of observing both queen alleles (Hyink et al. 2013). Samples were collected from 37 colonies from the NSW beekeeper in April 2021, 55 drone-producing colonies from Better Bees WA in October 2020, 97 colonies from the NSW Department of Primary Industries in 2021–2022, and 20 colonies from the Queensland beekeeper in 2018. Samples were preserved in 100% ethanol or dry ice and stored at  $-20\text{ }^{\circ}\text{C}$ .

DNA was extracted from the hind legs of the pupae. Twenty milligrams of tissue was homogenised in 400  $\mu\text{l}$  of 5% Chelex (Bio-Rad, United States) using a TissueLyser II (Qiagen, Germany). The tissue was then boiled for 10 min in a water bath (Oldroyd et al. 1997) and centrifuged at 1883 RCF for 20 min. One hundred microliters of supernatant was then stored at  $4\text{ }^{\circ}\text{C}$  and used as a DNA template for PCR.

### 2.2. *csd* sequencing

The HVR of *csd* was amplified using the primers M13Fconscsdrev and AD1genoRfw (Hyink et al. 2013). PCR was performed using 0.15  $\mu\text{l}$  KAPA polymerase (Merck, Germany), 3  $\mu\text{l}$  template DNA, 0.40  $\mu\text{M}$  primer, and 1.5 mM  $\text{MgCl}_2$  in 20  $\mu\text{l}$  reactions on a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA). The thermocycling protocol was  $95\text{ }^{\circ}\text{C}$  for 5 min, 35 cycles of  $95\text{ }^{\circ}\text{C}$  for 30 s,  $49\text{ }^{\circ}\text{C}$  for 15 s,  $72\text{ }^{\circ}\text{C}$  for 30 s, and a final extension of  $72\text{ }^{\circ}\text{C}$  for 10 min.

To distinguish between each of the queen's alleles, restriction digests were used, which resulted in restriction fragment length polymorphisms (RFLP), where bands of different sizes indicate different alleles to sequence. Five microliters of drone PCR product was digested with *ApoI* (New England BioLabs, USA). The restriction digest was incubated for 30 min at  $49.5\text{ }^{\circ}\text{C}$  and then run on a 1.5% agarose gel in Sodium Borate buffer, containing SYBR Safe DNA gel stain (Invitrogen, USA) and a 1 Kb Plus DNA Ladder (Invitrogen, USA) at 160 V for 25–30 min. The digests were visualised on the Molecular Imager<sup>R</sup> Gel Doc™ XR System (Bio-Rad, USA) on Image Lab software v6.1 (Bio-Rad). PCR products representing each of the queen's *csd* alleles were sent for sequencing in the forward direction at Macrogen (South Korea) using sequencing primer AD1 (Hyink et al. 2013). In some cases, only one RFLP profile was found from a colony, which may be due to only drones carrying the same queen allele being sampled by chance (expected 4% of the time where six drones were sampled per colony) or due to alleles not differing at *ApoI* sites. In some cases, more than two RFLP profiles were found from a colony. This could be due to the colony being queenless, resulting in the workers activating their ovaries and producing unfertilised eggs, rare workers activating their ovaries and producing unfertilised eggs in the presence of the queen (Ratnieks 1993), or mutation. Where more than two alleles were found from a colony, the sequences were aligned and assessed for mutation or recombination.

### 2.3. Data analysis

DNA sequences were manually checked for quality; 47 sequences were excluded as less than 25% of the contig was high quality under Geneious Prime's (version 2021.2, Biomatters Ltd., New Zealand) calculation of Phred quality score. The sequences were trimmed to the six amino acid flanking sequence upstream of the HVR and the highly conserved 'IEQI' sequence downstream following Bilodeau and Elsie (2021) and aligned using Clustal Omega alignment in Geneious Prime (Dotmatics).

The nucleotide sequence was translated to the amino acid sequence in Geneious Prime. Sequences with  $\geq 1$  amino acid variants were considered different alleles. The alleles were matched to the Hymenoptera Genome Database (Bilodeau and Elsik 2021). Alleles that did not have a 100% match to the database record were given new identification labels. Newly identified alleles (16) were aligned pairwise via a Needleman-Wunsch global alignment in Geneious Prime to obtain 120 alignments of potential combinations of new *csd* alleles and the number of amino acid differences between them was calculated.

### 3. RESULTS

For the NSW beekeeper, 15 out of 37 colonies had fewer than six pupae collected (mean = 4.9, range = 1–6), due to poor drone availability in April. Better Bees WA had six drone pupae collected from each colony; in the NSW Department of Primary Industries samples, 96 out of 97 colonies had six drones. The Queensland beekeeper had only one colony out of 20 with six drones collected (mean = 4, range = 1–6).

#### 3.1. Amino acids

Eighty-two unique *csd* amino acid sequences were identified (Supplementary Table 1). Sixteen had not been previously reported and the other 66 matched previously identified alleles in the Hymenoptera Genome Database (Bilodeau and Elsik 2021). The new alleles are deposited in GenBank under the accession numbers ON507758–ON507774.

Only a single amino acid sequence was characterised in nine colonies for the NSW beekeeper, six colonies for Better Bees WA, ten colonies for the NSW Department of Primary Industries, and five colonies for the Queensland beekeeper, where two were expected due to queens carrying two alleles. Eight colonies had three amino acid sequences characterised, while three had four amino acid sequences characterised. Sequence alignments indicated that the origin of these alleles was from workers laying eggs rather than mutations or recombination.

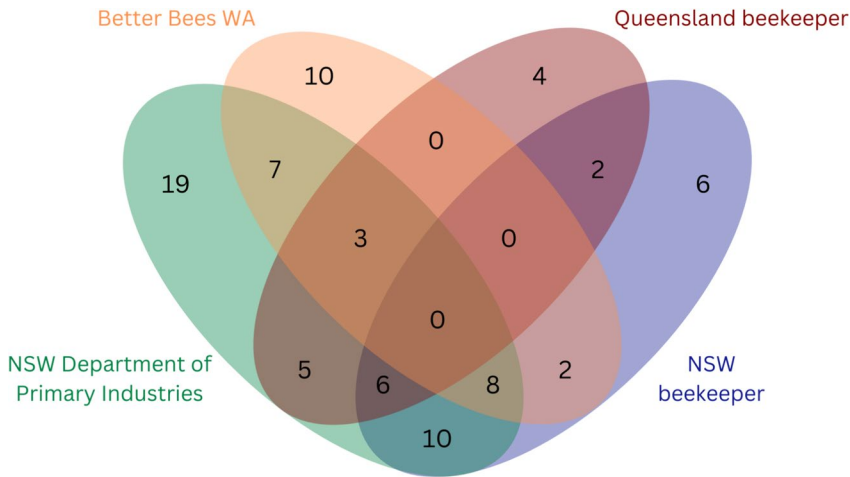
The NSW beekeeper had 34 unique alleles characterised from 37 colonies, Better Bees WA had 30 unique alleles from 55 colonies, the NSW Department of Primary Industries had 58 from 97 colonies, and the Queensland beekeeper had 19 alleles from 20 colonies.

No allele was found in all four populations (Figure 1). Better Bees WA and the Queensland beekeeper had the fewest alleles in common (3), while the NSW beekeeper and NSW Department of Primary Industries shared the most (24). Seven sequences occurred in both of the NSW Department of Primary Industry breeding populations. The black subpopulation had 36 unique alleles found from 33 colonies and the yellow subpopulation had 51 alleles found from 57 colonies.

The newly characterised alleles varied in length from 29 to 53 amino acids. Pairwise alignments revealed an average difference of  $14.46 \pm 0.47$  amino acids between pairs and a range of 1–29 (Figure 2; Supplementary Table 2).

### 4. DISCUSSION/CONCLUSION

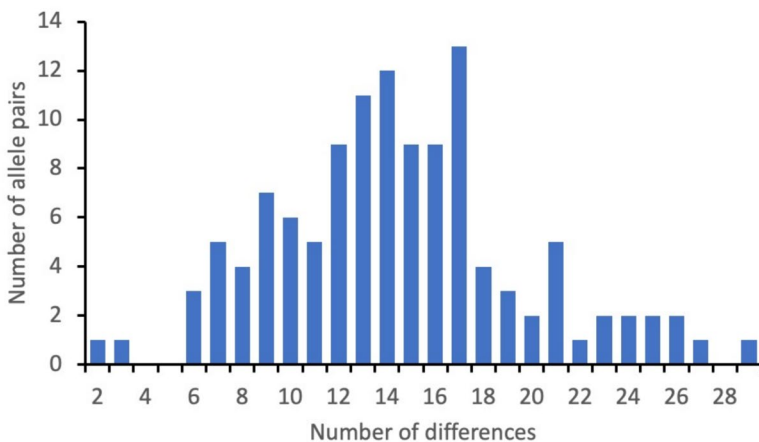
The results from this study demonstrate that there is sufficient diversity at *csd* in Australia, using other countries as comparison, boding well for the Plan Bee programme. Generally, allele diversity at *csd* is reported as the number of *csd* alleles found in the total colonies sampled. Here, we have reported the number of alleles found as a proportion of the possible number found assuming that only the queen produced drones (i.e. the number of colonies multiplied by two, the number of *csd* alleles the queen carries). The proportions found in this study for all four populations (27.3–47.5%) are within the range reported worldwide, (19.0–66.5%; Table 1). While this method enables comparison of diversity among populations, it is problematic. Firstly, workers are known to produce drones in the presence of the queen (Ratnieks 1993; this study) and this calculation cannot take that into consideration. Secondly, it does not take into consideration the probability of sampling both queen alleles due to differences in sample size per colony. Finally,



**Figure 1.** Venn diagram representing the overlap of alleles found in the four populations in this study: Better Bees WA Inc, individual bee breeders from New South Wales and Queensland, and the research population managed by the New South Wales Department of Primary Industries

it does not provide information about whether *csd* proportions are diverse enough to sustain long-term growth. Nevertheless, it does provide a means of comparing populations. The results of this study contain both underestimations due to missing queen alleles and overestimations due to worker reproduction; this however does not affect the main conclusion that there is sufficient

*csd* diversity (82 unique alleles) in Australia. New Zealand’s breeding population has the lowest recorded *csd* diversity at 19.0% and 16 unique alleles (Table I). As the New Zealand programme is sustaining normal breeding, it is used as a baseline for minimum viable *csd* population diversity (Hyink et al. 2013; Zareba et al. 2017; Kaskinova et al. 2019). Next-generation



**Figure 2.** Number of differences observed between pairs of 16 newly identified amino acid sequences for the hyper-variable region (HVR) of the complementary sex determiner (*csd*) gene in honey bees (*Apis mellifera*) when aligned pairwise (120 combinations in total)

sequencing on honey samples has revealed 160 alleles identified from 12 colonies (Bovo et al. 2021). Thus, Bovo et al. (2021) show that high *csd* diversity in populations makes it unlikely for diploid males to occur at high frequency.

The *csd* diversity in Western Australia could be expected to be low as the state banned the import of bees since the 1970s due to the risk of introducing exotic honey bee diseases from the eastern states, and rates of feral bee introgression are low (Chapman et al. 2008, 2016). Better Bees WA has not observed any signs of inbreeding and has been able to maintain *csd* allele diversity with 30 alleles from 55 colonies. This observation is consistent with Better Bees WA's high genetic diversity at microsatellites (Chapman et al. 2008). Better Bees WA uses a high number of drone-producing colonies each year from different sources for mating (Chapman et al. 2008; Oxley and Oldroyd 2010). In 2020, 55 drone-producing colonies were taken to Rottnest Island. Coupled with the rapid mutation of the *csd* gene which is also under balancing selection (Ding et al. 2021), it has allowed novel *csd* variants to not only emerge, but also persist in the population. The ability of *csd* variants to persist in populations was observed in *A. cerana* in Australia, where a single introduced colony was able to successfully establish an invasive population with the aid of strong balancing selection that equalised allele frequencies, keeping initially rare *csd* alleles in the population (Gloag et al. 2019; Ding et al. 2021). It is likely that the combination of management, mutation, and selection has helped maintained *csd* diversity.

Computer simulations have attempted to establish *csd* loss in closed breeding populations; an early study of 50 colonies and 10 *csd* alleles found a 95% chance of mean brood viability being at least 85% after 40 generations (years) (Page and Laidlaw 1982). This study found 30 alleles in Better Bees WA, three times that in the simulation study, and only a small proportion of the population was sampled. There were 48,978 registered hives in WA in 2019 (Clarke and Le Feuvre 2021). It therefore seems likely the programme can continue for another 40 years.

Both individual queen bee breeders in NSW and Queensland had a greater proportion of unique alleles compared to the number of alleles in the consortium of 11 beekeepers in Better Bees WA. Both individual queen bee breeders source their queens from a variety of stock from all over Australia and have been operating for less than 10 years.

NSW Department of Primary Industries' black and yellow subpopulations both display *csd* diversity comparable to other breeding populations, with black having 54.5% of the potential number of alleles given the number of colonies investigated and yellow having 44.8%, a higher individual average compared to the total NSW Department of Primary Industries population comparison (Table 1).

This study revealed alleles at high frequency in three populations. The NSW beekeeper had one allele present in 17.4% of samples; another allele was present in 13.6% of Better Bees WA samples. The Queensland beekeeper had three alleles at frequencies above 10%. The overuse of the offspring of these queens would result in further overrepresentation of these alleles. In naturally reproducing populations, *csd* alleles will be maintained via balancing selection (Cho et al. 2006; Hasselmann and Beye 2006; Lechner et al. 2014; Gloag et al. 2019; Ding et al. 2021). Breeding populations take the queens and drones from the colonies that perform the best commercially, and propagate their genetics in the next generation, potentially leading to narrowing in the number of *csd* alleles or some alleles appearing at high frequency. Taking *csd* alleles and inbreeding in general into consideration is important for the maintenance of breeding populations long term. In particular, breeding programmes that use artificial insemination should consider testing *csd* alleles as this technique is both time-consuming and requires expert skills (Khan et al. 2022). As it is costly to produce queens using artificial insemination, issues with low *csd* diversity would be detrimental and could result in loss of accumulated genetic gain. Queens could thus be chosen as drone or queen mothers and crossed such that diversity at *csd* is

maximised. Checking *csd* alleles of queens used in artificial insemination is currently applied in the closed breeding programme in New Zealand (Hyink et al 2013). The methods used in this study should also be used to estimate *csd* diversity in other Australian breeding populations of importance. For instance, Kangaroo Island lost over a quarter of its hives in the 2019–2020 Australian bushfires (Clarke 2020) and has had no import of bees since 1885 (Chapman et al. 2019b).

This study identified 16 new *csd* alleles, and without doubt, if more samples had been taken, more alleles would have been found as each study identifies new alleles. The new alleles identified in this study show a similar range in length and distribution in variation between pairwise alignment as in other studies (Zareba et al. 2017; Bovo et al. 2021). *csd* alleles are traditionally considered diverse enough to make a functional pairing if a pair has a minimum of five amino acid differences (Beye et al. 2013; Zareba et al. 2017). However, this rule has shortcomings as in *A. cerana*, a single amino acid difference in the HVR was sufficient to result in a functional pair (Ding et al. 2021). Whether a pair will be functional is more complex than the number of amino acid differences in the HVR, with the PSD also playing a role (Ding et al. 2021). A larger number of variations in the HVR may be indicative of an increased probability of a functional pairing, but it will not always be the case; the only way to be sure that a pair will result in viable offspring is to combine them in the field.

*Varroa destructor* was detected in Newcastle, NSW, in June 2022 (Department of Regional NSW 2023) and efforts to eradicate it ceased in September 2023. Most queen breeding outside Western Australia occurs in southeast Queensland, and central and northern NSW, due to favourable climate (Clarke and Le Feuvre 2021). The *V. destructor* incursion may lead to an increase in local breeding in which beekeepers source bees locally due to border closures and movement restrictions to prevent spread. Local bee populations have shown local adaptations, making them less prone to environmental impacts (Meixner et al. 2014; Walsh and Rangel 2016). If this were to occur, genetic diversity in these populations should be evaluated.

Moreover, it is expected that untreated honey bee colonies will die within 1–2 years (Le Conte et al. 2007), and some proportion of treated colonies may also die. This may be further exacerbated by Australia's honey bee stock having shown a lack of resistance against *V. destructor* when tested in the USA (Oldroyd 2012; Rinderer et al. 2013). Such a loss is likely to significantly reduce genetic diversity in general. More detailed knowledge of *csd* diversity across Australia may help to develop management plans to mitigate such losses. It will take some time for *V. destructor* to spread across such a large country; sampling of commercial and feral populations ahead of the invasion front and over time will allow not only the tracking of loss of genetic diversity, but also the identification of selective sweeps associated with strong selection on genes associated with resistance to *V. destructor* (Chapman et al. 2023).

## SUPPLEMENTARY INFORMATION

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## AUTHOR CONTRIBUTION

EYYM contributed to the study conception and design, sample collection, data collection and analysis, and the first draft of the manuscript. PU performed data collection. TB collected samples. JD collected samples. CEG contributed to the study conception and design and data analysis. JL performed data collection. EAF contributed to sample collection. NCC contributed to the study conception and design, sample collection, data collection and analysis, and the first draft of the manuscript. All authors read and approved the final manuscript.

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## DATA AVAILABILITY

Sequences are available on GenBank; all other data is available in the paper and supplementary material.

## CODE AVAILABILITY

Not applicable.

## DECLARATIONS

**Ethics approval** No ethics approval was required for this study.

**Consent to participate** Beekeepers provided consent to have their bees included in the analysis.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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## REFERENCES

- Aizen MA, Aguiar S, Biesmeijer JC, Jung C, Martins DJ, Garibaldi LA, Inouye DW, Medel R, Morales CL, Paxton RJ (2019) Global agricultural productivity is threatened by increasing pollinator dependence without a parallel increase in crop diversification. *Glob Change Biol* 25:3516–3527
- Aizen MA, Harder LD (2009) The global stock of domesticated honey bees in growing slower than agricultural demand for pollination. *Curr Biol* 19:915–918
- Banks RG, Frost E, Chapman N, Boerner V, Walkom S, Ferdosi M (2021) Progressing implementation of genetic selection in Australian honey bees. *AgriFutures Australia Wagga Wagga NSW Australia*
- Beye M, Hasselmann M, Fondrk MK, Page RE, Omholt SW (2003) The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* 114:419–429
- Beye M, Seelman C, Gempe T, Hasselmann M, Vekemans X, Fondrk MK, Page RE (2013) Gradual molecular evolution of a sex determination switch through incomplete penetrance of femaleness. *Curr Biol* 23:2559–2565
- Bienefeld K, Ehrhardt K, Reinhardt F (2008) Noticeable success in honey bee selection after the introduction of genetic evaluation using BLUP. *Am Bee J* 148:739–742
- Bilodeau L, Avalos A, Danka RG (2020) Genetic diversity of the complementary sex-determiner (*csd*) gene in two closed breeding stocks of *Varroa*-resistant honey bees. *Apidologie* 51:1125–1132
- Bilodeau L, Elsik C (2021) A scientific note defining allelic nomenclature standards for the highly diverse complementary sex-determiner (*csd*) locus in honey bees. *Apidologie* 52:749–754
- Bixby M, Baylis K, Hoover SE, Currie RW, Melathopoulos AP, Pernal SF, Foster LJ, Guarna MM (2017) A bio-economic case study of Canadian honey bee (Hymenoptera: Apidae) colonies: marker-assisted selection (MAS) in queen breeding affects beekeeper profits. *J Econ Entomol* 110:816–825
- Bovo S, Ribani A, Utzeri VJ, Taurisano V, Schiavo G, Bolner M, Fontansei L (2021) Application of next generation semiconductor-based sequencing for the identification of *Apis mellifera* complementary sex determiner (*csd*) alleles from honey DNA. *Insects* 12:868
- Breeze TD, Bailey AP, Balcombe KG, Potts SG (2011) Pollination services in the UK: how important are honeybees? *Agr Ecosyst Environ* 142:137–143
- Büchler R, Andonov S, Bienefeld K, Costa C, Hatjina F, Kezic N, Kryger P, Spivak M, Uzunov A, Wilde J (2013) Standard methods for rearing and selection of *Apis mellifera* queens. *J Apic Res* 52:1–30

- Büchler R, Berg S, Le Conte Y (2010) Breeding for resistance to *Varroa destructor* in Europe. *Apidologie* 41:393–408
- Chapman NC, Colin T, Cook J, da Silva CRB, Gloag R, Hogendoorn K, Howard SR, Remnant EJ, Roberts JMK, Tierney SM, Wilson RS, Mikheyev AS (2023) The final frontier: ecological and evolutionary dynamics of a global parasite invasion. *Biol Lett* 19:20220589
- Chapman NC, Frost EA (2021a) Plan Bee breeding manual, AgriFutures, Wagga Wagga, Australia
- Chapman NC, Frost EA (2021b) Plan Bee: beekeeper and queen bee breeder surveys 2020, AgriFutures, Wagga Wagga, Australia
- Chapman NC, Frost EA (2022) 2021 Plan Bee survey. AgriFutures Australia, Wagga Wagga, NSW Australia
- Chapman NC, Frost EA, Banks RG (2022) A model for, and early implementation of, genomic selection in the Australian honey bee population. *Proceedings of 12th World Congress on Genetics Applied to Livestock Production*: 2512–2515
- Chapman NC, Harpur BA, Lim J, Rinderer TE, Allsopp MH, Zayed A, Oldroyd BP (2016) Hybrid origins of Australian honeybees (*Apis mellifera*). *Apidologie* 47:26–34
- Chapman NC, Lim J, Oldroyd BP (2008) Population genetics of commercial and feral honey bees in Western Australia. *J Econ Entomol* 101:272–277
- Chapman NC, Dos Santos Cocenza R, Blanchard B, Nguyen LM, Buchmann G, Oldroyd BP (2019a) Genetic diversity in the progeny of commercial Australian queen honey bees (*Apis mellifera*) produced in autumn and early spring. *Econ Entomol* 112:33–39
- Chapman NC, Sheng J, Lim J, Malfroy SF, Harpur BA, Zayed A, Allsopp MH, Rinderer TE, Roberts JMK, Remnant EJ, Oldroyd BP (2019b) Genetic origins of honey bees (*Apis mellifera*) on Kangaroo Island and Norfolk Island (Australia) and the Kingdom of Tonga. *Apidologie* 50:28–39
- Cho SC, Huang ZY, Green DR, Smith DR, Zhang JZ (2006) Evolution of the complementary sex-determination gene of honey bees: balancing selection and trans-species polymorphisms. *Genome Res* 16:1366–1375
- Clarke M (2020) Bushfire Recovery Plan: understanding what needs to be done to ensure the honey bee and pollination industry recovers from the 2019–20 bushfire crisis, AgriFutures, Canberra, Australia
- Clarke M, Le Feuvre D (2021) Size and scope of the Australian honey bee and pollination industry: a snapshot, AgriFutures, Wagga Wagga, Australia
- Costa-Maia FC, Lourenco D, Tsuruta S, Martins E (2018) Selection criteria for improving honey production in Africanized honey bees. *J Anim Sci* 96(S3):115
- Department of Regional NSW (2023) Biosecurity (Varroa Mite) emergency order (No. 17) 2023, in: Government N. (Ed.), 242, Government Gazette
- Ding G, Hasselmann M, Huang J, Roberts J, Oldroyd BP, Gloag R (2021) Global allele polymorphism indicates a high rate of allele genesis at a locus under balancing selection. *Heredity* 126:163–177
- Frost EA, Chapman NC, Banks RG, Walkom SF, Hermes S (2022) Economic value and production characteristics of table honey. *Proceedings of 12th World Congress on Genetics Applied to Livestock Production* 2508–2511
- Frost EA, Chapman NC, Banks RG, Hermes S (2021) Breeding for improved fertility of honey bees, Breeding focus 2021 - improving reproduction. University of New England, Animal Genetics and Breeding Unit, pp 97–110
- Gallai N, Salles JM, Settele J, Vaissière BE (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol Econ* 68:810–821
- Gloag RS, Christie JR, Ding G, Stephens RE, Buchmann G, Oldroyd BP (2019) Workers' sons rescue genetic diversity at the sex locus in an invasive honey bee population. *Mol Ecol* 28:1585–1592
- Goulson D, Nicholls E, Botías C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* 347:2015
- Gray A, Adjlane N, Arab A, Ballis A, Brusbardis V, Douglas AB, Cadahía L, Charrière J-D, Chlebo R, Coffey MF, Cornelissen B, da Costa CA, Danneels E, D'anihlik J, Dobrescu C, Evans G, Fedoriak M, Forsythe I, Gregorc A, Arakelyan II, Johannesen J, Kauko L, Kristiansen P, Martikkala M, Martín-Hernández R, Mazur E, Medina-Flores CA, Mutinelli F, Omar EM, Patalano S, Raudmets A, San Martín G, Soroker V, Stahlmann-Brown P, Stevanović J, Uzunov A, Vejsnaes F, Williams A, Brodschneider R (2022) Honey bee colony loss rates in 37 countries using the COLOSS survey for winter 2019–2020: the combined effects of operation size, migration and queen replacement. *J Apic Res* 62:204–210
- Guichard M, Neuditschko M, Soland G, Fried P, Grandjean M, Gerster S, Dainat B, Bijma P, Brascamp EW (2020) Estimates of genetic parameters for production, behaviour, and health traits in two Swiss honey bee populations. *Apidologie* 51:876–891
- Hasselmann M, Beye M (2006) Pronounced differences of recombination activity at the sex determination locus of the honeybee, a locus under strong balancing selection. *Genetics* 174:1469–1480
- Hoppe A, Plate M, Bernstein R, Tiesler FK, Kärcher M, Bienefeld K (2020) Breeding value estimation yielded substantial progress in the international breeding of the honeybee *Apis mellifera carnica*. *Insects* 11:768
- Hristov P, Shumkova R, Palova N, Neov B (2020) Factors associated with honey bee colony losses: a mini-review. *Vet Sci* 7:166

- Hung K LJ, Kingston JM, Albrecht M, Holway DA, Kohn JR (2018) The worldwide importance of honey bees as pollinators in natural habitats. *Proc R Soc Lond B* 285:20172140
- Hyink O, Laas F, Dearden PK (2013) Genetic tests for alleles of *complementary-sex-determiner* to support honeybee breeding programmes. *Apidologie* 44:306–313
- Insolia L, Molinari R, Rogers SR, Williams GR, Chiaromonte F, Calovi M (2023) Honey bee colony loss linked to parasites, pesticides and extreme weather across the United States. *Sci Rep* 12:20787
- Kaskinova MD, Gataullin AR, Saltykova ES, Gaifullina LR, Poskryakov AV, Nikolenko AG (2019) Polymorphism of the hypervariable region of the *csd* gene in the *Apis mellifera* L. population in Southern Urals. *Russ J Genet* 55:267–270
- Khan KA, Rafique MK, Lashari MA, Iqbal A, Mahmood R, Ahmed AM, Khoso FN, Ahmad S, Al-Shehri BM, Mohammed MEA, Ghramh HA (2022) Instrumental inseminations: a nontraditional technique to produce superior quality honey bee (*Apis mellifera*) queens. *Journal of King Saud University - Science* 34:102077
- Klein AM, Vaissière BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T (2007) Importance of pollinators in changing landscapes for world crops. *Proc R Soc Lond B* 274:303–313
- Kühnert M, Carrick M, Allan L (1989) Use of homogenized drone semen in a bee breeding program in Western Australia. *Apidologie* 20:371–381
- Le Conte Y, De Vaublanc G, Crauser D, Jeanne F, Rousselle JC, Bécard JM (2007) Honey bee colonies that have survived *Varroa destructor*. *Apidologie* 38:566–572
- Lechner S, Ferretti L, Schöning C, Kinuthia W, Willemsen D, Hasselmann M (2014) Nucleotide variability at its limit? Insights into the number and evolutionary dynamics of the sex-determining specificities of the honey bee *Apis mellifera*. *Mol Biol Evol* 31:272–287
- Mackensen O (1951) Viability and sex determination in the honey bee (*Apis mellifera* L.). *Genetics* 36:500
- Mashilingi SK, Zhang H, Garibaldi LA, An J (2022) Honeybees are far too insufficient to supply optimum pollination services in agricultural systems worldwide. *Agric Ecosyst and Environ* 335:108003
- Maucourt S, Fortin F, Robert C, Giovenazzo P (2021) Genetic progress achieved during 10 years of selective breeding for honeybee traits of interest to the beekeeping industry. *Agriculture* 11:535
- Meixner MD, Francis RM, Gaida A, Kryger P, Nadoonov S, Uzunov A, Topolska G, Costa C, Amiri E, Berg S, Bienkowska M, Bouga S, Büchler R, Dyrba W, Gurgulova K, Hatjina F, Ivanova E, Janes M, Kezic N, Korpela S, Le Conte Y, Panasiuk B, Pechhacker H, Tsoktouridis G, Vaccari G, Wilde J (2014) Occurrence of parasites and pathogens in honey bee colonies used in a European genotype-environment interactions experiment. *J Apic Res* 53:215–229
- Neumann P, Moritz RF, van Praagh J (1999) Queen mating frequency in different types of honey bee mating apiaries. *J Apic Res* 38:11–18
- Oldroyd B (2012) How susceptible are Australian honeybee stocks to *Varroa*?, Rural Industries Research and Development Corporation
- Oldroyd BP, Morag JC, Wongsiri S, Rinderer TE, Sylvester HA, Crozier RH (1997) Polyandry in the genus *Apis*, particularly *Apis adeniformis*. *Behav Ecol Sociobiol* 40:17–26
- Oxley PR, Oldroyd BP (2010) The genetic architecture of honeybee breeding, *Advances in Insect Physiology*, Academic Press, Burlington, MA USA
- Page RE (1980) The evolution of multiple mating behaviour by honey bee queens (*Apis mellifera* L.). *Genetics* 96:263–273
- Page RE, Laidlaw HH (1982) Closed population honeybee breeding. 1. Population genetics of sex determination. *J Apic Res* 21:30–37
- Page RE, Laidlaw HH (1985) Closed population honeybee breeding. *Bee World* 66:63–72
- Paolillo G, De Iorio MG, Filipe JFS, Riva F, Stella A, Gandini G, Pagnacco G, Minozzi G (2022) Analysis of *complementary sex-determiner* (*csd*) allele diversity in different honeybee subspecies from Italy based on NGS data. *Genes* 13:991
- Petersen GEL, Fennessy PF, Amer PR, Dearden PK (2020) Designing and implementing a genetic improvement program in commercial beekeeping operations. *J Apic Res* 59:638–647
- Phiri BJ, Fèvre D, Hidano A (2022) Uptrend in global managed honey bee colonies and production based on a six-decade viewpoint, 1961–2017. *Sci Rep* 12:21298
- Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. *Trends Ecol Evol* 25:345–353
- Ratnieks FLW (1993) Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. *Behav Ecol Sociobiol* 32:191–198
- Rinderer TE, Oldroyd BP, Frake AM, de Guzman LI, Bourgeois L (2013) Responses to *Varroa destructor* and *Nosema ceranae* by several commercial strains of Australian and North American honeybees (Hymenoptera: Apidae). *Aust J Entomol* 52:156–163
- Simone-Finstrom M, Tarpay DR (2018) Honey bee queens do not count mates to assess their mating success. *J Insect Behav* 31:200–209
- Smith KM, Loh EH, Rostal MK, Zambrana-Torrel CM, Mendiola L, Daszak P (2013) Pathogens, pests, and economics: drivers of honey bee colony declines and losses. *EcoHealth* 10:434–445
- Steinhauer N, Kulhanek K, Antunez K, Human H, Chantawannakul P, Chauzat MP, VanEngelsdorp

- D (2018) Drivers of colony losses. *Curr Opin Insect Sci* 26:142–148
- Tahmasbi G, Kamali MA, Ebadi R, Nejati Javaremi A, Babaei M, Gharadaghi AA, Bahraimi R (2015) Genetic trends and parameters of honey production, swarming and defense behavior in Iranian honeybee (*Apis mellifera meda*) colonies. *J Agric Sci Technol* 17:1735–1742
- Tarpy DR, Caren JR, Delaney DA, Sammataro D, Finley J, Loper GM, DeGrandi-Hoffman G (2010) Mating frequencies of Africanized honey bees in the south western USA. *J Apic Res* 49:302–310
- Tarpy DR, Delaney DA, Seeley TD (2015) Mating frequencies of honey bee queens (*Apis mellifera* L.) in a population of feral colonies in the Northeastern United States. *PLoS ONE* 10:e0118734
- Tarpy DR, Nielsen R, Nielsen DI (2004) A scientific note on the revised estimates of effective paternity frequency in *Apis*. *Insectes Soc* 51:203–204
- Utaipanon P, Holmes MJ, Chapman NC, Oldroyd BP (2019) Estimating the density of honey bee (*Apis mellifera*) colonies using trapped drones: area sampled and drone mating flight distance. *Apidologie* 50:578–592
- Walsh E, Rangel J (2016) Local honey bee queen production and quality. *Bee World* 93:30–32
- Woyke J (1963) What happens to diploid drone larvae in a honeybee colony? *J Apic Res* 2:73–75
- Woyke J (1980) Effect of sex allele homo-heterozygosity on honeybee colony populations and on their honey production I. Favourable development conditions and unrestricted queens. *J Apic Res* 19:51–63
- Yang Y, Wu Y, Long H, Ma X, Shariati K, Webb J, Guo L, Pan Y, Ma M, Deng C, Cao P, Chen J (2023) Global honeybee health decline factors and potential conservation techniques. *Food Security* 15:855–875
- Zakour MK, Bienefeld K (2014) Basic considerations in the development of breeding plans for honey bees, illustrated by data on the native Syrian honey bee (*Apis mellifera syriaca*). *J Apic Res* 53:314–326
- Zareba J, Blazej P, Laszkiewicz A, Sniezewhki L, Majkowski M, Janik S, Cebrat M (2017) Uneven distribution of *complementary sex determiner (csd)* alleles in *Apis mellifera* population. *Sci Rep* 7:2317

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