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INVITED REVIEW

Assessing the density of honey bee colonies at ecosystem scales

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Abstract. 1. Information about the density of wild honey bee (*Apis* spp.) colonies in an ecosystem is central to understanding the functional role of honey bees in that ecosystem, necessary for effective biosecurity response planning, and useful for determining whether pollination services are adequate. However, direct visual surveys of colony locations are not practical at ecosystem scales. Thus, indirect methods based on population genetic analysis of trapped males have been proposed and implemented.

2. In this review, indirect methods of assessment of honey bee colony densities are described, which can be applied at ecosystem scales. The review also describes how to trap males in the field using the Williams drone trap (or virgin queens) the appropriate genetic markers and statistical analyses, and discusses issues surrounding sample size.

3. The review also discusses some outstanding issues concerning the methods and the conversion of estimated colony number to colony density per km^2. The appropriate conversion factor will require further research to determine the area over which a drone trap draws drones.

Key words. Biosecurity, colony density, drone trap, pollination, resource competition.

Introduction

In this paper we review methods for assessing the density of honey bee colonies, primarily the European honey bee (*Apis mellifera*), at ecosystem scales, based on population genetic approaches. Such estimates are important in various contexts, including crop pollination, conservation of natural environments, and planning efficient biosecurity responses. We begin our review by considering the importance of understanding honey bee colony densities. We then discuss the various approaches to sampling, genetic analysis and statistical analysis. We conclude by considering some outstanding research questions that must be addressed before these techniques can reach their full potential.

Understanding the population dynamics of wild honey bee populations is important in several contexts. First, in some agricultural industries, it is assumed that pollination services provided by wild bees (both native and feral honey bees) are adequate. Generally it is recommended that insect pollination-dependent crops should have four to five strong honey bee colonies per hectare (Free, 1970; McGregor, 1976; Delaplane *et al.*, 2000). The assumption that there are sufficient wild or feral honey bee colonies in agricultural ecosystems often has no scientific basis, and growers may be losing production and profit by not providing supplementary pollinators (Breeze *et al.*, 2011; Cunningham & Le Feuvre, 2013). Therefore, rapid methods for determining the density of honey bee colonies are needed to inform growers.

Second, when an exotic honey bee disease or parasite is introduced to a country, the appropriate biosecurity response is dependent in part on the extent of the extant honey bee population in the area where the incursion is first detected. In areas where the density of honey bees is low, eradication is more likely to be successful than in areas where the density of colonies is high. As eradication programmes are disruptive and expensive, information about colony density is crucial to determining the appropriate biosecurity response.

Third, in an ideal world there would be no feral animals, including feral honey bees, in areas of high conservation value. Feral honey bees can compete with native animals and bird species for nest sites (Saunders *et al.*, 1982; Coelho & Sullivan, 1994; Oldroyd *et al.*, 1994; Wood & Wallis, 1998a,b; Hudewenz & Klein, 2013) and displace native pollinators (Brittain *et al.*, 2013; Hudewenz & Klein, 2013; Lindström *et al.*, 2016),

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thereby disrupting co-adapted plant–pollinator relationships and potentially reducing seed set (Celebrezze & Paton, 2004). In other contexts, honey bees can successfully replace native pollinators that no longer provide adequate pollination services (Taylor & Whelan, 1988; Corlett, 2001). Accurate estimates of the density of feral colonies, both temporally and spatially, are essential to understanding the possible impacts of feral bees on conservation values (Tilman, 1987; Strauss, 1991; Paton, 1993; Goulson, 2003; Simpson *et al.*, 2005), and the feasibility of reducing feral bee numbers where this is deemed desirable (Oldroyd, 1998).

Fourth, some Asian honey bee species face local extinction in the face of deforestation, over-hunting, excessive pesticide use and reproductive competition (Oldroyd & Wongsiri, 2006; Oldroyd & Nanork, 2009; Rattanawannee *et al.*, 2013; Remnant *et al.*, 2014). Efficient methods for estimating population size and monitoring temporal changes in colony density may help to spur better conservation efforts if it can be shown that economically and ecologically important honey bee species like *A. dorsata*, *A. florea* and *A. cerana* are in decline. Conversely, should a strategy to help conserve a honey bee species have been implemented, it is desirable to monitor the effectiveness of that strategy by documenting any improvements to population size over time.

Estimating the density of insects in the field: why honey bees are a special case

There are numerous methods for assessing the density of insects in the field (Seber, 1982; King, 2014). For relatively immobile species, it is possible to use some sort of sampling scheme and directly count individual insects, thereby obtaining an estimate of insects per unit area. For mobile species, individuals can be marked, released, and then recaptured. The proportion of marked individuals that are recaptured provides an estimate of the population size. Pheromone traps, pitfall traps and light traps can determine whether a species is present in the area, but cannot be used to determine precise densities, because the area over which the insects are attracted is effectively unknown (Elkinton & Carde, 1988; Tobin *et al.*, 2011).

It is not possible to estimate the density of honey bee colonies in an area by any of the means described above. Honey bees are central-place foragers, and use their dance language to focus forager attention on areas of high reward (Visscher & Seeley, 1982; Von Frisch, 1967). A colony's foraging focus changes temporally at daily and weekly scales and so observations of foragers on flowers may indicate the number of foragers present on that floral resource, but will have almost no correlation with the density of colonies (Visscher & Seeley, 1982). For these reasons, techniques based on sampling or trapping of foraging workers in a small area are not suitable for eusocial species like honey bees.

Direct versus indirect methods for accessing honey bee colony density and their limitations

Assessing the density of feral colonies at ecosystem scales is difficult. Assessment by visual identification of individual colonies is rarely feasible because nests are cryptic and hard to find. For example, Oldroyd *et al.* (1997) required a team of 12 people for 1 week to visually assess the number of colonies in seven 0.05-km^2 plots in accessible open woodland in Wyperfeld National Park in Victoria, Australia. This experience showed us that even in this accessible woodland, such surveys are expensive, even with volunteers, and are prone to error when colonies are missed. Accurate inference of the density of colonies in the broader environment from a small number of plots requires that the plots were truly representative of the environment and that no colonies were missed. Indirect methods based on population genetics provide a practical alternative method for estimating colony densities at broad scales while ignoring any heterogeneity in colony densities across the landscape. Because workers fly up to 10 km to forage (Beekman & Ratnieks, 2000), the average density of colonies in an ecosystem is more ecologically relevant than the local density. Therefore indirect methods based on drone genotypes require less labour, are cheaper, are probably more accurate, and provide information that is more ecologically relevant than direct observations.

Population genetic methods for assessing colony densities

During the reproductive season (spring–autumn), honey bee colonies produce large numbers (500+) of males (drones). When they are about 2 weeks old, males commence daily mating flights. Large numbers of males from many colonies gather at drone congregation areas (DCAs; Loper *et al.*, 1992; Koeniger & Koeniger, 2000; Galindo-Cardona *et al.*, 2012). The time of mating flights and the location of the congregation areas are species-specific (Koeniger & Wijayagunasekera, 1976; Koeniger *et al.*, 1988; Rinderer *et al.*, 1993b; Hadisoesilo & Otis, 1996; Koeniger & Koeniger, 2000; Otis *et al.*, 2000; Oldroyd & Wongsiri, 2006). Mating takes place on the wing. Typically, a queen mates on one or two afternoons in her life, with 10–30 males on each occasion (Palmer & Oldroyd, 2000). Males are attracted to a queen by her shape, movement, and the sex pheromone she secretes from her mandibular glands, which has 9-oxo-2-decanoic acid (9-ODA) as a major component (Butler *et al.*, 1962; Gary, 1962). Drones fly to DCAs along flyways that follow major features in the landscape such as treelines (Loper *et al.*, 1987, 1992).

Aspects of this reproductive biology can be exploited to obtain estimates of colony density. Males can be sampled from an area either by an aerial trap baited with a pheromone lure (Kraus *et al.*, 2005b; Moritz *et al.*, 2007; Jaffé *et al.*, 2010; Arundel *et al.*, 2012; Hinson *et al.*, 2015) (see the Sampling methods section below and Fig. 1) or by sampling the worker progeny of queens that were allowed to mate at the site of interest (Jaffé *et al.*, 2010; Arundel *et al.*, 2014). In both cases, rather than searching for colonies, colonies are identified by inferring the minimum number of colonies that could generate the observed genotypes of the sampled males.

Honey bee genetics – how haplodiploidy facilitates the identification of colonies

Honey bees are haplo-diploid. Males are derived from unfertilised eggs and are haploid, whereas females are derived from

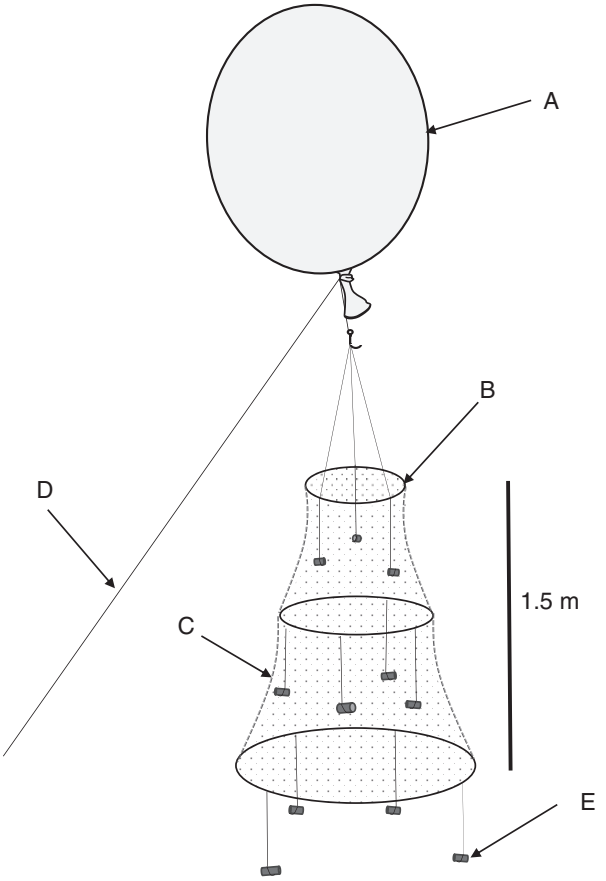

Fig. 1. A Williams drone trap (Williams, 1987). The trap comprises a weather balloon (A); a wire frame (B); net (C); queen lures made of black cigarette filters with synthetic queen pheromone placed on the surface (D); and a fishing line tether (A).

fertilised eggs and are diploid (Cook & Crozier, 1995; Crozier & Pamilo, 1996). As there is only one reproductive queen in a colony, all the drones in a colony are brothers, each carrying one of the queen's two alleles at every locus. If a sample of males is genotyped at a number of marker loci, it is straightforward to determine how many different mothers are necessary to explain the array of drone genotypes (Fig. 2; Baudry *et al.*, 1998; Kraus *et al.*, 2003; Wang, 2004; Kraus *et al.*, 2005b; Jones & Wang, 2010).

Sampling methods

Using virgin queens to 'catch' males

In this method, six to 10 nucleus colonies, each with a virgin queen, are placed at the site of interest (Jaffé *et al.*, 2010; Arundel *et al.*, 2014). The queens attract and mate with drones. The resulting worker progeny are sampled and genotyped. By subtracting the queen's genotype from those of the worker genotypes, the genotype of every male that mated with the queens is inferred. The number of source colonies that provided the males is then inferred from the male genotypes.

The advantage of this method is that it is unnecessary to physically trap males – the queens find them. The method is therefore less plagued by inclement weather and low sample size. The disadvantages are that the distance that drones and queens fly to mate is unknown and probably unknowable in all contexts, and it can be logistically difficult to put colonies with virgin queens in the field.

Trapping males using a Williams trap

In this method, a Williams drone trap is raised aloft using a helium balloon (Williams, 1987; Kraus *et al.*, 2005b; Moritz *et al.*, 2007; Jaffé *et al.*, 2010; Arundel *et al.*, 2012; Hinson *et al.*, 2015). The Williams trap comprises a tapered tulle cylinder 1.5 m long and 500 mm at the base (Fig. 1) (Williams, 1987). Drones are induced to enter the trap by the presence of queen dummies (typically blackened cigarette filters), and synthetic 9-ODA. Ideally, the sampling site should have DCA features: an open space surrounded by trees, or a treeline. Note that males of the Asian species *Apis cerana* refuse to enter a trap, even though they are attracted by 9-ODA. Instead they can be trapped by coating the line with insect glue (Crop Pro®, Kuala Lumpur, Malaysia; Fig. 3) (R. Gloag *et al.*, pers. comm.).

It is often possible to catch several hundred males within 30 min using a Williams trap. This method is therefore much more efficient than using virgin queens because it is logistically simpler, and the sample size is typically much larger. The drawback of this technique is that it requires appropriate weather for drone flight, traps cannot be deployed when there is any significant wind, the site needs to be open enough for the weather balloon to be raised without making contact with tree branches, and one needs a supply of helium, which is not always available in remote locations. (Sometimes a pole may make a satisfactory substitute for a balloon.) Note that it is not necessary to locate a DCA in order to trap drones, because the pheromone trap attracts males across a distance, and because males fly along treelines (Loper *et al.*, 1992). Practical experience shows that large numbers of males can be readily caught almost anywhere that has a treeline, although some trial and error may be necessary to find the best spot (Brockmann *et al.*, 2006).

Inferring the number of mothers from genetic markers

DNA microsatellites are the ideal genetic markers for inferring the maternity of individual males. A microsatellite locus comprises a sequence of nucleotide repeats. The number of repeats is highly variable at individual loci and between individual organisms, but also highly heritable (Ellegren, 2004). Microsatellite markers are co-dominant, reliable and repeatable, and relatively inexpensive to genotype. It is possible to multiplex several loci in one PCR reaction, greatly enhancing the efficiency of genotyping. They are ideal for inferring parentage and for calculating relatedness between individuals and colonies (Queller *et al.*, 1993).

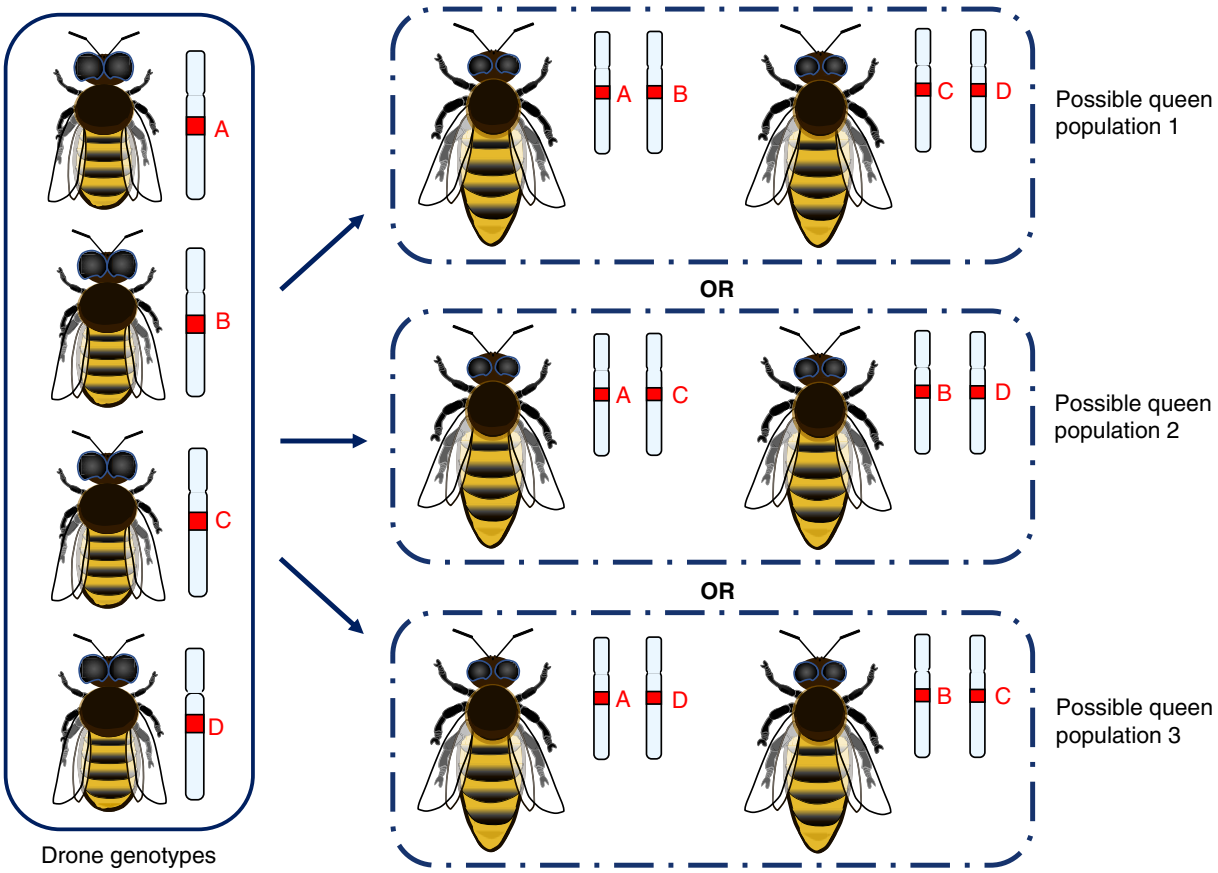

Fig. 2. Inference of the least number of possible mothers from drone genotypes. The minimum number of mothers that could produce the observed array of drone genotypes is two with three possibilities. Thus, these drones came from at least two colonies. [Colour figure can be viewed at wileyonlinelibrary.com].

There are three broad approaches to inferring the number of source colonies from drone genotype data: (i) determining the number of unique haplotypes from the analysis of tightly linked loci; (ii) analysis of unlinked loci using maximum likelihood; and (iii) analysis of independent groups of tightly linked markers using maximum likelihood.

Analysis of linked loci

If the microsatellite markers used for inferring brothers are tightly linked, any particular queen will produce two, and only two, haplotypes (Fig. 4) (Kraus *et al.*, 2003; Shaibi *et al.*, 2008; Arundel *et al.*, 2012, 2013, 2014; Hinson *et al.*, 2015). The analysis of such data is therefore simple; the number of unique haplotypes present in the male sample is divided by two. However, lack of recombination reduces the information content of the dataset because the genetic diversity is diminished relative to the same number of unlinked loci (Devlin *et al.*, 1988).

The power of linked markers can be increased if two or more sets of linked loci are used. Each set of linked markers must assort independently. If two sets of linked loci are used, the number of unique haplotypes present in the sample is divided not by two but by four (Fig. 4). The allelic richness of each linkage group should be equal; otherwise, it is more sensible to simply use the data from the most diverse linkage group, because the number of colonies estimated from the two sets of linked markers can actually be less than the number of colonies estimated from the most diverse set (Arundel *et al.*, 2014).

Unlinked loci

Here the minimum number of queens required to explain the array of drone genotypes sampled is inferred via maximum likelihood (Fig. 5; Wang, 2004). The use of unlinked loci has two major advantages over linked loci. First, let us assume that the number of loci to be analysed is equal, but one set is linked and the other is unlinked. With unlinked markers, each locus provides an independent genetic marker, whereas with linked loci, individual loci are not independent. The use of linked loci maximises allelic (haplotype) richness for the multi-marker locus, whereas the use of unlinked loci maximises the number of loci, albeit with some reduction in allelic richness per locus. Second, because of unrestricted recombination among unlinked loci, the possibility that two unrelated males will be inferred as being brothers by chance alone is very low. By contrast, with linked loci, queens that are related may transmit

Fig. 3. *Apis cerana* males will not enter a Williams balloon trap (see Fig. 1). Instead they can be caught on the line that tethers the balloon (left panel). The line is coated with glue (right panel) (photographs courtesy of Ros Gloag). [Colour figure can be viewed at wileyonlinelibrary.com].

identical multi-locus haplotypes to their sons, leading to the possibility that non-brothers will be assumed to come from the same colony.

The disadvantage of unlinked markers is that the analysis is conceptually more difficult because colony identity must be inferred, not by dividing by two, but from maximum likelihood. Fortunately, the COLONY program, developed by Jinliang Wang, provides a convenient analysis platform to infer mother genotypes from drone genotypes via maximum likelihood using a simulated annealing process (Wang, 2004, 2013, 2016).

Analysis of independent tightly linked groups using maximum likelihood

In this technique, two or more sets of linked markers are regarded as pseudo-loci (Devlin *et al.*, 1988). The technique harnesses the benefits of the extreme genetic diversity among haplotypes of linked loci. As the linkage groups are independent, the sibship reconstruction can be based on maximum likelihood using methods as in unlinked loci (Jaffé *et al.*, 2009, 2010).

Potential errors associated with population genetic methods

Non-detection errors

Non-detection errors occur when two males have genotypes that are compatible with descent from one queen, whereas in fact they come from different colonies (Foster *et al.*, 1999). Non-detection errors cause false negatives in which drones from two families are erroneously grouped as brothers, bringing about underestimation of colony number. Non-detection errors are most likely to occur when mothers of drones are full siblings or mother and daughter. A non-detection error is more likely to occur in linked-loci analysis. If two queens are full sisters or mother–daughter, approximately half of their offspring will have identical haplotypes. By contrast, when males are genotyped at a large number of unlinked loci, the likelihood of a non-detection error arising because non-brothers are indistinguishable is negligibly small. For example, if six loci are analysed, each of which has six alleles of equal frequency, the loci in combination can generate up to 46 656 unique male genotypes and $46\,656^2/2 = 1.09 \times 10^9$ potential queen genotypes. Even though, in reality, allele frequencies are not equal and vary

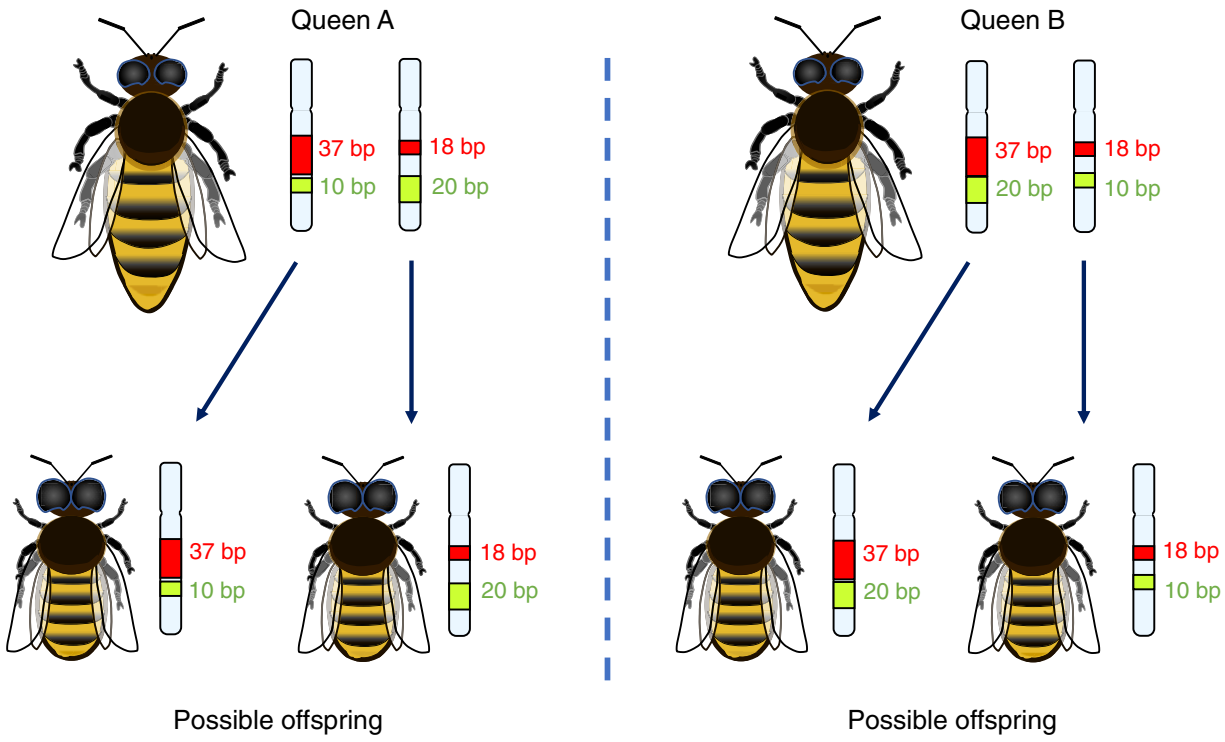

Fig. 4. The possible genotypes of drones that are produced by a queen when two loci are tightly linked. A queen can produce two haplotypes among her sons. [Colour figure can be viewed at wileyonlinelibrary.com].

between populations, most honey bee microsatellite loci typically have 10–20 alleles (Estoup *et al.*, 1994; Shaibi *et al.*, 2008; Beekman *et al.*, 2009). We recommend a minimum of six highly polymorphic markers. If similar results are obtained after removing one or more loci from a dataset, then one can be confident that the number of loci used was sufficient.

Typing errors

Allelic dropout (null alleles). Allelic dropout is an error that occurs when one or more alleles of a polymorphic locus do not amplify during polymerase chain reaction (Wang, 2004; Soulsbury *et al.*, 2007). Allelic dropout leads to missing data or, more significantly, scoring of a heterozygous genotype as homozygous. Allelic dropout can have a significant impact on pedigree reconstruction, especially when the group maximum likelihood method is used (Wang, 2004). Fortunately, as drones are hemizygous, a microsatellite dataset obtained directly from drones cannot suffer from allelic dropout in heterozygotes; allelic dropout only results in missing data. However, where drone genotypes are inferred from their worker offspring, the importance of allelic dropout is potentially significant (Wang, 2004, 2016).

Other typing errors. Other typing errors can come from many sources. They can occur in the DNA amplification process, allele calling, and from mutation (Jones & Ardren, 2003; Wang, 2004). Each genetic marker in an organism can have a different typing error rate. These errors can be accounted for by equations provided in Wang (2004) based on maximum likelihood, most of which have been implemented in COLONY.

Non-sampling errors

A colony does not produce trappable drones. In honey bees, drone production is seasonal and correlates with colony health (Allen, 1958, 1963). Small, unhealthy colonies may not be represented in a sample of trapped drones because such colonies produce few drones, if any. Therefore, any method that estimates colony densities based on drones will tend to miss such colonies. This is probably not an important source of error because small, weak colonies are unlikely to survive and are of little consequence ecologically or as pollinators.

Seasonal considerations. Colony density will tend to be underestimated if sampling is undertaken at an inappropriate time of year when most colonies do not have drones, or during inclement weather when few drones are flying. Observations of known colonies in the area can be used to plan the optimal sampling time.

Sampling site. Drones tend to aggregate in particular areas in the landscape (Gary & Marston, 1971; Loper *et al.*, 1987, 1992; Taylor & Rowell, 1988; Ayasse *et al.*, 2001). Even though the pheromone lure attracts males across distances of at least

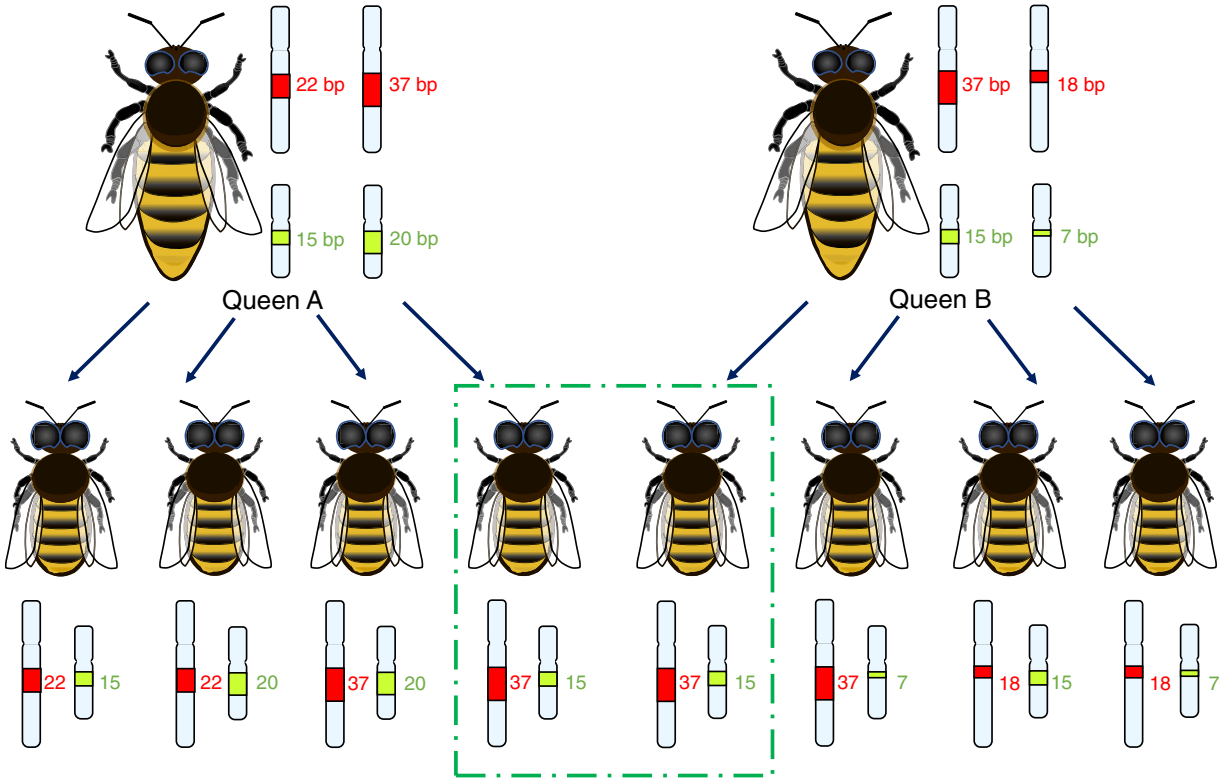

Fig. 5. The possible genotypes of drones that are produced by a queen when alleles are not linked. As the number of markers is increased, the probability that two drones will be inferred to have the same mother in error becomes vanishingly small, even if their mothers are related. [Colour figure can be viewed at wileyonlinelibrary.com].

100 m (Brockmann *et al.*, 2006), and probably much more, sampling away from aggregation areas or the flyways that lead to them may not sample all the males from all the colonies in an area, especially those from small, weak colonies. It is important to spend time identifying sites within the study area where large numbers of drones are trapped easily, indicating that the site is at or near a congregation area or drone flyway. Doing so will reduce the probability of non-sampling error due to heterogenous distributions of drones, and increase the probability of sampling drones from small weak colonies, provided that the sample size is large.

Sample size

In areas where the density of honey bee colonies is very large, a finite sample of drones may underestimate the number of colonies present because some colonies are not sampled. We emphasise that this kind of non-sampling error is best addressed by genotyping large numbers of drones (at least 200) so that all colonies are sampled. Unfortunately the appropriate number cannot be known *a priori* (Chapman *et al.*, 2003). *Post hoc*, it is possible to explore whether non-sampling is likely to have been a problem by determining the number of inferred colonies from random subsamples of drones (Fig. 6). As the subsample size increases, the number of inferred colonies will also increase (Fig. 6). At some point, the number of new colonies discovered by increasing the sample size should asymptote, and at this point the sample size is adequate (Fig. 6). If the number of new colonies discovered does not decline with sample size, then the total sample size was inadequate (Fig. 6).

In cases where the sample size turns out to have been smaller than was needed, one potential solution is to fit an appropriate statistical distribution to the dataset, and to then use the fitted distribution to estimate the total number of colonies in flight range, including those that were not sampled. Baudry *et al.* (1998) fitted the observed distribution of the number of drones drawn from different colonies to the truncated Poisson distribution. Similar analyses based on the Poisson distribution have been used in subsequent studies (e.g. Chapman *et al.*, 2003; Jaffé *et al.*, 2009, 2010). We therefore describe the use of the truncated Poisson distribution in the following section, before discussing potential alternative distributions in the next section. We again emphasise that obtaining a large sample size in the first place is a better option than fitting data to mathematical distributions.

Using a truncated Poisson distribution to estimate the number of missing colonies

Consider a set of experimental data where N_c distinct brother groups (each brother group representing a separate colony) have

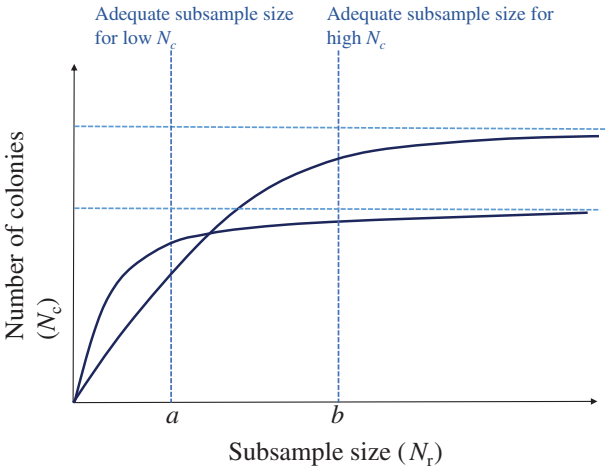

Fig. 6. The relationship between number of inferred colonies and subsample size (N_r). As the sample size increases, it is expected that the number of inferred colonies will increase. At the inflection point, the number of new colonies identified with increasing sample size asymptotes. Therefore, the most efficient sample size (a or b in the figure) is the sample size at the inflection point for N_c. By plotting the number of colonies identified against subsample size (N_r), it is possible to determine whether colony number asymptotes with increasing sample size. If not, then the sample size is likely to have been inadequate, and it will be necessary to genotype more drones or to fit the data to a mathematical distribution (the truncated Poisson distribution has proved satisfactory in the past) to obtain a better estimate of the number of colonies in the environment. [Colour figure can be viewed at wileyonlinelibrary.com].

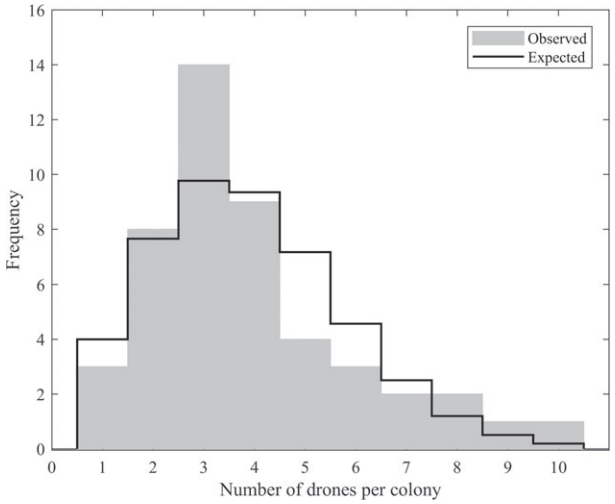

Fig. 7. Observed and expected frequency of number of captured drones per colonies which is fit by using truncated Poisson distribution as illustrated in Table 1.

been detected using the COLONY program based on maximum likelihood. Each detected colony, i, $i = 1, 2, \ldots, N_c$, is represented by k_i drones in the overall sample of N_d drones (for an example of such a dataset, see Table 1). From these data, we seek an estimate of the number of source colonies that were probably present in the sampled area, but were not all represented in the sample because the sample was too small. Assume that all colonies result in drones arriving at the DCA at the same average rate. Such a process can be modelled with a truncated Poisson process (David & Johnson, 1952), where the probability of a single source colony being represented by a count of r drones in the final sample is:

$$p_r = \frac{\lambda^r e^{-\lambda}}{r!\ (1 - e^{-\lambda})}, \qquad r = 1, 2, 3, \ldots$$

where λ is the single parameter of the truncated Poisson distribution to be determined from the data.

If the estimate for λ is obtained via a maximum likelihood method (David & Johnson, 1952; Baudry *et al.*, 1998), then denoting the maximum likelihood estimate for λ as $\hat{\lambda}$, a conditional maximum likelihood estimator for the total number of colonies in the range of the trap is simply:

$$N_t = \left\lfloor \frac{N_d}{\hat{\lambda}} \right\rfloor,$$

with no calculus required (Dahiya & Gross, 1973; Blumenthal *et al.*, 1978; Baudry *et al.*, 1998). […] denotes rounding down to the nearest integer. A formal derivation of the maximum likelihood estimate of $\hat{\lambda}$ is given in Appendix S1. A worked example of use of the truncated Poisson distribution to correct for inadequate sample size is given in Table 1 and Fig. 7.

Selecting the best distribution

The entire procedure for estimating the total number of colonies by correction for non-sampling error described earlier relies on the truncated Poisson distribution being a good fit to the data. It is unlikely that all datasets will match such a distribution. Further, some of the assumptions in fitting the truncated Poisson model are unlikely to hold all the time in reality. For example, feral honey bee colonies can vary greatly in size and the number of drones that they carry (see, for example, Free & Williams, 1975; Seeley & Morse, 1976; Smith *et al.*, 2014). This is contrary to the assumption that all colonies will have the same average number of drones arrive at a given DCA, as assumed when using a truncated Poisson model.

In a case where the truncated Poisson distribution is not a good fit, it may be worthwhile to apply a similar procedure to that suggested by Baudry *et al.* (1998) using a different underlying, left-truncated, discrete probability distribution. Candidate distributions for such analysis include compound Poisson processes (as in David & Johnson, 1952) and the truncated negative binomial distribution (Johnson *et al.*, 1992), whose parameters can be estimated via maximum likelihood. The truncated negative binomial distribution can be derived from a mixture of truncated Poisson distributions (Johnson *et al.*, 1992), and thus may be a reasonable model for the case where drones from different colonies arrive or are caught at a DCA at different rates. The next step is then to infer the number of non-sampled colonies from the expected count of the zero class (as in Al-Saleh &

Table 1. A hypothetical dataset showing the observed number of drones from each of N_c colonies.

			Goodness-of-fit test to the Poisson distribution		
	Number of drones per colony	Observed number of colonies	$p_r = \frac{\lambda^r e^{-\lambda}}{r!\ (1-e^{-\lambda})}$	Expected number of colonies = $\sum N_c p_r$ for each bin	$\chi^2 = \frac{(Observed-expected)^2}{Expected}$
	1	3	0.0850	11.65	0.0357
	2	8	0.1628		
	3	14	0.2078	9.77	1.8351
	4	9	0.1990	9.35	0.0132
	5	4	0.1524	7.16	1.3966
	6	3	0.0973	8.98	0.0001
	7	2	0.0532		
	8	2	0.0255		
	9	1	0.0108		
	10	1	0.0042		
Total drones N_d	186			χ^2 (d.f. = 3 (no. bins (5) − no. estimated parameters (1) - 1))	3.2807
Total colonies observed N_c		47		$p = 0.3503$ (χ^2 test)	
Corrected number of colonies N_t		48			
Maximum likelihood estimate for $\widehat{\lambda}$	3.8299				

Note: here $\widehat{\lambda}$ is the same for all colonies, and must be estimated via maximum- likelihood, as described in Appendix S1.
The total number of colonies, N_t, applies the correction for unsampled colonies, assuming that the data follows a truncated Poisson distribution. The last three columns apply a goodness-of-fit test to assess whether the data are reasonably fit by the truncated Poisson distribution. Bin widths for the χ^2 test were automatically selected via MATLAB's *chi2gof* function (MATLAB and Statistics Toolbox Release 2017b, The MathWorks, Inc., Natick, Massachusetts, USA) to avoid expected counts below 5 in outer bins (Fig. 7). See text for further details.

AL-Batainah, 2003). Such an analysis is not necessarily easy to perform [see, for example, the methods for obtaining estimates of the zero class for a truncated Poisson sample in Blumenthal *et al.* (1978) and Dahiya and Gross (1973)].

Using goodness-of-fit to choose the appropriate statistical distribution for determining the likely number of missing colonies

The accuracy of the final estimate of the number of colonies obtained by fitting a statistical distribution to the data relies heavily on the assumption that the fitted distribution is a reasonable approximation of the actual distribution. If the fit to the data is poor, then the estimate could be inaccurate. Baudry *et al.* (1998) and Chapman *et al.* (2003) used a standard χ^2 test (Pearson, 1900) to test the assumption that the truncated Poisson distribution was a good fit to their data. The χ^2 goodness-of-fit test is a good general-purpose test that can work well for most datasets and is often described in text books of statistical ecology (Ludwig & Reynolds, 1988; Young & Young, 1988). We provide a worked example in Table 1. However, there are instances where the χ^2 goodness-of-fit test cannot be applied, particularly when the data cannot be separated into more bins than the number of parameters estimated during fitting plus one (this is connected to the degrees of freedom associated with the test). In the case that the χ^2 goodness-of-fit test cannot be applied, an appropriate alternative is the one-sample Kolmogorov–Smirnov test applied via a parametric bootstrap algorithm (a necessary approach in the case that parameters of the hypothesised distribution are estimated from the data) (Kolmogorov, 1933; Smirnov, 1948; Durbin, 1973, 1975; Stute *et al.*, 1993; Szűcs, 2008). The test statistic for the χ^2 test is based on differences between observed and expected frequencies over a set of bins that cover all possible data outputs, whereas the test statistic for the Kolmogorov–Smirnov test is based on the maximum difference between empirical and fitted cumulative density functions. Application of the one-sample Kolmogorov–Smirnov test via a parametric bootstrap algorithm is explained in Appendix S2.

What is the area sampled? Converting the number of colonies to the number of colonies per unit area

The number of colonies identifiable from the array of male genotypes can be used as a relative measure of colony density in the landscape. Obviously, a drone sample from an area with many colonies per hectare will show a greater diversity of genotypes than a sample acquired in an area where colonies are rare. However, absolute measures are more useful than relative measures. To obtain absolute measures, it is necessary to know the area from which a drone trap or a virgin queen draws a sample of drones. Generally, it is assumed that drone trap draws drones from an area of radius 900 m from the sampling site. This distance is based on the drone flight range in Taylor and Rowell (1988), but the basis of this estimate is unclear. When the virgin queen technique is used, the area over which drones are drawn is typically assumed to be of radius 1800 m by conservatively assuming that queen and drone flight ranges are equal (Moritz *et al.*, 2007; Arundel *et al.*, 2013, 2014; Hinson *et al.*, 2015). However, this assumption is also based on limited data.

In an attempt to solve the problem of limited information on drone flight distance and the effects of the spatial distribution of colonies, Arundel *et al.* (2013) used all available information on honey bee mating biology to develop agent-based models of the likely distribution of drone haplotypes given a range of colony densities and spatial aggregations. The results of this modelling suggested that drones are trapped from a much larger area than the 2.5 km^2 assumed on the basis of a 900-m drone flight range, and resulted in much lower estimates of the densities of colonies. The estimates of Arundel *et al.* (2013) are based on 'normal' mating behaviour. They do not consider the possibility of changes in the mating behaviour of drones as a consequence of a trap baited with a super-stimulus of many times the usual concentration of 9-ODA. Furthermore, drone flight range is sensitive to the physical landscape and the maximum range can be up to 5 km (Ruttner & Ruttner, 1972). Therefore, for the drone trapping method to realise its full potential as a means to estimate the absolute density of wild honey bee colonies in the environment, we will need to empirically assess the distances over which a Williams trap will lure drones in a variety of habitats. This research is difficult to conduct, but it needs to be done.

Results to date

Table S1 provides estimates of honey bee colony densities derived from direct survey and drone trapping surveys based on Hinson *et al.* (2015). These studies show that the density of colonies varies hugely with the environment and assessment technique.

Outstanding research questions

Although many of the protocols required to obtain estimates of colony densities from genotypic data have been developed, two important questions remain. First, we do not know empirically the distance over which a pheromone trap can trap drones and the effects of the environment on this distance. It is likely that drones travel further in some environments than in others, and the strength and direction of the wind may influence the distance over which drones are attracted (Elkinton & Carde, 1988). This issue could be addressed by sampling along a 5-km transect, and determining the maximum flight distance of drones. Second, the contribution of worker-laid drones to DCAs is unknown. Although the number of worker-laid drones is negligibly low in queenright colonies (Visscher, 1989), queenless colonies produce males in large numbers (Page & Erickson, 1988). Males produced by queenless colonies are likely to be classified as non-brothers by COLONY (as they should be). If so, the number of colonies present in the dataset would be over-estimated. Potentially, worker-laid drones could be identified morphologically by their small size and discarded from the dataset.

These problems need to be addressed before the full potential of the technique can be realised. Nonetheless, drone trapping provides a convenient and powerful method for estimating relative, and perhaps absolute, abundance of honey bee colonies.

Future perspectives

An additional application of drone trapping is to help detect and eradicate incursions of exotic honey bee species. In Australia there are two to three incidents per year in which colonies or swarms of exotic honey bee species are detected on shipping or aircraft. On three separate occasions since 2007, the Asian hive bee *Apis cerana* has established breeding populations on the Australian mainland (Cairns in 2007, Townsville in 2016 and Darwin in 2018). The Townsville and Darwin populations have been successfully eradicated [the Cairns population remains extant (Koetz, 2013; Gloag *et al.*, 2016)]. Drone trapping, using the techniques described earlier, provides an efficient method for determining when eradication has been successful, and provides a cheap and efficient method for ongoing monitoring.

Two subspecies of *A. mellifera* from southern Africa are generally regarded as having behavioural traits that make them less suitable for commercial beekeeping than subspecies from elsewhere (Needham *et al.*, 1988; Rinderer, 1988; Rinderer *et al.*, 1993a). *Apis mellifera scutellata* and its hybrid (called 'Africanised' honey bees in the Americas) are extremely defensive and prone to excessive reproductive swarming (Needham *et al.*, 1988; Winston, 1992). *Apis mellifera capensis* is prone to social parasitism and causes losses of up to 10 000 commercial bee colonies in South Africa every year (Allsopp, 1992, 1993; Beekman *et al.*, 2008). Drone trapping provides a convenient method for broadly sampling a honey bee population (Moritz *et al.*, 2007; Collet *et al.*, 2009). Single nucleotide polymorphism genotyping can be used to determine the likely subspecies of the sampled males (Chapman *et al.*, 2015; Harpur *et al.*, 2015) and could potentially be used to determine the extent of a new incursion.

Although the techniques discussed here have been developed for the Western honey bee (*A. mellifera*) they have the potential to be applied to other social insects that also have lek mating. There are at least 10 other *Apis* species (Lo *et al.*, 2010), all of which are potentially amenable to drone trapping because drones are attracted to 9-ODA (Plettner *et al.*, 1997; Kraus *et al.*, 2005b; Beaurepaire *et al.*, 2014). It has already been shown that *A. dorsata* (Kraus *et al.*, 2005a) and *A. cerana* (R. Gloag *et al.*, pers. comm.) drones can be trapped using pheromone lures.

Stingless bee (tribe Meliponini) drones can be trapped in the mating swarms that form outside colonies containing a virgin queen (Sommeijer & de Bruijn, 1995; Cameron *et al.*, 2004; Kraus *et al.*, 2008; Mueller *et al.*, 2012). However, at this time, we do not know how far stingless bee males travel from their natal nest, so getting estimates of colony densities from the genotypes of aggregated males may be problematic. Ants and termites generate mating swarms only infrequently and mating is triggered by environmental conditions. It is therefore unlikely that ants and termites will be amenable to pheromone trapping of males to determine population structure.

Broader applications

Whenever an insect species can be attracted in the field using synthetic sex or aggregation pheromones, there is the potential to use pheromones to assess whether a species is present

at a location using pheromone-baited traps (Jacobson, 1972; Widemo & Johansson, 2006; Cabrera & Jaffe, 2007). If so, it should also be possible to assess whether population size is increasing or contracting (Tewari *et al.*, 2014). As with honey bee males, it may be possible to assess the distance over which insects of some species are attracted to traps, and by this means determine the likely number of insects per unit area. We hope that our review on honey bees may inspire entomologists from other fields to think laterally about new applications of pheromone traps in the assessment of insect population size at ecosystem scales.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Estimates of the density of wild (native) and feral (exotic) honey bee colonies from across the world, ordered from lowest to highest density.

Appendix S1. A formal derivation of the maximum likelihood estimate of λ for the truncated Poisson distribution

Appendix S2. Parametric bootstrap procedure for the one-sample Kolmogorov–Smirnov test for goodness-of-fit

Video S1. Williams drone trap in action.

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