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FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Faculty of Bioscience Engineering (FBE)

Departement of crop protection

Microsatellites to identify the impact of genetic parameters on bumblebee decline and genes associated with foraging

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Thesis submitted in fulfillment of the requirements for the degree of

Doctor (PhD) in Applied Biological Sciences

Academic year: 2014-2015

Dutch translation of the title:

Het gebruik van microsatellieten om de impact van genetische parameters op de achteruitgang van hommels en genen geassocieerd met foerageren te identificeren

Please cite as:

Maebe, K. (2015) Microsatellites to identify the impact of genetic parameters on bumblebee decline and genes associated with foraging. PhD thesis, Ghent University. Ghent, Belgium. pp 237.

Cover photo:

An ABgene PCR-plate with film (VWR), DNA strands (dnatestingexpert.com), and a bumblebee, probably *Bombus terrestris*, photographed by Trounce on 19 April 2007 in Ireland.

Printing:

University Press

ISBN-number:

978-90-5989-777-9

Acknowledgements

ACKNOWLEDGEMENTS

Bijna is het doctoraat volledig af. Inderdaad, “bijna” want ik moet hier nu wel nog eventjes de tijd nemen om een dankwoord neer te schrijven, want heel veel mensen hebben hun steentje bijgedragen tot dit werk.

Bovenaan de lijst van de mensen die ik zou willen bedanken, staan mijn twee promotoren Prof. Dr. ir. Guy Smagghe en Dr. Ivan meeus.
Bedankt Guy, voor de gegeven kans om dit doctoraat te kunnen afleggen binnen het Labo. Agrozoölogie. Hierdoor kreeg uw groep ook een volledig nieuw luik, namelijk populatie genetisch onderzoek.
Bedankt Ivan, zonder deze zeer gedreven, inspirerende post-doc, was mijn thesis er zeker nooit gekomen. De urenlange vergaderingen, soms wel eens discussies, het oneindig keer verbeteren en aanvullen van papers, maar voornamelijk het enthousiasme waarmee je dingen overbrengt, chapeau! Echt iets wat ik ook zou willen kunnen in mijn toekomstige onderzoek of carrière.

Mijn dank gaat ook uit naar Prof. Rasmont van de Universiteit van Bergen, Prof. Biesmiejer van Naturalis in Leiden, en Prof. Grootaerts van het Koninklijk Belgisch Instituut voor Natuurwetenschappen voor het openstellen van hun hommel collecties. Met hun hulp kon ik een unieke staalname uitvoeren op historische museumstalen.

Daarnaast wil ik ook Dr. Jan De Riek van het ILVO bedanken die me hielp met de QTL-paper.

Begin 2011 waren ik en Ivan de enigen die binnen de groep onderzoek op hommels uitvoerden, maar in de loop van de 4 jaar zijn er heel wat collega’s van verschillende nationaliteiten bijgekomen, deze wil ik ook bedanken voor de aangename werksfeer zowel in het labo als in de bureau. Maar mijn speciale dank gaat toch wel uit naar Jafar: “Thank you Jafar for being, besides a good colleague, also a good friend.”

Mijn ouders wil ik uiteraard ook bedanken en dit voor de mogelijkheid die ze me gegeven hebben om me te laten studeren, en ook al vlotte het de eerste jaren niet zo goed, om in mij te blijven geloven, me te steunen, naar mijn gezaag te luisteren wanneer een bepaald experiment niet wilde lukken, maar voornamelijk om die lieve schatten van ouders te zijn!

Verder wil ik ook mijn broers en daarnaast ook mijn familie, ‘schoon’ familie en vrienden bedanken om er te zijn, me een optie te bieden voor ontspanning en te helpen ontsnappen aan alle stress.

En ten laatste, maar als meest belangrijkste, wil ik ook men lieve schat bedanken. Bedanken voor al de steun en hulp die ze gedurende die laatste jaren (en misschien wel de meest zware jaren) gegeven heeft. Bedankt!!

Kevin Maebe
8 februari 2015

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List of non-standard abbreviations

ANL	Average Number of Loci successfully amplified per individual per species
A_R	Allelic richness
BM	Basement Membrane
bp	base pairs
Br	*Brachycephalibombus*
C	Cornea
Cc	*Coccineobombus*
CC	Crystalline Cone
CCEP	Crystalline Cone Extensions
CI	Confidence Interval
CLS	Critical Light Sensitivity of a colony
cM	centiMorgan
Cr	*Crotchiibombus*
CZ	Clear Zone
D*est*	Jost' *D*
Ds	*Dasybombus*
E_L	length of compound eye
E_S	total surface of compound eye
E_W	width of compound eye
F_a^s	initial foraging activity of a colony in strong light conditions
F_a^w	initial foraging activity of a colony in weak light conditions
F_c	initial nest-leaving capacity
F_{IS}	inbreeding coefficient
F_{IS} **IIM**	the for null alleles corrected inbreeding coefficient
Fm_L	femur length
Fm_W	femur width
Fn	*Funebribombus*
Fr	*Fraternobombus*
FS	number of Full Sibs
F_{ST}	genetic differentiation/ genetic structure values
H_E	expected Heterozygosity
H_O	observed Heterozygosity
HW	Hardy-Weinberg equilibrium

IAM	Initial Alleles Model
IIM	Individual Inbreeding Model
IM	composite Interval Mapping analysis
IUCN	International Union for Conservation of Nature
KAM	K-Alleles Model
KW	Kruskal-Wallis
L	maximum number of Loci used in further analysis
LF	Long Faced clade
LG	Linkage Group
LOD	Logarithm of the Odds
M	Garza and Williamson M-statistic
MAS	Marker Assisted Selection/ Breeding
MOc	diameter of Median Ocellus
MP	Multiplex PCR Master Mix
MT_L	metatarsus length
MT_W	metatarsus width
MW	Molecular Weight
MQM	Multiple QTL model Mapping
N	Number of alleles
n	number of workers
NA	number of specimens that were Not Amplifiable
N_{Ae}	ancestral effective population sizes
Ne	effective population size
NW	New World Clade
Om	total numbers of Ommatidia
P	Probability
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PC-QTL	Principal Components Quantitative Trait Loci
PPC	Primary Pigment Cells
PUA	Proportion of Unsuccessfully Amplified individuals per locus
r	relatedness
Rb	*Robustobombus*

Rc	*Rubicundobombus*
RetC	Retinula Cells
RC	forewing Radial Cell length
RCA	Retinula Cell axons
RCP	Retinula Cell Pigments
Rh	Rhabdom
r_s	correlation coefficient
SD	Standard Deviation
SDL	Single sex Determination Locus
SE	Standard Error
SF	Short Faced clade
SMM	Stepwise Mutations Model
Sp	*Separatobombus*
SPC	Secondary Pigment Cells
SSR	Simple Sequence Repeat
STEP	Status and Trends of European Pollinators project
Ta	annealing Temperature
Ti_L	tibia length
Ti_W	tibia width
TPM	Two Phase Mutation Model
Tr_L	trochanter length
Tr_W	trochanter width
VNTR	Variable Number Tandem Repeats
QA	Quality Assurance
QTL	Quantitative Trait Loci

Objectives and outline of this study

Bumblebees are, as generalist foragers, essential pollinators in natural and managed ecosystems (Heinrich, 1979; Goulson, 2003; 2010). Like for many pollinator species, most bumblebee species undergo a worldwide observed decline (e.g. Williams & Osborne, 2009; Potts *et al.,* 2010; Cameron *et al.,* 2011; Carvalheiro *et al.,* 2013). This general phenomena, which is observed to have a distinct impact on bumblebees, is instigating both ecological and economic concerns (Kremen *et al*., 2002; Steffan-Dewenter *et al*., 2005; Klein *et al*., 2007; Goulson & Osborne, 2010). Several hypotheses have been proposed to explain the observed declines in bee populations, e.g. the impact of pathogen infections and possible pathogen spill-over from managed pollinators, the use of pesticides, diet specialization, landscape modification and loss of forage (e.g. Potts *et al.*, 2010; Goulson, 2010; Vanbergen & the Insect Pollinators Initiative 2013). These factors and their interactions with each other, influence pollinator populations on different locations and on different scales. Also population genetic aspects will play a role in bee declines with genetic threats such as inbreeding and loss of genetic diversity (Reed & Frankham, 2003; Spielman *et al.*, 2004; Frankham, 2005; Goulson *et al.,* 2008; Zayed, 2009). In order to secure the pollination services of wild bumblebees and improve conservation strategies, a better understanding of genetic factors influencing natural bumblebee populations is vital (Goulson *et al.*, 2008; Zayed, 2009).

Furthermore, several bumblebees species, such as *Bombus terrestris,* are intensively reared and used in agriculture, as they can provide an improved pollination of several important greenhouse vegetables, such as tomatoes and peppers, compared to other pollinators (Velthuis & van Doorn, 2006; Goulson, 2010). This commercially valuable service of bumblebees can be improved by associating genotypes with commercially interesting properties. Enhanced foraging was chosen to be an interesting study target for its dual importance, with obvious commercial benefits but also as it is very important in the ecological context. In this thesis, the main goal is to implement microsatellite technology to assess the pollination service in both natural and managed ecosystems.

Chapter 1 is a general introduction of bumblebees: their life-cycle, sex determination, morphology, foraging behaviour and economic value. As there is no red list of bumblebee species in Belgium, the red list status of the 29 bumblebee species in The Netherlands will be discussed and placed in the European context. Furthermore, the several hypotheses (partially)

explaining the observed declines will be described. Finally, a brief overview of the microsatellite technology, its limitations, applications and in particularly their use in quantitative trait loci (QTL) analyses will be given.

In a first part of results within this dissertation, we studied the loss of the pollination service (chapter 2 and 3) by focussing on bumblebee decline and the genetic parameters associated with it. With the use of microsatellite DNA markers, we will examine the genetic diversity of pin-mounted bumblebee specimens sampled from extensive historical collections of wild bumblebees. Museum collections provide a unique opportunity to examine the population structure and the genetic diversity of past populations (Wandeler *et al.*, 2007). This approach will allow us to check for currently formulated hypothesis which are based on assessments of contemporary specimens of both declined and stable bumblebee species (Goulson *et al.*, 2008; Lozier *et al.*, 2011). Knowing the population structure and the genetic parameters before the actual decline began, will provide an increased insight into the importance of population genetic parameters in the decline of bumblebees (Wandeler *et al.*, 2007; Goulson *et al.*, 2008; Lozier *et al.*, 2011).

One goal is to examine how genetic diversity and population structure are correlated with species extinction. More specically in **chapter 2** we will use the developed PCR multiplexes of microsatellites to study the impact of genetic parameters on natural populations of the in Belgium almost extinct bumblebee species, *Bombus veteranus*. After this case study, we will verify these initial findings in their bigger context and compare the historical genetic diversity between declining and stable *Bombus* species in **chapter 3**.

Aside from describing the genetic viability of natural populations, microsatellite analyses can also be used to search for genetic markers associated with a specific phenotype (Wilfert *et al.*, 2007a; 2007b). This phenotype can be an enhanced feature of an interesting commercial characteristic of bumblebees. In chapter 4 and 5, we will use the microsatellite technology to identify genes correlated with foraging behaviour. Microsatellite markers linked with a phenotype of interest could then be used for selective breeding or marker-assisted selection (MAS; Williams, 2005). In this way the foraging service, or an phenotype associated with the commercial potential of this service can be enhanced. We will focus on two phenotypes: the impact of light intensity and body size.

In **chapter 4**, we will investigate the connection between light sensitivity and foraging. We will assess the foraging behavior of different *B. terrestris* colonies in changing light conditions and investigate if differences could be explained by an improved vision of the workers. To achieve this, we developed bioassays that could distinguish light sensitivity differences between colonies (colony level) and between individuals (individual level). Furthermore, we will test if bumblebee body size, weight and morphological parameters of the eye correlate with the measured light sensitivity of the workers. Finally, we will perform a QTL analysis to search for one or more microsatellite marker(s) linked with light sensitivity, body weight, body size, and morphological eye parameters in **chapter 5**. Thereby identifying potential markers for MAS.

Finally, in **chapter 6**, we show a direct application of the microsatellite technology in bumblebee breeding facilities. Microsatellites can be integrated within a bumblebee mass-breeding to detect diploid drones. After all, the presence of diploid drones can be used as a validation of their production process.

Chapter I

General introduction

1.1 Bumblebees - *Bombus*

1.1.1 *Taxonomy and phylogeny*

Kingdom:	**Animalia**	Linnaeus, 1758
Subkingdom:	**Eumetazoa**	Buetschli, 1910
unranked,	**Bilateria**	Hatschel, 1888
Infrakingdom:	**Protostomia**	Grobben, 1908
Superphylum:	**Ecdysozoa**	Aguinaldo *et al.* 1997
Phylum:	**Arthropoda**	von Siebold, 1848
Subphylum:	**Hexapoda**	Latreille, 1802
Class:	**Insecta**	Linnaeus, 1758
Subclass:	**Pterygota**	Lang, 1888
Infraclass:	**Neoptera**	Martynov, 1923
Superorder:	**Endopterygota**	Sharp, 1898
Order:	**Hymenoptera**	Linnaeus, 1758
Suborder:	**Apocrita**	Gerstaecker, 1867
Infraorder:	**Aculeata**	Latreille, 1802
Superfamily:	**Apoidea**	Latreille, 1802
Family:	**Apidae**	Latreille, 1802
Subfamily:	**Apinae**	Latreille, 1802
Supertribe:	**Apiti**	Latreille, 1802
Tribe:	**Bombini**	Latreille, 1802
Genus:	***Bombus***	Latreille, 1802

Bumblebees are insects belonging to the Hexapoda. Furthermore, they are holometabolous insects or Endopterygoya as they undergo a metamorphosis during their pupal stage resulting in adults which have huge morphological differences compared to their larval stage. Together with bees, wasps, sawflies and ants, bumblebees belong to the large and successful insect order of Hymenoptera. Currently, there are over 150,000 known species of Hymenoptera of which approximately 25,000 known species of bee, belonging to over 4,000 genera (Goulson, 2010).

Within the large order of Hymenoptera, bumblebees, bees, wasps and ants, belong to the suborder Apocrita. Species belonging to this suborder are characterized by the presence of a narrow "waist' formed between the first two segments of the abdomen (the petiole), and the fusion of the first abdominal segment with the thorax (the propodeum). The Apocrita have been split into two groups, the "Parasitica" and the 'Aculeata". The phylogenetic relationships within the group of aculeate Hymenoptera were for a long time uncertain (Brothers, 1999; Pilgrim *et al.,* 2008; Debevec e*t al.,* 2012). However, recent research based on genomic data revealed the phylogenetic relationships between the major lineages (Johnson *et al.,* 2013; Figure 1.1).

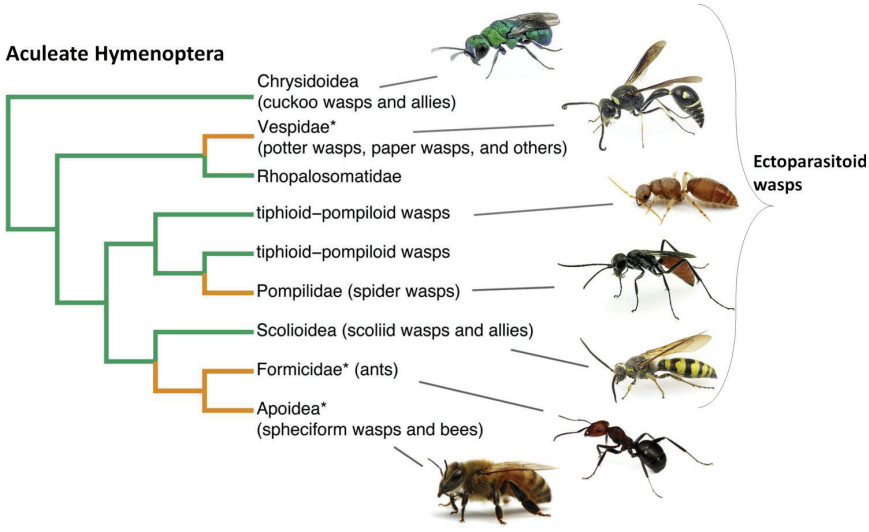


Figure 1.1 Evolution of the Aculeate Hymenoptera. Branch colours: green means parasitoidism; orange means nest construction and/or predation. Asterisks indicates for lineages containing eusocial species. Picture adapted from Johnson *et al.* (2013).

In contradiction with the earlier idea that ants are more closely related to ectoparasitoid wasps, they found that Formicidae (ants) and Apoidea (spheciform wasps and bees) were sister groups. The other lineages are clades of ectoparasitoid wasps (Johnson *et al.,* 2013; Figure 1.1).

Within the Apoidea, bees belong to the Apidae. This family has a common ancestor with predatory and parasitic wasps (Spheciform wasps) belonging to the Sphecoidea (Goulson, 2010; Johnson *et al.,* 2013; Figure 1.2).

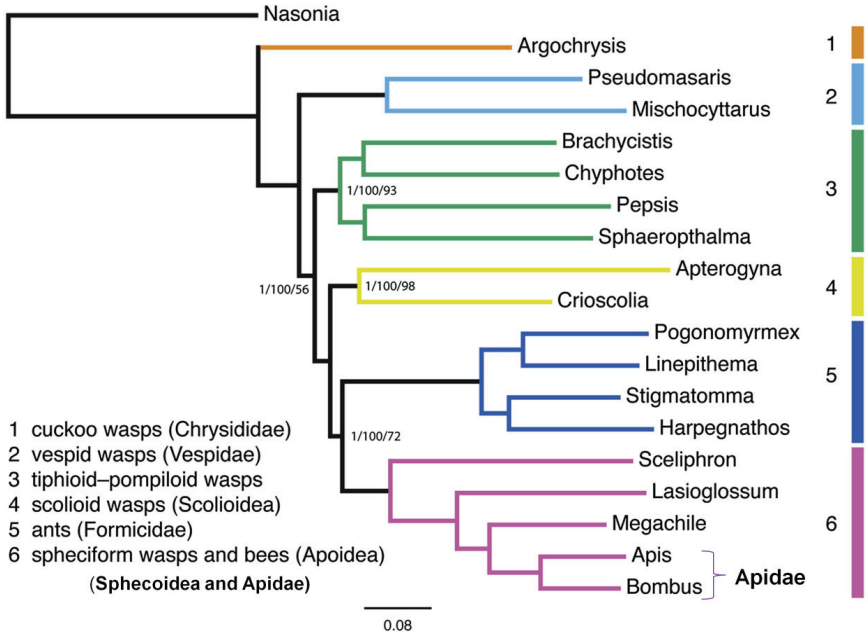


Figure 1.2 Maximum-Likelihood Tree of Aculeate Hymenoptera, with three different settings: Bayesian posterior probabilities, bootstrap values based on 1,000 replicates and bootstrap values from a separate species tree analysis, respectively. Unlabeled nodes have maximum support values (1/100/100). Scale bar indicates number of substitutions per site. Picture adapted from Johnson *et al.* (2013).

All bumblebee species are classified in a single genus *Bombus* (Williams, 1994; 1998; Goulson, 2010). Most bumblebee species are 'true' bumblebees which means that they have a sterile social worker caste (although they can produce unfertile eggs or haploid males). The other 45 species are "cuckoo" bumblebees. These social parasitic bees live within the nests of

true bumblebees feeding on the food gathered by their hosts (Goulson, 2010). Formerly, they were placed in a separate genus *Psithyrus*. However, this genus is now regarded as one of many *Bombus* subgenera (Williams, 1994; Cameron *et al.,* 2007; Goulson, 2010).

In the past scientists attempted to divide the genus *Bombus* in several subgenera based on coat colour patterns (Dalla Torre, 1880; 1882; Goulson, 2010) and male genitalia (Kruger, 1917; Skorikov, 1922; Goulson, 2010). As most bumblebee species have different colour patterns both within and between populations the first subdivision was of limited value, while the latter subdivision was more useful. Although there were still problems with the phylogenetic relationships between these subgenera (Cameron *et al.,* 2007; Goulson, 2010). Today, the genus *Bombus* can be divided into two clades: a 'short-faced' clade (SF) and a 'long-faced' clade (LF). This division is based on sequencing data for four nuclear and one mitochondrial gene (Cameron *et al.,* 2007; Goulson, 2010; Figure 1.3). Furthermore, this subdivision supported most of the existing subgenera on the basis of morphological characters (Cameron *et al.,* 2007; Goulson, 2010).

There are now approximately 250 bumblebee species described of which 29 known for Belgium and The Netherlands. Although most scientists presume that most bumblebee species are known, it is probable that some species remain undiscovered. For instance, the widespread species *B. cryptarum*, remained undetected until 2005 due to its morphological similarities with *B. lucorum* (Bertsch *et al.,* 2005; Murray *et al.,* 2008; Goulson, 2010). A phylogenetic tree of 218 different bumblebee species is presented in Supplementary File S1, Supplementary File S2, and Supplementary File S3 following Cameron *et al.* (2007).

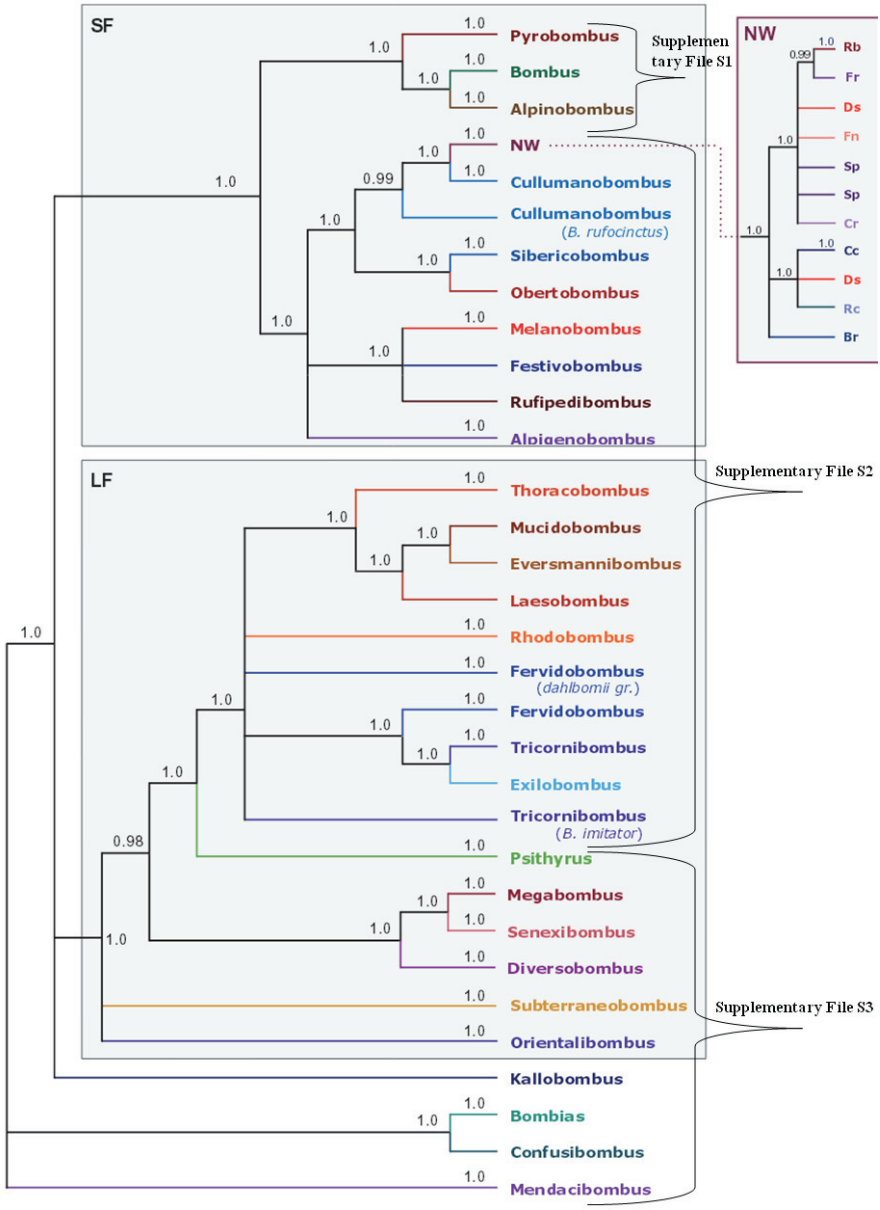


Figure 1.3 Bumblebee phylogeny showing only the subgeneric relationships with strong support (P = 0.95). The values on the branches are Bayesian posterior probability values. The abbreviations stand for: SF Short faced clade; LF, Long faced clade; and NW; New World Clade. Within NW: *Rb*, *Robustobombus*; Fr, *Fraternobombus*; Ds, *Dasybombus*; *Fn*, *Funebribombus*; *Sp, Separatobombus*; *Cr, Crotchiibombus*; Cc, *Coccineobombus*; *Rc*, *Rubicundobombus*; and *Br*, *Brachycephalibombus*. Figure adapted from Cameron *et al.* (2007).

1.1.2 *Life-cycle*

Here the life-cycle of *Bombus* species is described, largely based on the detailed descriptions given by Alford (1975) and Goulson (2010), but with the exclusion of the Cuckoo bumblebees (subgenus *Psithyrus*). In general, for most bumblebees this is an annual life cycle (Figure 1.4). After a hibernation period, fertilized queens emerge in late winter or spring depending on: (i) species, (ii) weather conditions, and (iii) location. These newly emerged queens start foraging for pollen and nectar to replenish their loss of fat during hibernation. In a next step, she starts searching for suitable nest sites, which are highly variable between different bumblebee species (Osborne *et al.,* 2008; Goulson, 2010). Some bumblebee species prefer to build their nest on or just above the surface of the ground, some prefer to nest in trees, while other species nest underground. Abandoned holes of small mammals or nests of birds are often used. Generally, the nest consists out of a central chamber with a single entrance and insulating material found within the abandoned nest such as moss, hair, dry grass and/or feathers. The first days or even weeks, the forming queen gathers pollen in which she will lay her first batch of eggs (between 8 and 16 eggs). On the outside, this pollen is covered with a mixture of pollen and wax secreted by the queen.

The brood is incubated by the queen sitting on top of this pollen lump. To ensure the high amount of energy needed for the maintenance of incubation heat, the queen creates a wax pot stored with nectar at the entrance of her nest. Furthermore, in this stage of nest making (see Figure 1.4) the queen will still forage to provide sufficient nectar and pollen.

Based on the way of feeding of the larvae, bumblebees can be divided in 2 groups; the ‘pocket makers’ (corresponds to the ‘long faced’ clade of Cameron *et al.*, 2007, as described in chapter 1.1.1) and the ‘pollen storers’ (‘short faced’ clade; see chapter 1.1.1). The larvae of the ‘pocket makers’ feed all together from the pollen clump. New pollen are given to the larvae first collectively from the underside of the pollen clump and later regurgitated food will be given directly through the wax cap. In the ‘pollen storers’ the larvae are fed regurgitated pollen initially together and later separately in self-made cells from wax and silk. As ‘pocket makers’ are more difficult to rear, mostly species of ‘pollen-storers’ are intensively reared commercially, which biases our knowledge of bumblebee ecology towards the latter group (Goulson, 2010). The total development time from larvae to adults is about 4

to 5 weeks: two weeks for the larvae to go through 4 instars and starts to pupate in a silk cocoon, and then another two weeks to hatch. The first batch of eggs are normally all workers. A part of the workers take over the foraging task of the queen, using the empty cocoons for storage of pollen and/or nectar, while others help the queen with the care and nursing of the next batches of offspring. In this way the nest grows rapidly, to a 10 times increase in weight within 3 to 4 weeks (Goulson *et al.,* 2001). Colonies of the buff-tailed bumblebee, *B. terrestris* can contain even up to 350 workers (Goulson *et al.,* 2001). While for bumblebee species belonging to the subgenera *Alpinobombus* (*B. polaris*, *B. balteatus*, and *B. hyperboreus*) and the mountain species *B.* (*Thoracobombus*) *inexspectatus,* it is known that nests can be very small containing only a few workers or even none (Yarrow, 1970; Løken, 1973; Richard, 1973; Gjershaug, 2009; Hines & Cameron, 2010). As these species live only in Artic and high mountain regions, the reduced colony production and the bias to the production of reproductive stages may be caused by the brief window of favourable climatic conditions (Hines & Cameron, 2010).

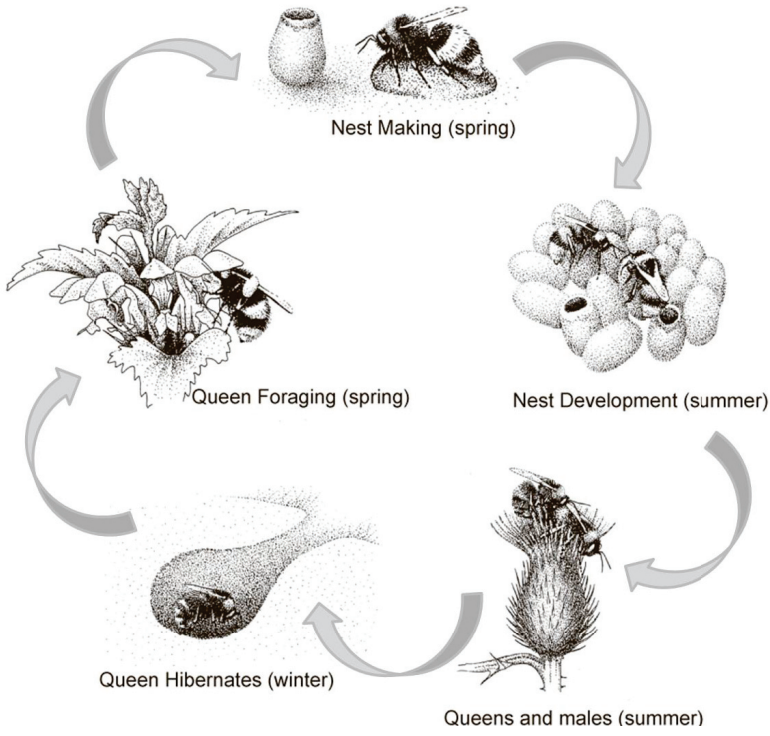


Figure 1.4 The bumblebee life-cycle. Picture adapted from Prŷs-Jones & Corbet (2011).

At a certain colony size, the density of workers in the nest triggers the queen to switch to the production of reproductives: drones and daughter queens. After this 'switching point', no more workers are produced. As developing daughter queens require more food over a longer period, they are produced when enough food and workers are available. The number of reproductives produced in a colony depends largely on the nest size. Small nests may rear no reproductives, moderate-sized nests only males, while both males and daughter queens are only produced by the largest nests (Schmid-Hempel & Schmid-Hempel, 1998). In contrast to daughter queens which stay a period in the nest, regularly foraging for pollen and nectar for themselves to build up their fat reserves, males do not contribute to tasks in the colony and after a few days they leave the colony. Once left, they feed on pollen and nectar of flowers, and search for a virgin queen. Molecular studies showed that the offspring of most bumblebee species were full sibs, which indicates that queens mate only once (= monoandrous) (Estoup *et al.,* 1995; Schmid-Hempel & Schmid-Hempel, 2000). However, queens of some species such as *B. hypnorum* mate more frequently (Paxton *et al.,* 2001). After mating, the queens start searching for a suitable hibernation site. Queens survive this dormancy period burning their fat reserves. In *B. terrestris* the critical weight of fat reserves to survive hibernation is about 0.6 g (Beekman *et al.,* 1998). After the departure of the reproductives, the nest degenerates rapidly. The former queen and the remaining workers will die and the remains of the comb will be consumed by parasites and commensals. Nests have last for 14 to 25 weeks in *B. pratorum* and *B. pascuorum,* respectively (Goodwin, 1995).

1.1.3 *Ploidy, sex determination and sociality*

Like other Hymenoptera, such as ants and wasps, bumblebees are haplodiploid insects in which the fertilized eggs of the queen will develop in diploid female offspring (workers and daughter queens), while the unfertilized eggs will develop in haploid males (drones). The queen has the ability to control whether her eggs are fertilized, and thus if her eggs will develop into sons or daughters.

The consequence of this sex-determination system is that all sisters within a nest are more related than when they would be in case of diploid organisms. In diploid species, all offspring from the same 2 parents share 50% of each other's genes. Their relatedness (r) is 0.5. In haplodiploid organisms the genetic relationship between sisters is higher, r = 0.75. Indeed, in haplodiploid species, diploid specimens (= sisters) receive half of the genes of their mother

(queen) and all the genes of the father (haploid male). As their father develops from an unfertilised egg, he has only one set of chromosomes. Every sperm contains the same set of chromosomes, thus all diploid offspring will receive the same genetic material. Their relatedness is thus minimal 50%. From the mother's side, sisters receive one of the two sets of chromosomes. So, they will receive either the same genes or either different genes from their mother ($r = 0.5$ or $r = 0$, respectively). In general, sisters have a mean relatedness of: $r = (0.5 + 1.0) / 2 = 0.75$. Furthermore, the relatedness between a female and her offspring will be $r = 0.5$; and between workers and their brothers only 0.25 (Figure 1.5).

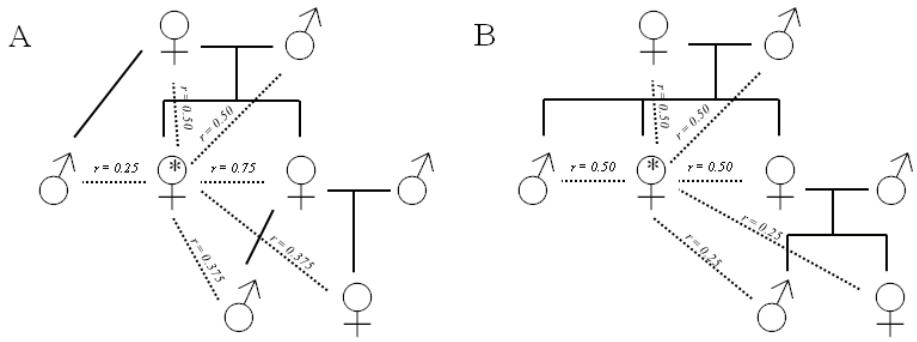


Figure 1.5 (a) An example of a haplodiploid, and (b) a diploid family tree indicated by the full lines. The dotted lines are indications of the relatedness of a female (indicated with a star) to her kin assuming monoandrous species.

This implies that females are more related to a sister ($r = 0.75$) than they would be to her own daughters ($r = 0.50$). Thus, a worker will profit more by helping her mother to produce more sisters than by producing her own daughters. Haplodiploid females are also more related to their nieces ($r = 0.375$) than diploid females are to their nieces ($r = 0.25$; Figure 1.5). The consequence of the haplodiploid sex-determination system predispose bumblebees, and Hymenopterans in general, to evolve sociality (Goulson, 2010). Actually, the estimation of relatedness between nest mates is or could be even more complex, as it depends heavily on the number of patrilines within a colony (Schmid-Hempel & Schmid-Hempel, 2000).

The above mentioned calculations of relatedness were based on a monoandrous mating system, as the majority of bumblebee species appears to be monoandrous (Goulson, 2010). However, also polyandrous bumblebee species exist (such as *B. hypnorum*; Paxton *et al.,*

2001). If in a polyandrous colony, sisters have the same father, $r = 0.75$; but if they have unrelated fathers their relatedness will be between 0.25 and 0.5, depending on the number of males the queen has mated with (Figure 1.6).

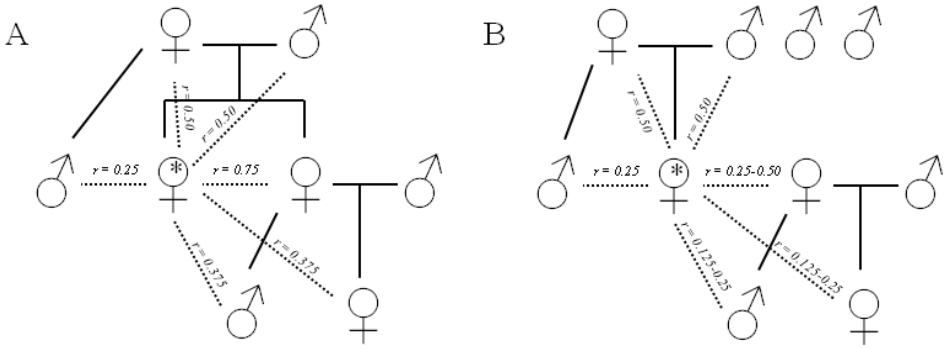


Figure 1.6 (a) An example of a monoandrous versus (b) a polyandrous haplodiploid family tree with indication of the relatedness of a female (indicated with a star) to her kin.

Another consequence of this haplodiploid sex-determination system is that all females can produce male offspring without ever mating. Thus, even workers have the ability to produce male offspring from their unfertilized eggs, a phenomena which can sometimes be seen at the 'switching point' (see 1.1.2). A worker will then have a greater genetic 'interest' in raising her own and/or her sister's sons ($r = 0.50$, $r = 0.375$; respectively) than she will have with raising her brothers ($r = 0.25$) (see Figure 1.5 and Figure 1.6A; Goulson, 2010).

In Hymenoptera the fertilized eggs develop into diploid females and unfertilized eggs in haploid males. However, this is not always true. Indeed, in Hymenopterans the sex is determined by the presence of complementary alleles at a single sex-determining locus (Cook & Crozier, 1995). As unfertilised haploid eggs are hemizygous (having only one gene copy) they will all develop in males. Bumblebees, heterozygous at this locus (having two different alleles) will develop in females, while bees homozygous at this locus will develop in diploid males (Duchateau *et al.,* 1994; Whitehorn *et al.,* 2009). The occurrence of these diploid males will depend on the number of alleles at this loci (at least 46 alleles for *B. terrestris*; Duchateau *et al.,* 1994). In a healthy population the probability of matched-pair matings at

the sex locus is low, however in small inbred populations this probability is much higher. The presence of diploid males is seen as a negative 'burden' for the colony, because: half of the workers will develop in diploid males. These males will not contribute to colony tasks, and have also a low fertility (Duchateau & Marien, 1995). Queens who mate with these diploid males are normally unable to initiate a colony (Cook & Crozier, 1995; Gerloff & Schmid-Hempel, 2005; Whitehorn *et al.*, 2009).

However, several research papers have shown that successful mating between diploid males and queens does occur and then this leads to the formation of triploid offspring which in turn is sterile (Ayabe *et al.*, 2004; Darvill *et al.*, 2012). This triploid offspring will develop either in workers when one of the three alleles at the sex determination locus is different (comparable with 'heterozygous') or either in drones when all alleles at this locus are the same (comparable with 'homozygous') (Ayabe *et al.*, 2004; Darvill *et al.*, 2012).

This observation triggered a recent study to investigate if queens have the ability to avoid mating with diploid males (Lecocq *et al.*, 2014). Although no differentiation between diploids and haploids males was found for male cephalic labial gland secretion (CLGS, a main chemical reproductive trait), which argues that there is no diploid male discrimination by queens through CLGS compositions, no precise conclusions can be made yet (Lecocq *et al.*, 2014).

1.1.4 *Morphology*

The bumblebees' body consists out of an exoskelet. These are hard plates of chitin which deny bumblebees the ability to grow as an adult (Wigglesworth, 2008). As in other insects, the bumblebee body can be divided into three typical tagmata: (i) the head, with eyes, mouthparts and antennae; (ii) the thorax, with legs and wings; and (iii) the abdomen, which contains the digestive and reproductive organs and the sting (Sladen, 1912; Figure 1.7).

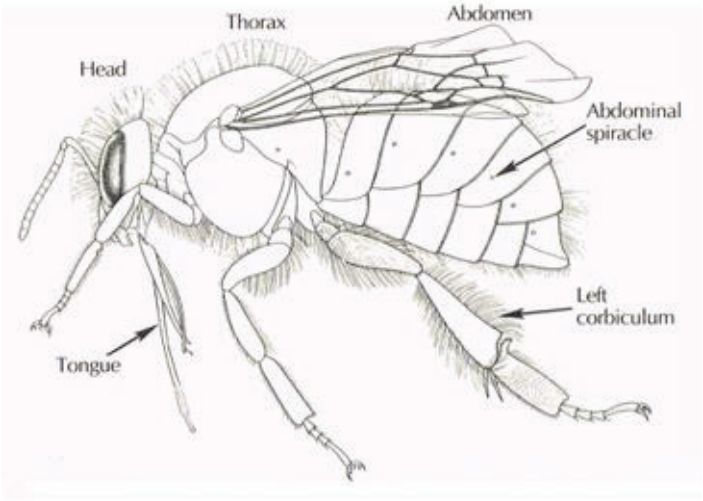


Figure 1.7 Drawing of a bumblebee with indication of the three tagmata: (i) the head, (ii) the thorax, and (iii) the abdomen (image from Heinrich, 1979).

Bumblebees have 3 pairs of legs. A figure of the legs is shown in Figure 1.7. These legs are fairly unspecialized, especially the claws, femur, trochanter and coxa, which have similar design as found in many other insects (Figure 1.7). However, like honeybees, bumblebee workers have also specialized morphological structures on their legs, especially the hind legs, for the collection of nectar and pollen (Sladen, 1912; Michener, 1999; Thorp, 2000; Figure 1.8). Indeed, workers and queens have a pollen basket or corbicula on the outside surface of the tibia of each hind leg. The tibia surface is concave and hairless, but is also bordered by a fringe of long and stiff hairs which forms the pollen basket (Figure 1.8). Also the tarsus, which consists out of 5 segments of which the first 4 segments are similar, has special hairs and combs on the much larger fifth segment or metatarsus (Figure 1.8). The female bee uses the combs and brushes on her legs to gather pollen that sticks to her hair and body, and stores this in her corbicula (Michener, 1999; Thorp, 2000). Male bumblebees have no corbicula.

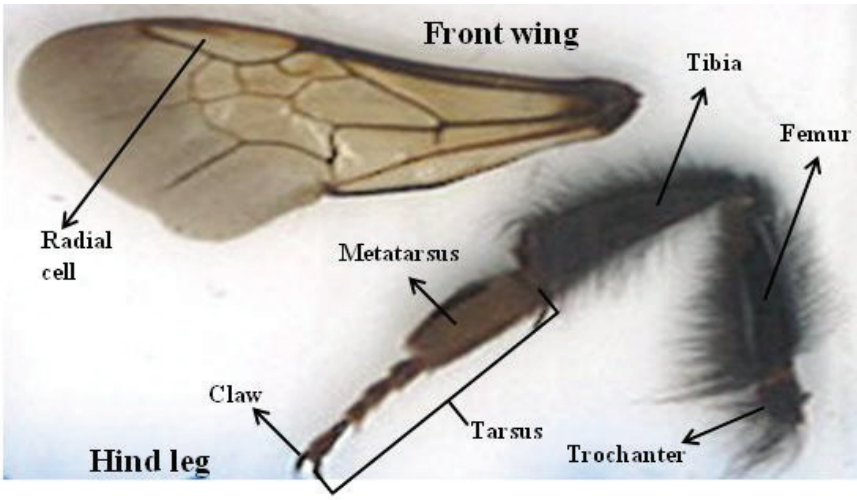


Figure 1.8 Picture of the fore wing and hind leg of a *Bombus terrestris* male. Coxa not shown.

Furthermore, bumblebees have on each front leg a pair of antennal cleaners which are used to remove dirt or pollen from the antennae (Sladen, 1912; Beattie, 1971). These antennae consist out of a long pedicel and 12 smaller segments, which form the flagellum (Figure 1.9). This is true for queens and workers. However, males have 13 segments in their flagellum (Sladen, 1912). On top of the final segment of the flagellum bumblebees have pore plates for detecting odours (Agren & Hallberg, 1996; Spaethe *et al.*, 2007; Figure 1.9). The pore plates sensilla are the most abundant antennal olfactory sensilla, with connection to 13-20 sensory neurons (Agren & Hallberg, 1996; Spaethe *et al.*, 2007).

Bumblebees have two pairs of wings. The rear wings are small and attached to the front wings by a row of hooks or hamulae (Slade, 1912). The big wing muscles take all thorax space and need a temperature of 30°C (Heinrich, 1975; 1979; Goulson, 2010). In flight, the muscle temperature is regulated to stay between 30 - 44°C.

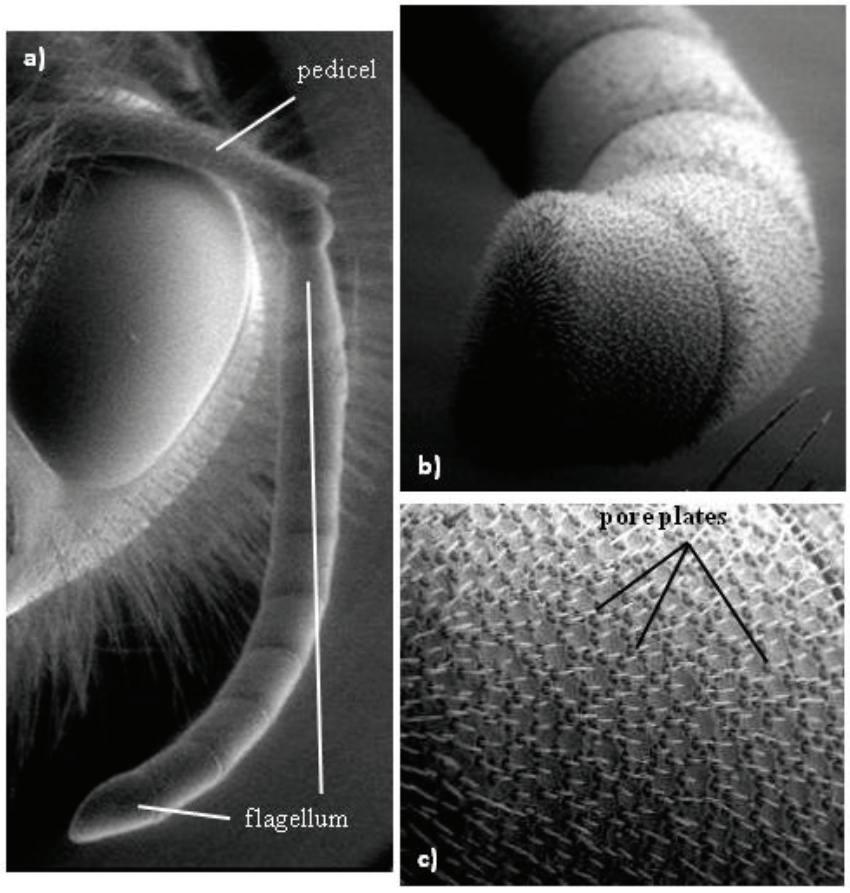


Figure 1.9 Picture with: (a) frontal view of a antenna, (b) detailed view of the five top segments of the flagellum, and (c) detailed view of the pore plate of the top segment of a *Bombus terrestris* female. Picture adapted from www.bumblebee.org.

Bumblebees generate heat (i) through shivering the flight muscles, and (ii) through substrate cycling in the flight muscles (Heinrich, 1975; 1979; Goulson, 2010). (i) The two sets of powerful wing muscles contract alternately during flight. However, during warm-up they will contract at the same time, generating heat, and little or no movement (Heinrich 1979; Goulson, 2010). (ii) bumblebees are able to burn sugars to generate heat in the flight muscles through substrate cycling. The key enzyme in this process is fructose bisphosphatase and this enzyme has an unusually high activity in the flight muscles of bumblebees. This enables the

bees to maintain a stable internal temperature when inactive. Once they are attacked by a predator, they need to generate heat rapidly to take off, and they do so through substrate cycling (Goulson, 2010). Furthermore, the thorax is more than 20°C warmer than ambient and 10°C warmer than the abdomen. Heat loss from the thorax to the abdomen is reduced by the narrow waist (the petiole) separating the two, and by an insulating air sac in the anterior section of the abdomen where it contacts the thorax.

The petiole acts as a countercurrent heat exchanger. Cool haemolymph in the heart flows forwards from the abdomen to the head, and in the petiole is forced into intimate contact with the warm haemolymph flowing backwards from the thorax. Inevitably, heat will be transferred between the two as they pass alongside each other, so that rather little heat is lost to the abdomen. Furthermore, from colder regions have much longer hairs then species from warmer climates (Peat *et al.*, 2005). Just as there must be a minimum temperature (30°C) at which bumblebees can fly, there is also a maximum (42-44°C). The larger the insect, the more heat is generated, and the less surface area (proportionally) is available through which to lose it. Thus queens and large foragers are liable to overheat at high ambient temperatures (Heinrich 1975; 1979; Goulson, 2010). Due to this thermoregulation system, bumblebees are capable of foraging on days when it is too cold to forage for other pollinators (Heinrich, 1975; 1979; Goulson, 2010).

Structurally, queen and worker bumblebees are identical in their external morphology, although queens are remarkable bigger than workers (Michener, 1974; Alford, 1975; Cnaani & Hefetz, 2001; Goulson, 2010). The abdomen of young queens is full of fat; while workers have very little fat. As their main task is foraging, workers need more place for the honey stomach in which nectar can be stored on their foraging trips. This is also why queens are heavier for their size than workers (Goulson, 2010). Bumblebee workers weigh mostly between 0.2 g to 0.4 g, while queen are normally more than 0.6 g with some large queens can reach 0.89 g (Alford, 1975; Michener, 1974; Přidal & Hofbauer, 1996; Hagen *et al.,* 2011).

1.1.5 *Bumblebee vision*

Bumblebees have two types of eyes: simple and compound eyes (Meyer-Rochow, 1981; Warrant *et al.,* 2006; Wcislo & Tierney, 2009; Figure 1.10). The three simple eyes or ocelli, which looks like shiny bumps, are arranged in a triangular pattern located dorsally on top of the head (Warrant *et al.,* 2006; Wcislo & Tierney, 2009; Figure 1.10). They focus light through a single lens (cornea) with underneath a layer of photoreceptors (Wcislo & Tierney, 2009; Figure 1.10). Bees use their ocelli to stabilize the flight, to navigate and to orientate themselves towards the sun (Warrant *et al.,* 2006; Wcislo & Tierney, 2009).

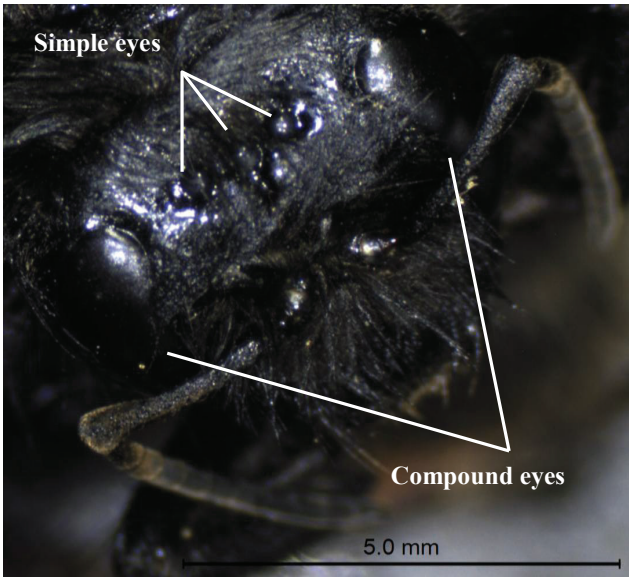


Figure 1.10 Picture of the head of a bumblebee *Bombus terrestris*, with indication of the two apposition compound eyes and the three simple eyes or ocelli.

Bumblebees also have apposition compound eyes, which are typical for diurnal insects (insects which are mostly active during daytime) (Warrant *et al.,* 2004; Somanathan *et al.,* 2008; Kelber *et al.,* 2011). In general, compound eyes consist out of a large number of individual hexagonal visual units called ommatidia, each equipped with a tiny single lens (Meyer-Rochow, 1981; Nilson, 1989; Warrant *et al.,* 2004; Greiner, 2006; Kelber *et al.,* 2006; Warrant, 2008; Kelber *et al.,* 2011; Figure 1.11). Apposition compound eyes consist

out of thousands of these tiny individual optical units, also called facets (Warrant *et al.,* 2004; 2006; Greiner, 2006; Kelber *et al.,* 2006; Somanathan *et al.,* 2008; Warrant, 2008).

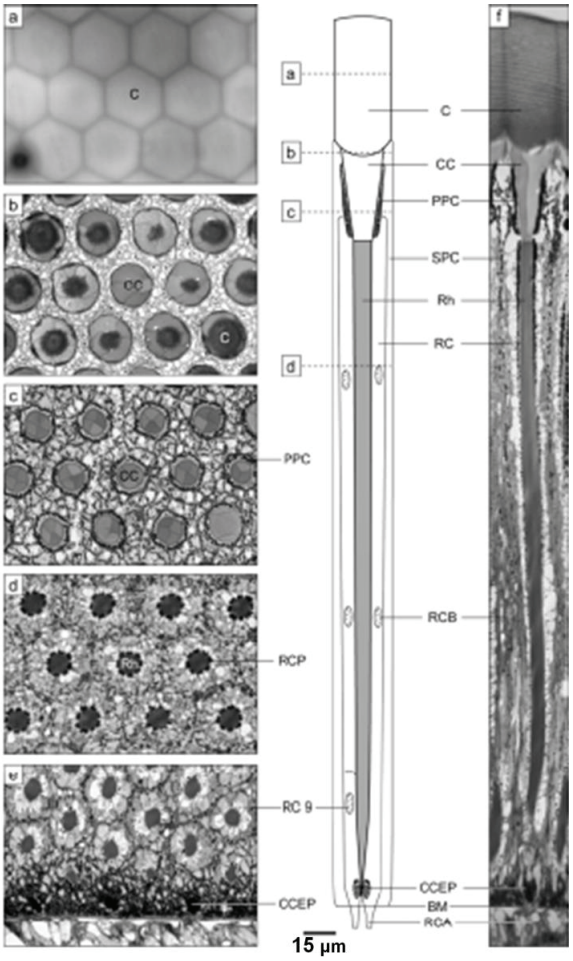


Figure 1.11 Drawing and picture of an ommatidium of an apposition compound eye of the nocturnal wasp *Apoica pallens* and several longitudinal sections. With indication of the different structures of an ommatidium: the dioptric apparatus which consists out of the corneal facet (C) and the crystalline cone (CC); primary pigment cells (PPC); secondary pigment cells (SPC); and the fused rhabdom (Rh) which contains nine retinula cells (RC). The ninth retinula cell (RC9) an the crystalline cone extensions (CCEP) appears only in the proximal end of the rhabdom. The axons of the retinula cell axons (RCA) pass as bundles through the basement membrane (BM). When in light-adapted state, the pigments of the retinula cells (RCP) tightly surround the rhabdom (adapted from Greiner, 2006).

Underneath each facet lies the crystalline cone, generally formed by four Semper cells. Both corneal lens and crystalline cone build up the dioptric apparatus of the compound eye. Under the crystalline cone are the visual cells which are connected to a nerve axon and thus the brain. In bees there are 8 to 9 retinula cells or photoreceptors within each ommatidium which collectively form a central axis or transparent tube, called the rhabdom (Meyer-Rochow, 1981; Nilson, 1989; Greiner, 2006; Figure 1.11). There are three types of retinula cells: ultraviolet-sensitive (347-353 nm), blue-sensitive (430-436 nm) and green-sensitive (533-548 nm) (Meyer-Rochow, 1981; Skorupski *et al.*, 2007; Dyer *et al.*, 2011; Figure 1.11).

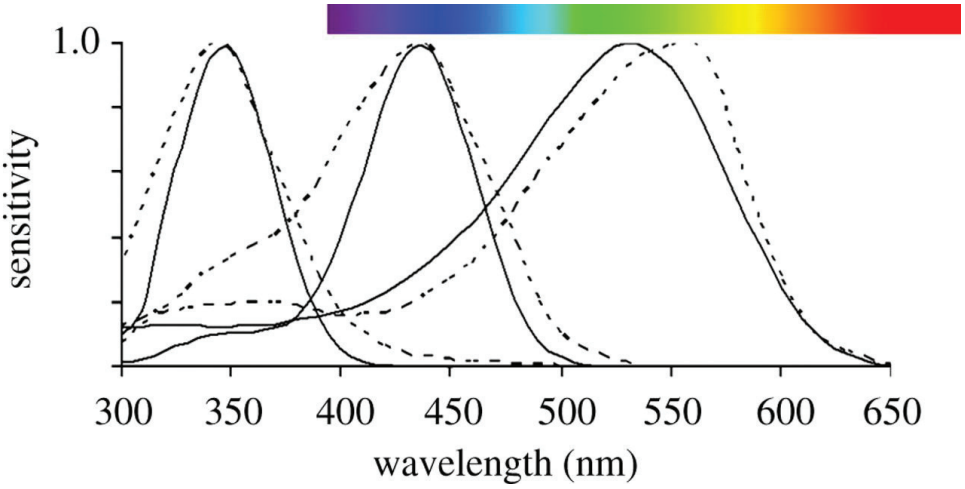


Figure 1.12 The representative sensitivity of the three photoreceptors of bumblebees (solid line) and honeybees (dotted line). In contrast, humans can perceive longer-wavelength radiation which is indicated by the visible light spectral bar above the graph. Figure adapted from Dyer *et al.*, 2011.

The rhabdom is made of 8 to 9 open or fused rhabodmeres which consist out of specially photon-absorbing, visual pigments arranged in microvilli (Meyer-Rochow, 1981; Figure 1.13). These microvilli are bristle-like membrane projections from the photoreceptor cells which increase the membrane surface area, and thus increase the amount of visual pigments in the cell (Meyer-Rochow, 1981; Land, 1997; Figure 1.13). The microvilli of a single retinula cell collectively form a rhabdomere.

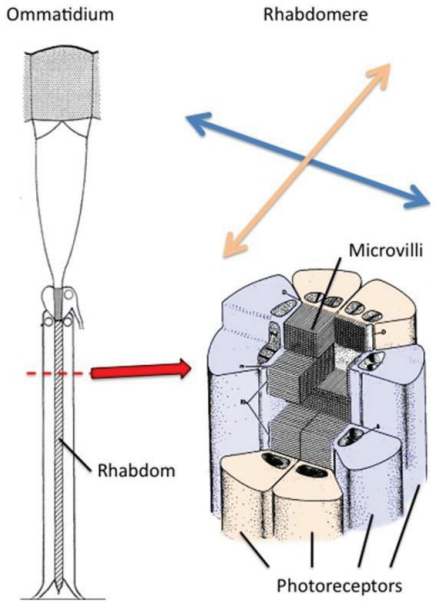


Figure 1.13 Longitudinal drawing of a rhabdom from the ommatidum of an apposition compound eye, with indication of the individual rhabdomere which consists out of the microvilli from the photoreceptors or retinula cells.

Furthermore, each ommatidium contains several types of pigment cells: (i) two primary pigment cells which surround the crystalline cone; (ii) a varying number of secondary pigment cells which ensheath the entire ommatidium, and (iii) retinula cell pigments which are present within the retinula cells (Meyer-Rochow, 1981; Greiner, 2006; Kelber *et al.,* 2011; Figure 1.13).

In general, (i) the tight apposition of the crystalline cone and the rhabdom, together with (ii) the thick sheath of pigments present in the secondary pigment cells and (iii) the crystalline cone extensions which covers the basement membrane, are the major characteristics of apposition eyes to absorb stray light (Greiner, 2006; Figure 1.13). Axial light from a single facet is thus focused onto the respective rhabdom underneath (Warrant *et al.,* 2004; Kelber *et al.,* 2006; Warrant, 2008). Light reaching the eye off-axis will be absorbed by the pigments (Warrant, 2004; Greiner, 2006; Somanathan *et al.,* 2009a).

The other types of compound eyes are: neural superposition and refracting superposition eyes (Nilson, 1989; Greiner, 2006; Figure 1.14). The division in three major groups are based on variations in eye optics or neural wiring between the eye and the first optic ganglion or lamina (Nilson, 1989; Greiner, 2006; Figure 1.14).

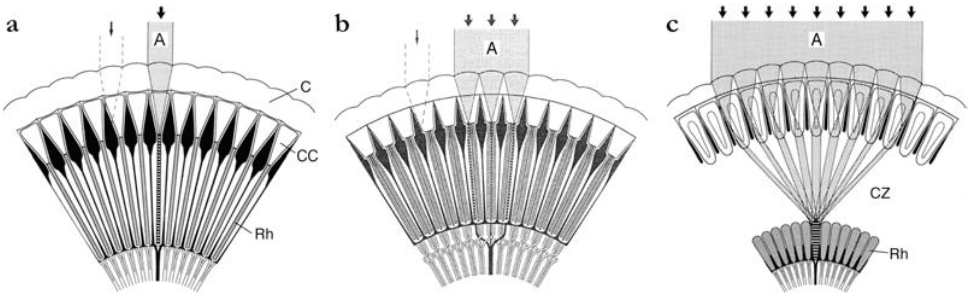


Figure 1.14 Longitudinal sections of the three major types of compound eyes: (a) apposition, (b) neural superposition and (c) refracting superposition eyes. The size of the aperture (A) reflects differences in sensitivity whereas the arrows in the grey shade are showing the path of light absorbed by the photoreceptor. C = cornea, CC = crystalline cone, CZ = clear zone, and Rh = rhabdom (adapted from Greiner, 2006).

In neural superposition eyes, which can be found in flies (Diptera, suborder Brachycera), the rhabdomeres are separated and can receive light from slightly different angles. The retinula cell axons of the rhabdomeres, which receive light from the same angle but originated from different ommatidia, converge onto the same neural unit of the lamina (Figure 1.14b). In this way, sensitivity can be increased 6-fold in these diurnal insects (Greiner, 2006).

In refracting superposition eyes, typically for nocturnal insects, the optics and the light-absorbing rhabdom layer are separated by a pigment-free or 'clear' zone (Figure 1.14c). Through special optics, the light rays from a large number of facets can be focused onto a single rhabdom (Figure 1.14c). Thus, each rhabdom receives light through the 'clear zone' from hundreds or thousands of facets. This greatly improves photon catch and thus sensitivity (Greiner, 2006).

Animals with apposition compound eyes are usually restricting to a diurnal lifestyle, because their eye design works best at bright light intensities (Warrant *et al.,* 2004; Kelber *et al.,* 2006; Somanathan *et al.,* 2008; Wcislo & Tierney, 2009). Their small aperture limits the absolute sensitivity of their eyes and therefore the use at night or under dimmed light conditions (Warrant *et al.,* 2004; Kelber *et al.,* 2006; Warrant, 2008; Somanathan *et al.,* 2009). Indeed, low light intensities result in a poor photon catch and unreliable visual signals (Warrant, 2004). However, an increase in ommatidial diameter can improve the sensitivity towards lower light conditions and higher spatial acuity, as the photoreceptors of these ommatidia will capture more photons (Spaethe & Chittka, 2003; Warrant, 2004; Kelber *et al.,* 2006; Kapustjanskij *et al.,* 2007). Therefore, bees and other Hymenopterans which show a nocturnal or crepuscular lifestyle, and thus become active when light conditions are poorer, possess (i) relatively larger eyes with reasonably larger ommatidial facets, (ii) larger ocellar diameters, and (iii) unusual wide rhabdoms (compared to diurnal species of similar size) (Kerfoot 1967; Jander & Jander, 2002; Warrant *et al.,* 2004; Kelber *et al.,* 2006; Somanathan *et al.*, 2009a) (for a review, see Warrant, 2008; Wcislo & Tierney, 2009). However, not all dim-light foraging bees have enlarged ocelli and compound eyes (Wcislo & Tierney, 2009). Furthermore, apposition eyes have to search for a balance between spatial resolution (by increasing the number of ommatidia) and absolute sensitivity (by larger ommatidia) (Warrant *et al.,* 2004; Somanathan *et al.,* 2008).

Aside from type of lifestyle, also body size usually correlates with the eye size (Spaethe & Chittka, 2003), facet and ocellar diameters and thus presents a good predictor of overall light sensitivity of the visual system in Hymenopterans (Jander & Jander, 2002; Kelber *et al.,* 2006). Furthermore, Kapustjanskij *et al.* (2007) showed that random sampled bumblebees with a larger eye morphology have a higher ability to fly in weaker light conditions.

In conclusion, several studies with nocturnal sweat bee *Megalopa genalis* (Warrant *et al.,* 2004; 2006; Kelber *et al.,* 2006), nocturnal and diurnal paper wasps (Warrant *et al.,* 2006), crepuscular bees (Kelber *et al.,* 2006), and Indian carpenter bees (Somanathan *et al.*, 2008, 2009) showed that morphological parameters of the eye can affect the sensitivity in different light conditions. Furthermore, bumblebees with larger ocelli and/or ommatidia will be more light sensitive (Kapustjanskij *et al.,* 2007, for review: Warrant, 2008; Wcislo & Tierney, 2009).

1.1.6 *Cast determination and division of labour*

Bumblebees do not have a strict age-related division of labor as honeybees (*A. mellifera)* do (Cameron, 1989; O'Donnell *et al.,* 2000). Young honeybees perform in-hive tasks, whereas older bees undergo a transition from a nurse to a forager worker bee, collecting food outside the nest (Robinson, 1992). The division of labor for bumblebees is mainly based on worker size, as several studies have revealed a correlation between workers size and their probability for a certain task, a phenomenon known as alloethism (O'Donnell *et al.,* 2000; Jandt & Dornhaus, 2009). Small workers are more found to stay inside the nest and fulfill nest duties whereas large workers have a higher probability of foraging (Goulson *et al.,* 2002; Yerushalmi *et al.,* 2006; Jandt & Dornhaus, 2009), although task switching is possible (Jandt & Dornhaus, 2009). In contrast, some indication of age-related division of labor was found in *B. terrestris* colonies by Yerushamli *et al.,* (2006) and Jandt & Dornhaus, (2009). They observed that younger bumblebees are more likely to perform brood care and 'in nest' tasks, whereas older bees are more likely to forage. However, those age effects are not strict, as many bumblebees never initiate foraging and stay in their nest throughout their entire life (Brian, 1952; Free, 1955; Yerushalmi *et al.,* 2006). The division between forager and nester is not strict, and bumblebees can already start to forage as early as 2 days after emergence (Pouvreau, 1989; Yerushalmi *et al.,* 2006) with large bees performing foraging flights earlier than small bees (Yerushalmi *et al.,* 2006). Also Robinson (1992) described that the age of foraging depends on the needs of the colony. In the absence of foragers, the smaller bees that normally stay inside will start foraging to comply with the nutritional needs of the bumblebee colony (Goulson, 2010).

So, a correlation between worker size and caste determination is found, but what causes this size variation in bumblebee workers? In pollen-storing species, larvae are fed directly on nectar and pollen mixes regurgitated by the adults (Alford, 1975; Goulson, 2003). Thus, adults could determine the size attained by each larva (Ribeiro, 1994) as well-fed larvae will eventually become larger adults than less-fed larvae (Spaethe & Weidenmuller, 2002). However, given the fact that larvae are reared in a controlled environment by a team of specialized nest workers, it seems implausible that a 10-fold variation in worker mass could result from the accidental neglect of some larvae at the expense of others (Alford, 1975; Sutcliffe & Plowright, 1988; 1990; Goulson *et al.,* 2002; Goulson, 2010). So, most research

was concentrated towards size variation as an adaptive function, in which colonies will benefit from rearing workers of a range of sizes (Goulson *et al.,* 2002; Spaethe & Weidenmuller, 2002; Powell & Franks, 2006; Spaethe *et al.,* 2007). However, Couvillon & Dornhaus, (2009) recently showed that size differences in pupae of *Bombus impatiens* were indeed made by intentional neglect of the larvae at the periphery of the nest which received less care than those in the centre.

1.1.7 *Foraging, light sensitivity and size*

The visual system of bumblebees consist out of two apposition compound eyes and three ocelli (Wcislo & Tierney, 2009), while the olfactory system consist out of several pore plate sensillae on their antennae (Spaethe *et al.,* 2007; as described in 1.1.4 and 1.1.5). Both sensory systems determine the foraging abilities of an individual bumblebee. Thus, an improvement of one or both sensory systems will increase the foraging efficiency (Chittka *et al.,* 1999). Indeed, bumblebees use a combination of color and spatial relationships to learn from which flowers to forage (Spaethe *et al.,* 2001; Goulson, 2010). They normally visit the same patches of flowers every day, which is called 'flower constancy' (Free, 1970; Chittka *et al.,* 1999). Moreover, dependent on the species, they can visit patches of flowers up to 2.4 km from their nest (Walther-Hellwig & Frankl, 2000; Chapman *et al.*, 2003; Wolf & Moritz, 2008; Charman *et al.*, 2010). Nectar can be extracted from the flower using their long tongues (or glossae), or by "nectar robbing", biting at the base of the flower to extract nectar (Irwin & Brody, 1999). After visiting a flower some bumblebee species leave a scent mark on the flower which marks visitation of the flower to other bumblebees (Schmitt & Bertsch, 1990).

Furthermore, bumblebees have an thermoregulation system (Heinrich, 1975; 1979; Goulson, 2010). This make them capable of foraging in bad weather conditions and on cold days, even when it is too cold to forage for other pollinators (Heinrich, 1975; 1979; Goulson, 2010). Next to temperature and weather conditions also other environmental conditions like humidity and light intensity determines bumblebee foraging activity (Corbet *et al.,* 1993; Peat & Goulson, 2005; Goulson, 2010). Also external factors such as food quality play a role in this (Chittka *et al.,* 1997; Roldán-Serrano & Guerra-Sanz, 2005; Goulson, 2010).

In social insects, the food influx of a colony is determined by how work is allocated among the members of the colony (Goulson, 2003). The size-dependent division of labor, discussed in chapter 1.1.6, could help to maximize the nectar and pollen influx of a colony (Goulson *et al.*, 2002; Spaethe Weidenmüller, 2002). Larger workers are able to forage early in the morning (and also late at dusk) when small workers and other small bees are prevented from foraging due to low temperatures and/or inadequate light conditions (Heinrich, 1975; 1979; Heinrich & Heinrich, 1983). Furthermore, large bumblebees have bigger eyes and can see better in lower light conditions than small conspecifics (Kapustjanskij *et al.*, 2007). Larger bumblebees exhibit also an increased odor (Spaethe *et al.,* 2007), are faster learning (Worden *et al.,* 2005) and have a better visual resolution (Spaethe & Chittka, 2003). As many flowers accumulate nectar and pollen overnight a colony might be able to significantly increase its overall food intake rate by allocating large workers to forage, especially at dawn and dusk (Corbet *et al.,* 1995).

1.2 The value of pollination and bumblebee decline

1.2.1 *The value of pollination*

Many wild flowers and agricultural crops depend heavily on insects for their pollination. Pollination, the transfer of pollen from the anther of a flower to the stigma of the same (self pollination) or of a different flower (cross pollination), is a key step in the sexual reproduction of plants (Free, 1993). Cross pollination is essential for the production, quality, earliness and uniformity of seed set and fruit quality (Corbet *et al.,* 1991; Free, 1993). Pollination is a crucial process in the persistence and viability of both wild and managed plant populations (Kevan *et al.,* 1990; Kearns & Inouye, 1997; Allen-Wardell *et al.,* 1998).
Pollinators contribute for more than €22 billion to European agriculture per year (STEP-project), of which bumblebees are extremely important as three of the five most important pollinator species of European crops are bumblebee species. Bumblebees pollinate several main agricultural crops such as: pepper (*Capsicum annuum*), melon (*Cucumis melo*), watermelon *(Citrullus lanatus*), cucumber (*Cucumis sativa*), strawberry (*Fragaria x ananassa*), raspberry (*Rubus idaeus*) and apple (*Malus domestica*) and the greenhouse tomato (*Solanum esculentum*) as the main agricultural crop (Velthuis & van Doorn, 2006). Velthuis & van Doorn (2006) reported in 2006 that worldwide, 95% of all bumblebee sales were made for tomatoes. Although now, bumblebee breeders are diversifying the use of bumblebees for

pollination purposes. In 2006, more than 40,000 hectares of bumblebee pollinated tomatoes were cultured in greenhouses with a total estimated crop value of €12,000 million per year (Velthuis & van Doorn, 2006). Within a few years after the introduction of the use bumblebees for their pollination service in greenhouses, nearly 100% of growers in Belgium and the Netherlands switched to bumblebee pollination in their greenhouses. Honeybees (*Apis mellifera*) can also pollinate most of the above mentioned crops, but they are often less efficient than bumblebees (e.g. Velthuis & van Doorn, 2006; Goulson, 2010). That is because, commercial bumblebees perform better in the artificial environment of the greenhouse than honeybees, as they can cope with lower temperatures and/or lower light intensities. While honeybees normally do not forage in temperatures less than 16 °C, bumblebees forage even in temperatures lower than 10 °C (Heinrich, 1979; Goulson, 2010). Bumblebees also can stay active in temperatures up to 32-35°C (Heinrich, 1979; Goulson, 2010). Furthermore, bumblebees are capable of "buzz pollination", which honeybees cannot. Indeed, as some plant species release their pollen from small holes in the anther and do not split open to release pollen, a bumblebee is still able to collect the pollen by producing a strong vibration that shakes the pollen out of the anthers due to rapidly contracting of the flight muscles (Buchmann, 1983). In addition, honey bees fly out of greenhouse vents when other more rewarding flowers are available outside the greenhouse, while bumblebees will remain working in the greenhouse as they are not able to communicate about a food source outside the greenhouse (Griffiths & Robberts, 1996).

The major bumblebees species being commercially reared and being used are *B. terrestris* in Europe, *B. ignitus* for Asia, and *B. impatiens* in the U.S.A and Canada. The loss of bumblebee species, certainly when the species is a key pollinator, could lead to a decrease in plant seed set, genetic diversity and ultimately to extinction of these plants (flower). In turn, this could lead to a cascade of effects on other animals dependent on the plant for food and shelter (Kearns & Inouye, 1997). The severity of the plants extinction depends on whether the plant is pollinated by one or more pollinator species, on self compatibility and/or on seed production (Kearns & Inouye, 1997).

1.2.2 *Red list*

All over the world different pollinator species are undergoing major declines (e.g. Potts *et al.,* 2010). Many bumblebee species, essential pollinators in natural and managed ecosystems (as

described in chapter 1.1.8), are no exception to this general phenomenon (Williams & Osborne, 2009; Cameron *et al.*, 2011, Carvalheiro *et al.*, 2013).

Currently, a red list of bee species in Belgium is not existing. However, in The Netherlands they had already a red list of bee species in 2003 (Peeters & Reemer, 2003). In this work, 16 of the 29 *Bombus* species received a red list status corresponding to the decline in their distribution before and after 1970. Two bumblebee species were described as 'vulnerable', 5 species as 'endangered', 5 species as critically endangered, and even 4 species 'disappeared' from the Netherlands (see Table 1.1; Peeters & Reemer, 2003). The other 13 bumblebee species were considered as stable and or 'Least Concern'.

Table 1.1 List of *Bombus* species on the Red list of the Netherlands following Peeters & Reemer (2003).

Species	Red list status	Species	Red list status
Bombus barbutellus	Critically Endangered	*Bombus pomorum*	Disappeared
Bombus confusus	Disappeared	*Bombus ruderarius*	Vulnerable
Bombus cullumanus	Disappeared	*Bombus ruderatus*	Critically Endangered
Bombus distinguendus	Critically Endangered	*Bombus rupestris*	Endangered
Bombus humilis	Endangered	*Bombus soroeensis*	Critically Endangered
Bombus jonellus	Vulnerable	*Bombus subterraneus*	Disappeared
Bombus magnus	Endangered	*Bombus sylvarum*	Critically Endangered
Bombus muscorum	Endangered	*Bombus veteranus*	Endangered

In a recent study, researchers belonging to the Status and Trends of European Pollinators (STEP) project examined all known bumblebee species of Europe. In this study, which also contributes to the European Red List of pollinators, they found that 24% of the 68 European bumblebee species are threatened with extinction (The IUCN Red List of Threatened Species™, 2014; Table 1.2). Furthermore, most bumblebee species had a declining population trend (46%), while 42% had a stable or increasing population trend (29% and 13%, respectively) (The IUCN Red List of Threatened Species™, 2014; Table 1.2). Information on criteria and rules concerning the different red list classifications used by the IUCN can be found in Figure 1.15, in the document (IUCN, 2012) and the IUCN website (http://www.iucnredlist.org/technical-documents/red-list-documents).

A comparison between the red list of the Netherlands and the European red list showed that from the 16 Red List species of The Netherlands, only 4 species were indicated as

'vulnerable' and 1 species as 'Critically Endangered' on the European Red List, while all other bumblebee species had a status of 'Least Concern' in Europe (Table 1.1 and Table 1.2; Peeters & Reemer, 2003; The IUCN Red List of Threatened Species™, 2014). The difference in status of the red list species between both lists was most strikingly seen in the case of *B. subterraneus*, this bumblebee disappeared from The Netherlands, but on an European scale this species received only a status of 'Least Concern' (Table 1.1 and Table 1.2; Peeters & Reemer, 2003; The IUCN Red List of Threatened Species™, 2014).

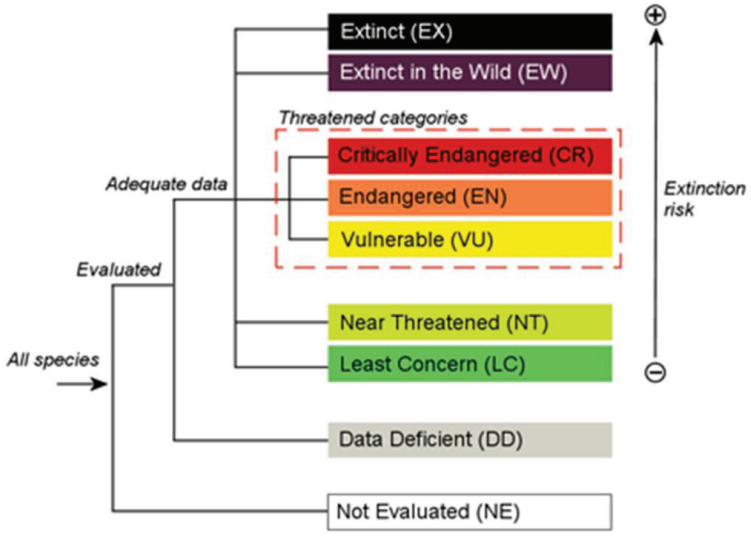


Figure 1.15 Structure of the different red list categories. Picture from IUCN, (2012). With the extinction risk going from low (indicated with a "-") to high (indicated with a "+").

Different hypotheses aim to explain the observed declines in bee populations (as reviewed in: Williams & Osborne, 2009; Potts *et al.,* 2010; Cameron *et al.,* 2011, Carvalheiro *et al.,* 2013; Vanbergen & the Insect Pollinators Initiative, 2013). In the next chapter 1.2.3, the most important hypotheses of bumblebee decline will be briefly discussed.

Table 1.2 Red list status and population trend for all known European bumblebee species (The IUCN Red List of Threatened SpeciesTM, 2014).

Species	Red list status	Population trend	Species	Red list status	Population trend	Species	Red list status	Population trend
Bombus alpinus	Vulnerable	Decreasing	*Bombus hypnorum*	Least Concern	Increasing	*Bombus pomorum*	Vulnerable	Decreasing
Bombus argillaceus	Least Concern	Stable	*Bombus inexspectatus*	Endangered	Decreasing	*Bombus pratorum*	Least Concern	Increasing
Bombus armeniacus	Endangered	Decreasing	*Bombus jonellus*	Least Concern	Stable	*Bombus pyrenaeus*	Least Concern	Stable
Bombus balteatus	Least Concern	Stable	*Bombus laesus*	Near Threatened	Decreasing	*Bombus quadricolor*	Least Concern	Decreasing
Bombus barbutellus	Least Concern	Decreasing	*Bombus lapidarius*	Least Concern	Increasing	*Bombus reinigiellus*	Endangered	Decreasing
Bombus bohemicus	Least Concern	Stable	*Bombus lapponicus*	Least Concern	Unknown	*Bombus ruderarius*	Least Concern	Decreasing
Bombus brodmannicus	Endangered	Stable	*Bombus lucorum*	Least Concern	Stable	*Bombus ruderatus*	Least Concern	Decreasing
Bombus campestris	Least Concern	Stable	*Bombus magnus*	Least Concern	Unknown	*Bombus rupestris*	Least Concern	Unknown
Bombus cingulatus	Least Concern	Stable	*Bombus mendax*	Near Threatened	Decreasing	*Bombus saltuarius*	Data Deficient	Unknown
Bombus confusus	Vulnerable	Decreasing	*Bombus mesomelas*	Least Concern	Decreasing	*Bombus schrencki*	Least Concern	Increasing
Bombus consobrinus	Least Concern	Stable	*Bombus mlokosievitzii*	Data Deficient	Unknown	*Bombus semenoviellus*	Least Concern	Increasing
Bombus cryptarum	Least Concern	Unknown	*Bombus mocsaryi*	Endangered	Decreasing	*Bombus sichelii*	Least Concern	Stable
Bombus cullumanus	Critically Endangered	Decreasing	*Bombus modestus*	Data Deficient	Unknown	*Bombus soroeensis*	Least Concern	Decreasing
Bombus deuteronymus	Data Deficient	Decreasing	*Bombus monticola*	Least Concern	Decreasing	*Bombus sporadicus*	Least Concern	Stable
Bombus distinguendus	Vulnerable	Decreasing	*Bombus mucidus*	Near Threatened	Decreasing	*Bombus subterraneus*	Least Concern	Decreasing
Bombus flavidus	Least Concern	Stable	*Bombus muscorum*	Vulnerable	Decreasing	*Bombus sylvarum*	Least Concern	Decreasing
Bombus fragrans	Endangered	Decreasing	*Bombus niveatus*	Least Concern	Stable	*Bombus sylvestris*	Least Concern	Stable
Bombus gerstaeckeri	Vulnerable	Stable	*Bombus norvegicus*	Least Concern	Stable	*Bombus terrestris*	Least Concern	Increasing
Bombus glacialis	Data Deficient	Unknown	*Bombus pascuorum*	Least Concern	Increasing	*Bombus vestalis*	Least Concern	Increasing
Bombus haematurus	Least Concern	Increasing	*Bombus patagiatus*	Data Deficient	Decreasing	*Bombus veteranus*	Least Concern	Decreasing
Bombus hortorum	Least Concern	Stable	*Bombus perezi*	Least Concern	Stable	*Bombus wurflenii*	Least Concern	Decreasing
Bombus humilis	Least Concern	Decreasing	*Bombus pereziellus*	Least Concern	Stable	*Bombus zonatus*	Endangered	Decreasing
Bombus hyperboreus	Vulnerable	Decreasing	*Bombus polaris*	Least Concern	Decreasing			

1.2.3 *Causes of bumblebee decline*

Many potential drivers can affect pollinator abundance and diversity in particular (Natural Research Council, 2006). The most important drivers are: (i) land-use change with a decreased resource diversity (Biesmeijer *et al.*, 2006), and the loss and fragmentation of habitats (Goulson *et al.*, 2008; Winfree *et al.*, 2009; Steffan-Dewenter *et al.*, 2002; Hendrickx *et al.*, 2007); (ii) use of pesticides (Kevan *et al.*, 1997; Rortais *et al.*, 2005); (iii) non-native species and the spread of pathogens (Thomson, 2006; Cox-Foster *et al.*, 2007; Stout & Morales, 2009; Neumann & Carreck, 2010); and (iv) climate change (Williams *et al.*, 2007; Dormann *et al.,* 2008). These different environmental drivers rarely act alone (Didham *et al.,* 2007). Indeed, these factors and their interactions with each other, influence pollinator populations on different locations and on different scales (Potts *et al.,* 2010, Figure 1.16). However, as most studies have analyzed the impacts of specific drivers in isolation (Tylianakis *et al.,* 2008; Schweiger *et al.,* 2010), they will also be briefly described here.

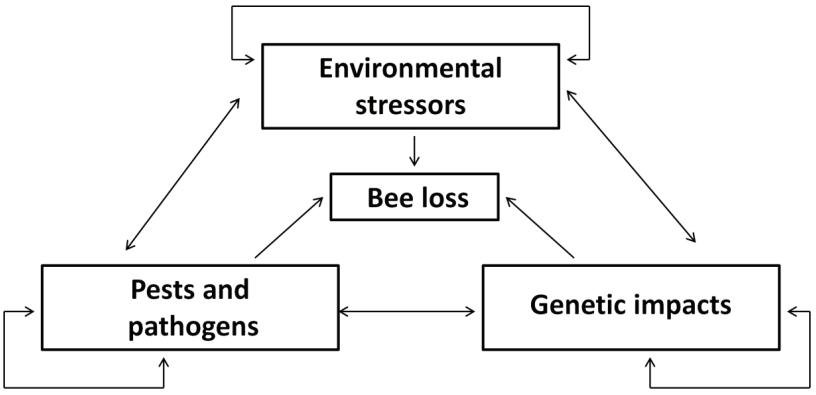


Figure 1.16 Interactions among the three main groups of drivers of bee loss. Here, blue boxes represent the three main groups of drivers; red arrows represent direct effects of drivers; green arrows represent interactions between drivers, and blue arrows represent interactions within drivers, adapted from Potts *et al.* (2010).

1.2.3.1 Loss of habitat and food resources

The agricultural intensification occurring during the latter half of the twentieth century, is presumed to be one of the main causes of bumblebee decline and the loss of biodiversity in general (Williams, 1986; Osborne & Corbet, 1994; Goulson *et al.,* 2006; Goulson, 2010). By

the development of artificial fertilizers and new grass varieties, farmers could improve their lands productivity by changing unimproved grassland to monocultures of grasses (Stapledon, 1935; Waller, 1962, Goulson, 2010). This practice has lead to major losses of unimproved grassland in whole Europe and North America (Fuller, 1987; Howard *et al.*, 2003; Wilcove *et al.,* 1998; Hines & Hendrix, 2005). For instance, in the United Kingdom over 90% of unimproved lowland grassland is lost between 1932 and 1984 (Fuller, 1987; Howard *et al.*, 2003).

Furthermore, since the introduction of cheap artificial fertilizers, crop rotations involving legumes (mostly *Trifolium* spp.) have been almost entirely abandoned (Goulson, 2010). The abandoning of the use of these leguminous crops, which are highly preferred food sources for long-tongued bumblebees, is one major cause responsible for the observed decline of long-tongued bumblebees (Rasmont, 1988; Rasmont and Mersch, 1988; Goulson, 2010). Hence, an increase in use of selective herbicides, which could entirely eliminate broad-leaved weeds within the crop further reduced the botanical diversity (Haughton *et al.,* 2003; Hawes *et al.,* 2003; Goulson, 2010).

As bees are entirely dependent on flowers, the decline of the European flora and changed crop rotations which decreased the food availability for bees, inevitable had negative effects on bumblebee populations and their distribution (Goulson, 2010). Several studies showed a direct correlation between the floral diversity and the number of bee species within an area (Banaszak, 1983; Kells *et al.,* 2001; Backman & Tiainen, 2002). Indeed, uncropped areas of farmland, such as hedgerows, old fields, scrublands, forests, roadside verges, shelterbelts, borders of streams and ponds, green lanes and unimproved grasslands can provide flowers throughout the season, and tend to support far greater numbers of foraging bumblebees than cultivated areas (Goulson, 2010).

On farmland, the crops themselves may provide an abundance of food during their brief flowering periods (Goulson, 2010). However, bumblebees require a continuous succession of flowers from April to July, something which flowering crops alone cannot provide. Bumblebees do not store large quantities of honey in the way honeybees do, which makes them more vulnerable to discontinuities in the food supply (Shelly *et al.,* 1991; Williams & Christian, 1991). Thus, unless farms contain areas of wild flowers, there may be gaps in the

succession of flowering plants during which bumblebee colonies will starve and die. In turn, the plants normally pollinated by bumblebees will set less seed, and therefore have less progeny the following years resulting in even less food for the bumblebees which is described as an 'extinction vortex' (Corbet, 1987; Osborne *et al.,* 1991; Osborne & Corbet, 1994).

In addition, the scarcity of weeds and field flowers means that there are also fewer seeds to eat for rodents, such as voles and mice. Lower populations of these mammals will lead to fewer nest sites for both below and above-ground nesting bumblebee species (Goulson, 2010). McFrederick & LeBuhn (2006) found a positive correlation between the number of rodent holes and bumblebee abundance in urban parks, indicating that the need for nest sites may be a limiting factor. Bumblebees need also suitable hibernation sites where young queens can remain undisturbed through the autumn and winter. And as these hibernation sites are quite different from nesting sites, the decreased availability of these sites can also contribute to the observed bee declines (Goulson, 2010). Indeed, as nesting site bumblebees prefer abandoned holes of small mammals or nests of birds under or above ground or even in trees, while for a hibernation site bumblebee queens prefer loose soil, such as in a mole hill or compost in a flower pot, as their digging abilities are not very well developed (Goulson, 2010). Furthermore, modern farming practices also have an impact on bumblebee suitable nesting sites. The loss of hedgerows and unimproved pastures have reduced the availability of nest sites for both above-ground and below-ground nesting bumblebee species (Banaszak, 1983; von Hagen, 1994), and nests above the ground are frequently destroyed by farm machinery. Although exact empirical data of the latter is missing, the loss of nests or hibernation queens caused by farm machinery, for instance plowing, would be an interesting research topic.

Habitat fragmentation, emerging discontinuities in habitat, probably also has a negative affect on wild pollinator populations (Stefan-Dewenter *et al.,* 2006; Winfree *et al.*, 2009). Indeed, Stefan-Dewenter *et al.* (2006) reported a declining species richness and abundance for bees related to a decreased fragment size. However, several other studies did not find an effect of fragmention on overall community richness or abundance of bee pollinators (Donaldson *et al.,* 2002; Cane *et al.*, 2006; Brosi *et al.,* 2008). Although these studies also showed that some bee species were favored by increased native habitat, while others were favored by an increased non-native matrix area (Donaldson *et al.,* 2002; Cane *et al.*, 2006; Brosi *et al.,*

2008). Furthermore, other studies demonstrated positive effects of urbanization or agriculture probably by the introduction of novel foraging and/or nesting resources or micro-habitats (Cane *et al.*, 2006; Winfree *et al.*, 2007; Carre *et al.,* 2009). Thus, habitat fragmentation probably has a negative effect on some bees species but not all as certain species can tolerate or benefit from a moderate level of disturbance, including moderate levels of habitat loss (Winfree *et al.*, 2009, Carre *et al.,* 2009).

1.2.3.2 Use of pesticides

The widespread introduction of insecticides is another plausible cause of bumblebee decline. Neonicotinoids, nicotinic acetylcholine receptor agonists, are now the most commonly used insecticides (Goulson, 2013). Three possible exposure routes to pesticides are possible: (i) through direct contact of sprays on foragers; (ii) through contact with contaminated plants; and (iii) through the uptake of contaminated food (nectar or pollen). The latter one is probably the most important route of exposure for bumblebees. Indeed, neonicotinoids can be found in the nectar and pollen (Goulson, 2013). The concentrations in nectar range from <1 to 23 ppb, with concentrations in pollen ranging from <1 to 66 ppb, depending on the way the neonicotinoids are applied on to the crops (as seed dressings, or by irrigation water direct in the soil) (Goulson, 2013). The fact that a large volume of nectar is consumed by bumblebees and their offspring, these pesticides can accumulate in bees (Goulson, 2013).

Most insecticides are broadly toxic for both, honeybees and bumblebees, and in high doses will lead to bee mortality (Thompson & Hunt, 1999). Although it is unlikely that a normal field-realistic application of neonicotinoids will cause direct bumblebee mortality, there is now strong evidence for sublethal effects (Goulson, 2013). Chronical exposure to sublethal doses of neonicotinoids is known to reduce bumblebee learning, foraging and homing ability (Mommaerts *et al.*, 2010, and as reviewed in Goulson, 2013). Indeed, Whitehorn *et al.* (2012) found an reduced queen production in queenright (= a colony with a properly functioning queen) *B. terrestris* colonies exposed to field realistic doses of imidacloprid produced, which could be caused by an reduced fecundity of the queen or foraging efficiency of the workers (Goulson, 2013). Hence, Gill *et al.* (2012) showed that exposure to 10 ppb imidacloprid in sugar water reduced the foraging success of workers. Indeed, they observed a higher proportion of foragers that did not return to the colony, fewer workers emerged from pupae,

and bees exhibited increased foraging activity (Gill *et al.* 2012). A study with field-realistic doses of imidacloprid confirmed the impact on foraging ability, when collecting pollen (Feltham *et al.*, 2014). Recently, Scholer & Krischik (2014) showed that chronical exposure to two neonicotinoids: imidacloprid and clothianidin significantly reduced colony health of queenright colonies of *B. impatiens*. They observed higher queen mortality starting at 20 ppb, as a result of an decreased foraging ability of the workers (reduced worker movement, consumption, wax production, and nectar storage) (Scholer & Krischik, 2014). Also for other insecticides such as: λ-cyhalothrin, negative (sub)lethal effects are observed. Indeed, *B. terrestris* colonies exposed to the pyrethroid pesticide lambda (λ)-cyhalothrin showed higher mortality of worker bees in the nest during the early stages of colony development (Gill *et al.,* 2012). However, a longer term only reduced body mass was observed (Baron *et al.,* 2014). Thus, that pesticides have a sublethal effect on bumblebee populations is certain, but their interaction with other stressors and the severity of their impact on the observed declines is yet unclear (Goulson, 2013).

However, recent studies already tried to fill in this gap in our knowledge on pesticides (Fauser-Misslin *et al.*, 2014 and Baron *et al.*, 2014). Both studies searched for the influence of combined pesticides and parasite exposures on bumblebee colonies (Fauser-Misslin *et al.*, 2014 and Baron *et al.*, 2014). Combined exposure to thiamethoxam and clothianidin under laboratory circumstances reduced worker production, life duration of workers and colony reproductive success (Fauser-Misslin *et al.*, 2014). The combined exposure of a trypanosome gut parasite *Crithidia bombi* with these two neonicotinoids reduced queen survival (Fauser-Misslin *et al.*, 2014), but the combination of *C. bombi* with λ-cyhalothrin had no additional effects (Baron *et al.*, 2014). Further research is needed to study the influence of combined pesticides and parasite exposures on bumblebee colonies.

1.2.3.3 Impact of non-native species and the spread of pathogens

As discussed in chapter 1.1.8, a low number of bumblebee species is commercially reared for pollination in greenhouses. For *B. terrestris* alone, probably more than one million colonies are shipped to 60 countries worldwide. These bumblebee colonies and also widely shipped honeybees (*Apis mellifera*), which are native to Europe, Africa and the Middle East, can have negative effects on the presence and distribution of other pollinator species by: (i)

competitive displacement; (ii) introgression (hybridization); (iii) introduction of pathogens or 'pathogens spill over' into wild populations (Goulson, 2010; Pott *et al*., 2010).

Competitive displacement at the preferred host plants can lead to declines in native pollinator populations, particularly specialist species (Traveset & Richardson, 2006). Indeed, honeybees which have been introduced by man to almost every country in the world are highly polylectic (flower generalist) (e.g. Butz Huryn, 1997; Coffey and Breen, 1997) and have the potential to displace native organisms from preferred forage sources (Goulson, 2010). There is increasing evidence that honeybees have indeed negative effects on bumblebees by competition for food (Thomson, 2004; 2006; Forup & Memmott, 2005; Walther-Hellwig *et al.,* 2006; Goulson & Sparrow, 2009).

Introgression is the interbreeding of managed bee species with endemic populations. This is also called genetic dilution and could thereby erode the genetic diversity of the native populations (Franck *et al.,* 1998).

The spread of pathogens from managed bumblebees or honeybees to the wild bee populations is not yet proven but quite possible. Indeed, it is shown that in the past commercial bumblebee colonies could be infested with *Crithidia bombi* and *Locustacarus buchneri* (Colla *et al.,* 2006; Otterstatter & Thomson, 2008; Yoneda *et al.,* 2008) and honeybees are infected with multiple parasites and viruses (Genersch, 2010). Furthermore, they can act as dispersal vectors for parasites and pathogens, for example: *Varroa* mites in *Apis*, *Nosema* spp. in *Bombus,* and *Ascosphaera apis* fungus in *Megachile* (Potts *et al*., 2010). Furthermore viruses, notorious in honeybees, can invade multiple host species and have thus the potential to infect other pollinator species (Genersch *et al.,* 2006; Eyer *et al.,* 2009; Meeus *et al.*, 2014). Especially in North America the rapid decline of several wild bumblebee species during the last 15 years fueled the speculation that an non-native pathogen or strain got accidentally imported and is causing the decline (Thorp, 2005; Thorp & Shepherd, 2005; Rao & Stephen, 2007; Goulson, 2010). However, no evidence is presented to proof a causal link, like there is for the other stressors, which is of course also very difficult to accomplish. On the other hand, the mechanism and the potential of spillover is getting clear. Fürst *et al.* (2014), showed that managed bees can disrupt host parasite and virus interaction in sympatric bumblebee species. Murray *et al.* (2013) showed that spillover of a protozoan parasite is

possible from bumblebees escaping the greenhouse. Thus, it is clear that there are risks associated with these kind of spillovers, especially for endangered pollinator communities, as reviewed by Meeus *et al.* (2011).

1.2.3.4 Climate change

Finally, also climate change has an impact on the decline of pollinators (Williams et *al.,* 2007; Dormann *et al.,* 2008; Potts *et al.,* 2010; Iserbyt & Rasmont, 2012). Climate change can have direct and indirect effects on bumblebee species, colonies, populations and communities. Hence, climate change can have a direct impact by: (i) changing the temporal activity of bees (Stone & Willmer, 1989), (ii) changes in phenology, and by shifting climatic niches (Williams et *al.,* 2007; Hegland *et al.,* 2009; Iserbyt & Rasmont, 2012) and (iii) changing composition and functioning of pollinator communities (Memmott *et al.,* 2007; Iserbyt & Rasmont, 2012). An example of an indirect effect of climate change is mismatches in temporal and spatial co-occurrence of species (Schweiger *et al.,* 2008; Hegland *et al.,* 2009).

1.2.4 *Genetic impacts*

Also genetic factors can have an impact on the observed declines of bumblebee populations. Due to the different drivers discussed in chapter 1.2.2, bumblebee populations can become increasingly small and isolated. These small (bumble)bee populations will disappear, despite the apparent suitability of the remaining habitat. They will have a reduced genetic diversity as a result of genetic drift, and will be more vulnerable than genetic rich populations to inbreeding (mating with relatives) and inbreeding depression (Reed & Frankham, 2003; Spielman *et al.,* 2004; Frankam, 2005; Zayed, 2009; Goulson, 2010). This will in turn lead to low adaptive ability in response to current and future changes in the environment, such as new pathogens, climate change and habitat loss, and so this can ultimately lead to extinction (Frankham, 2005; Zayed, 2009; Goulson & Osborne, 2010).

In a normal metapopulation, local extinctions of populations will be balanced with recolonization. By dispersal or gene flow genetic cohesion and diversity will be maintained (Goulson, 2010). However, in fragmented populations dispersal will be limited or absent. In

turn, extincted patches may never be repopulated and small populations will lose genetic diversity through drift (Goulson, 2010). The rate of genetic drift is determined by the effective population size (*N*e). In social insects, *N*e will be low as it depends not on the amount of workers, but on the colonies reproductive success: the number of egg-laying queens and their mates from each individual colony. As most bumblebee species have colonies which consist out of one founder queen and are mostly monoandrous, *N*e will be even very low (Estoup *et al.,* 1995; Schmid-Hempel & Schmid-Hempel, 2000).

Furthermore, bumblebees' dispersal ability, between 3 to 140 km in one year, differs greatly between species and the study (Hopkins, 1914; Stout & Goulson, 2000; Hingston, 2006, Kraus *et al.,* 2009; Goulson, 2010; Darvill *et al.*, 2010; Lepais *et al.*, 2010). Although bumblebees can colonize islands up to 30 km off shore (Macfarlane & Griffin, 1990), sea barriers of more than 10 km can already restrict gene flow (MacFarlane & Gurr, 1995). Indeed, the latter could even lead to the development of subspecies (Rasmont, 1983). For example, *B. terrestris canaeriensis* and *B. terrestris xanthopus,* two subspecies of *B. terrestris* occur on the Canarian islands and Corsica, Capraia Island and Elba Island, respectively (Rasmont, 1983; Widmer *et al.*, 1998; Rasmont *et al.*, 2008). The dispersal ranges of the reproductives stages of bumblebees (daughter queens and males) may differ between subgenera (Darvill *et al.*, 2010; Goulson *et al.*, 2011). Bumblebees of the subgenera *Pyrobombus* (*B. pratorum*, *B. jonellus*, and *B. hypnorum*) may have a high dispersal ability than bumblebees belonging to the subgenera *Thoracobombus* (*B. pascuorum*, *B. muscorum*, *B. sylvarum*, *B. humilis*, and *B. ruderarius*) (Darvill *et al.*, 2010; Goulson *et al.*, 2011). Populations of bumblebee species with a more limited dispersal rate will have less chance of a successful recolonization event and will be more vulnerable to inbreeding.

Inbreeding can cause a decrease in polymorphism of the loci involved in the sex determination which leads to the presence of sterile diploid or triploid males (Duchateau *et al.,* 1994; Whitehorn *et al.,* 2009). Furthermore, inbreeding can lead to inbreeding depression caused by the expression of deleterious recessive alleles (Frankham, 2005; Zayed, 2009). Although, it has been reported that haplo-diploid species, as is the case for bumblebees, are considered not to be as sensitive to genetic pauperization and inbreeding depression as diploid species do, because deleterious alleles are purged from the population in the haploid males (Sorati *et al.,* 1996; Packer & Owen, 2001).

1.2.5 *Conservation*

The conservation of bumblebee species demands big and different efforts. Viable bumblebee populations need large areas of suitable habitat. It is not enough to protect and manage a few small areas of suitable habitat surrounded by unsuitable farm land. However, many small patches (such as field margin strips) may also be sufficient to support viable populations. The connection of these habitat 'islands' could increase the population size and so reduce inbreeding and even extinction (Goulson, 2010). Furthermore, the conservation of bumblebee populations can be supported by: (i) the restoration of areas with unimproved flower-rich grassland, (ii) the reintroduction of clover (e.g. *Trifolium pratense*), (iii) decreasing the use of artificial fertilizers which promotes rapid growth of grasses, (iv) changes in pesticide use, and (v) increased restrictions on transportation of bees and for stricter quarantine and monitoring systems or (vi) the use of native bumblebee species (Carvell, 2002; Winter *et al.,* 2006; Carvell *et al.*, 2007; Pywell *et al*., 2006; 2007; Rundlof *et al.,* 2008; Goulson, 2010). In addition, long-term monitoring and recording of bumblebee populations is required to be able to follow these populations and bumblebee species (Goulson, 2010).

1.3 Microsatellites

1.3.1 *General*

Microsatellites, also called simple sequence repeats (SSRs), variable number tandem repeats (VNTRs), or short tandem repeats (STRs), are short tandemly repeated DNA sequences present in the genomes of eukaryotic and prokaryotic organisms (Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006; Selkoe & Toonen, 2006; Leclercq *et al.,* 2010; Miah *et al.*, 2013; Figure 1.17). These short DNA fragments are usually 1 to 6 base-pairs long (Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006; Selkoe & Toonen, 2006; Leclercq *et al.,* 2010). Typically, these are repeated 5 to 40 times, but this can also be longer (Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Figure 1.17).

Microsatellites will be classified as mono-, di-, tri-, tetra-, penta- or hexa-nucleotide repeats based on the number of nucleotides per repeated unit (Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006; Selkoe & Toonen, 2006; Miah *et al.*, 2013). Although in most species, the majority of microsatellites is dinucleotide repeats (Chambers & MacAvoy, 2000; Selkoe &

Toonen, 2006; Miah *et al.*, 2013). Microsatellites were for many years considered to be selectively neutral, it is now known that they are also present in coding regions and influenced by selective pressures. Indeed, for instance changes in the number of repeats can cause diseases in humans (Oliveira *et al.*, 2006 and see references therein). In coding regions, especially tri-, and tetra-nucleotide repeats are found (Oliviera *et al.*, 2006).

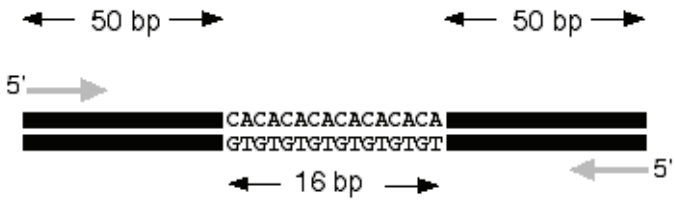


Figure 1.17 Example of a 116 bp microsatellite fragment, which consists out of an dinucleotide repeat CA, eight times repeated and two flanking regions of 50bp each. The grey sequences at both 5' ends, flanking these microsatellite loci, are the PCR primers which allow amplification through PCR.

Furthermore, microsatellites can also be classified as being perfect, imperfect, interrupted or composite based on the constancy of their repeated unit (Oliveira *et al.*, 2006; Miah *et al.*, 2013). Perfect microsatellites consist out of one tandemly repeated unit (e.g. ACACACACAC), while composite microsatellites consist out of the combination of two or more tandemly repeated units (e.g. ACACACACACTCTCTCTCTC). Imperfect and interrupted microsatellites have their tandemly repeat unit interrupted by one pair of bases or by a small non-repeated sequence, respectively (e.g. ACACACCTACACAC and ACACACCTAGACACAC, respectively; Oliveira *et al.*, 2006; Miah *et al.*, 2013). A particular microsatellite locus can often be identified by its flanking DNA sequences, which are generally conserved across individuals of the same species, populations and/or even between species (Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Figure 1.17).

One important characteristic of microsatellites is that they have a high mutation rate, which is estimated to be between 10^{-2} and 10^{-4} per generation (Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006; Selkoe & Toonen, 2006; Leclercq *et al.,* 2010; Miah *et al.,* 2013). As this high rate of mutation slippage within short evolutionary times will lead to multiple alleles of different length per locus, microsatellites have often high levels of polymorphism (Oliveira *et*

al., 2006; Selkoe & Toonen, 2006; Leclercq *et al.,* 2010). The number of repeats in the repeated region generates the polymorphism of that microsatellite locus (Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Figure 1.18).

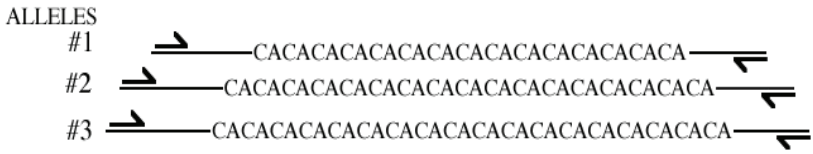


Figure 1.18 Example of three alleles for a certain microsatellite loci, each with a different number of CA repeat. In 1: CA is repeated 15 times; in 2, 17 times; and in 3, CA is repeated 18 times.

Due to their high variability within species, microsatellites are useful for discriminating between individuals within populations, and populations among each other. Microsatellites have become state-of-the-art markers for a large number of studies, for instance: in population genetics, QTL mapping, genome mapping, conservation genetics, marker-assisted rearing or breeding (MAS), and even forensic research through genetic fingerprinting (Estoup *et al.*, 1995; Solignac *et al.*, 2004; Kraus *et al.,* 2009; Wilfert *et al.*, 2007; Stolle *et al.*, 2011; and reviewed in: Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006; Selkoe & Toonen, 2006; Miah *et al.,* 2013, and see chapter 1.3.2).

For microsatellites, four mutation models are described: (i) the Initial Alleles Model (or IAM), (ii) the Stepwise Mutations Model (or SMM), (iii) the Two Phase Mutation Model" (or TPM), and (iv) the K-alleles model (or KAM) (Di Rienzo *et al.,* 1994; Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006). They differ in how mutations are formed. Following IAM, a certain repeat can result in a random repeat. This because of mutation slippage a random number of tandem repeats are added or lost, while following SMM, mutation slippage will occur only in small steps of adding or losing one single tandem repeat at the time (Figure 1.19). The TPM is a combination of these two mutation models, which consist out of a proportion p of single step mutations, and 1-p larger step mutations (Di Rienzo *et al.,* 1994; Chambers & MacAvoy, 2000; Oliveira *et al.*, 2006). Finally, following KAM the probability of a given allel to mutate in another allel is μ/k-1,in which μ is the mutation rate and k the exact number of possible alleles at the given locus (Oliveira *et al.*, 2006).

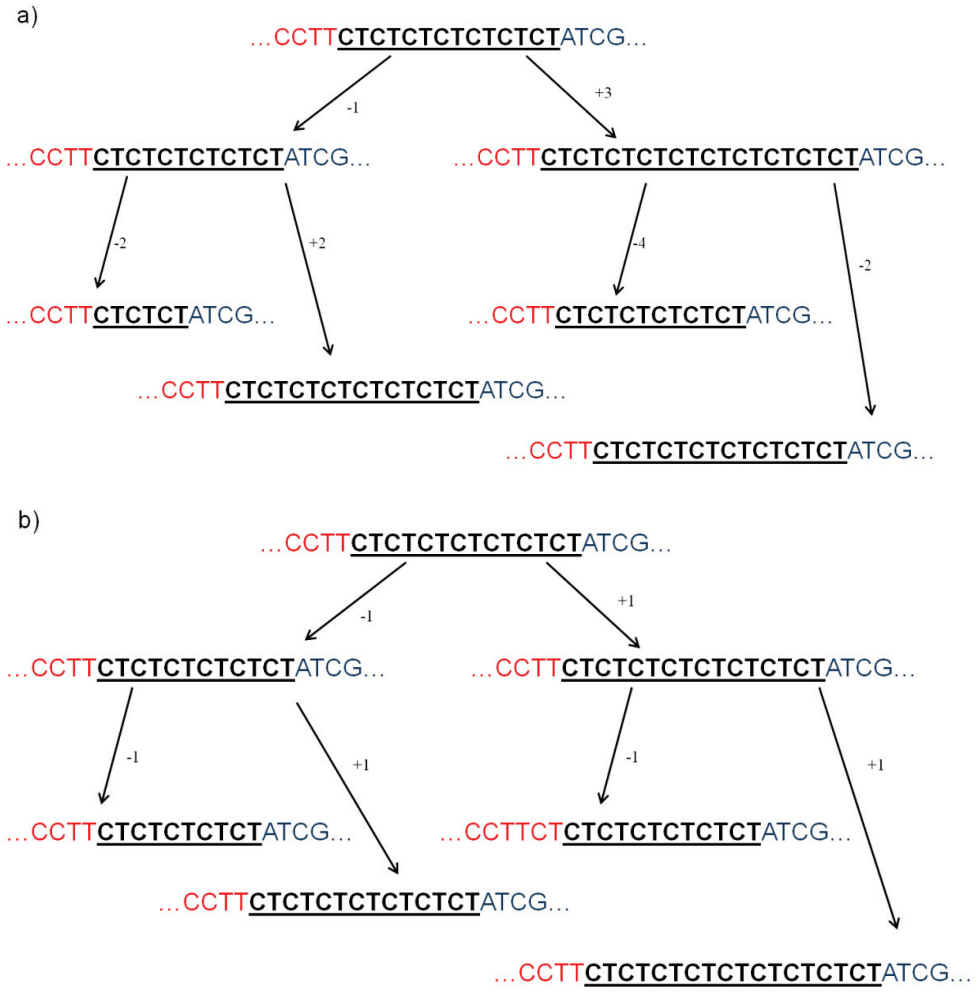


Figure 1.19 Schematic presentation of the different mutation steps between: a) the IAM and b) the SMM mutation models. The underlined sequence represent the tandemly dinucleotide repeat "CT". The numbers next to the arrows indicate the number of repeats that are added or lost during one step, starting from a 7 repeated dinucleotide repeat "CT".

To detect microsatellites, one needs to design polymerase chain reaction (PCR) primers with matching sequences in the conserved flanking regions unique to one locus in the genome. A single pair of PCR primers should work for all individuals of a species and even better also in closely related species. Each primer couple produces different sized products for each of the different length microsatellites. As microsatellites can be amplified with PCR, identifying

them is easy and fast, (Benson, 1999; Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Miah *et al.*, 2013; Figure 1.20).

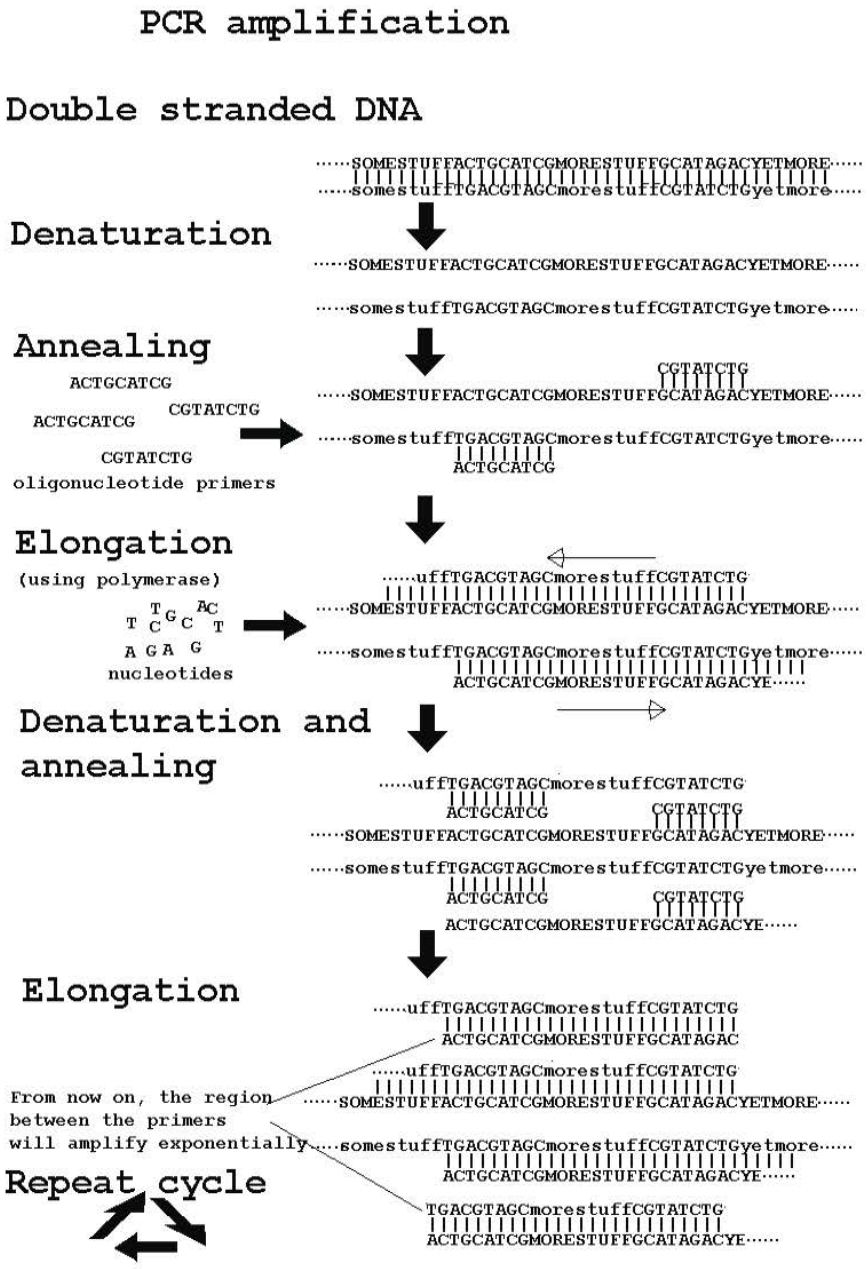


Figure 1.20 PCR amplification process.

During PCR, extracted DNA is repeatedly denatured at a high temperature to separate the two strands, then cooled to allow annealing of the primers, and the extension of nucleotide sequences through the microsatellite. This exponential process results in the production of high amounts of DNA and thus only a small start concentration of DNA is needed for amplification (Figure 1.20).

The amplified microsatellite PCR fragments can then be separated and visualized through high resolution gel electrophoresis or capillary electrophoresis (Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Figure 1.21). Although traditional agarose or acrylamide gel electrophoresis methods are cumbersome and toxic, the use of recyclable superfine resolution gel (SFR) can be an cheaper and reasonable alternative capable of resolving DNA bands that differ by only 2% in the range of 100-1000bp (Seng *et al.*, 2013). However, capillary electrophoresis is now the standardized method of microsatellite visualization (Guichoux *et al.*, 2011).

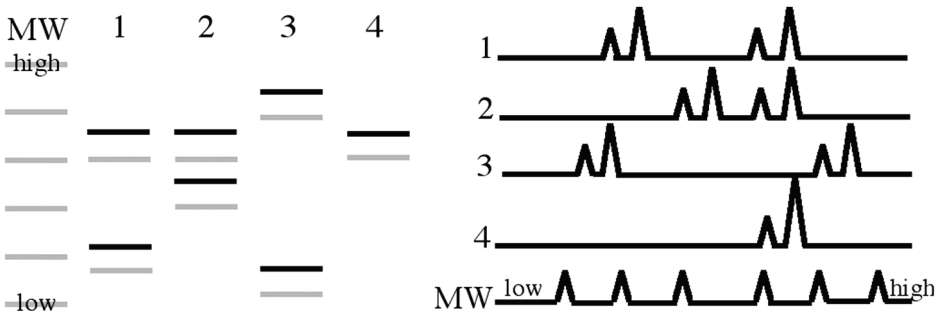


Figure 1.21 Visualization of microsatellites, comparison between the bands of gel electrophoresis (left) versus the peakes of capillary electrophoresis (right), with MW = molecular weight size marker. The grey bands (left) and the smaller peakes (right) are "stutter peakes". These artifacts occur due to DNA-replication slippage during PCR amplification of the microsatellites. Most stutter bands are shorter than the actual microsatelilite allele (Schlötterer, 2004). Number 1 to 3 are examples of heterozygote specimens, while number 4 is an homozygote specimen.

To visualize the different DNA fragments in capillary electrophoresis fluorescent dyes are used, by fluorophore labelling of the oligonucleotides (primers) for PCR. This will enable the detection of multiple microsatellite loci in one reaction. One will be able to distinguish

between the results or peaks of each primer by their transmitted colour. Fluorescent dyes used in the dissertation are: 6-FAM (blue), NED (yellow), PET (red), and VIC (green).

In diploid organisms microsatellites are co-dominant, each microsatellite on the coupled homologous chromosomes is amplified during PCR, and will be visualized in the electrophoresis. The different lengths a microsatellite can generate are called alleles, in analogy with gene nomenclature. When this organism is heterozygous, which means having two different alleles for a certain locus, this will result in two separate bands on the gel or two peaks on the electropherogram in capillary electrophoresis, and this while homozygotes will produce only one band or peak. In this way, heterozygotes can be differentiated from homozygotes (Caterino *et al.,* 2000; Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Figure 1.21).

1.3.2 *Limitations*

Table 1.3 gives an overview of the advantages and weaknesses of microsatellite markers (Miah *et al.,* 2013). One major limitation of microsatellites is their incapacity for higher-level systematic, which is due to their high mutation rate (Oliveira *et al.*, 2006; Selkoe & Toonen, 2006; Miah *et al.,* 2013). The microsatellite primer sites may not be conserved anymore, due to possible point mutation(s) between different classes. Indeed, microsatellites developed for a particular species can often be used for closely related species, but the percentage of loci that amplifies decreases with increasing genetic distance (Jarne & Lagoda, 1996; Chambers & MacAvoy, 2000; Dakin & Avise, 2004; Oliveira *et al.*, 2006; Miah *et al.,* 2013).

Table 1.3 The advantages and disadvantages of microsatellite markers (adapted from Miah *et al.*, 2013).

Benefits	Weakness
-Easy to automate	-Not well-examined
-Genomic abundance high	-Sometimes not suitable across species
-Highly reproducible	-Sequence information needed
-High polymorphism	
-Multiple alleles	
-Moderate genome coverage	
-No radioactive labeling	

Furthermore, because of the high mutation rate of microsatellites, 'homoplasy' is more likely to occur. This means that we cannot assume that two alleles identical in state are identical by descent, as explained in Figure 1.22 (Estoup *et al.,* 1995; Chambers & MacAvoy, 2000; Selkoe & Toonen, 2006; Miah *et al.,* 2013).

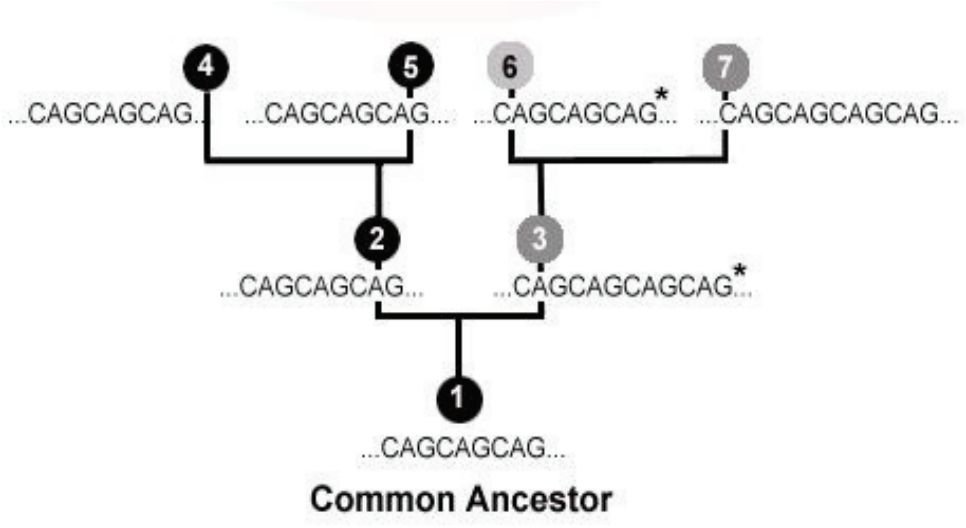


Figure 1.22 Example of 'homoplasy': from a common ancestor (species 1), species 2 and 3 arose with the difference that species 3 obtained an extra CAG repeat through mutation. Species 6 and 7 are descendents of species 3, and species 6 has lost one CAG repeat. Therefore, when studying these different species one would assume that species 6 has a closer common ancestry with species 4 and 5 opposed to species 7, which is not the case. Mutation steps are marked with an asterisk.

Another limitation is the occurrence of 'null alleles', which is the absence of one or both alleles after PCR. This phenomena, which can heavily complicate the interpretation of microsatellite allele frequencies, can be caused by: (i) poor primer annealing due to sequence divergence in flanking regions, or (ii) preferential amplification of alleles with a particular size (Selkoe & Toonen, 2006; Miah *et al.,* 2013). This could lead to PCR failure of a particular loci or the differential amplification of only one allele (homozygous), when in reality the specimen has two alleles (heterozygous) (Selkoe & Toonen, 2006; Chapuis & Estoup, 2007; Miah *et al.,* 2013).

1.3.3 *Applications*

Microsatellite markers are used in different types of research (Figure 1.23). In forensics, microsatellite markers have become the primary marker for DNA testing (estimating of the relatedness between individuals or groups and for parentage analysis) due to their high specificity (Evett & Weir, 1998; Selkoe & Toonen, 2006). Indeed, the probability of matching microsatellite profiles (the alleles of a combination of different microsatellites markers) can be very low (probability of a match is less than one in millions).

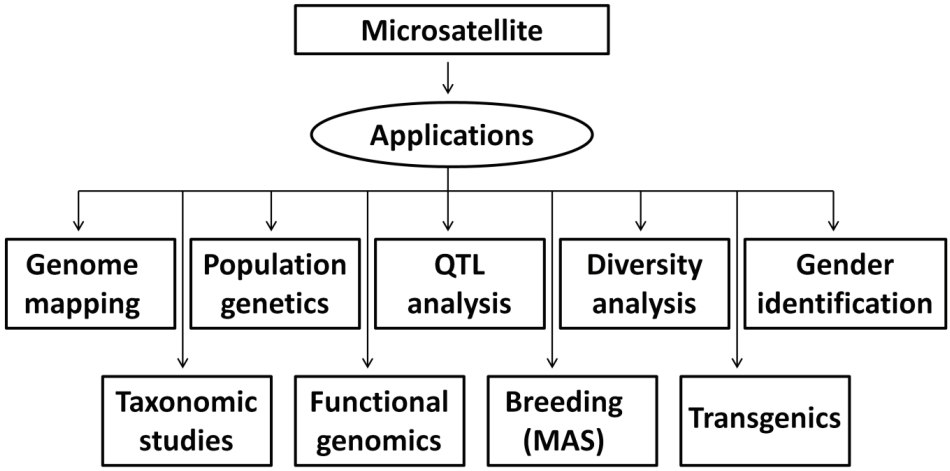


Figure 1.23 List of applications where microsatellites are used (adapted from Miah *et al.*, 2013).

In population genetics, microsatellites are used to estimate the genetic diversity, inbreeding levels, and the genetic structure of subpopulations and populations (e.g. Selkoe & Toonen, 2006; Zayed, 2009). Generally, the genetic diversity of a population is determined by the calculation of two genetic parameters: the allelic richness (A_R) and the expected heterozygosity (H_E). The allelic richness is the number of alleles corrected for sample size. H_E is calculated based on the allele frequencies, and range between 0 and 1. It is an estimation of the amount of heterozygous specimens you should normally find in your population following Hardy-Weinberg's equilibrium of random mating. Often population genetic studies also estimate the observed heterozygosity (H_O), which also ranges between 0 and 1, and is the proportion of homozygous specimens in the population. Both parameters of

heterozyosity can be used to estimate the inbreeding coefficient or $F_{IS} = (H_E\text{-}H_O)/H_E$. This genetic parameter, which ranges from -1 to 1, indicates if a population is under inbreeding (F_{IS}-values significant different from 0 and towards 1) or outbreeding (F_{IS}-values significant different from 0 and towards -1).

Furthermore, the demographic history can be assessed by: (i) searching for evidence of population bottlenecks, (ii) assess the effective population size, and (iii) investigate the magnitude and directionality of gene flow between populations (e.g. Selkoe & Toonen, 2006; Zayed, 2009). Population genetic studies also often calculated *F*-statistics to determine the population structuring by calculation of F_{ST} (Weir & Cockerham, 1984; Nei, 1987). For microsatellites this parameter range from 0 to 1, with zero representing no differentiation and a value of $F_{ST} = 1$, means fixation of different alleles between the populations and thus population structuring (Meirmans & Hedrick, 2011). Recently, the use and accuracy of F_{ST}-values were under debate (Jost, 2008; Whitlock, 2011; and as reviewed in Meirmans & Hedrick, 2011). Indeed, due to its dependency on within-population diversity, F_{ST}-values are not always trustworthy. Therefore, a new estimated parameter (D_{est}) was described based on the effective number of alleles (Jost, 2008). Currently, both parameters are estimated and used together in population genetic studies (Meirmans & Hedrick, 2011; Cameron *et al.*, 2011; Lozier *et al.*, 2011, Jha & Kremen, 2013).

For bumblebees, several population genetic studies have been performed on different *Bombus* species. Most studies in Europe were done on *B. terrestris* (Estoup *et al.*, 1995; Schmid-Hempel *et al.,* 2007; Wilfert *et al.*, 2007; Whitehorn *et al.,* 2009; Kraus *et al.*, 2009; 2011), but also other bumblebee species were genetically studied such as: *B. muscorum* (Darvill *et al.,* 2006; Darvill *et al.,* 2010), *B. jonellus* (Darvill *et al.,* 2010), *B. humilis* (Connop *et al.*, 2010), *B. sylvarum* (Ellis *et al.,* 2006; Connop *et al.,* 2010), and *B. hortorum* (Goulson *et al.*, 2011). In America and Japan the most studied bumblebee species are *B. impatiens* (Lozier & Cameron, 2009; Cameron *et al.,* 2011) and *B. ignitus* (Shao *et al.*, 2004; Takahashi *et al.*, 2008), respectively. Although in America also many other *Bombus* species were studied (Lozier & Cameron, 2009, Cameron *et al.,* 2011; Lozier *et al.*, 2011, Jha & kremen, 2013). The genetic diversity parameters observed in populations of declining bumblebee species were lower than in the populations of more stable bumblebee species (Charman *et al.*, 2010; Cameron *et al.,* 2011; Lozier *et al.*, 2011, and reviewed in Goulson *et al.,* 2008). While

several studies show population structure for island populations (Darvill *et al.*, 2006; Ellis *et al.*, 2006; Goulson *et al.*, 2008; Darvill *et al.,* 2010; Goulson *et al.*, 2011), no population structuring was found for continental populations of the more stable and abundant bumblebee species such as *B. terrestris* and *B. pascuorum* (Widmer *et al.,* 1998; Widmer & Schmid-Hempel, 1999; Goulson *et al.*, 2008). Although Widmer & Schmid-Hempel (1999) detected two isolated gene pools for *B. pascuorum* separated by the alps.

All studies, with the exception of Lozier & Cameron (2009) used contemporary bumblebee specimens. Lozier & Cameron (2009) compared the genetic variation between recent and historical populations of the declining and stable bumblebee species, *B. pensylvanicus* and *B. impatiens* respectively, in America. With the exception of this study, comparison of the genetic variation between the historical and current situation is still undiscovered territory, certainly for European bumblebee species.

Microsatellite markers are also useful markers for genome mapping. Indeed, the high number of available microsatellites in bumblebees (Estoup *et al.,* 1995, Reber-Funk *et al.,* 2006; Wilfert *et al.* 2009; Stolle *et al.,* 2011) allowed for the construction of several linkage maps in *B. terrestris* (Gadau *et al.*, 2001; Wilfert *et al.*, 2006; Stolle *et al.*, 2011). In Hymenoptera, like *B. terrestris*, a genetic linkage map can be easily constructed as the queens meiotic recombination rates can be reliably measured from her male offspring (Gadau *et al.*, 2001; Wilfert *et al.*, 2006; 2007a,b; Stolle *et al.,* 2011). Furthermore, the construction of a genetic linkage map allows quantitative trait loci (QTL) analysis (Slate, 2005). The goal of a QTL analysis is to determine the genes responsible for the phenotypic variation of a certain trait, by identification of the markers linked with these genes (Slate, 2005; Wilfert *et al.*, 2007a,b). In *B. terrestris* several QTLs for important traits have been discovered, such as: QTLs for immune defence, reproduction (Wilfert *et al.*, 2007b), host-parasite interactions and body size (Wilfert *et al.,* 2007a). Hence, the publication of the genome of both *B. terrestris* and *B. impatiens* allow us to go even a step further, and thus not only to identify the QTL region and the markers linked to a certain trait but also to identify the genes associated with these linked markers (Consortium IBG, 2014).

Chapter II

Low genetic diversity and inbreeding in the bumblebee *B. veteranus*, a case study

Redrafted after:

Maebe, K., Meeus, I., Maharramov, J., Grootaert, P., Michez, D., Rasmont, P., Smagghe, G. (2013) Microsatellite analysis in museum samples reveals inbreeding before the regression of *Bombus veteranus*. Apidologie 44(2), 188-197.

2.1 Introduction

Just as many other pollinator species, also many bumblebees species are under decline (e.g. Klein *et al.*, 2007; Goulson & Osborne, 2010; Potts et al., 2010). The main hypotheses to explain these observed declines in bee populations were already told in chapter 1.2.2 (reviewed in Potts *et al.,* 2010,). As a consequence of this decline, bumblebee populations gradually become smaller, generating new genetic threats, such as: (i) a reduced genetic diversity which may lead to a more limited evolutionary potential against future changes in the environment (Frankham, 2005; Zayed, 2009; Goulson & Osborne, 2010), and (ii) inbreeding (mating with relatives) which can lead to the presence of sterile diploid or triploid males (Duchateau *et al.*, 1994; Whitehorn *et al.*, 2009), and to inbreeding depression caused by the expression of deleterious recessive alleles (Frankham, 2005; Zayed, 2009; see also chapter 1.2.3). However, due to purging of deleterious alleles from the population in the haploid males, bumblebees are, as haplo-diploid species, not as sensitive to genetic pauperization as diploid species (Sorati *et al.*, 1996; Packer & Owen, 2001). So the question remains: whether populations of haplo-diploid bumblebees are under danger of extinction when severe inbreeding is detected as has been reported before for mammals (or diploid species) (i.e. Keller & Waller, 2002), or can bumblebees sustain several years of inbreeding?

In this chapter, we will develop PCR multiplexes of microsatellites DNA markers which we then will use to study the impact of genetic parameters, inbreeding and genetic diversity, on natural populations of declined bumblebee species. Therefore, we examine the genetic diversity of pin-mounted bumblebee specimens sampled from extensive bumblebee collections which allows a comparison of more recent populations with those sampled in the past. This approach may increase the power to detect recent changes in population structure and diversity. Our goals are to examine how genetic diversity and population structure are correlated with species extinction and to learn more about bumblebee decline worldwide. Here, we used historical populations of the declined species *Bombus veteranus* (*Thoracobombus*) (Fabricius, 1793) as a case study to study the impact of genetic parameters in bumblebee decline. This species is a good example of a declining bumblebee species in Belgium. Indeed, *B. veteranus*, which lives in the plains of Northern Europe and has a highly patchy distribution, (Rasmont & Iserbyt, 2010), was one of the most abundant bumblebees in Belgium one century ago, but it started to decline in 1950 and to date this species is almost vanished (Rasmont & Mersch, 1988; Rasmont *et al.*, 1993). Samples were collected spanning

a period of three decades (1895-1923), before the decline occurred, and we analyzed how the allelic richness, heterozygosity and inbreeding coefficients responded over this period. These findings can increase our understanding of genetic parameters of bumblebee populations before their actual decline or extinction.

2.2 Material and methods

2.2.1 *Museum specimens*

Belgian specimens of *B. veteranus* were selected from the Banque de Données Fauniques de Gembloux & Mons (Pauly & Rasmont, 2010). Between 1890-1950, *B. veteranus* represented 10% of all bumblebees. This proportion decreased rapidly towards 2% between 1950-1970 and less than 0.5% after 1970. Multiple bumblebee workers (BV1-BV111; Supplementary File S4) present in the museum collection of The Royal Belgian Institute of Natural Sciences (RBINS) were chosen for three different time periods before the actual decline in 1950: 1895 (n = 10), 1915 (n = 47) and 1923 (n = 32). For each of these time periods a maximum distribution of this species was created, see Figure 2.1.

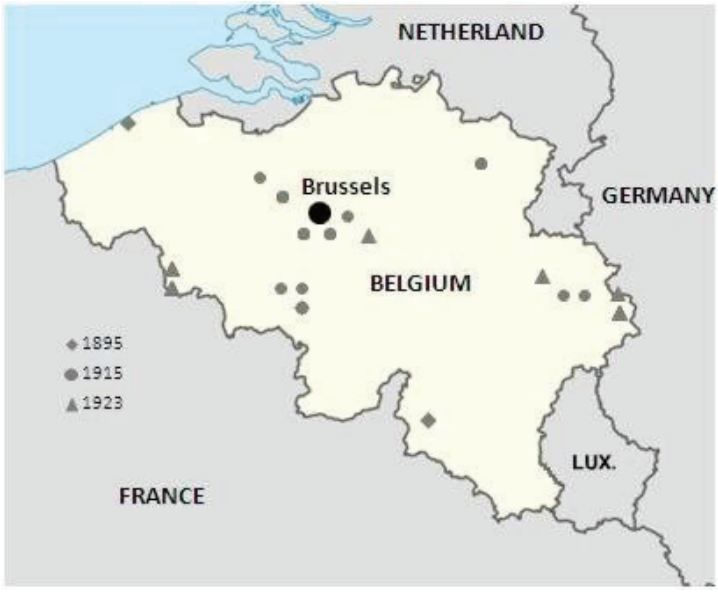


Figure 2.1 Distribution of the *Bombus veteranus* specimen collected for each year in the microsatellite analysis.

Although, our setup was not perfect we created a maximum distribution in Belgium over the three time periods with the low amount of available specimens within each year. In this way, the specimens were sampled from different but in general comparable environments. Furthermore, the specimens are collected between March and September. As bumblebee colonies stay in the neighbourhood of their nest, this will not have an effect on the genetic diversity measurement of bumblebees sampled in one location. In addition, 10, 20 and 20 drones were selected out of each respective time period.

2.2.2 *DNA extraction and microsatellite protocol*

Bumblebee DNA was extracted from one middle leg of each selected pin-mounted museum specimen using sterilized forceps. Before each extraction, the area and equipment were treated to remove potential contaminants. The DNA extractions were performed with 5% Chelex (InstaGeneTM Matrix, BioRad) using a modification of the Chelex protocol (Walsh *et al.,* 1991) adding of 400 µl of InstaGene^{TM} matrix and 20 µl of proteinase K (20 mg/ml) to the sample followed by a first incubation step at 37°C overnight (17 h) and a second incubation step at 97°C for 1 h. Amicon Ulta-0.5 Centrifugal Filter Devices (Millipore) were used for purification and to concentrate the extracted DNA following the manufacturer's guidelines, as they were essential for obtaining enough good quality DNA out of museum samples of more than 90 years old. All extractions and subsequent polymerase chain reactions (PCRs) were accompanied by negative controls. Extractions were stored at -20°C.

Workers were then genotyped at 8 microsatellite loci that have a range lower or around 200 bp to avoid the chance of null alleles (Wandeler *et al.*, 2007) and that gave a reliable signal in the museum samples. Microsatellite loci used here were: B11, B126 and B132 (Estoup *et al.*, 1993) and BT04, BT08, BT10, BT11 (Reber-Funk *et al.*, 2006) originally developed from *B. terrestris*, and BL02 (Reber-Funk *et al.*, 2006) derived from *Bombus lucorum*.

Microsatellites were amplified by PCR in 15 µl volumes using the Type-it QIAGEN PCR kit. Each reaction contained 2 µl template DNA, Type-it Multiplex PCR Master Mix (2x, Qiagen), and 0.5 µM of the forward and reverse primers for mix 1 (MP1) and mix 2 (MP2) (Table 2.1). The forward primer of each microsatellite loci was 5'-end labeled with fluorescent labels for capillary electrophoresis. Samples were initially denatured at 95°C for 5

min, followed by 28 cycles of denaturing at 94°C for 30 s, annealing at 49-52°C for 30 s for mix 2 and mix 1, respectively, and extension at 72°C for 30 s. The PCR protocol ended with a final extension step at 72°C for 30 min. Final PCR products were visualized on a ABI-3130xl or ABI-3730xl sequencer (Applied Biosystems) using an internal size standard (Genescan 500 LIZ, Applied Biosystems). The fragments were then examined and scored manually using Peak Scanner Software v 1.0 (Applied Biosystems). To ensure data quality, museum specimens were amplified twice at each locus; there was no evidence of amplification or scoring errors based on those repeated genotyping.

2.2.3 *Data analysis*

Because there is a possibility of sampling multiple sisters from the same colony, which could potentially affect estimates of population genetic parameters, we used the program Colony 1.2 (Wang, 2004) to examine family relationships for each time period, employing corrections for genotyping errors (5% per locus). We checked our data also with the program Kinalyzer (Ashley *et al.*, 2009) with both the '2 allele' algorithm and the 'consensus' method to exclude problems using Colony 1.2 on populations with low genetic variability (Ashley *et al.*, 2008). All further analyses were made after removal of the identified sisters.

Tests for genotypic linkage disequilibrium and departures from Hardy-Weinberg (HW) equilibrium were performed for each population with randomization methods implemented in FSTAT 2.9.3 (Goudet, 2001). The program GENALEX 6.3 (Peakall & Smouse, 2006) was used for testing genotype frequencies against HW equilibrium expectations. When excess homozygosity was found, the program MICROCHECKER 2.2.3 (van Oosterhout *et al.*, 2004) was used to check for evidence of null alleles and their frequencies at different loci were estimated with the program FREENA (Chapuis & Estoup, 2007).

2.2.4 *Genetic diversity and inbreeding*

Estimation of the population genetic diversity was performed by calculating the expected and observed heterozygosities (H_E and H_O, respectively), and the allelic richness (A_R). The program GENALEX 6.3 (Peakall & Smouse, 2006) was also used to calculate H_E and H_O for each microsatellite loci. We estimated H_E in each population using Nei's unbiased expected heterozygosity (Nei, 1978) because this statistic is unbiased by sample size and does not

appear to be seriously affected by null alleles (Chapuis *et al.*, 2008). The allelic richness (A_R) corrected for sampling size (El Mousadik & Petit, 1996) and the inbreeding coefficient (F_{IS}) were estimated in FSTAT 2.9.3 (Goudet, 2001). We used a paired Student's *t*-test in SPSS (version 20.0.0.1) to examine whether the mean genetic diversity and allelic richness significantly differed between different time periods. As null alleles can reach high levels when studying old museum specimens (Wandeler *et al.*, 2007; Strange *et al.*, 2009), the inbreeding coefficients were corrected for null allele frequencies based on the individual inbreeding model (IIM) using the program INEst (Chybicki & Burczyk, 2009). The estimated distribution was used to estimate corrected allele frequencies and inbreeding coefficients using 10000 iterations (Chybicki & Burczyk, 2009).

2.2.5 *Population structure*

Pairwise differentiation values (F_{ST}) among the different time periods were calculated using 1000 permutations in FSTAT 2.9.3 (Goudet, 2001). Because null alleles may affect *F*-statistics (Chapuis & Estoup, 2007) the pairwise F_{ST}-values were re-calculated after applying the ENA correction for null alleles as implemented in FREENA. We also estimated Jost's D (D_{est}; Jost, 2008). This recently developed statistic provides a true measure of differentiation for highly variable markers, such as microsatellites, using the software SMOGD v2.6 (Crawford, 2010).

2.2.6 *Bottleneck presence*

Evidence of recent genetic bottlenecks in the temporal samples was tested using Garza & Williamson (M) statistic (Garza & Williamson, 2001). The program assumes that a reduction in population size has a stronger effect on the number of alleles (*k*) than the range of allele sizes (*rs*). This leads to a smaller M-ratio (= *k*/*rs*) in size-reduced populations compared to equilibrium populations (Garza & Williamson, 2001). In order to evaluate the likelihood of a bottleneck occurrence (95% criterion), the M-ratios calculated and averaged across loci were compared with the distribution of simulated M_C-ratios of a population in equilibrium. The M_C-ratios were simulated based on parameters describing the evolution of the analyzed microsatellite loci (μ: the mutation rate/locus/generation, Δ_g: the mean size of larger mutations and p_s: fraction of mutations larger than a single step) and the effective population size of pre-bottlenecked populations (N_e). Each sample estimate of M-ratio (M critical or M_C)

was thus tested under different evolutionary scenarios as suggested by Guinand & Scribner (2003).

2.2.7 *Simulation of gene diversity over time*

We observed no significant decrease of the genetic diversity in *B. veteranus*. So, we made simulations of how the genetic variation would be affected by a change (decline) in population size. And this in an equivalent data set and time periods like we found in our studied *B. veteranus* populations.

Therefore, we created models of populations that have sample sizes equivalent to our *B. veteranus* samples. For those created populations we constructed a simple model of decline in population size of 28 generations starting from a stable population. We used the program BayeSSC, a modification of the simulation program Serial SimCoal (Excoffier *et al.*, 2000; Anderson *et al.*, 2005), for coalescent simulations of data collected at multiple time points. We used the same parameters as described in Lozier & Cameron (2009) except that we let the growth factor range from 0% to 5% over 28 generations and the ancestral effective population sizes (N_{Ae}) from 15000 to 100. Indeed, we changed the negative growth factor (as for a decline) range from 0%, 1%, 2% and 5% over 28 generations and the ancestral effective population sizes (N_{Ae}) from 15000, 10000, 5000, 1000, 500 to 100. Other parameters we used were: a mutations probability for microsatellite loci of 5×10^{-4} per generation (average mutation rate, Selkoe & Toonen, 2006) according to a stepwise mutation model and a limit of 40 allele states per locus. We performed 7000 simulations for each population size combination or 1000 simulations for each microsatellite loci. To evaluate the loss of genetic diversity between the different time points we averaged the H_E estimates across loci and determined the drop in H_E.

2.3 Results

2.3.1 *Microsatellite data*

Of the eight microsatellite loci screened, seven (B11, B126, B132, BT04, BT10, BT11 and BL02) amplified strongly and were consistent across replicates. The locus BT08 could not be scored in a reliable manner and was therefore excluded from further analyses (Table 2.1).

Table 2.1 Overview of the selected microsatellite loci for the two multiplexes, their range, number of alleles and fluorescent dyes used. Label = fluorescent dye; *N* = number of alleles.

Locus	Label	Multiplex	Range	N_A
BL02	NED	MP1	148-158	5
BT04	NED	MP1	154-180	10
BT08	PET	MP1	160-210*	3*
BT10	VIC	MP1	112-140	13
BT11	6-FAM	MP1	92-118	14
B11	NED	MP2	124-136	6
B126	PET	MP2	146-176	13
B132	VIC	MP2	144-158	6

*** = not completed because of scoring difficulties.**

Analysis with Colony 1.2, and controlled with Kinalyzer, revealed that most of the populations contained some full-sib pairs. For populations with identified sisters, we randomly selected one individual for further analysis. Of the originally selected numbers of bumblebees: 1895 (n = 10), 1915 (n = 47) and 1923 (n = 32), we used in all further analyses only the numbers after removal of the identified sisters: 1895 (n = 6), 1915 (n = 34) and 1923 (n = 18). Furthermore, we based all our analyses and conclusions on the time periods 1915 and 1923 as the numbers of specimens in the time period 1895 became too low. However, we still find the information obtained for the time period 1895 indicative.

Six of the seven loci displayed heterozygote deficits under the Hardy-Weinberg equilibrium that could be indicative for inbreeding or the presence of null alleles. However, MICROCHECKER 2.2.3 revealed only low null allele frequencies for those loci over the different time periods (<10%). A significant linkage disequilibrium ($P < 0.05$) was found between 3 pairs of loci: BL02-BT10, BL02-B11 and B11-B132, when testing each locus pair across populations. The exclusion of locus BL02 and/or B11 had no major effect on the results.

2.3.2 *Changes in genetic diversity*

The allelic richness (A_R) and expected heterozygosity (H_E) varied widely among loci, although differences between time periods were less pronounced (Table 2.2). The mean H_E was 0.607 ± 0.164 (mean ± SD) in 1895, 0.577 ± 0.310 in 1915, and 0.578 ± 0.313 in 1923,

with the difference being not significant for 1915-1923 (paired *t*-test, t = -0.034, d.f. = 6, P = 0.98). The allelic richness estimate showed a slight increase from 3.47 ± 0.91 in 1895 over 3.68 ± 1.66 in 1915 to 3.71 ± 1.71 in 1923, although this difference was not significant for 1915-1923 (paired *t*-test, t = -0.119, d.f. = 6, P = 0.91).

Table 2.2 After removal of identified sisters, the number of workers (n), the number of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) and the for null alleles corrected inbreeding coefficient (F_{IS} IIM; Cybicki & Burczyk, 2009) for all microsatellite loci over the populations for each time period, with mean values and SD.

Population	BT11	BL02	BT10	BT04	B11	B132	B126	Mean	SD
1895 (n=6)									
N_A	4.0	2.0	6.0	3.0	4.0	4.0	4.0	**3.9**	±1.2
A_R	4.00	1.91	4.89	3.00	3.58	3.33	3.58	**3.47**	±0.91
H_O	0.000	0.000	0.833	0.250	0.600	0.500	0.600	**0.398**	±0.322
H_E	0.750	0.278	0.778	0.656	0.580	0.625	0.580	**0.607**	±0.164
F_{IS}	1.000	1.000	-0.071	0.619	-0.034	0.200	-0.034	**0.383**	±0.484
F_{IS} IIM	0.530	0.464	0.095	0.266	0.132	0.178	0.153	**0.260***	±0.171
1915 (n=34)									
N_A	10.0	3.0	11.0	7.0	5.0	4.0	10.0	**7.1**	±3.2
A_R	5.60	1.77	5.18	4.19	2.57	1.64	4.79	**3.68**	±1.66
H_O	0.545	0.000	0.706	0.421	0.379	0.042	0.654	**0.392**	±0.279
H_E	0.872	0.213	0.841	0.745	0.406	0.157	0.804	**0.577**	±0.301
F_{IS}	0.374	1.000	0.161	0.435	0.066	0.765	0.187	**0.423***	±0.338
F_{IS} IIM	0.194	0.718	0.068	0.245	0.087	0.256	0.117	**0.241***	±0.223
1923 (n=18)									
N_A	9.0	3.0	7.0	8.0	5.0	2.0	7.0	**5.9**	±2.6
A_R	5.00	1.62	4.84	5.87	2.80	1.61	4.24	**3.71**	±1.71
H_O	0.231	0.056	0.529	0.500	0.529	0.000	0.692	**0.362**	±0.267
H_E	0.820	0.156	0.824	0.859	0.471	0.165	0.749	**0.578**	±0.313
F_{IS}	0.718	0.644	0.357	0.418	-0.125	1.000	0.075	**0.441***	±0.386
F_{IS} IIM	0.445	0.342	0.195	0.228	0.061	0.647	0.071	**0.284***	±0.211

*** = Inbreeding coefficient significantly different from 0 (P < 0.05).**

2.3.3 *Population structure*

Comparison of the different time periods revealed no significant genetic differentiation (F_{ST}) between the years (Table 2.3). In agreement, the genetic differentiation grouped over all different time periods was also small and not significantly different from zero (F_{ST} = 0.039, Confidence Interval (CI): -0.008-0.090, one sample *t*-test against 0, t = 0.861, d.f. = 6, P =

0.42). Correction for the occurrence of null alleles, i.e. the ENA correction, had no effect on the genetic differentiation and was not significantly different from zero (F_{ST} = 0.024, CI: -0.058-0.071, one sample *t*-test against 0, t = 0.739, d.f. = 6, P = 0.48). Calculation of Jost *D*, another statistic to measure differentiation, among the different time periods was 0.034 and this was not significantly different from zero (CI: -0.047-0.113, one sample *t*-test against 0, t = 1.768, d.f. = 6, P = 0.13).

Table 2.3 Pairwise F_{ST} for the different time periods (with ENA correction) under the diagonal and the harmonic mean of *D*est across loci above the diagonal.

F_{ST}/ D*est*	1895	1915	1923
1895	-	0.039	0.045
1915	0.055	-	0.005
1923	0.037	-0.003	-

2.3.4 *Inbreeding and presence of diploid males*

We detected high inbreeding coefficients (F_{IS}) across all loci (0.415 ± 0.387, mean ± SD) (Table 2.2). Both the year 1915 and 1923 were significantly different from zero (one sample *t*-test against 0, d.f. = 6, t = 3.31, P = 0.028 and t = 3.03, P = 0.038, respectively). The inbreeding corrected for null alleles based on IIM (F_{IS} IIM) across all loci was much lower: 0.262 ± 0.194 (Table 2.2), but was still significantly different from zero for each time period (one sample *t*-test against 0, d.f. = 6, t = 5.75, P = 0.001 for 1915; t = 4.86, P = 0.003 for 1923; and t = 5.81, P = 0.001 for 1895) (Table 2.2). Significant inbreeding was supported by the occurrence of diploid males in each time period: one in 1895 (n = 10) and three in both the years 1915 (n = 20) and 1923 (n = 20).

2.3.5 *Test for bottleneck presence*

The calculated M-ratios averaged across loci, were 0.650 in 1895, 0.673 in 1915 and 0.662 in 1923. Based on the generally accepted critical M-ratio of $M_C \leq 0.680$ as described by Garza & Williamson (2001), the population of all three time periods showed evidence of a bottleneck. When comparing the calculated M-ratios averaged across loci with the here simulated M_C-ratios, which ranged from 0.639 to 0.831, each population showed also signs of having passed through a bottleneck except for combinations using extreme parameter values.

The parameter settings of the calculation were Δ_g=3.5, μ=0.20 and p_s=5 and 10, and the resulting M_C-ratios were 0.639 and 0.643, respectively. It should be noticed that for small data sizes, as is here the case for the year 1895, the interpretation of the results can be problematic because of stochastic effects (Garza & Williamson, 2001), however, the generated data are valid for the other time periods.

2.3.6 *Simulation of H_E evolution in declining populations*

The simulations for different ancestral effective population sizes (N_{Ae}=100 to 15000) over 28 generations resulted in marginal losses of H_E of around or less than 0.05 even when starting with a strong negative growth factor of 5%.

2.4 Discussion

In this chapter, we used a set of eight microsatellites to genotype museum specimens of *B. veteranus*. Then, we analyzed how genetic parameters of bumblebee populations (i.e. allelic richness, observed and expected heterozygosities, genetic differentiation and inbreeding) evolved over a period of three decades (1895-1923). In all the time periods, we detected low heterozygosities and positive inbreeding coefficients (the F_{IS}-values ranged from 0.383 to 0.441) which can be caused by several factors such as the presence of null alleles, population subdivision and inbreeding (Callen *et al.*, 1993). For null alleles, the program MICROCHECKER 2.2.3 confirmed the presence of null alleles in our data, but the frequencies were low in all loci. After we corrected for null alleles based on the individual inbreeding model IIM, the inbreeding coefficients stayed high (F_{IS} IIM ranging from 0.241 to 0.284) which is indicating that the high inbreeding coefficients cannot be explained by the occurrences of null alleles. Similarly, population subdivision can be excluded as a factor here for our data since the genetic differentiation observed in *B. veteranus* (F_{ST} = 0.024) was small. In continuation of our analysis, it seemed to be more likely that the significant positive inbreeding coefficients have been influenced by high levels of inbreeding. Indeed the presence of inbreeding was confirmed by the occurrence of sterile diploid males in the three time periods. Our data demonstrated that the population of *B. veteranus* in Belgium showed inbreeding between 1915-1923, with the indication that this phenomenon was already present since 1895. Thus inbreeding was already present 25-30 years before the actual decline of *B.*

veteranus that started in Belgium around 1950 (Rasmont & Mersch, 1988; Rasmont *et al.*, 1993). As a consequence, we believe that the data obtained here suggests that the observed inbreeding did not directly result in the collapse of *B. veteranus*.

As reported by Goulson *et al.* (2008), it is expected that populations of declining species become rare and isolated. As a consequence, populations of declining species exhibit a loss of genetic diversity (drop in heterozygosity and allelic richness) and gene flow over time, while for stable populations such changes are less likely to occur (Goulson *et al.*, 2008). In this context we ran a simulation over 28 generations with *B. veteranus*. However, these simulations demonstrated that in most of the cases a reduction in population size (simulating bumblebee decline) resulted in a marginal loss of H_E of around or less than 0.05. Interestingly, our simulation data agree with those of Lozier and Cameron (2009) as these authors could also not detect a major drop in H_E in a simulation over 38 generations in the declining bumblebee species *B. pensylvanicus*. So both simulations do not show a major drop in H_E over time. In their review, Goulson *et al.* (2008) presented the hypothesis that the genetic diversity (A_R and H_E) in current declined species is reduced as compared to other common *Bombus* species. But without actually knowing the ancestral H_E, it is difficult to conclude if a drop of H_E really occurred. Indeed our data are strong indicatives that *B. veteranus* already had a low H_E before its decline. This agrees with a low H_E in the old specimens of *B. pensylvanicus* that is a declined bumblebee species in the USA (Lozier & Cameron, 2009).

As reported by Rasmont & Mersch (1988), Rasmont *et al.* (1993) and Goulson *et al.* (2008), general drivers like the reduction in floral resources by agricultural intensification acted around 1950 for bumblebee decline. With the data obtained in this chapter, we can postulate the hypothesis that bumblebees with a low genetic diversity were then the first to decline. Hence, they were less prepared to face these troubled times or less adapted to this new environment. Furthermore, the low H_E we found in all populations could also be explained by the fact that *B. veteranus* was a source-sink population in Belgium, as this species is well known for its sudden appearance in different parts in Europe (Söderman, 1999; Rasmont & Iserbyt, 2010). Indeed, in agreement with the low genetic diversity, each time period demonstrated signs of the occurrence of a genetic bottleneck. Here, the presence of a bottleneck is based on the M-values; however we notice here that some M-values should be

interpreted with some caution since they can be sensitive to outliers in small data sets.

In addition to those general drivers affecting the bumblebee populations, Voveikov (1953) described that *B. veteranus* is often inquilines of other *Thoracobombus* species such as *B. sylvarum*, *B. muscuorum*, *B. humilis* and *B. ruderarius.* This phenomenon of *B. veteranus* being dependent on the nesting behavior of other species, could have made this species even more vulnerable toward extinction. Our data showed that *B. veteranus* remained abundantly present in Belgium until the fifties and this in spite of the high inbreeding coefficients and the low genetic diversity presented in the population. This is unexpected in the case that inbreeding would have had major effects on the species success. Nonetheless, no inbreeding depression was detected here. These results are similar to those of *B. terrestris* in Tasmania (Schmid-Hempel *et al.*, 2007). The latter study demonstrated that, despite a drastic genetic bottleneck, *B. terrestris* could successfully invade and colonize Tasmania. Therefore, we believe that this population was robust against the possible effects of a low genetic diversity and/or associated inbreeding. But it has also to be noted that in Tasmania there was a very favorable environment with no direct inter-species competition and no pathogens. However, negative effects of inbreeding have been reported, like the production of diploid males. In the case this happens, the queens which mate with diploid males are unable to initiate a colony and also diploid males do not work for the colony which will also have a negative effect on the population growth rate (Cook & Crozier, 1995; Gerloff & Schmid-Hempel, 2005; Whitehorn *et al.*, 2009). Furthermore, our result of detecting inbreeding without further inbreeding depression could be explained by the hypothesis that the haplo-diploid sex determination system of Hymenoptera is leading to a strong effect of purging selection against recessive deleterious alleles in the haploid males (Sorati *et al.,* 1996; Packer & Owen, 2001).

In conclusion, our data with *B. veteranus* demonstrated inbreeding over a period of 1895 to 1923 while the population remained stable, implying that inbreeding does not directly trigger the actual decline and/or extinction of bumblebees. However, inbreeding might still play an indirect role in the decline of bumblebee populations because of the appearance of diploid males and because a low H_E might reduce the capacity of the bumblebee population to react on environmental changes.

Chapter III

Historical low genetic diversity in declining Bombus species: a case-study with 11 species in the Netherlands

Redrafted after:

Maebe, K., Meeus, I., Ganne, M., De Meulenmeester, T., Biesmeijer, K., Smagghe, G. (2015) Microsatellite analysis of museum specimens reveals historical differences in genetic diversity between declining versus stable *Bombus* species. PLoS ONE. Submitted, under review.

3.1 Introduction

All over the world different pollinator species are undergoing major declines (e.g. Potts *et al.,* 2010). Generalist foragers like many bumblebees, that are essential pollinators in natural and managed ecosystems, are no exception to this general phenomenon (Williams & Osborne, 2009; Cameron *et al.,* 2011; Carvalheiro *et al.,* 2013). Different hypotheses aim to explain the observed declines in bee populations, as explained in chapter 1.2.2.

Genetic processes can also play a role in this observed decline (chapter 1.2.3). For example, there are two mechanisms through which low genetic diversity might contribute to declines. Firstly, low genetic diversity might threaten populations by limiting their ability to adapt to future environmental changes (Spielman *et al.,* 2004; Frankham, 2005; Goulson & Osborne, 2010). For instant, low diversity may predispose populations to disease epidemics (Cameron *et al.,* 2011; Whitehorn *et al.*, 2011). Secondly, low diversity may result in inbreeding, thereby reducing individual fitness and threatening population extinction (Reed & Frankham, 2003; Spielman *et al.*, 2004; Frankham, 2005; Zayed, 2009). Based on contemporary specimens, several studies have shown that populations of declining bumblebee species have lower levels of genetic diversity compared to stable species (Darvill *et al.*, 2006; Ellis *et al.*, 2006; Goulson *et al.*, 2008; Charman *et al.*, 2010; Cameron *et al.*, 2011). This reduction in genetic diversity is thought to be caused by population decline or recent bottlenecks (e.g. Goulson *et al.*, 2008; Charman *et al.*, 2010). However, as discussed by Lozier *et al.*, (2011), without information on the historic situation, the question remains: is this low diversity actually the result of recent declines, or is it due to historical, e.g. pre-decline, differences in genetic variation among species?

In this chapter, we compared the genetic diversity of declining and more stable bumblebee species before their major recent decline. We used microsatellites to genotype a set of pin-mounted museum specimens of 4 more stable bumblebee species: *Bombus pascuorum*, *B. hortorum*, *B. pratorum* and *B. lapidarius*, and 7 declining species: *B. muscorum, B. veteranus*, *B. ruderarius*, *B. sylvarum*, *B. humilis*, *B. ruderatus* and *B. subterraneus* (Peeters & Reemer, 2003). Samples were all collected in the Netherlands (1918-1926) before the recent declines started (between 1950-1980) (Rasmont & Mersch, 1988; Rasmont *et al.,* 1993; Biesmeijer *et al.,* 2006; Carvalheiro *et al.,* 2013). Furthermore, we compared our results with currently available data (time period: 1975-2010) on genetic diversity in

bumblebees (Ellis *et al.*, 2006; Schmid-Hempel *et al.*, 2007; Kraus *et al.*, 2009; 2011; Darvill *et al.,* 2010; Connop *et al.,* 2010; Goulson *et al.,* 2011; see also chapter 2) to obtain further insights whether the genetic diversity is similar in historical and current populations of declining and stable species. Together, these findings contribute to our understanding of the role of genetic parameters of bumblebee populations in population shifts and can provide valuable information for future conservation strategies.

3.2 Material and methods

3.2.1 *Museum specimens and their distribution*

Museum specimens of 11 bumblebee species were selected from the Hymenoptera collection of the Naturalis Biodiversity Center in Leiden taking into consideration their distribution in the Netherlands (Figure 3.1).

We divided the selected species in groups based on their presence and status on the red list of the Netherlands (Peeters & Reemer, 2003) (Supplementary File S5). Bumblebee species grouped as 'declining' have been given a red list status of 'vulnerable', 'endangered', 'critically endangered' or 'disappeared', while species grouped as 'stable' did not have a special red list status although these species also had range reductions. This first division of the species according to their red list status corresponds to the decline in their distribution (= trend, Table S1). Here, species distribution is calculated as the relative areal size (i.e. the amount of hour blocks a species has been found / the total amount of hour blocks checked) x 100%, with a hour block representing a 5 x 5 km square area. The decline in distribution is calculated as: (the relative areal size of after 1970 - relative areal size before 1970) / relative areal size before 1970) x 100% (Peeters & Reemer, 2003). The species assigned to the 'declining' group showed a decline in distribution of 65% or more between 1970 and 2003, while for the 'stable' species the decline in distribution was less than 40% ((Peeters & Reemer, 2003), see Supplementary File S5). Furthermore, we divided the group of declining species in two based on their distribution before 1970: species with a distribution lower than 10% were considered as restricted (with mean (SD): 6.1% (2.8%)) while declining species with a distribution between 15-25% were considered as widespread (19.1% (2.4%); *T*-test, $t = -6.465$, d.f. = 5, $P < 0.001$). The group of declining and widespread species was not

significantly different in range from the group of widespread but stable species (23.2% (2.8%); *T*-test, $t = 1.937$, d.f. = 4, $P = 0.125$; Peeters & Reemer, 2003).

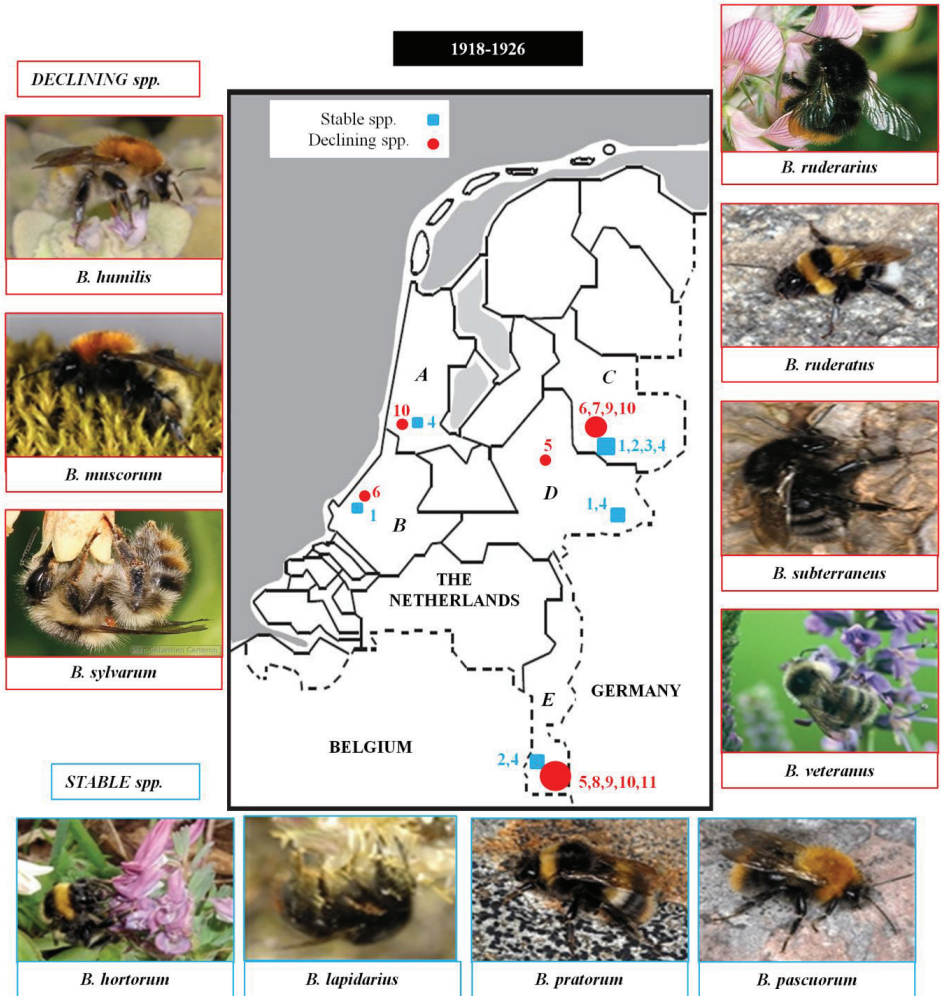


Figure 3.1 Distribution of the specimens of the declining and more stable *Bombus* spp. Specimens collected in The Netherlands between the years 1918-1926 before the recent bumblebee declines started (1950-1980), with a picture of each *Bombus* spp. used in the analysis. Species pictures from Rasmont & Iserbyt (2010). The letters refer to each sampling location: A = N-Holland, B = Z-Holland, C = Overrijssel, D = Gelderland and E = Limburg. Symbol size refers to the number of species sampled at that location, while the numbers refer to which species: 1 = *B. hortorum*, 2 = *B. lapidarius*, 3 = *B. pratorum*, 4 = *B. pascuorum*, 5 = *B. humilis*, 6 = *B. ruderatus*, 7 = *B. subterraneus*, 8 = *B. sylvarum*, 9 = *B. muscorum*, 10 = *B. ruderarius*, and 11 = *B. veteranus*.

Based on these criteria, we identified 4 stable and widespread bumblebee species: *B. pascuorum*; *B. hortorum*, *B. pratorum* and *B. lapidarius*, 3 declining but widespread species: *B. muscorum, B. veteranus* and *B. ruderarius,* and 4 declining but restricted species: *B. sylvarum*, *B. humilis*, *B. ruderatus* and *B. subterraneus*. Populations were collected in the period 1918-1926 and in 5 Dutch provinces: North-Holland, South-Holland, Gelderland, Overijssel and Limburg (Figure 3.1). Samples from a province were from one locality or different localities close together (within a 5 x 5 km frame). Before analyzing all *Bombus* species, we estimated the genetic diversity of one stable species: *B. pascuorum*. As this species was and still is abundantly present in the Netherlands, we suspected the genetic diversity to be fairly stable in space and time. If we detect low genetic diversity in the past for *B. pascuorum*, this could suggest artefacts associated with the genotyping of museum specimens, such as the presence of null-alleles. For this species, we selected additional specimens from two more recent time periods, 1949-1955 and 1975-1990 and from one additional province: Drenthe. For all populations, 7 to 10 bumblebee workers were chosen and genotyped.

3.2.2 *DNA extraction and microsatellite protocol*

Bumblebee DNA was extracted from one middle leg of each selected museum specimen with the same method as described in chapter 2. Workers were genotyped at 10 microsatellite loci that have a size range lower or around 200 bp to avoid the chance of null alleles (Wandeler *et al.,* 2007): B11, B100, B121, B126 and B132 (Estoup *et al.,* 1993) and BT04, BT08, BT10, BT11 (Reber-Funk, 2006) originally developed from *B. terrestris*, and BL02 (Reber-Funk, 2006) derived from *B. lucorum*. Microsatellites were then amplified by PCR and visualized with capillary electrophoreses as described in chapter 2. Genotype replications of random individuals (n = 48 or 16%) were conducted of which only 4 specimens showed an error at 1 of the 10 loci. We have thus a correct repetition of a single microsatellite locus of 99.2%.

3.2.3 *Data analysis*

Not all genotyped individuals of a population were included in the analysis due to several extra validation steps. First, specimens which could not be scored in a reliable manner for a minimum of 5 microsatellite loci, were excluded. Second, we used the program Colony 2.0 (Wang, 2004) employing corrections for genotyping errors (5% per locus) to search for the

presence of multiple sisters from the same colony. To exclude problems using Colony 2.0 on populations with low genetic variability (Ashley *et al.,* 2008), we checked our data also with the program Kinalyzer (Ashley *et al.,* 2009) with both the '2 allele' algorithm and the 'consensus' method.

As the microsatellites used here were developed from *B. terrestris* and *B. lucorum,* we needed to validate if they could be used in a reliable manner in the different *Bombus* spp. We tested for genotypic linkage disequilibrium with FSTAT 2.9.3 (Goudet, 2001) and for genotype frequencies against HW equilibrium expectations with GENALEX 6.3 (Peakall & Smouse, 2006). When excess homozygosity was found, the program MICROCHECKER 2.2.3 (Van Oosterhout *et al.,* 2004) was used to check for evidence of null alleles. We randomly selected one individual per sibship for further analysis.

3.2.4 *Genetic diversity*

We estimated genetic diversity in each population using the allelic richness (A_R) and Nei's unbiased expected heterozygosity (H_E; Nei, 1978). The latter statistic is not biased by sample size and appears not to be affected by null alleles (Chapuis *et al.,* 2008). The program HP-RARE (Kalinowski, 2005), with hierarchical rarefaction to correct for sampling size, and GENALEX 6.3 (Peakall & Smouse, 2006) were used to estimate A_R and calculate H_E for each microsatellite locus, respectively. As some of our groups did not pass the Levene test, we used only nonparametric tests (e.g. Independent samples Mann-Whitney U test) in SPSS (version 21.0.0.0) to examine if the genetic diversity differed significantly between the widespread stable versus the restricted and widespread declining species and an ANOVA with Repeated Measures Factors was used to examine the genetic diversity between populations of *B. pascuorum*.

We conducted a sensitivity analysis of the calculated mean expected heterozygosity (H_E) for each population of the different *Bombus* spp. in the time period 1918-1926 based on more stringent exclusion policies for missing data. We started this analysis from a maximum of 50% missing values (or 5 loci) within one specimen towards a more stringent exclusion step of only 10% (or one locus) missing data.

3.2.5 *Population structure and inbreeding*

Genetic differentiation values (F_{ST}) between the *B. pascuorum* populations within years and within a location between years were calculated using 1000 permutations in FSTAT 2.9.3 (Goudet, 2001) and re-calculated after applying the ENA correction for null alleles as implemented in FREENA (Chapuis & Estoup, 2007). We also estimated the true measure of differentiation, D_{est} (Jost, 2008), using the software SMOGD v2.6 (Crawford, 2010).

Inbreeding coefficient (F_{IS}) were estimated in FSTAT 2.9.3 (Goudet, 2001). The inbreeding coefficients were also corrected for null allele frequencies based on the individual inbreeding model (IIM) using the program INEst (Chybicki & Burczyk, 2009). The estimated distribution was used to estimate corrected allele frequencies and inbreeding coefficients using 10000 iterations (Chybicki & Burczyk, 2009).

3.3 Results

3.3.1 *Data analysis*

Genotype replications of random individuals showed only 4 specimens with an error at 1 of the 10 loci. Thus, we have a correct repetition of a single microsatellite locus of 99.2%.

Almost all microsatellite loci amplified strongly in each *Bombus* species and were consistent across replicates (Supplementary File S6). Analysis with Colony 2.0, controlled with Kinalyzer, revealed that most populations contained some full-sib pairs (Supplementary File S6). We randomly selected one individual per sibship for further analysis. Of the 302 specimens (116 of 7 declining bumblebee species and 186 of the more stable species), 234 specimens were kept for further analyses after removal of sisters (86 specimens of 7 declining species and 148 specimens of the stable species; Supplementary File S6).

No significant linkage disequilibrium was found between the pairs of loci, when testing each locus pair across populations. All loci displayed heterozygote deficits under the Hardy-Weinberg equilibrium which is indicative for the presence of null alleles. However, MICROCHECKER 2.2.3 revealed low null allele frequencies for those loci.

3.3.2 *Genetic diversity, inbreeding and differentiation of* B. pascuorum

The genetic diversity of the *B. pascuorum* populations was stable over the different locations (ANOVA with Repeated Measures Factors; A_R, $F = 1.032$, df = 4, $p = 0.408$; H_E, $F = 1.262$, df = 4, $p = 0.308$) and the three time periods (ANOVA with Repeated Measures Factors, A_R, $F = 0.0116$, df = 1, $p = 0.743$; and H_E, $F = 0.276$, df = 1, $p = 0.615$; Figure 3.2). Thus, the genetic diversity of *B. pascuorum* populations in the Netherlands can be regarded as stable across locations and time periods, and the microsatellite analysis of old specimens is reliable.

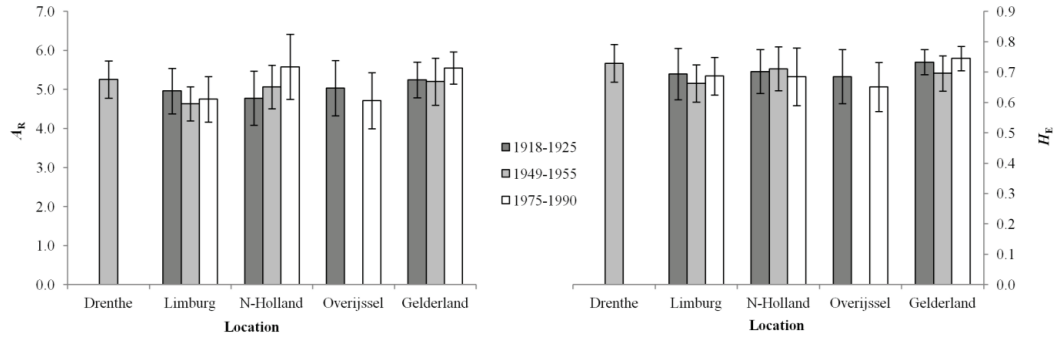


Figure 3.2 Genetic diversity of the *Bombus pascuorum* populations. The mean allelic richness (A_R) and expected heterozygosity (H_E) averaged across loci (and S.E.) between the *B. pascuorum* populations over the different locations and the three time periods.

Comparison of the *B. pascuorum* populations within and between the different time periods revealed only in a few cases significant genetic differentiation (F_{ST}) (Supplementary File S9). Correction for the occurrence of null alleles, i.e. the ENA correction, had no effect on the genetic differentiation. Furthermore, the calculation of D_{est}, another statistic to measure differentiation, within each time period was low: 0.057 for 1918-1926, 0.060 in 1949-1955, and 0.013 in 1975-1990, and not significantly different from zero (one sample T-test against 0, $t = 2.202$, $p = 0.064$; $t = 1.742$, $p = 0.125$; and $t = 1.204$, $p = 0.268$; respectively). So, *B. pascuorum* populations showed no or only marginal genetic differentiation.

Within each population of *B. pascuorum,* we detected low inbreeding coefficients (F_{IS}) across all loci (0.100 ± 0.232, mean ± SD). Both F_{IS} and the inbreeding corrected for null alleles (F_{IS} IIM) were not significantly different from zero for each population (one sample *t*-test against 0, d.f. = 7, $P > 0.05$).

3.3.3 *Genetic diversity in declining versus stable species*

For each population of the declining and more stable species, we estimated the genetic diversity Table 3.1). Next, we assessed whether declining *Bombus* species (B) had a lower genetic diversity than stable species (A) before their recent decline (Figure 3.3). The allelic richness (A_R) and expected heterozygosity (H_E) of the declining species: 3.281 (SE = 0.199) and 0.476 (SE = 0.038), were significantly lower than that of the stable bumblebee species with 4.696 (SE = 0.293) and 0.672 (SE = 0.032) (A_R and H_E, respectively) (Mann-Whitney U test, Z = -2.646, p = 0.008; and Z = -2.268, p = 0.023; Table 3.1). Although two declining species (*B. ruderatus* and *B. subterraneus*) had a comparable mean H_E as some of the stable species (Figure 3.3).

Table 3.1 Historical genetic diversity within all *Bombus* species. Here, we describe the mean values (and SE) of the allelic richness, and the expected heterozygosity for each *Bombus* spp. over all the microsatellite loci and populations within the time period 1918-1926. With n: the number of samples used for this analysis after removal of the identified sisters.

				A_R^x		H_E^y	
Group	***Abundance***	***Species***	***n***	**Mean**	**SE**	**Mean**	**SE**
Stable (A)	**Widespread**	*B. hortorum*	22	5.362		0.746	
		B. lapidarius	12	4.302		0.632	
		B. pratorum	8	4.114		0.604	
		B. pascuorum	33	5.006		0.704	
		Total	**75**	**4.696[a]**	**0.293**	**0.672[a]**	**0.032**
Declining (B)	**Restricted**	*B. humilis*	16	2.717		0.396	
		B. ruderatus	12	3.808		0.606	
		B. subterraneus	7	4.111		0.625	
		B. sylvarum	11	2.947		0.455	
		Subtotal	***46***	***3.396[b]***	***0.335***	***0.521[ab]***	***0.056***
	Widespread	*B. muscorum*	15	3.486		0.452	
		B. ruderarius	18	2.957		0.413	
		B. veteranus	7	2.942		0.382	
		Subtotal	***40***	***3.128[b]***	***0.416***	***0.416[b]***	***0.020***
		Total (declining)	**86**	**3.281[b]**	**0.476**	**0.476[b]**	**0.038**

[x] = allelic richness

[y] = expected heterozygosity

[abc] = significance level, $P < 0.05$

The lower genetic diversity within the declining species as reported here could be the result of the smaller distribution range of some species in the declining group (B). This was not the case. Indeed, when we divided the group of declining species (B) in restricted and widespread species following Peeters & Reemer (2003), the result remained the same. The genetic diversity of the widespread & declining group was significantly lower than that of the stable species (A_R, Z = -2.121, p = 0.034; and H_E, Z = -2.121, p = 0.034) and the restricted & declining group was also significantly lower than that of the stable species for A_R (Z = -2.309, p = 0.021) and showed the same but not significant trend for H_E (Z = -1.732, p = 0.083, Figure 3.3). Both groups of declining species were not different from each other (A_R, Z = -0.354, p = 0.857 and H_E, Z = -1.414, p = 0.229; Table 3.1 and Figure 3.3). This indicates that historically declining species already had a lower genetic diversity than bumblebee species with stable populations.

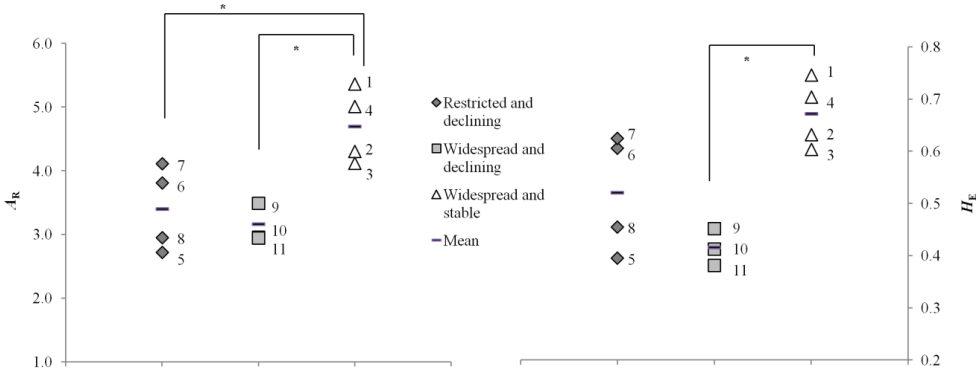


Figure 3.3 Historical genetic diversity of declining versus stable bumblebee species. Comparison of the mean allelic richness (A_R) and expected heterozygosity (H_E) averaged across loci between the populations of the declining and more stable *Bombus* species within the time period 1918-1926. With indication of the significance level, * = P < 0.05.

The sensitivity analysis of the calculated mean heterozygosity showed that H_E was stable over the different exclusion steps (Supplementary File S7). Furthermore, the differences of H_E between stable and declining species remained. A few populations had non-amplifications for a certain microsatellite loci for all their individuals, which could have a possible impact on our estimate of genetic diversity. After removal of three species (*B. subterraneus*, *B. ruderatus* and *B. lapidarius*) and some populations which had non-amplifications for a

certain microsatellite loci we re-analyzed the genetic diversity with the same 8 microsatellites (B11, B121, B126, B132, BT04, BT08, BT10, and BT11). This analysis showed no major impact of these non-amplifications on our dataset (Supplementary File S8).

3.4 Discussion

3.4.1 *Genetic diversity in declining versus stable species*

Our results showed that historical populations of declining bumblebee species had a significantly lower genetic diversity than found within the historical populations of co-distributed more stable species (Figure 3.3). This result is relevant for the interpretation of other studies which solely used recent specimens to assess genetic diversity (Darvill *et al.*, 2006; Ellis *et al.*, 2006; Goulson *et al.*, 2008; Charman *et al.,* 2010; Lozier *et al.,* 2011). Indeed, when we compared the genetic diversity of declining versus stable bumblebee species based on historical and recent data from the study performed in this chapter and the literature (Supplementary File S10), we observed the same trend in genetic diversity (Figure 3.4).

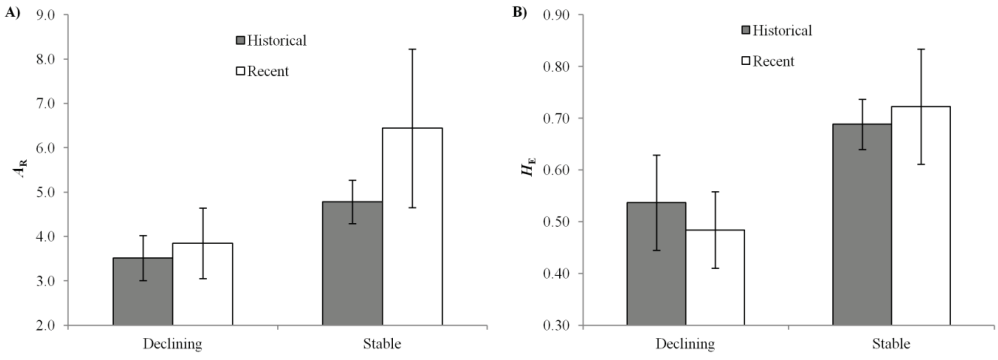


Figure 3.4 Comparison of the genetic diversity as the mean allelic richness (A_R) and the expected heterozygosity (H_E) averaged across loci (± S.D.) between the historical and recent data of a) the declining and b) the more stable bumblebee spp., with data from our project and from the literature. See also Supplementary File S10 for referees and genetic parameters of these populations. With time periods: 'historical' = 1895-1930; and 'recent '= 1975-2010'.

In studies with recent specimens, this lower genetic diversity in declining bumblebee species is sometimes explained as a reduction in genetic diversity in response to environmental drivers (e.g. Goulson *et al.*, 2008; Charman *et al.*, 2010). Interestingly, our results were obtained with museum specimens of nine decades ago, that is two to three decades before the declines of most bumblebees started. As reported for Belgium by Rasmont & Mersch, (1988) and Rasmont *et al.*, (1993) and for the Netherlands and Britain by Biesmeijer *et al.,* (2006) and reviewed in Goulson *et al.*, (2008), general drivers like the reduction in floral resources by agricultural intensification started around 1950. Thus here, the observed difference in genetic variation between declining and stable bumblebee species was not due to a recent reduction in genetic diversity but was already present in the years 1918-1926.

3.4.2 *Comparison of genetic diversity between groups of species*

Here, we compared the genetic diversity of several declining and stable bumblebee species. Such comparison of intra-population genetic diversity levels between different bumblebee species, could be a promising step in the detection of populations at risk of decline (Goulson *et al.,* 2008; Lozier *et al.,* 2011). However, the interpretation of the observed inter-specific differences cannot be made easily due to: (i) mutation rates which may vary at different microsatellites loci and (ii) differences in polymorphism of the microsatellite loci. To remedy these effects, we used the same microsatellite loci for each species and bumblebee specimens with similar distribution in The Netherlands. In addition, we compared a group of 7 declining species with a group of 4 stable (or less declining) bumblebee species instead of single species. Furthermore, each group consisted of bumblebee species of multiple subgenera. In this way we minimize inconsistencies and perform a valid comparison between groups of species (Goulson *et al.,* 2008; Charman *et al.,* 2010; Lozier *et al.,* 2011), while admitting that one can never rule out biases from undetected problems completely.

3.4.3 *Genetic diversity and rarity*

One possible explanation for low genetic diversity of the declining species in the early 20th century could be a lower abundance of these species in this time period. Indeed, small bumblebee populations can have a reduced genetic diversity as a result of higher genetic drift (Frankham, 2005; Zayed, 2009). However, there are indications that rarity alone cannot totally explain the observed low genetic diversity of the declining species: (i) some declining

species were present in the collection with a magnitude comparable to some of the stable species between the years 1900-1940. However, this method is not fully reliable as it has caveats, e.g. collector biases and preference for collecting rare species over common ones (Wandeler *et al.,* 2007), (ii) by referring to historical publications or expert judgement indicating a fairly common status. No historical information of the Netherlands is present but some of these declining species were reported as abundant in Belgium (Ball, 1914; 1920). For example: *B. veteranus* (then called *B. equestris*) ranked with second lowest allelic richness (2.942) was described as "assez commun" (= fairly common) in Belgium (Ball, 1914; 1920). While other species like *B. subterranus* with a relatively high allelic richness (4.111) comparable with the very common species is described as rare. However, as both indications have their own drawbacks, rarity is still a valid explanation of the low genetic diversity observed in the declining species.

There are also some other possible explanations of the low genetic diversity in the declining bumblebees: (i) having small effective population sizes could be an intrinsic characteristic of those species. If this would be the case it makes those species originally more vulnerable for the major drivers of bumblebee decline; (ii) the genetic diversity in the populations of the declining species could be altered due to habitat fragmentation or population isolation events before the dates used in this chapter (1918-1926). Therefore we could search for a genetic bottleneck. However, the use of bottleneck tests for haplodiploid species is somewhat dubious, as there are many violations of the model assumptions certainly when the power is low due to low samples size (Peery e*t al.,*2012). So, we cannot exclude that the declining species had undergone a historical decline before 1918-1926; (iii) the populations of the declining species could be at the edge of their ecological range in The Netherlands. Indeed, Williams *et al.*, (2009) found a link between bumblebee species decline and being at the edge of their climatic tolerance. The differences in species' ecological range could cause thus the results observed here. We found that the distribution and thus the ecological range of the stable species (IUCN, 2014) was further to the North than those of the declining species (Supplementary File S6). Indeed, the declining species have a distribution until the middle of Scandinavia, while most of the more stable species have a distribution until North Scandinavia (IUCN, 2014). Although the range of the declining species is thus smaller than the range of the more stable species, their range is not so much smaller (Supplementary File S6). Thus we believe that the populations of the declining species are, in The Netherlands,

not at the peripheral of their range. Or certainly not that close to the peripheral to cause the much lower genetic diversity levels within the declining species versus the stable species.

3.4.4 *Implications of low levels of genetic diversity*

Whatever the cause of the low genetic diversity in the declining bumblebee species may be, populations with low levels of genetic diversity will be more sensitive to local extinction. Firstly, the low levels of genetic diversity may result in inbreeding and inbreeding depression, reducing the individual fitness. Although for bumblebees individual negative effects of low levels of inbreeding are not proven, the production of diploid males in a colony is a clear negative effect of higher levels of inbreeding (Duchateau *et al.,* 1994; Whitehorn *et al.*, 2009). Secondly, populations with a lower genetic variation will be more vulnerable to changes and stressors in the environment, such as climate change, habitat loss and new pathogens (Reed & Frankham, 2003; Spielman *et al.,* 2004; Frankham, 2005; Zayed, 2009; Goulson *et al.,* 2011). Genetically pauperized bumblebees are also more susceptible to disease. Whitehorn *et al*., (2009) demonstrated a link between the gut trypanosome *Crithidia bombi* and genetic diversity. In the UK, populations of *B. muscorum* with a lower level of heterozygosity showed a higher prevalence of this gut parasite. Furthermore, declining bumblebee species with low levels of genetic diversity had a higher prevalence for the microsporidian *Nosema bombi* in northern America (Cameron *et al.,* 2011). So, the link between the level of genetic diversity and bumblebee decline as we found here, could also be due to an increased vulnerability to pathogens.

3.4.5 *Conservation*

Our results have strong implications for conservation strategies. Determination of the genetic diversity of bumblebees can reveal which species are more vulnerable to local extinction in the longer term. Indeed, as shown is Figure 3.3, all bumblebee species with a low genetic diversity and thus predicted to be vulnerable to decline, suffered more severe declines than the other species. However, it should be remarked that knowing the genetic diversity will not always identify which population is threatened. Indeed, two declining species showed similar levels of expected heterozygosity but had stronger declines than stable species with similar levels of heterozygosity (Figure 3.3). Thus clearly also other factors than genetic diversity can play a role in the observed bumblebee declines. However and in general, these results

suggest that determination of the genetic diversity is still a very good tool to predict bumblebee decline, as all five species with historically low genetic diversity levels (H_E lower than 0.550 and a A_R lower than 3.5) have subsequently suffered strong declines in their distribution.

As bumblebee populations with high genetic diversity may be less likely to decline or to undergo local extinction, improving the genetic diversity of the populations of restricted bumblebee species is a valuable strategy. Populations can be restored by connecting neighbouring populations as for example this will reduce the loss of diversity through drift and thus eventually result in an increase in diversity. Another, potentially risky, strategy is the introduction of bumblebees from foreign ranges. To increase success, introduced bees need to be from geographical and climatically comparable regions. Release of new pathogens in the habitat needs to be avoided, thus screening for pathogens prior to the introduction is needed (Meeus *et al*., 2011). But as probably not all pathogens are known, this could still impose a risk. A good recent introduction example is the second attempt of reintroducing *B. subterraneus* in the UK with specimens from Sweden (The Bumblebee Conservation Trust, 2009-2013).

Our results demonstrate that species with a lower genetic diversity are the ones that are currently endangered. However, species with a high genetic diversity could still be at risk for extinction. Indeed, the more stable species also underwent distribution declines but not as severe as the declining group. So, to preserve bumblebee diversity one must tackle also the current drivers of bumblebee decline, to ensure that these low and even high genetic diversity species will not go extinct. It is therefore recommended that conservation strategies create more suitable habitat for sustaining bumblebee populations.

Chapter IV

Recruitment to forage of bumblebees in artificial low light is less impaired in light sensitive colonies

Redrafted after:

Maebe, K., Meeus, I., Smagghe, G. (2013) Recruitment to forage of bumblebees in artificial low light is less impaired in light sensitive colonies, and not only determined by external morphological parameters. J. Insect Physiol. 59, 913-918.

4.1 Introduction

Bumblebees are essential pollinators in natural and managed ecosystems (Heinrich, 1979; Goulson, 2003). Like honeybees, bumblebee workers have specialized morphological structures for the collection of nectar and pollen such as a corbicula and adapted mouthparts (Michener, 1999; Inouye, 1980; Thorp, 2000). Due to their thermoregulation system, bumblebees are capable of foraging on days when it is too cold to forage for other pollinators (Heinrich, 1975; 1979; Goulson, 2010). The foraging abilities of bumblebees also rely on their sensory systems, the visual and the olfactory system, which consist out of two apposition compound eyes and three ocelli (Wcislo & Tierney, 2009) and several pore plate sensillae on their antennae as described for *Bombus terrestris* L. by Spaethe *et al.* (2007), respectively. An increase in size of the morphological parameters of both sensory systems increases the ability to detect and discriminate between different flowers which in turn can increase their foraging efficiency (Chittka *et al.*, 1999).

As bumblebees are social insects, the food influx of a colony is affected by how the work is allocated among all members of the colony (Goulson, 2003). Typically, bumblebee colonies consist out of hundred workers that differ in size (Goulson, 2010). The size differences within a colony are related to a specific task, a phenomenon known as alloethism (O'Donnell *et al.,* 2000; Jandt and Dornhaus, 2009). Small workers are found more inside the nest where they fulfill all kinds of nest tasks, whereas large workers are more likely to become foragers (Goulson *et al.*, 2002; Spaethe & Weidenmüller, 2002; Jandt &Dornhaus, 2009). However, this size-dependent division of labor is not strict and task-switching is possible (Jandt & Dornhaus, 2009). For instance, when there is a shortage of foragers, the smaller bees can be recruited or start foraging to comply with the nutritional needs of the bumblebee colony (Dornhaus & Chittka, 2005; Molet *et al.*, 2008; Kitaoka & Nieh, 2009).

Bumblebee foraging activity depends also on external factors such as food quality (Chittka *et al.*, 1997; Roldán-Serrano & Guerra-Sanz, 2005; Goulson, 2010) and environmental conditions like temperature, humidity, weather conditions and light intensity (Corbet *et al.,* 1993; Peat & Goulson, 2005; Goulson, 2010). The latter parameter turned out to be of importance in relation to foraging activity and foraging initiation of bumblebees in greenhouses (Blacquière *et al.*, 2007; Roman & Szczęsna, 2008; Johansen *et al.,* 2011).

Bumblebees (*B. terrestris*) are used worldwide in greenhouses for the pollination of different crops (Velthuis & van Doorn, 2006). Although commercial bumblebees perform better in the artificial light environment of the greenhouse than honeybees, they also show some problems particularly when the artificial light environment of a greenhouse deviates from the natural light environment in intensity and spectral composition (Morandin *et al.*, 2001, Blacquière *et al.*, 2006; 2007; Johansen *et al.,* 2011). Indeed, under these reduced artificial light conditions the activity of the bumblebees is decreased (Roman & Szczęsna, 2008).

Here in this chapter, we wanted to determine which parameters of individual bumblebees are linked with the lower performance of colonies in artificial light conditions. Therefore, we used eight queenright bumblebee colonies from a mass-rearing program and developed a new bioassay which determines the number of workers triggered to forage in two different standardized light intensities. Furthermore, we measured different external morphological parameters and the light sensitivity of 15-20 individual bumblebees of each of those colonies. In this way, we obtained more insights in the plasticity or variability of these parameters within the same colony and between colonies. The data obtained may help to improve the criteria for selecting towards light sensitive bumblebees and their link with the foraging capacity of these bumblebees.

4.2 Material and methods

4.2.1 *Laboratory conditions for maintenance of bumblebee colonies*

In this project we used 8 commercial queenright colonies of *B. terrestris* from a mass-rearing program at Biobest (Westerlo, Belgium) (Figure 4.1a,b). These colonies were provided with commercial sugar water (BioGluc, Biobest) and pollen (Apihurdes, Spain) *ad libitum*. All experiments were performed in a controlled laboratory environment at 28-30°C and 60-65% air humidity.

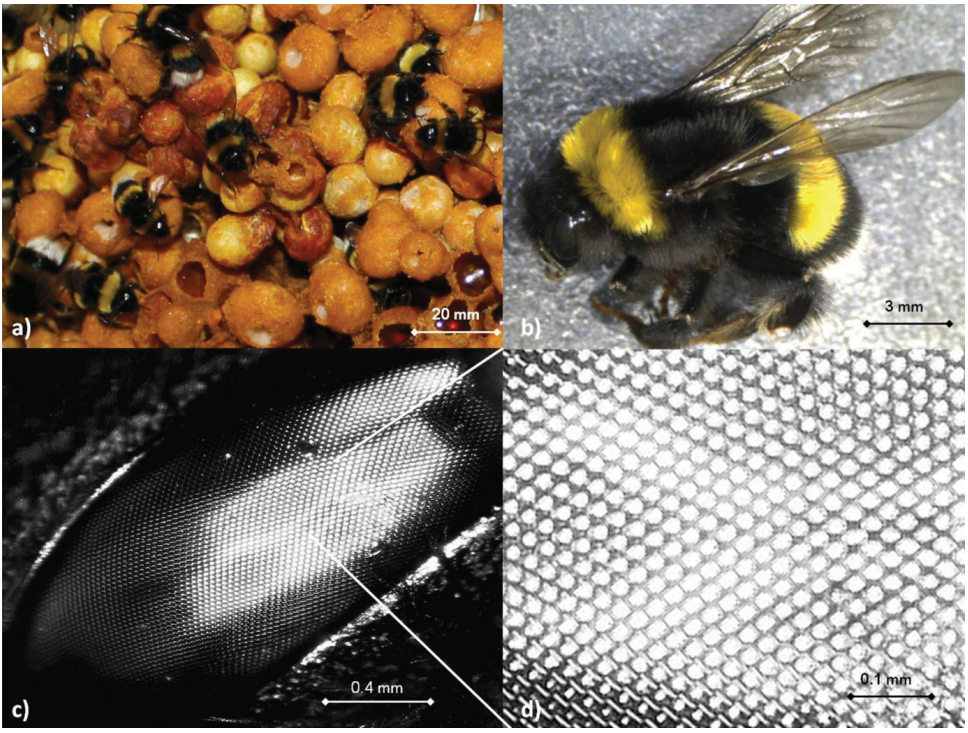


Figure 4.1 Panel with (a) *Bombus terrestris* colony, (b) *B. terrestris* worker, (c) compound eye and (d) facets.

4.2.2 *Determination of the initial nest-leaving capacity under different light conditions*

We developed a new bioassay to determine the initial foraging activity (F_a), which is the number of bumblebees leaving the colony in a time period of 1 h divided by the total number of workers in the colony at that moment. With the use of this bioassay we measured both the initial foraging activity of a colony in weak and strong light conditions (F_a^w and F_a^s, respectively). The initial nest-leaving, F_c, was calculated as the ratio of the initial foraging activities at weak and strong light intensity, F_a^w/F_a^s. This parameter (F_c) is a measure for the ability of a colony to keep its baseline initial foraging activity even with a decrease in light intensity.

In detail, for the 8 different queenright colonies we measured the foraging activity by placing each colony individually in a meshed fly cage (60 x 60 x 60cm, BugDorm-2, MegaView Ltd,

Taichung, Taiwan) in strong light intensity (F_a^s, 14000-14500 lux or 2.2- 2.3 x 10^{20} photons $m^{-2}s^{-1}$) and weak light intensity (F_a^w, 4000-4500 lux or 5.3 - 6.0 x 10^{19} photons $m^{-2}s^{-1}$; Figure 4.2). Light was provided by a Halogen Floodlight (PowerPlus Light, Varo, PowLI023, W400/500) which was placed at 30 cm in front of the entrance of the colony. The light intensity was measured with a calibrated luxmeter (Taschen-Luxmeter LM37, Karlsruhe, Germany) at the opening of the colony. As foragers are only active during the period of day, due to a robust internal circadian clock (Stelzer *et al.,* 2010; Stelzer & Chittka, 2010), both measurements were performed during 1 hour each, on the same day between 10 a.m. and 12 p.m., and by alternating weak and strong light intensities as first measurement. Bumblebee activity was recorded by manually counting the workers leaving their nest. All foragers were placed back in the colony before the light conditions were changed. As bumblebees cannot see in the red part of the visual spectrum, they become inactive when being exposed to red light conditions (see chapter 1.1.5). In this way, we were able to easily catch and place the workers back in their colony. Furthermore, colonies were placed in continuous darkness outside the test periods.

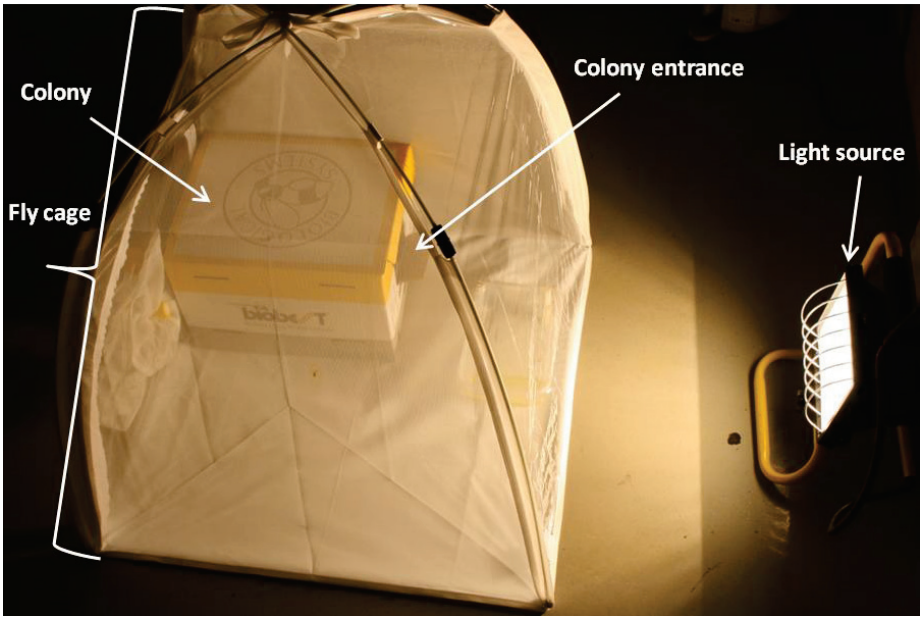


Figure 4.2 Picture of the developed bioassay to determine the initial foraging activity (F_a) in alternating light conditions.

The foraging test was performed 20 times for each colony, following a three days cycle of overnight starvation, one day of measurement and a day of recuperation. In the latter step, colonies were allowed to feed on sugar water. Colonies were starved overnight to trigger each nest towards maximal foraging. During the experiment we determined F_c and measured F_a^w and F_a^s, while the colonies developed from a workforce of 20 until 99 workers. For each colony these values of F_c, F_a^w and F_a^s were placed in different classes based on the size of the workforce in the colony at the moment of measurement (with class 1: a workforce from 20 until 29 workers; class 2: a workforce of 30 until 39 workers; …; class 8: from 90 until 99 workers). Thereafter we calculated F_c, F_a^w and F_a^s as the mean (±SE) over all the classes.
After logarithm transformation of the measured F_c, F_a^w and F_a^s values, the data were tested for normal distribution and analyzed by one-way ANOVA followed by a *post-hoc* Tukey test.

4.2.3 *Measurement of different morphology parameters of bumblebee workers*

For 15-20 workers of the 8 different bumblebee colonies, we determined 8 morphology parameters: (i) thorax width (intertegular span) of workers; (ii) total fresh weight; (iii) dorsal-ventral length of compound eye; (iv) width of compound eye; (v) total surface of compound eye; (vi) diameter of facet; (vii) total numbers of ommatidia of the compound eye; and (viii) diameter of median ocellus (Figure 4.1c,d).

Each bumblebee and its left compound eye were photographed with a Leica DFC295 (Leica Microsystems Ltd, Switzerland) digital camera mounted on a Leica S6D microscope by using the software LAS vs 3.6.0 (Leica Application Suite). Measurements of all the morphological parameters were done on the images with the free software program Image J (http://rsb.info.nih.gov/ij/index.html) (Figure 4.1c,d). Worker size was measured as the thorax width (Goulson *et al.*, 2002) and the total surface of the compound eye (S) was estimated by using the formula of measuring an ellipse surface as described by Jander & Jander (2002). We calculated the diameter of a facet as the mean of a row of 10 facets measured in three dimensions (w, y and z) (Kapustjanskij *et al.,* 2007) and always at the centre of the compound eye (Jander & Jander, 2002). The ommatidia surface, a hexagon, was calculated using the formula $S=3\sqrt{3}/2*z^2$ with z as the radius of the ommatidia. Ommatidia numbers were then estimated by dividing the eye surface with the ommatidia surface. As ommaditia diameter is not uniform across the eye, measurements of the ommatidia number at

the centre of the eye will be an estimate and not the actual ommatidia number. Correlations between morphological characters were tested by the Pearson correlation test in SPSS (version 21.0.0.0) and we also performed sequential Bonferroni corrections for multiple significance tests (Rice, 1989).

4.2.4 *Determination of the critical light sensitivity for flight*

The critical light sensitivity (CLS) is defined as the lowest light intensity at which a worker of a colony is able to fly. This parameter could be measured with use of the bioassay as described by Kapustjanskij *et al.,* (2007) with some small modifications (Figure 4.3). In brief, an individual worker was placed on a platform (9 cm in diameter) and exposed to light. A JC-G4 W/20 lamp positioned at 55 cm above the platform was used and the light intensity was measured at the centre of the platform with a calibrated luxmeter (Taschen-Luxmeter LM37, Karlsruhe, Germany; Figure 4.3). The bees were encouraged to fly with the help of tweezers.

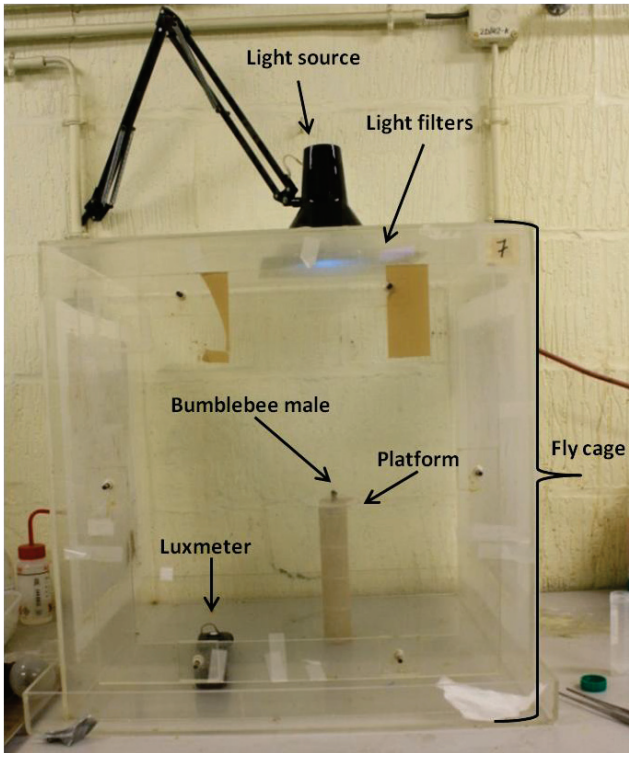


Figure 4.3 Picture of the developed bioassay to determine the critical light sensitivity.

The first evaluation if a bumblebee could fly in a certain light intensity was done at 50 lux. A bumblebee was scored as flying when the bee could lift up from the platform towards the light. We repeated the test 5 times for each light intensity. If a bumblebee could fly at least 3 out of 5 times, the light intensity was lowered. If not, the light intensity was increased until the lowest intensity at which the bumblebee could fly was found. Due to these stepwise measurements, individual bumblebees were measured at different light intensities: 5, 10, 20, 30, 40, 50, 60, 70 and 80 lux. A dimming device (EMD200, Elix) was used to change the light intensity between 0.25 lux (9.3 x 10^{14} photons $m^{-2}s^{-1}$) and 235 lux (2.1 x 10^{18} photons $m^{-2}s^{-1}$). After measuring the CLS for 15-20 workers of each of the 8 bumblebee colonies, the data were logarithm transformed and analyzed with a one-way ANOVA followed by a *post-hoc* Tukey test.

4.3 Results

4.3.1 *Initial nest-leaving capacity of the colonies*

During the experiment we determined F_c and measured F_a^w and F_a^s, while the colonies developed from a workforce of 20 until 99 workers. The colony size increased but the F_a and F_c values stayed constant. Indeed the dividing in classes showed no significant differences (One-way ANOVA, Tukey HSD post hoc tests, $F = 0.415$, d.f. = 88, $P = 0.890$ for F_a^s; One-way ANOVA, $F = 0.610$, d.f. = 88, $P = 0.746$, for F_a^w; One-way ANOVA, $F = 0.803$, d.f. = 88, $P = 0.587$ for F_c). So, colony size did not have an effect on F_a and F_c. The nest-leaving capacity (F_c) was significantly different between the colonies (One-way ANOVA, $F = 3.598$, d.f. = 49, $P = 0.004$; Table 4.1).

Table 4.1 The grouping of the mean and standard error of the nest-leaving capacity (F_c) of each colony.

Colony number	Mean	SE
3	0.616^{a}	0.068
8	0.565^{ab}	0.141
4	0.553^{ab}	0.221
6	0.495^{ab}	0.269
1	0.470^{ab}	0.131
7	0.459^{ab}	0.098
2	0.420^{b}	0.036
5	0.292^{c}	0.066

^{abc} = significance level, $P < 0.05$

Furthermore, the foraging activities in strong and weak light intensity (F_a^s and F_a^w) were also significantly different between the colonies (F_a^s, One-way ANOVA, $F = 6.265$, d.f. = 49, $P = 0.000$ and F_a^w, One-way ANOVA, $F = 4.293$, d.f. = 49, $P = 0.001$).

4.3.2 *Correlations between eye morphology and whole body parameters*

Fifteen to twenty workers were measured per colony and this was done for the 8 colonies. Typically, the parameters of body size correlated significantly with the body mass and the different eye morphology parameters both within and between the colonies (Table 4.2). The only exception was the number of ommatidia as this eye parameter did not correlate with the bumblebee size within all colonies (Table 4.2) and also not between colonies ($r_s = 0.146$, $P = 0.082$; Table 4.2).

Table 4.2 The correlations between the thorax length (as parameter of bumblebee size) and the different morphological parameters of the workers on the intra and inter colony level. With *N* = number of workers tested for each colony and r_s = the correlation coefficient.

Thorax length			Weight	Eye length[1]	Eye width[1]	Eye surface[1]	Facet diameter	Ommatidia number[1]	Ocellus diameter
	Colony	*N*	r_s	r_s	r_s	r_s	r_s	r_s	r_s
Intra colony	1	19	0.888**	0.814**	0.756*	0.860**	0.823**	0.612*	0.469**
	2	16	0.928**	0.810*	0.689*	0.808*	0.549*	0.492	0.700**
	3	20	0.949**	0.936**	0.826**	0.919**	0.810**	0.045	0.766**
	4	15	0.834**	0.593	0.698*	0.733*	0.647*	0.291	0.830**
	5	15	0.893**	0.655*	0.706*	0.721*	0.736**	0.422	0.713**
	6	16	0.676**	0.580	0.701*	0.694*	0.505*	0.530	0.556*
	7	19	0.915**	0.784*	0.709*	0.795*	0.780**	0.379	0.876**
	8	20	0.904**	0.891**	0.900**	0.937**	0.875**	0.201	0.725**
Inter colony	All	143	0.831**	0.730**	0.658**	0.750**	0.694**	0.146	0.681**

With indication of the significance level, ** = $P < 0.01$ and * = $P < 0.05$ and [1] after sequential Bonferroni corrections.

4.3.3 *Determination of the critical light sensitivity for flight and correlations with body size, mass and eye morphology*

The mean CLS of 4 days-old workers (n = 15-20, for each colony) was determined for the 8 colonies and ranged from 6.50 ± 0.91 lux (colony 3) to 15.88 ± 1.91 lux (colony 2) (Table 4.3). Significant differences between colonies were found (One-way ANOVA, $F = 5.731$, d.f. = 142, $P < 0.001$). Due to those significant differences we categorized the colonies as low, medium and high light sensitive colonies (Table 4.3).

Table 4.3 The grouping of the light sensitiveness of each colony. Based on the critical light sensitivity (= CLS) of each colony as the mean of the CLS of the individual workers, with indication of the standard error.

Colony number	CLS (Lux)		
	Category	Mean	SE
3	High [a]	6.50	0.91
8	Medium [ab]	8.70	0.62
1	Medium [abc]	9.21	1.48
6	Medium [abc]	9.38	0.90
7	Medium [abc]	11.37	1.87
4	Medium [bc]	11.47	1.19
5	Medium [bc]	13.22	1.59
2	Low [c]	15.88	1.91

[abc] = significance level, $P < 0.05$

Within a colony the morphological parameters were negatively correlated with the CLS (Table 4.4). But this negative correlation was not significantly present for all colonies tested. Indeed when comparing the means of the different parameters (worker mass, worker size and eye morphology) with the mean critical light sensitivity over the different colonies, we found no significant correlation. The correlation coefficients r_s ranged from -0.057 to 0.614 for weight and facet diameter ($P = 0.894$ and $P = 0.105$, respectively).

Table 4.4 The correlations between the critical light sensitivity (= CLS) and the morphological parameters of the workers of each colony on the intra colony level and the inter colony level. Furthermore, we presented here also the correlation of the morphological parameters and the nest-leaving capacity (*F*c). With *N* = number of workers tested for each colony and r_s = the correlation coefficient.

				Thorax length	Weight	Eye length[1]	Eye width[1]	Eye surface[1]	Facet diameter	Ommatidia number[1]	Ocellus diameter
		Colony number	*N*	r_s	r_s	r_s	r_s	r_s	r_s	r_s	r_s
CLS	Intra colony	1	19	-0.484*	-0.487*	-0.499	-0.528	-0.551	-0.311	-0.612*	-0.356*
		2	16	-0.785**	-0.627**	-0.540	-0.565	-0.591	-0.479*	-0.238	-0.506*
		3	20	-0.504*	-0.452*	-0.497	-0.505	-0.506	-0.363	-0.196	-0.340
		4	15	-0.480*	-0.524*	-0.505	-0.259	-0.443	-0.364	-0.139	-0.472*
		5	15	-0.699**	-0.770**	-0.653*	-0.627*	-0.666*	-0.567*	-0.528	-0.344
		6	16	-0.521*	-0.366	-0.552	-0.63	-0.641	-0.465*	-0.297	-0.357
		7	19	-0.363	-0.292	-0.470	-0.443	-0.463	-0.324	-0.442	-0.424*
		8	20	-0.269	-0.323	-0.325	-0.346	-0.358	-0.245	-0.224	-0.421*
	Inter colony	All	8 col.	0.446	0.614	0.233	0.311	0.316	-0.057	0.340	0.418
F_c	Inter colony	All	8 Col.	-0.274	-0.470	-0.360	-0.202	-0.315	-0.117	-0.457	-0.088

Indication of the significance level, ** = $P < 0.01$ and * = $P < 0.05$ and [1] after sequential Bonferroni corrections.

4.3.4 *Correlation with the nest-leaving capacity and foraging activity*

The only strong significant correlation we found was between the mean CLS and the initial nest-leaving capacity of the colonies (r_s = -0.724, P = 0.042, Figure 4.4). No significant correlation was found between F_a and CLS with r_s = 0.496, P = 0.211 for F_a^s and r_s = -0.194, P = 0.645 for F_a^w.

We checked also for differences between F_c and the mean of the morphological parameters of the workers for each colony. None of those parameters were significantly correlated with F_c, with r_s ranging from -0.470 to -0.088 for weight and ocelli diameter (P = 0.240; P = 0.836, respectively) (Table 4.4).

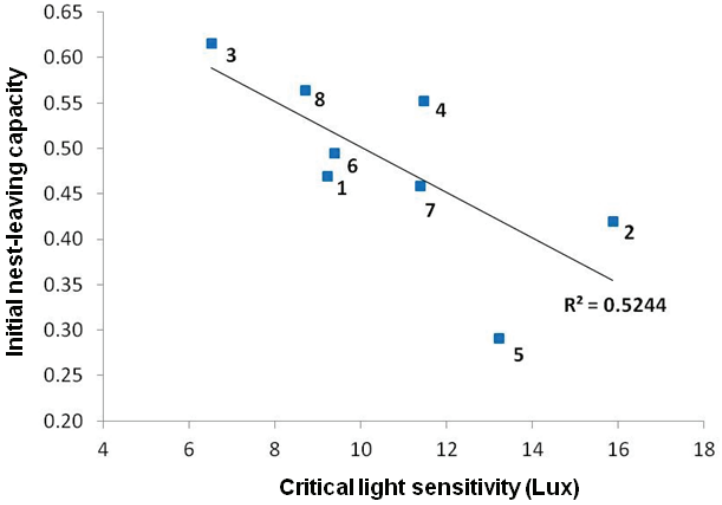


Figure 4.4 Correlation of the nest-leaving capacity (*F*c) with the critical light sensitivity (CLS) of each colony, with indication of the colony number.

4.4 Discussion

Here in this chapter, all eye morphological parameters, except the ommatidia numbers, were positively correlated with bumblebee body size and weight. These results confirmed the correlations of these morphological parameters with body size described by Kapustjanskij *et al.* (2007), for review see Wcislo & Tierney (2009). So both on the intra and inter colony level, we saw that bigger bees have bigger eyes, and these bigger eyes are mainly a consequence of bigger facets and not by an increase in the numbers of ommatidia.

Several studies showed that the morphological parameters of the eye affect the sensitivity in different light conditions as is described for the nocturnal sweat bee *Megalopa genalis* (Warrant *et al.,* 2004, 2006; Kelber *et al.*, 2006), nocturnal and diurnal paper wasps (Warrant *et al.,* 2006), crepuscular bees (Kelber *et al.*, 2006), and Indian carpenter bees (Somanathan *et al.*, 2008, 2009). So, larger bumblebees would have larger eye parameters and should thus have a better light perception. Kapustjanskij *et al.* (2007) described that bigger is better, meaning that bigger bumblebees have bigger eyes and are more light sensitive, which in turns means being able to fly in weaker light conditions. Indeed, looking to the individuals within

one colony our results confirmed this. But between colonies this correlation was lost. It was striking that some colonies containing small bumblebees had a better light perception compared to colonies with bigger specimens (Table 4.4). Thus, within one bumblebee family, size is an important parameter for better light perception. But it should be remarked that improved vision is not only a consequence of improved light perception. Therefore, we expect that between bumblebee families other morphological parameters such as larger photoreceptors (rhabdomeres) or genetic parameters like the molecular capturing of photons, signal transduction and neuron composition can play a more important role as has also been discussed by Warrant (2004) and Kapustjanskij *et al.* (2007).

The ability to capture more light and being able to fly at weak light intensities is a first step towards foraging but it does not necessarily mean that these bumblebees will indeed leave their nest and forage in these conditions. We therefore tested if colonies with different critical light intensities had a different foraging behaviour in changing light conditions. For this we developed a bioassay measuring the number of workers allocated to forage without the presence of a food stimulus. This bioassay measures a subset of the complex behaviour of foraging. In our opinion our assay describes the number of workers that a colony is willing to sacrifice to explore the environment, as these workers are sent out to forage without a reward being present or brought back to the hive. Our bioassay was performed in-house with artificial lighting to exclude other parameters influencing the results. Indeed when placing colonies outside different external parameters are not kept under control. For instance, light conditions are also correlated with temperature.

We determined how our different light sensitive bumblebee colonies (see Table 4.3) are triggered to forage in different light conditions. A striking observation was that colonies, which consisted out of light sensitive bumblebees (which had a low CLS), were not the colonies with a high initial foraging activity in weak light intensity, as F_a^{w} did not correlate with the mean CLS. Thus, the initial foraging activity in weak light intensity is not strictly a consequence of light perception alone. Other parameters such as the intrinsic characteristic to be less reluctant to leave the nest for foraging will also play an important role. We corrected for colony activity by calculating the initial nest-leaving capacity as the ratio of the initial foraging activities of a colony in weak and strong light intensities ($F_c = F_a^w/F_a^s$). When comparing the initial nest-leaving capacities of the colonies with the critical light sensitivity

scores, we showed that both parameters are significantly correlated (r_s = -0.727, P = 0.041, Figure 4.4). Thus, showing that the recruitment to forage of bumblebees in artificial low light is less impaired in light sensitive colonies. Bumblebee size, nor the different morphological parameters of the eye correlated with the initial nest-leaving capacity (F_c).

Our results have important implications for rearing strategies to select for more light sensitive bumblebees. For instance a simple morphology-based selection strategy towards bigger bumblebees will not necessarily results in more light sensitive bumblebees or better foragers in weaker light conditions. Although these bumblebee workers will be better equipped to capture light, other genetic parameters are also crucial for optimal light perception. Further research is needed to identify suitable markers which could be used for the selection of bumblebees towards improved foraging in artificial light.

Chapter V

QTL analysis for light sensitivity, body weight, body size, and morphological eye parameters

Redrafted after:

Maebe, K., Meeus, I., De Riek, J., Smagghe, G. (2015) Quantitative trait loci for light sensitivity, body weight, body size, and morphological eye parameters in the bumblebee, *Bombus terrestris*. PLoS ONE. Submitted, under review.

5.1 Introduction

Bumblebees are essential pollinators in natural and managed ecosystems (Heinrich, 1979; Goulson, 2003). Several bumblebee species, such as the buff-tailed bumblebee *Bombus terrestris* L., are used worldwide in greenhouses for the pollination of different crops (Velthuis & van Doorn, 2006). In the artificial light environment of a greenhouse bumblebees perform better than honeybees (*Apis mellifera*). However, when the artificial light environment of a greenhouse deviates from the natural light environment in intensity and spectral composition, bumblebees also have troubles finding their way back to the colony and have decreased foraging activity (Morandin *et al.,* 2001, Blacquière *et al.,* 2006; 2007; Roman & Szczęsna, 2008, Johansen *et al.,* 2011).

Bumblebee performance in greenhouses with artificial light could be enhanced by selection towards more light sensitive bumblebees. One rearing strategy could be simple morphology-based selection towards bigger bumblebees. Larger bumblebees have bigger eyes which should have better light perception and thus should be more light sensitive (Kapustjanskij *et al.,* 2007; Wcislo & Tierney, 2009). Indeed, an increase in the size of the morphological parameters of the sensory system enhances the ability to detect and discriminate between different flowers which in turn can increase foraging efficiency (Chittka *et al.,* 1999). In chapter 4 we found that at both intra and inter colony levels, larger *B. terrestris* individuals had larger eyes. However, some colonies containing smaller bumblebees also had better light perception compared to colonies with larger specimens. Thus, a large body size did not necessarily correlate with greater light sensitivity or increase foraging efficiency in weak light conditions. Indeed, other morphological parameters, such as larger photoreceptors (rhabdomeres), better molecular photon capture, signal transduction and neuronal composition can play a more important role in optimizing light perception (see chapter 4) as has also been discussed by Warrant (2004) and Kapustjanskij *et al.* (2007).

An alternative strategy could be a marker based selection for more light sensitive bumblebees. For marker-assisted selection (MAS) we need to identify at least one marker linked to the gene or genes responsible for light sensitivity (Dekker, 2004; Williams, 2005). Identification of markers linked with the genes responsible for the phenotypic variation of a certain trait can be determined by quantitative trait loci (QTL) analysis (Slate, 2005; Wilfert *et al.*, 2007a,b). The first step in a QTL analysis is the construction of a genetic linkage map

(Slate, 2005). In social Hymenoptera, like *B. terrestris*, a genetic linkage map can be easily constructed as the queen's meiotic recombination rates can be reliably measured from her male offspring (drones) (Gadau *et al.*, 2001; Wilfert *et al.*, 2006; 2007a,b; Stolle *et al.*, 2011). For *B. terrestris* several linkage maps have already been constructed (Gadau *et al.*, 2001; Wilfert *et al.*, 2006; Stolle *et al.*, 2011). Stolle *et al.* (2011) created a second generation linkage map which showed 18 linkage groups (LGs) with a total length of 2047 cM, representing the 18 chromosomes of haploid bumblebee males (Ayabe *et al.*, 2004). QTLs have been discovered for several important traits related to immune defence, reproduction (Wilfert *et al.*, 2007b), host-parasite interactions and body size of *B. terrestris* (Wilfert *et al*., 2007a).

Here, we performed a QTL analysis on drones of *B. terrestris* to determine QTL regions and to identify markers linked with light sensitivity and body size. To this end, we measured the light sensitivity under both blue and UV light conditions of each drone, as well as body size, body mass and several other morphological parameters of the eye and the hind leg for each individual. Furthermore, we genotyped each drone using 136 microsatellite markers. The QTLs and markers identified here show the first promise to be used in marker assisted breeding to improve selection for light sensitive bumblebees.

5.2 Material and methods

5.2.1 *Mapping population*

For this project we received 10 commercial queenright colonies of *B. terrestris* from a mass-rearing program (Biobest, Westerlo, Belgium). From each colony we randomly selected 10 workers and determined their critical light sensitivity (CLS), the lowest light intensity at which an individual bumblebee is able to fly, as described in chapter 4. From the colony with the most variation in CLS, we selected additional workers with whose we created 4 micro-colonies consisting of 5 workers each. Micro-colonies are nests made of a small group of new-born worker bees. Within 2 days, one worker becomes dominant, i.e. pseudo-queen, and starts laying unfertilized or haploid eggs that develop into drones while the other workers take care of the brood. The pace of colony development follows a well-defined pattern (i.e., time until first oviposition, first larvae developed, and first pupae) for colonies receiving the same diet *ad libitum* (Mommaerts *et al.*, 2010; Blacquière *et al.*, 2012). The 96 drones

produced by these 4 micro-colonies were used for genetic linkage mapping (Figure 5.1). All queenright colonies and micro-colonies were provided with commercial sugar water (BioGluc; Biobest, Westerlo, Belgium) and pollen (Apihurdes, Cáceres, Spain) *ad libitum* in a controlled laboratory environment at 28-30 °C and 60-65 % air humidity and in continuous darkness.

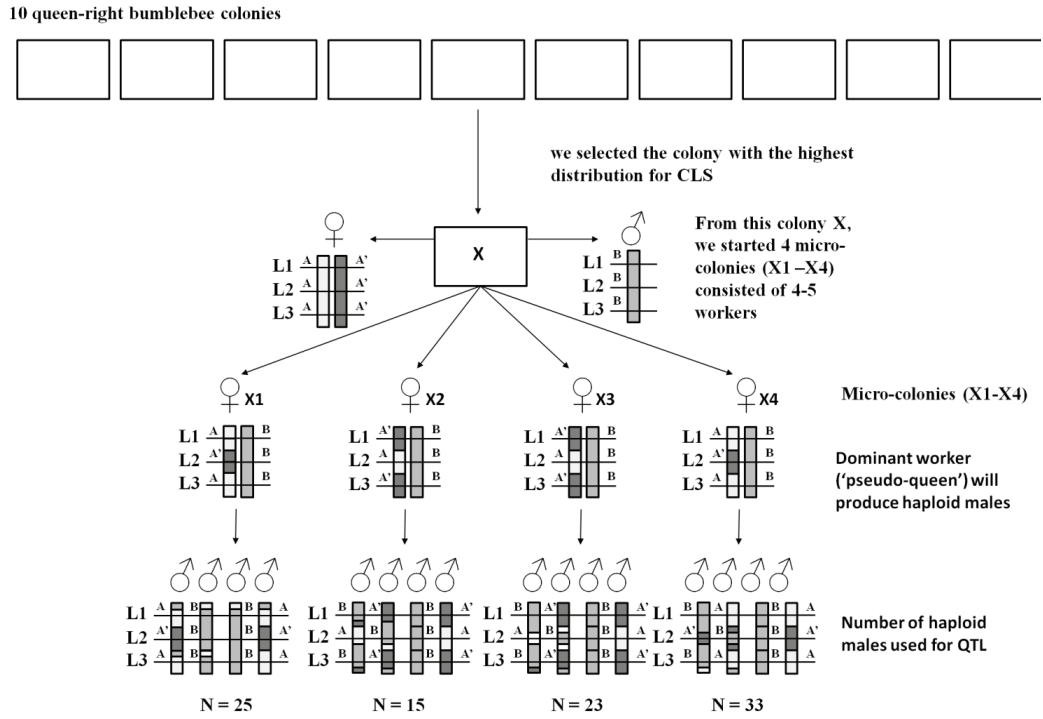


Figure 5.1 Genetic mapping population. From 10 queenright bumblebee colonies we selected 1 colony (X). Four micro-colonies were developed with 4-5 workers of colony X (X1-X4). The unfertilized eggs (haploid males) produced by the 'pseudo-queen' of these micro-colonies were used for the QTL analysis. In addition, the heritability of three hypothetical loci (L1-L3) are shown, base on the maternal alleles (A and A') of the queen in colony X, and the paternal allele B of the drone the queen of colony X has mated with.

5.2.2 *Critical light sensitivity in blue and ultraviolet light*

For each drone we determined, under blue and ultraviolet (UV) light conditions, the lowest

light intensity at which it is able to fly, applying the bioassay for determination of CLS described in Kapustjanskij *et al.* (2007) and in chapter 4, with some small modifications. An individual drone was placed on a platform (9 cm in diameter) and exposed to blue or UV light. For the blue light condition we positioned a JC-G4 W/20 lamp at 55 cm above the platform and in front of the lamp we placed a Tokyo Blue LEE colour filter (Phlippo Showlights, Lier, Belgium) allowing the transmission of light in the blue spectrum (400-500 nm) together with a LEE UV filter (Phlippo Showlights) to ensure no transmission of UV light. For the ultraviolet light condition, we used a Mini-Lynx 20W BL350 lamp (Havells Sylvania, Tienen, Belgium) allowing the transmission of UV light between 315 and 400 nm with a peak at 352 nm. LEE Neutral Density filters of 0.15, 0.3, 0.6, 0.9 and 1.2 (Phlippo Showlights) were used to reduce the light intensity without altering the spectral composition of the light. Light intensities were measured at the centre of the platform with a calibrated luxmeter (Taschen-Luxmeter LM37, Karlsruhe, Germany). When the drone, encouraged to fly with the help of tweezers, could lift up from the platform towards the light, he was scored as "flying". If he could not, the light intensity was increased until we found the lowest intensity at which he was still able to fly. For further analyses, the CLS values were log transformed.

5.2.3 *Morphological characteristics*

For each drone we measured several parameters related to body size and eye morphology as described in chapter 4: total fresh body mass, forewing radial cell length, dorsal-ventral length of compound eye, width of compound eye, total surface of compound eye, diameter of facet, total numbers of ommatidia of compound eye, diameter of median ocellus, length of hind leg, trochanter length, trochanter width, femur length, femur width, tibia length, tibia width, metatarsus length, metatarsus width, and tarsus length.

The right forewing and hind leg of each drone were dissected from the body, taped on a transparent paper, and scanned to allow measurements of the wing and different leg parameters with Image J (Abramoff *et al.,* 2004). The forewing radial cell length was considered as representative for bumblebee size as radial cell length correlates well with head width, body mass and wing length (Gerloff *et al.,* 2003; Owen, 2012).

5.2.4 *Correlations*

Correlations between the different morphological characters were tested by the Spearman correlation test in SPSS (version 22.0.0.0). Instead of the more conservative sequential Bonferroni corrections for multiple significance tests (Rice, 1989), we calculated the false discovery rate by the Benjamini & Hochberg (1995) formula [$P(i) \leq (\alpha * i) / m$], with α being the significance threshold value, m the number of performed tests and i the number of null hypotheses arranged by ascending *P*-values. Instead of the significance threshold of $\alpha = 0.05$, we created with this formulae a 'new threshold value' for rejection of the null hypothesis, and this for the first i-value which has a lower calculated *P*-value than *P*(i). To achieve this, we searched for the first *P*-value which follows this formula. Here, with $\alpha = 0.05$ and m =190, we compared each *P*(i) with 0.05(i)/190, starting from *P*(190). As *P*(156) = 0.034 < (0.05*156)/190, our new significance threshold was 0.041.

For datasets with many correlated traits, multivariate methods, like PCA, are often performed to reduce the dimensionality of the dataset without losing much of the original variation (Choe & Rocheford, 2012). Thereby, the principal components (PCs) can serve as traits in the QTL analysis (Choe & Rocheford, 2012). Here, we performed a PCA for the different body size traits and also for the different eye traits with Primer 6 (Clarke & Gorley, 2006). The PCs with the largest eigenvalues were used for PC-QTL mapping.

5.2.5 *DNA extraction and microsatellites protocol*

Bumblebee DNA was extracted from one middle leg of each drone as described in chapter 2. Bumblebees were genotyped at 131 microsatellite loci developed for *B. terrestris*: 12 loci from Stolle *et al.* (2009), 11 loci from Reber-Funk *et al.* (2006), 106 loci developed from a BAC-library (Wilfert *et al.*, 2009) by Stolle *et al.* (2011), one new locus by Stolle *et al.* (2011) and one locus from Estoup *et al.* (1993; 1995) (Supplementary File S12). Additionally, we used 4 loci derived from *B. lucorum* (Reber-Funk *et al.*, 2006) and one locus from honeybee, *Apis mellifera* (Solignac *et al.,* 2007) (Supplementary File S12). All 136 microsatellite loci, used in this chapter, were already used before to construct a second generation genetic map of *B. terrestris* (Stolle *et al.,* 2011).

For detection of the microsatellite alleles, we used a tailed-primer approach (Schuelke, 2000): a universal M13-primer (= tail, 5'-GAGTTTTCCCAGTCACGAC-3') is coupled to a HEX, 6-FAM, VIC or NED fluorescent label to allow detection of the microsatellite alleles by capillary electrophoreses. Furthermore, for incorporation of this universal tail during PCR, the specific forward primers are prolonged at its 5'-end with the same (but unlabeled) sequence as the tail.

Each microsatellite locus was amplified in simplex by PCR. PCR reactions were carried out in 10 µl total volume. Each reaction contained 1.5 µl template DNA, 1 µl of 10x PCR buffer (Qiagen), 0.2 µl of 10 mM dNTP's (Qiagen), 0.1 µl of 10 µM forward primer, 0.4 µl of 10 µM reverse primer, 0.4 µl of 10 µM labeled M13-primer and 0.05 µl of 2.5 units/reaction Hotstar Taq DNA Polymerase (Qiagen). Samples were initially denatured at 95 °C for 15 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 48, 52 or 58 °C for 30 s, and extension at 72 °C for 30 s. The PCR protocol ended with a final extension step at 72 °C for 10 min. After pooling the final PCR products, they were visualized on a ABI-3730xl sequencer (Applied Biosystems) using an internal size standard (Genescan 500 LIZ, Applied Biosystems). The fragments were examined and scored manually using Peak Scanner Software v 1.0 (Applied Biosystems).

5.2.6 *Linkage mapping and phase determination*

First, a preliminary linkage mapping was established using 100 microsatellite loci with Kosambi's mapping function. These loci were chosen based on their known distribution on the 18 linkage groups described in Stolle *et al.* (2011) to obtain an as high as possible cover of the bumblebee genome. The mean number of markers on each linkage group was 5.55 (range: 2 - 9), with the minimum and maximum distance between two markers ranging between 2.72 cM and 65.56 cM (Supplementary Table S13). After identifying different QTL regions with this mapping on 16 linkage groups (LG), we conducted a fine mapping with 36 additional SSR markers, specifically chosen to cover better the preliminary QTL regions. Furthermore, knowing that the bumblebee genome size is 2047.09 cM (Stolle *et al.*, 2011), a power estimation of 136 markers based on the formula $c = 1\text{-}e^{-2md/L}$ with m = number of markers, d = distance between markers (in cM), L = genome length and c = proportion of the genome within this distance d, as described in (Lange & Boehnke, 1982) and used in ref.

Stolle *et al.* (2011), showed that 93.0% of the bumblebee genome is at average located within 20 cM of a marker and 73.5% within 10 cM of a marker.

Linkage analysis was performed with JoinMap software version 4.0 (Van Ooijen, 2006). Linkage groups were estimated by applying independent Logarithm of the Odds (LOD) threshold ranges from 1.0 to 10.0 in steps of 1.0. The initial grouping for mapping was selected from the groupings tree, preferentially by taking (smaller) nodes that showed a stable number of markers at the higher LOD score. We preferred to start from smaller but highly stable linkage groups. Regression linkage maps were established under the standard calculation settings of JoinMap 4.0 (linkages with a recombination frequency smaller than 0.45 and LOD higher than 1; goodness-of-fit jump threshold for removal of loci 5 and performing a ripple after adding one locus). The order of the SSR-markers in our grouping was compared with their order in the second generation linkage map constructed on 577 males of one *B. terrestris* colony as described by Stolle *et al.* (2011). Linkage phases were then estimated by JoinMap 4.0.

5.2.7 *QTL analysis*

First, we performed the Kruskal-Wallis (KW) test, a single marker non-parametric method imbedded in the software program MapQTL5.0 (Van Ooijen, 2004) to detect possible QTL's as is done in several other studies (e.g., Moghaddam *et al.,* 2012; De Keyser *et al.,* 2013). Secondly, we performed a composite Interval Mapping analysis (IM) with MapQTL 5 (Van Ooijen, 2004). The LOD thresholds for declaring a linkage group wide significant QTL were obtained by standard permutation tests (1000 iterations) with MapQTL 5.0 (Van Ooijen, 2004) for the significance level $p = 0.05$ and $p = 0.01$. This permutation test reduces the environmentally-induced variation. Third, we performed also a multiple QTL model mapping (MQM) within MapQTL 5.0. The selection of obtained QTLs in IM were used as cofactors during MQM-mapping, which allowed for the detecting of additional QTLs (Wilfert *et al.,* 2007a). When the LOD value of the QTL, assigned as cofactor, dropped during the MQM mapping below the threshold value, then the QTL was removed as cofactor and MQM was run again. We repeated this procedure until the list of cofactors remained stable. For both IM and MQM, the traits need to follow a normal distribution. Most traits were significantly different from normality (Supplementary Table S14). However, the Box-Cox transformation

had none or only very small effects on the size of the observed QTL regions. For the graphical presentation of the QTLs and markers we employed the software MapChart version 2.2 (Voorrips, 2006).

5.2.8 *Identification of candidate genes*

Candidate genes for light sensitivity were selected around the 95% confidence interval (= C.I.) of the QTL. The two SSR markers which determined the 95% C.I. of the QTL, were found in the bumblebee genome (http://www.ncbi.nlm.nih.gov/genome/2739) and all genes on this sequence (± 500k bp) were selected as candidate genes. We searched in UniProt (http://www.uniprot.org/) for the known function of those candidate genes, and selected the candidate gene which function could be directly linked with vision or light perception as primary target gene.

5.3 Results

5.3.1 *Correlation between traits*

In total, 96 drones were measured for 20 different traits (Table 5.1). The distribution of each of these traits can be seen in Supplementary File S15. There were no indications of significant colony effects (for all traits: Kruskal-Wallis test, $P > 0.05$).

Most morphological parameters of the leg and the body size correlated significantly with body mass and the different eye morphology parameters (Table 5.2). The only two exceptions were: (i) the number of ommatidia did not correlate with facet diameter (r_s = -0.171, P = 0.098); and (ii) body mass did not correlate with tibia length and width (r_s = 0.156, P = 0.128; r_s = 0. 207, P = 0.043, respectively), femur width (r_s = 0.146, P = 0.157), and both the trochanter length and width (r_s = 0.088, P = 0.395; r_s = -0.020, P = 0.846, respectively). Furthermore, we detected no correlation between birth order of the males and both bumblebee body size and body weight.

The mean critical light sensitivity (CLS), being the lowest light intensity at which a bumblebee is able to fly, of 4 days-old drones (n = 96) in blue and UV light conditions was 3.58 ± 2.89 lux and 1.73 ± 0.47 lux, respectively (Table 5.1).

Table 5.1 Means (± S.D.), skewness and kurtosis of the investigated traits.

	Code	N	Mean	±SD	Skewness	Kurtosis
Radial cell (cm)	RC	95	0.319	0.035	-0.449	-0.511
Metatarsus length (cm)	MT_L	96	0.285	0.038	-0.564	-0.273
Metatarsus width (cm)	MT_W	96	0.090	0.012	-0.096	0.211
Tibia length (cm)	Ti_L	96	0.429	0.054	-0.705	-0.037
Tibia width (cm)	Ti_W	96	0.119	0.018	0.020	-0.527
Femur length (cm)	Fe_L	96	0.372	0.056	-0.640	-0.276
Femur width (cm)	Fe_W	96	0.124	0.063	5.993	43.288
Trochanter length (cm)	Tr_L	96	0.067	0.012	-0.030	-0.295
Trochanter width (cm)	Tr_W	96	0.091	0.021	-0.889	2.889
Tarsus length (cm)	Tarsus	92	0.585	0.079	-0.665	-0.323
Leg length (cm)	Leg	92	1.452	0.187	-0.619	-0.423
Eye length (mm)	E_L	95	2.554	0.214	-0.994	0.542
Eye width (mm)	E_B	95	1.080	0.088	-1.199	1.298
Facet length (mm)	Facet	94	0.025	0.002	-0.161	-0.052
Median ocellus (mm)	MOc	94	0.279	0.031	-0.436	-0.430
Eye surface (mm^2)	E_S	95	2.180	0.340	-0.974	0.479
Ommatida number	Om	94	5587	760.7	0.695	1.177
Dry weight (g)	Weight	96	0.211	0.064	0.038	-0.314
CLS under blue light*	CLS_Blue	96	0.431	0.317	0.278	-0.834
CLS under UV light*	CLS_UV	96	0.223	0.117	0.248	-0.454

* after log transformation

As light sensitivity could be linked with size parameters (Kapustjanskij *et al.*, 2007; Wcislo & Tierney, 2009; see also chapter 4), we searched for correlations between different parameters of bumblebee body size, eye and hind leg with CLS. For most of these morphological parameters we found no significant correlation with the CLS in blue or UV conditions ($P > 0.041$). The CLS in blue and UV light conditions correlated only with the metatarsus length ($r_s = -0.228$, $P = 0.025$; $r_s = -0.218$, $P = 0.033$; respectively), the metatarsus width ($r_s = -0.227$, $P = 0.026$; $r_s = -0.265$, $P = 0.009$; respectively), and the tibia width ($r_s = -0.238$, $P = 0.020$; $r_s = -0.241$, $P = 0.018$; respectively). Furthermore, CLS in blue light sensitivity correlated also with the tarsus length ($r_s = -0.221$, $P = 0.034$; Table 5.2).

Table 5.2 Correlation coefficients between the investigated traits.

		RC	MT_L	MT_W	Ti_L	Ti_W	Fe_L	Fe_W	Tr_L	Tr_W	Tarsus	Leg	E_L	E_W	Facet	MOc	E_S	Om	Weight	CLS_Blue	CLS_UV
RC	r	-																			
MT_L	r	0.891**	-																		
MT_W	r	0.753**	0.811**	-																	
Ti_L	r	0.837**	0.897**	0.835**	-																
Ti_W	r	0.679**	0.727**	0.827**	0.860**	-															
Fe_L	r	0.794**	0.878**	0.778**	0.868**	0.758**	-														
Fe_W	r	0.760**	0.783**	0.732**	0.808**	0.763**	0.792**	-													
Tr_L	r	0.412**	0.439**	0.453**	0.504**	0.450**	0.397**	0.391**	-												
Tr_W	r	0.572**	0.559**	0.505**	0.597**	0.462**	0.461**	0.592**	0.455**	-											
Tarsus	r	0.875**	0.948**	0.827**	0.915**	0.758**	0.877**	0.766**	0.456**	0.552**	-										
Leg	r	0.859**	0.938**	0.855**	0.956**	0.826**	0.939**	0.824**	0.518**	0.554**	0.963**	-									
E_L	r	0.766**	0.829**	0.766**	0.798**	0.703**	0.825**	0.707**	0.455**	0.471**	0.836**	0.859**	-								
E_W	r	0.744**	0.781**	0.699**	0.741**	0.606**	0.744**	0.717**	0.389**	0.439**	0.750**	0.776**	0.807**	-							
Facet	r	0.565**	0.579**	0.456**	0.571**	0.530**	0.595**	0.627**	0.316**	0.405**	0.563**	0.598**	0.587**	0.563**	-						
MOc	r	0.790**	0.847**	0.785**	0.802**	0.657**	0.833**	0.749**	0.378**	0.508**	0.846**	0.864**	0.779**	0.759**	0.502**	-					
E_S	r	0.786**	0.842**	0.766**	0.803**	0.690**	0.828**	0.738**	0.443**	0.456**	0.830**	0.859**	0.958**	0.931**	0.596**	0.802**	-				
Om	r	0.443**	0.478**	0.514**	0.422**	0.341**	0.402**	0.305**	0.237*	0.222*	0.487**	0.467**	0.541**	0.552**	-0.171	0.488**	0.564**	-			
Weight	r	0.313**	0.280**	0.293**	0.156	0.207	0.260*	0.146	0.088	-0.020	0.276**	0.266*	0.339**	0.326**	0.221*	0.291**	0.349**	0.258*	-		
CLS_Blue	r	-0.172	-0.228*	-0.227*	-0.157	-0.238*	-0.086	-0.166	-0.126	-0.042	-0.221*	-0.182	-0.143	-0.082	-0.183	-0.164	-0.112	0.073	-0.398**	-	
CLS_UV	r	-0.169	-0.218*	-0.265*	-0.128	-0.241*	-0.080	-0.165	-0.004	-0.096	-0.202	-0.141	-0.192	-0.145	-0.107	-0.191	-0.163	-0.073	-0.388**	0.829**	-

**** Correlation is significant at the 0.01 level (2-tailed) after corrections following Benjamini and Hochberg (1995), with as new *P* = 0.008.**

*** Correlation is significant at the 0.05 level (2-tailed)) after corrections following Benjamini and Hochberg (1995), with as new *P* = 0.041.**

5.3.2 *QTL analysis*

Of the 136 SSR markers, 111 were polymorphic across our population (Supplementary File S12). By composite interval mapping (IM) we found 88 QTLs for 19 of the 20 traits evaluated (Table 5.3), with the only exception being for the CLS under UV light conditions. Individual QTLs accounted for 7.5-53.3% of the phenotypic variation and were distributed in 16 LGs (Table 5.3, Figure 5.2). We found one QTL for CLS in blue light conditions (*qBLU3*) explaining 10.6% of the genotypic variation, seven QTLs for body mass, five QTLs for radial cell length, 12 QTLs for eye traits, and 7 QTLs for leg traits (Table 5.3). Of those 88 QTLs significant at the LG specific significance level of 0.05 %, 34 QTLs were also significant at the 0.01% LG specific significance level (Table 5.3).

When considering the 19 traits for which we found a QTL with IM, 15 traits had at least 1 QTL with multiple QTL model mapping (MQM). Indeed, with the MQM mapping we identified 29 and 20 QTLs significant at the LG specific significance level of 0.05% and 0.01%, respectively (Table 5.3). These QTLs, distributed in 7 LGs, explained 6.7-41.2% of the phenotypic variation. For the CLS under blue light conditions we found one significant QTL explaining 8.7% of the genotypic variation, while for CLS under UV light we found no significant QTL. For body mass of drones we found three significant QTLs (*qDWE6*, *qDWE10* and *qDWE15*) while for the length of the radial cell we found only two significant QTLs (*qRAC1* and *qRAC15.2*), cumulatively explaining 40.7% and 23.8% of the phenotypic variation, respectively. With MQM, we detected 2 or 3 significant QTLs for most of the eye traits: for the dorsal-ventral length (*qEYL1.1*, *qEYL9*, *qEYL15.2*), width (*qEYW1.3*, *qEYW9*) and total surface of the compound eye (*qEYS1.1*, *qEYS9*, *qEYS15.2*), the amount of ommatidia of a compound eye (*qONN3.2*, *qONN9*), and the diameter of median ocellus (*qMOc9*, *qMOc15.2*) cumulatively explaining 40.1%, 57.2%, 33.9%, 46.4% and 23.8% of the phenotypic variation, respectively.

Table 5.3 List of identified QTL's with IM and/or MQM ranked by trait and linkage group (LG), with the respective Kruskal-Wallis significance level and the closest marker useful for Marker Assisted Breeding.

	Name	Location			IM				MQM				Mean allelic value	
Trait	QTL	LG	Closest marker	KW[a]	LOD[b]	R^2	0.05[c]	0.01[c]	LOD[a]	R^2	0.05[d]	0.01[d]	A[e]	B[e]
Radial cell	qRAC1	1	0801_67f8	****	2.41	20.1	0.0-17.9	0.0-17.9	2.52	9.4	10.4-10.4	10.4-10.4	0.30	0.33
	qRAC6	6	0810_65a23	**	2.26	38.7	8.4-15.4	-	-	-	-	-	0.34	0.32
	qRAC7	7	0607_19k14	***	1.57	7.5	84.4-84.5	-	-	-	-	-	0.34	0.31
	qRAC15.1	15	BTMS0103	***	2.06	9.6	10.8-12.6	-	-	-	-	-	0.30	0.33
	qRAC15.2	15	0583_22I4	****	3.18	16.4	80.8-96.6	94.2-96.6	3.47	14.4	90.2-96.6	96.6-96.6	0.34	0.32
Metatarsus length	qMTL1	1	0801_g7f8	**	2.06	16.7	0.0-14.9	-	3.41	14.1	10.4-10.4	10.4-10.4	0.28	0.30
	qMTL6	6	0810_65a23	**	2.42	37.5	8.37-18.4	-	3.09	20.1	27.4-31.8	27.4-31.8	0.32	0.29
	qMTL15.1	15	BTMS0103	*****	3.07	13.7	9.48-14.6	10.8-12.6	-	-	-	-	0.27	0.30
	qMTL15.2	15	0583_22I4	***	2.12	10.2	88.8-96.6	-	-	-	-	-	0.26	0.29
Metatarsus width	qMTW6	6	0810_65a23	*****	2.98	33.4	4.09-33.0	8.37-30.4	2.74	22.0	27.4-31.8	27.4-30.4	0.10	0.09
	qMTW9	9	0553_18c8	*****	1.99	15.6	47.6-53.3	-	-	-	-	-	0.10	0.09
	qMTB10	10	BTMS0129	**	1.79	12.0	12.2-19.2	-	-	-	-	-	0.10	0.09
	qMTL15	15	0583_22I4	***	1.81	8.7	96.2-96.6	-	-	-	-	-	0.08	0.09
Tibia length	qTIL15.1	15	BTMS0103	**	1.84	9.4	10.8-12.6	-	-	-	-	-	0.41	0.44
	qTIL15.2	15	0583_22I4	***	2.10	9.7	89.8-96.6	-	2.10	9.7	91.2-96.6	-	0.38	0.43
Tibia width	qTIW6	6	0810_65a23	****	2.73	53.3	11.4-34.0	15.4-19.4	2.39	17.8	27.4-31.8	-	0.13	0.12
	qTIW13	13	0244_81I8	***	1.54	15.5	16.5-21.1	-	-	-	-	-	0.13	0.12
Femur length	qFML7	7	0607_19k14	*****	2.19	13.3	73.7-85.5	80.4-84.5	2.17	13.0	75.4-86.5	76.4-85.5	0.41	0.36
	qFML9	9	0553_18c8	*****	2.35	22.4	46.6-56.9	49.6-49.6	-	-	-	-	0.41	0.36
	qTIL15	15	BTMS0103	***	2.15	12.3	11.6-12.6	-	2.30	15.8	10.9-14.6	-	0.35	0.38
Femur width	qFMW11	11	0930_40o1	***	2.03	9.3	70.9-70.9	-	2.03	9.3	70.9-70.9	-	0.11	0.12

Trait	Name	Location			IM				MQM				Mean allelic value	
	QTL	LG	Closest marker	KW[a]	LOD[b]	R^2	0.05[c]	0.01[c]	LOD[a]	R^2	0.05[d]	0.01[d]	A[e]	B[e]
Trochanter length	qTRL6	6	0810_65a23	****	2.89	35.1	13.4-33.0	-	2.61	23.4	27.4-31.8	27.4-30.8	0.08	0.07
Trochanter width	qTRW1	1	0196_69p16	**	1.54	7.8	69.4-70.7	-	1.54	7.8	69.4-70.7	-	0.10	0.09
Tarsus length	qTAR1.1	1	0801_g7f8	**	1.99	18.8	13.7-16.9	-	-	-	-	-	0.55	0.60
	qTAR1.2	1	0196_69p16	***	1.97	11.8	67.5-71.7	-	2.94	41.2	67.5-82.3	75.6-82.0	0.62	0.57
	qTAR6	6	0810_65a23	***	3.38	49.0	7.37-12.4	-	-	-	-	-	0.64	0.58
	qTAR9	9	0553_18c8	****	2.09	19.2	46.6-55.3	48.6-53.3	3.47	15.4	51.1-52.3	51.1-52.3	0.63	0.57
	qTAR10	10	BTMS0129	*****	2.16	13.1	15.4-19.2	-	-	-	-	-	0.63	0.57
	qTAR15.1	15	BTMS0103	******	2.88	14.5	9.48-13.6	10.8-12.6	2.88	16.3	9.85-13.6	9.85-13.6	0.54	0.60
	qTAR15.2	15	0583_22I4	****	1.78	8.8	94.2-96.6	-	-	-	-	-	0.51	0.59
Leg length	qLEG6	6	0810_65a23	****	3.35	48.9	9.37-11.4	-	-	-	-	-	1.59	1.43
	qLEG15.1	15	BTMS0103	****	2.40	13.6	9.85-13.6	-	-	-	-	-	1.35	1.49
	qLEG15.2	15	0583_22I4	****	1.95	9.5	94.2-96.6	-	-	-	-	-	1.28	1.45
Eye length	qEYL1.1	1	0801_g7f8	**	2.43	19.5	0.0-18.9	12.7-14.9	3.89	14.3	10.4-10.4	10.4-10.4	2.46	2.61
	qEYL1.2	1	0360_2n11	***	2.97	31.7	27.7-31.7	27.7-30.7	-	-	-	-	2.40	2.57
	qEYL3	3	0795_67k24	****	2.00	10.4	42.2-62.6	-	-	-	-	-	2.69	2.54
	sEYL8	8	0869_70d5	**	2.30	16.6	65.0-87.2	-	-	-	-	-	2.47	2.60
	qEYL9	9	0553_18c8	*****	2.38	21.7	41.0-58.9	45.6-50.4	3.61	17.3	51.1-52.3	51.1-52.3	2.70	2.53
	qEYL15.1	15	BTMS0103	**	2.22	11.3	10.8-12.6	-	-	-	-	-	2.46	2.60
	qEYL15.2	15	0583_22I4	****	2.44	11.4	93.2-96.6	-	2.71	8.5	92.2-96.6	-	2.35	2.56
Eye width	qEYW1.1	1	0801_g7f8	*	2.06	16.5	0.0-18.9	13.7-15.9	-	-	-	-	1.04	1.10
	qEYW1.2	1	0360_2n11	**	2.92	31.4	27.7-33.7	27.7-32.7	-	-	-	-	1.02	1.08
	qEYW1.3	1	0196_69p16	******	2.81	16.0	60.5-77.3	62.5-75.6	4.00	32.3	61.5-82.3	62.4.5-81.0	1.13	1.06
	qEYW8	8	0869_70d5	***	2.75	18.8	63.0-91.6	-	-	-	-	-	1.04	1.10
	qEYW9	9	0152_56e6	**	2.28	19.3	38.0-51.1	44.6-47.6	3.86	24.9	35.0-49.6	36.0-49.6	1.04	1.10

Trait	Name	Location			IM				MQM				Mean allelic value	
	QTL	LG	Closest marker	KW[a]	LOD[b]	R^2	0.05[c]	0.01[c]	LOD[a]	R^2	0.05[d]	0.01[d]	A[e]	B[e]
	qEYW15.1	15	BTMS0103	****	2.54	12.3	9.85-13.6	11.6-12.6	-	-	-	-	1.04	1.10
	qEYW15.2	15	0583_22I4	******	2.04	9.6	91.2-96.6	-	-	-	-	-	0.99	1.08
Facet length	qFAC11	11	0930_40o1	****	2.14	11.3	70.9-77.1	-	1.99	9.7	70.9-71.9	-	0.02	0.02
	qFAC15	15	BTMS0103	****	1.86	9.7	10.8-13.6	11.6-11.6	-	-	-	-	0.02	0.02
Median Ocellus	qMOc1	1	0360_2n11	*	2.25	29.6	28.7-31.7	-	-	-	-	-	0.26	0.28
	qMOC2	2	0956_26c17	***	1.68	12.9	0.0-17.6	-	-	-	-	-	0.29	0.27
	qMOc5.1	5	0357_2o10	**	1.59	8.5	6.47-8.57	-	-	-	-	-	0.31	0.28
	qMOc5.2	5	0216_63a9	***	1.69	11.0	29.8-37.4	-	-	-	-	-	0.29	0.27
	qMOc6	6	0810_65a23	**	3.08	45.9	6.37-21.4	-	-	-	-	-	0.29	0.28
	qMOc7	7	0607_19k14	******	2.47	13.4	73.7-86.5	83.4-84.5	-	-	-	-	0.30	0.27
	sMOc8	8	0627_20n22	*	1.56	12.2	77.2-82.2	-	-	-	-	-	0.29	0.28
	qMOc9	9	0553_18c8	****	2.90	25.9	39.0-51.1	46.6-46.6	2.54	17.1	51.1-52.3	-	0.30	0.27
	qMOc12	12	0867_70k14	***	1.78	13.9	37.2-42.0	-	-	-	-	-	0.30	0.28
	qMOc13	13	BL16	****	1.91	13.7	0.0-19.1	10.1-12.7	-	-	-	-	0.30	0.27
	qMOc15.1	15	BTMS0103	***	1.82	8.5	11.6-11.6	-	-	-	-	-	0.26	0.28
	qMOc15.2	15	0583_22I4	******	1.84	9.0	96.2-96.6	-	1.77	6.7	96.6-96.6	-	0.25	0.28
Eye surface	qEYS1.1	1	0801_g7f8	**	2.24	19.0	0.0-17.9	-	3.54	11.7	10.4-10.4	10.4-10.4	2.04	2.26
	qEYS1.2	1	0360_2n11	**	3.19	34.4	27.7-32.7	27.7-31.7	-	-	-	-	1.94	2.20
	qEYS1.3	1	0196_69p16	**	2.24	13.3	64.5-72.7	-	-	-	-	-	2.35	2.11
	sEYS8	8	0627_20n22	**	2.63	18.7	60.0-91.6	61.0-91.6	-	-	-	-	2.37	2.20
	qEYS9	9	0553_18c8	****	2.53	22.6	38.0-57.9	45.6-49.6	3.52	13.5	51.1-52.3	51.1-52.3	2.40	2.14
	qEYS15.1	15	BTMS0103	****	2.48	12.5	10.8-13.6	-	-	-	-	-	2.02	2.25
	qEYS15.2	15	0583_22I4	*****	2.44	11.4	91.2-96.6	-	2.68	8.7	96.6-96.6	96.6-96.6	1.84	2.19
Ommatida number	qOMN1	1	0360_2n11	***	2.39	23.1	27.7-31.7	-	-	-	-	-	5132.62	5655.78

Trait	Name QTL	Location LG	Closest marker	KW[a]	IM LOD[b]	R^2	0.05[c]	0.01[c]	MQM LOD[a]	R^2	0.05[d]	0.01[d]	Mean allelic value A[e]	B[e]
	qOMN3.1	3	0365_7n6	****	2.50	20.2	40.2-65.6	45.3-63.6	-	-	-	-	6077.68	5509.39
	qOMN3.2	3	0207_63e15	*****	2.15	13.2	88.2-96.4	89.2-96.4	2.62	21.0	78.5-87.3	80.5-87.3	5254.83	5772.37
	qOMN4	4	0304_9i13	****	2.71	35.7	63.1-80.7	64.7-80.7	-	-	-	-	6344.49	5559.78
	qOMN6.1	6	0810_65a23	****	2.41	26.4	20.4-30.4	-	-	-	-	-	6108.91	5517.56
	qOMN6.2	6	0725_82m14	****	2.39	26.1	73.4-87.8	-	-	-	-	-	5233.04	5767.28
	qOMN7	7	0338_2i5	***	1.75	16.5	113.4-132.2	-	-	-	-	-	6167.46	5545.42
	qOMN9	9	0553_18c8	*****	3.42	24.6	44.6-67.4	47.6-64.4	2.48	16.7	52.3-53.3	53.3-53.3	6142.32	5461.04
	qOMN12	12	0867_70k14	******	2.57	18.8	38.9-46.5	39.9-45.5	-	-	-	-	6243.95	5526.64
	qOMN13	13	BL16	****	2.18	19.6	0.0-13.8	0.0-11.7	-	-	-	-	6163.92	5522.71
	qOMN14	14	0655_82m17	****	2.12	10.7	52.0-56.9	-	-	-	-	-	5319.78	5803.45
	qOMN17	17	0608_19h1	*	1.10	8.4	46.4-57.6	-	-	-	-	-	5435.19	5773.70
CLS _ blue light	qBLU3	3	BT08	***	1.96	10.6	12.3-25.3	-	1.89	8.7	12.3-12.9	-	0.31	0.53
Dry weight	qDWE2	2	0956_26c17	**	1.74	18.3	20.6-23.5	-	-	-	-	-	0.24	0.20
	qDWE3	3	0795_67k24	****	2.27	18.0	39.2-56.0	40.2-53.0	-	-	-	-	0.26	0.20
	qDWE5	5	0357_2o10	*	1.50	9.2	4.47-8.57	-	-	-	-	-	0.27	0.21
	qDWE6	6	0810_65a23	*****	3.28	26.1	17.4-34.0	24.4-33.0	3.58	18.4	27.4-31.7	27.4-31.7	0.27	0.20
	qDWE9	9	0553_18c8	****	3.00	20.8	46.6-57.9	48.6-56.9	-	-	-	-	0.25	0.20
	qDWE10	10	BT20	******	2.07	14.5	103.6-126.5	-	2.47	8.1	116.0-116.0	-	0.18	0.23
	qDWE15	15	BTMS0103	****	3.05	14.1	9.85-13.6	10.8-11.6	3.87	14.2	9.85-14.6	10.8-13.6	0.18	0.23
Eye_PCA_1	qEPC1_1.1	1	0801_67f8	***	2.59	12.2	0.0-16.9	0.0-14.9	3.57	14.4	10.4-10.4	10.4-10.4	-0.75	0.36
	qEPC1_1.2	1	0360_2n11	*	1.68	9.2	26.8-27.7	-	-	-	-	-	-0.53	0.11
	qEPC1_7	7	BL05	**	1.48	7.3	152.7-157.4	-	-	-	-	-	-0.61	0.28
	qEPC1_9	9	0152_56e6	**	1.91	9.0	39.0-44.6	42.6-42.6	9.51	41.5	33.2-49.6	33.2-47.6	-0.82	0.26
Eye_PCA_2	qEPC2_6	6	0281_20d1	****	2.59	16.5	35.0-48.2	-	-	-	-	-	0.28	-0.17

	Name	Location			IM				MQM				Mean allelic value	
Trait	QTL	LG	Closest marker	KW[a]	LOD[b]	R^2	0.05[c]	0.01[c]	LOD[a]	R^2	0.05[d]	0.01[d]	A[e]	B[e]
	qEPC2_7	7	0338_2i5	**	2.03	21.6	105.1-146.2	114.4-117.9	1.86	18.5	136.1-141.1	-	-0.35	0.05
	qEPC2_12	12	0867_70k14	*****	3.68	18.9	34.4-46.5	36.2-45.5	3.06	22.7	39.9-44.5	41.1-44.5	-0.72	0.11
Size_PCA_1	qSPC1_6	6	0810_65a23	**	2.47	36.0	8.37-15.4	-	-	-	-	-	-1.27	0.25
	qSPC1_10	10	BTMS0129	***	1.62	7.5	18.2-18.2	-	-	-	-	-	-1.25	0.35
	qSPC1_15.1	15	BTMS0103	**	1.65	7.6	11.6-11.6	-	-	-	-	-	1.07	-0.34
	qSPC1_15.2	15	0583_22I4	**	1.74	8.6	94.2-96.5	-	1.74	8.6	96.5-96.5	-	1.92	0.08
Size_PCA_4	qSPC4_3	3	0795_67k24	**	2.06	10.6	41.2-55.0	-	1.80	7.6	42.2-48.2	-	-0.54	0.12
	qSPC4_15	15	0222_63d21	*******	2.39	10.8	36.9-49.8	-	13.4	47.2	36.9-68.7	42.2-68.7	0.70	-0.11
Size_PCA_5	qSPC5_13	13	0071_59g6	**	-	-	-	-	1.93	8.8	91.7-93.7	91.7-93.7	-0.26	0.10
	qSPC5_18	18	0187_69g1	**	1.23	8.3	27.0-51.0	-	4.60	34.8	45.0-46.0	45.0-46.0	-0.12	0.18

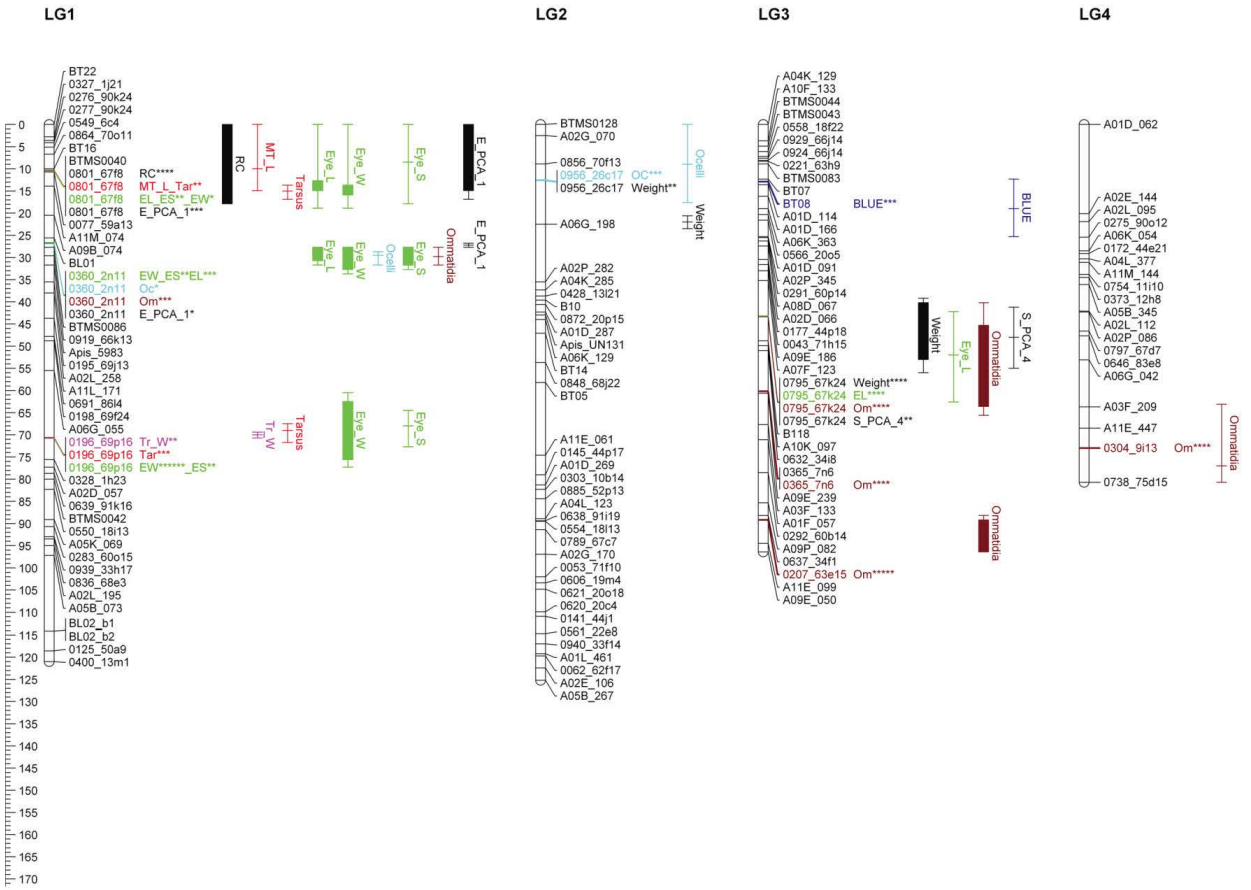
[a]Kruskal Wallis significance levels: * = 0.1, ** = 0.05, * = 0.01, **** = 0.005, ***** = 0.001, ****** = 0.0005 and ******* = 0.0001.**

[b]LOD-scores higher than the LG specific 0.05% LOD-threshold indicates a significant QTL.

[c]The with composite interval mapping (IM) detected QTL interval under linkage group wide significant levels of $p = 0.05$ and $p = 0.01$.

[d]The with multiple QTL model mapping (MQM) detected QTL interval under linkage group wide significant levels of $p = 0.05$ and $p = 0.01$.

[e]The mean allelic value of allele 'A' refers to the mean phenotypic value for the maternal allele (A or A') and allele 'B' for the paternal allele, respectively.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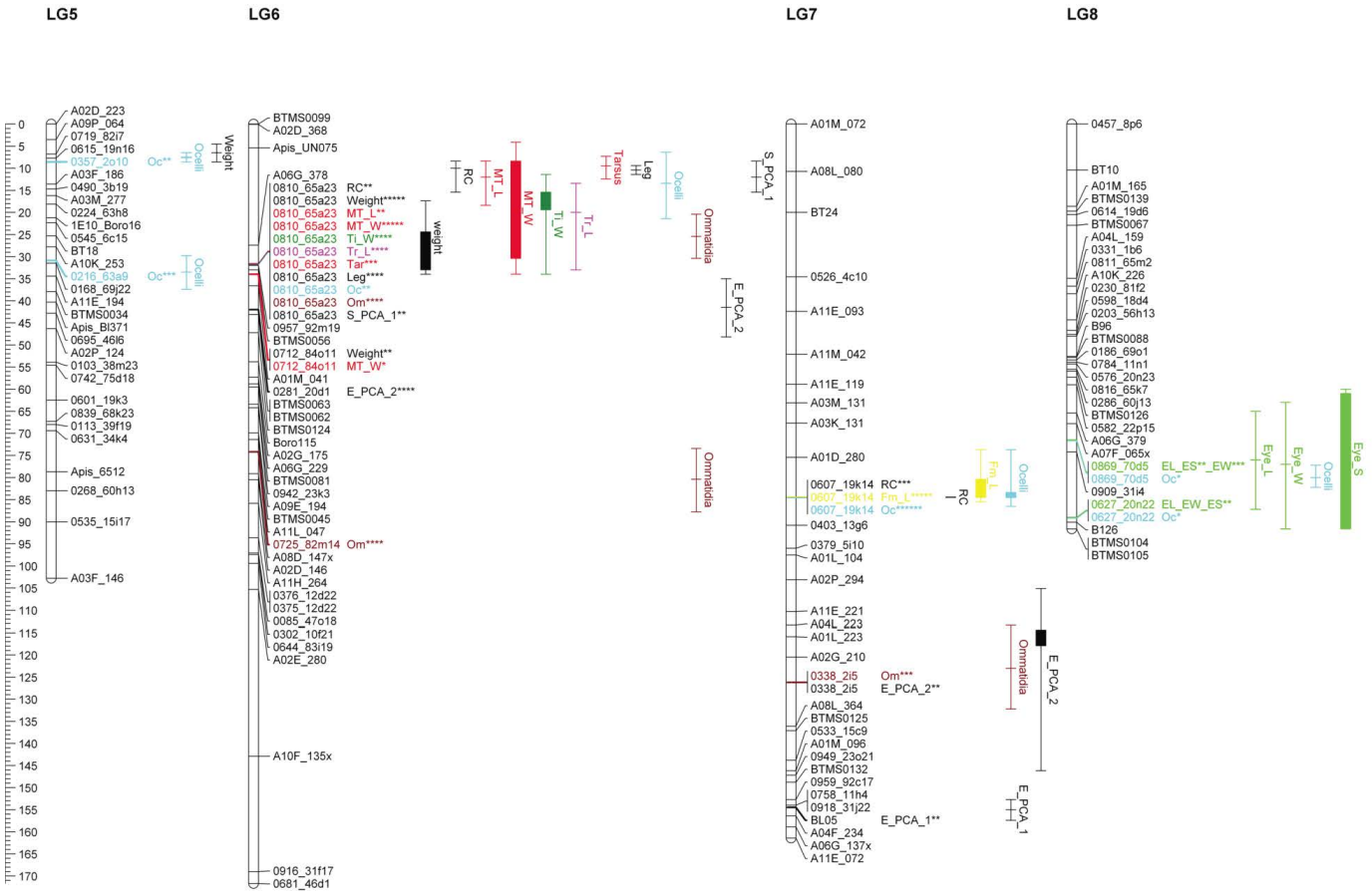
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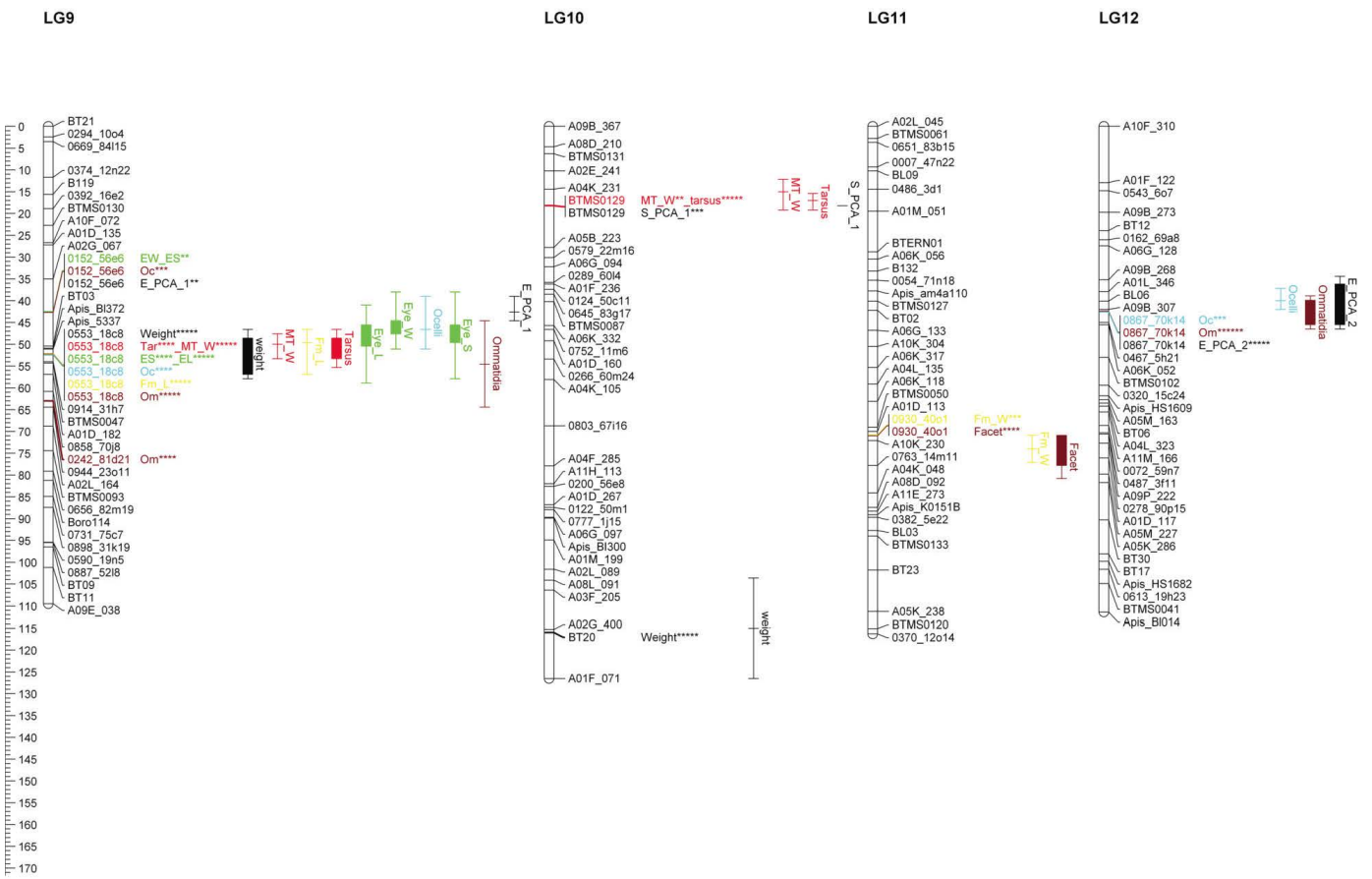
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EW_ES**
Oc***
E_PCA_1**
Weight*****
Tar****_MT_W*****
ES****_EL*****
Oc*****
Fm_L*****
Om*****
Om*****
weight
MT_W
Fm_L
Tarsus
Eye_L
Eye_W
Ocelli
Eye_S
Ommatidia
E_PCA_1
A09B_367
A08D_210
BTMS0131
A02E_241
A04K_231
BTMS0129
BTMS0129
A05B_223
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A06G_094
0289_60l4
A01F_236
0124_50c11
0645_83g17
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A01D_160
0266_60m24
A04K_105
0803_67i16
A04F_285
A11H_113
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A01D_267
0122_50m1
0777_1j15
A06G_097
Apis_Bl300
A01M_199
A02L_089
A08L_091
A03F_205
A02G_400
BT20
A01F_071
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S_PCA_1***
Weight*****
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Tarsus
S_PCA_1
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BL09
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BTERN01
A06K_056
B132
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Apis_am4a110
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A06G_133
A10K_304
A06K_317
A04L_135
A06K_118
BTMS0050
A01D_113
0930_40o1
0930_40o1
A10K_230
0763_14m11
A04K_048
A08D_092
A11E_273
Apis_K0151B
0382_5e22
BL03
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BT23
A05K_238
BTMS0120
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Facet****
Fm_W
Facet
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A01F_122
0543_6o7
A09B_273
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A01L_346
BL06
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0867_70k14
0467_5h21
A06K_052
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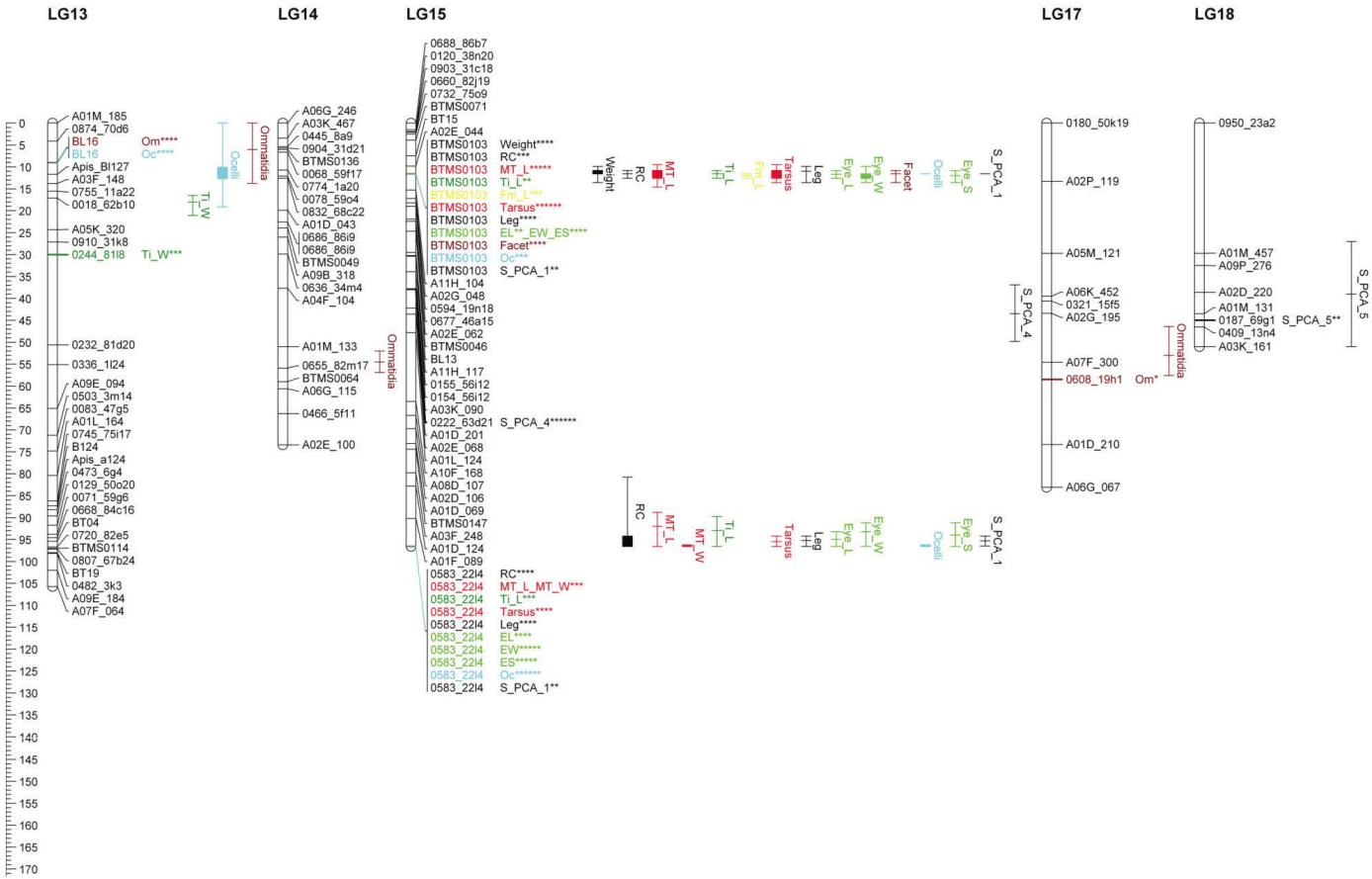
0320_15c24
Apis_HS1609
A05M_163
BT06
A04L_323
A11M_166
0072_59n7
0487_3f11
A09P_222
0278_90p15
A01D_117
A05M_227
A05K_286
BT30
BT17
Apis_HS1682
0613_19h23
BTMS0041
Apis_Bl014
Oc***
Om******
E_PCA_2*****
Ocelli
Ommatidia
E_PCA_2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LG18
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0232_81d20
0336_1I24
A09E_094
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A01L_164
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B124
Apis_a124
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0129_50o20
0071_59g6
0668_84c16
BT04
0720_82e5
BTMS0114
0807_67b24
BT19
0482_3k3
A09E_184
A07F_064
Om****
Oc****
Ti_W***
Ocelli
Ommatidia
Ti_W
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A03K_467
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0904_31d21
BTMS0136
0068_59f17
0774_1a20
0078_59o4
0832_68c22
A01D_043
0686_86i9
0686_86i9
BTMS0049
A09B_318
0636_34m4
A04F_104
A01M_133
0655_82m17
BTMS0064
A06G_115
0466_5f11
A02E_100
Ommatidia
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0120_38n20
0903_31c18
0660_82j19
0732_75o9
BTMS0071
BT15
A02E_044
BTMS0103 Weight****
BTMS0103 RC***
BTMS0103 MT_L*****
BTMS0103 Ti_L**
BTMS0103 Fm_L***
BTMS0103 Tarsus******
BTMS0103 Leg****
BTMS0103 EL**_EW_ES****
BTMS0103 Facet*****
BTMS0103 Oc***
BTMS0103 S_PCA_1**
A11H_104
A02G_048
0594_19n18
0677_46a15
A02E_062
BTMS0046
BL13
A11H_117
0155_56i12
0154_56i12
A03K_090
0222_63d21 S_PCA_4******
A01D_201
A02E_068
A01L_124
A10F_168
A08D_107
A02D_106
A01D_069
BTMS0147
A03F_248
A01D_124
A01F_089
0583_22I4 RC****
0583_22I4 MT_L_MT_W***
0583_22I4 Ti_L***
0583_22I4 Tarsus****
0583_22I4 Leg****
0583_22I4 EL****
0583_22I4 EW******
0583_22I4 ES******
0583_22I4 Oc******
0583_22I4 S_PCA_1**
Weight
RC
MT_L
Ti_L
Fm_L
Tarsus
Leg
Eye_L
Eye_W
Facet
Ocelli
Eye_S
S_PCA_1
MT_W
S_PCA_4
0180_50k19
A02P_119
A05M_121
A06K_452
0321_15f5
A02G_195
A07F_300
0608_19h1
A01D_210
A06G_067
Om*
0950_23a2
A01M_457
A09P_276
A02D_220
A01M_131
0187_69g1 S_PCA_5**
0409_13n4
A03K_161
S_PCA_5

Figure 5.2 Genetic linkage map showing the distribution of the QTLs. QTLs for each trait are colour coded: (i) forewing radial cell length (RC), body mass (weight), and length of hind leg (Leg) in black; (ii) metatarsus length (MT_L), metatarsus width (MT_W), and tarsus length (tarsus) in red; (iii) trochanter length (Tr_L), and trochanter width (Tr_W) in fuchsia; (iv) femur length (Fm_L), and femur width (Fm_W) in yellow; (v) tibia length (Ti_L), and tibia width (Ti_W), length of compound eye (E_L), width of compound eye (E_W), and total surface of compound eye (E_S) in green; (vi) diameter of facet (Facet), and total numbers of ommatidia (Om) in maroon; and (vii) diameter of median ocellus (MOc) in light blue. PC-QTLs of the eye parameters and body size are all coloured black: for eye size (E_PCA_1 and E_PCA_2) and for body size (S_PCA1, S_PCA_4 and S_PCA_5). Linkage group number are shown on top of the groups, and map distance (cM) is shown on the left margin of the figure. The genetic map originated from Stoll *et al.*, 2011. The significant markers within QTL regions are shown with there corresponding Kruskal-Wallis significance level (* = 0.10; ** = 0.05; * = 0.01; **** = 0.005; ***** = 0.001; ****** = 0.0005; and ******* = 0.0001).**

For facet diameter (*qFAC11*) we found one QTL explaining 9.7% of the variation. For the different hind leg traits we found only significant QTLs for: (i) metatarsus length and width (*qMTL1*, *qMTL6* and *qMTW6*; respectively) explaining 34.2% and 22.0% of variation, (ii) tibia length and width (*qTIL15.2* and *qTIW6*, respectively) explaining 9.7% and 17.8% of variation, (iii) femur length (*qFML7* and *qFML15*) cumulatively explaining 28.8% of variation, and finally (iv) three QTLs for tarsus length (*qTAR1.2*, *qTAR9*, *qTAR15.1*) explaining 72.9% of variation.

5.3.3 *PC-QTL*

The PCA for body size parameters showed 5 PCs of which two had eigenvalues higher than 1: 5.91 and 1.57 (PC1 and PC2, respectively; in Figure 5.3, Supplementary File S16). Together, these 5 PCs accounted for 89.5% of the total variance over these traits (Supplementary File S16). In total, we found 8 QTLs for three PCs: PC1 (4), PC4 (2) and PC5 (2). The most informative PC is PC1 with 53.8% of the total variance of the trait while PC4 and PC5 accounted only for 6.5% and 6% of the total variance, respectively. Three of the four QTLs (*qSPC1_6*, *qSPC1_15.1* and *qSPC1_15.2*) of PC1 are linked with body size in general as confirmed by the QTLs of the individual body size traits (Table 5.3 and Figure 52). QTL *qSPC1_10* was only confirmed by the traits linked with tarsus size (Table 5.3 and Figure 5.2).

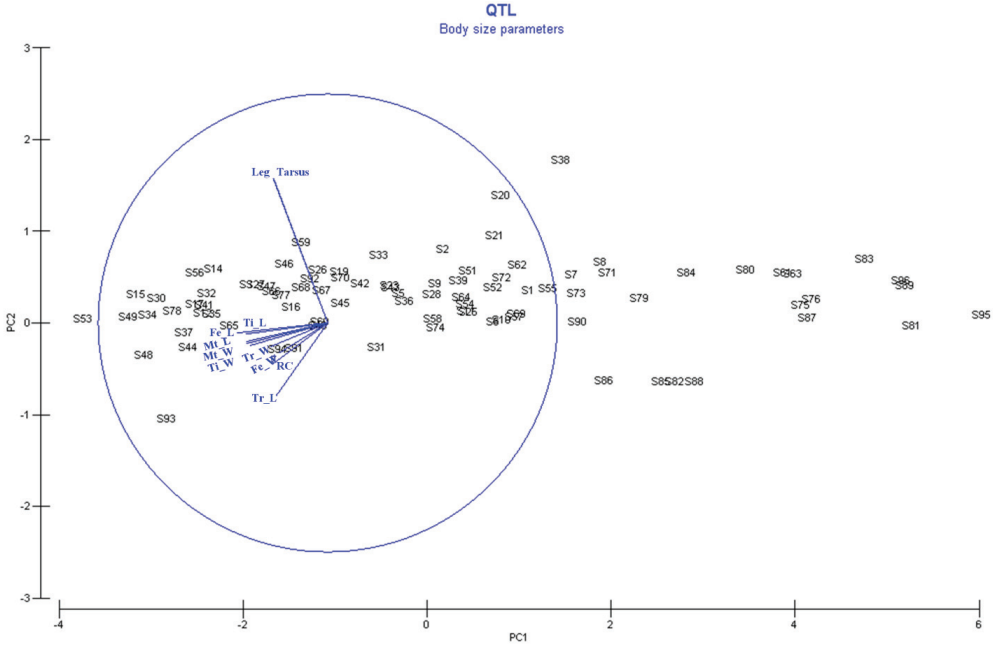

Figure 5.3 PCA graph of the different body size parameters.

The PCA on the different eye parameters showed 3 PCs which accounted for 74.1% (PC1), 10.9% (PC2) and 8.1% (PC3) of the total variance (Figure 5.4, Supplementary File S16). Only PC1 had an eigenvalue higher than 1: 4.45 (Supplementary File S16). All eye parameters showed negative correlations with PC1, ranging from -0.458 to -0.325. For compound eye length, eye width and eye surface, we found the highest correlations: -0.458, -0.456 and -0.453, respectively. Three of the 4 QTLs found for PC1 (*qEPC1_1.1, qEPC1_1.2* and *qEPC1_9*) were confirmed by the univariate QTLs for these three eye parameters, while QTL *qEPC1_7* was only confirmed by ommatidia number (Table 5.3 and Figure 5.2). The three QTLs for PC2 (*qEPC2_6, qEPC2_7* and *qEPC2_12*) correlated with the univariate QTLs found for median occelus and ommatida number on LG6, LG7 and LG12 (Table 5.3 and Figure 5.2).

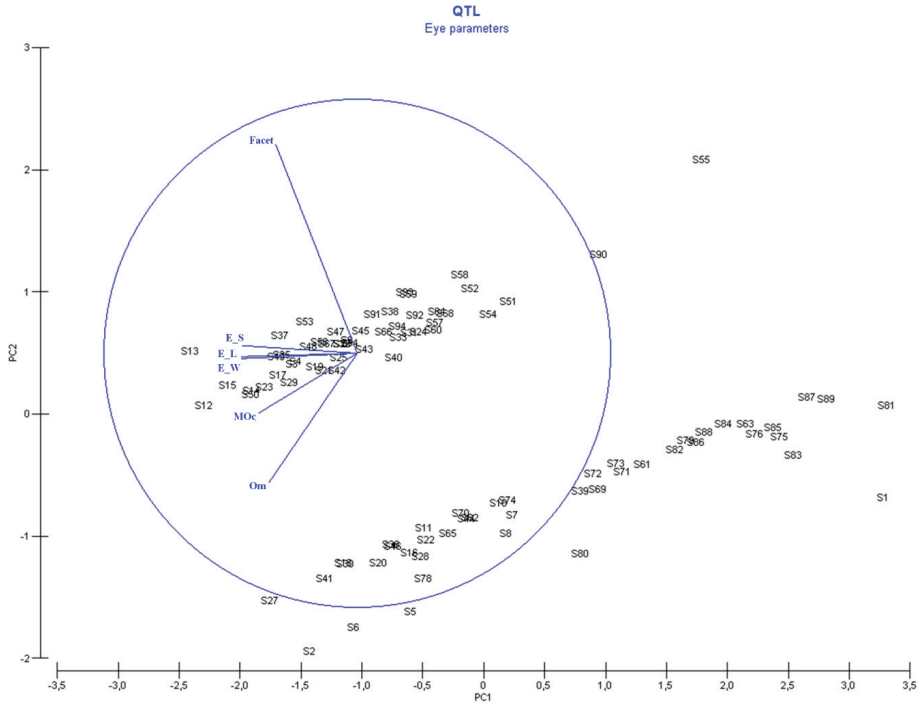

Figure 5.4 PCA graph of the different eye parameters.

5.3.4 *Candidate genes of light sensitivity*

Candidate genes were identified for the QTL *qBLU3*. Therefore, we used SSR-marker BT08 which determine the QTL region, and the markers BT07 and 0291_60p14 as borders for the 95% C.I. of the QTL. The 64 genes within the range created by the markers BT07 and 0291_60p14 on linkage group 3, were all identified as candidate genes (Supplementary File S17). Based on the possible function in phototransduction and visual perception, locus Loc100650954, with as description a *Phosrestin-1-like* gene, was selected as the primary candidate gene.

5.4 Discussion

Here, we have identified several significant QTLs for morphological traits related to bumblebee light sensitivity, body mass, body size and several eye and hind leg traits (Table

5.3). The presence of multiple QTLs for 16 of the 20 traits clearly demonstrate their polygenic genetic character. For three traits: i.e. femur width, trochanter length and trochanter width, we identified only one QTL. We were unable to find a QTL for only light sensitivity under UV light conditions. As UV light is important for bumblebee foraging (Raine & Chittka, 2007) and UV receptors are present in bumblebees (Skorupski *et al.,* 2007), loci linked with UV detection could be under strong selection resulting in low genetic variation. Hence, it is quite possible that in our population with maximum 3 alleles for each locus, these loci could be present as homozygous. Furthermore, developmental and environmental factors could have caused no detection of QTLs for UV light. Finally, it is also possible that small effect QTLs are not detected here.

Our sample size (n = 92 to 96) was comparable or smaller in comparison with the sample sizes of other QTL studies in bumblebees, such as in Wilfert *et al.* (2007a;b) where sample size ranged from n = 76 to 359 and n= 153 to 173 respectively, depending on which trait and population was investigated. Our sample size was also consistent with the sample size of other QTL studies, e.g. in plants (n = 90 or less; Moghaddam *et al.,* 2012). However, due to the Beavis effect, which causes biases in QTL effects, it is possible that small QTLs were not detected even with an increased sample size (Xu *et al.,* 2003). Thus only remarkably increasing the population size would increase the detection of yet unfound small effect QTLs. Although detection of all possible QTLs should be the ultimate target, the goal of the study performed in this chapter was to identify genetic markers linked to some specific phenotypes for their later use in MAS. For this purpose, small effect QTLs are not as useful.

In this chapter, we found a significant QTL for light sensitivity under blue light conditions in a region where there is no QTL linked with body size or any other related morphological parameter. We already showed before that although larger bumblebees are better equipped to capture light, other genetic parameters influence bumblebee light sensitivity (see chapter 4). For this trait, we identified 64 candidate genes of which we identified the *Phosrestin-1-like* gene as the primary candidate gene due to the known phototransduction function of *Phosrestin-1* (Xiong & Bellen, 2013). Indeed, in the Fruit fly (*Drosophila*) *Phosrestin-1*, also known as *Arrestin-B* or *Arrestin-2*, is identified as interacting directly with light-activated rhodopsin thereby activating the phosphorylation of metarhodopsin (Xiong & Bellen, 2013). Furthermore, low and high levels of *Arrestin-2* in the rhabdomeres will enhance the

photoreceptor sensitivity in weak light conditions, and prevent hyperactivity of the photoreceptors in strong light conditions (Xiong & Bellen, 2013). Further research is necessary to validate this gene's impact on improved light sensitivity in bumblebees and its effect on foraging activity in diminished light conditions.

Not surprisingly we also found several overlapping univariate QTLs between the length of the radial cell, as measurement of bumblebee body size, and most of the other measured size related morphological parameters (Table 5.3 and Figure 5.2). Several QTLs overlapped also between drone body mass and body size: e.g. one QTL region at LG6, LG9 and LG15, but a more interesting result was that not all QTLs overlapped for these parameters (Table 5.3 and Figure 5.2). Indeed, drone body mass showed unique QTL regions at LG2 (*qDWE2*), LG3 (*qDWE3*), LG5 (*qDWE5*), and LG 10 (*qDWE10*), while radial cell and body size parameters had unique QTL regions at LG1, LG7 and LG15. These regions were confirmed by the PC-QTL. Indeed, PCs showed size related QTLs on LG6, LG10 and LG15. Only one QTL on PC4 overlapped with one of the unique univariate body mass QTLs on LG3 (Figure 5.2). The presence of these specific genetic regions for drone body mass and body size indicates regulation of different genes.

Although preliminary, these results support the idea of marker assisted breeding towards larger bumblebees, with the use of the identified markers at those unique QTLs. However, before these QTLs could be used they need to be validated in a broader genetic background, using multiple bumblebee populations. For QTL studies it is common that most of the QTLs found in one population will not withstand this validation, even if there are only very small differences in the experimental setup (Wilfert *et al.,* 2007a). Indeed, in Wilfert *et al.* (2007a) the authors used three bumblebee populations in which they detected several QTLs for the traits: *Crithidia* infection intensity, general immune response (encapsulation of a novel antigen), and body size (measured by the length of the radial cell of the forewing) at different places and on different linkage groups. Wilfert *et al.* (2007a) found 10 QTLs for body size measured as the size of the radial cell of the forewing, with only low phenotypic effects (between 2% and 15%). Of those 10 QTLs, only one QTL (*BS-8*) was recovered in our study (*qRAC15.1*). This QTL, which accounts in our study only for 9.6% of the phenotypic variation, is a potential candidate for use as a genetic marker in MAS. Thus, in our study we were not only able to confirm a minor QTL for body size from Wilfert *et al.* (2007a), but we

also found several major QTLs explaining more than 15% to even 50% of the phenotypic variation within a certain trait which are restricted to our bumblebee population and need validation in a broader genetic background.

In conclusion, our study identified one QTL for light sensitivity under blue light conditions explaining 10.6% of the phenotypic variation of the trait. Furthermore, we identified a list of 64 possible candidate genes for this trait of which the *Phosrestin-1-like* gene is identified as the primary candidate gene. Finally, we also found several QTLs for body weight, body size and the morphological parameters of the eye and hind leg. Further research needs to determine if the QTLs found here, resist validation in a broader genetic background and if some of the SSR markers linked with those QTLs could be used as genetic markers in marker assisted breeding, to improve the pollination service of bumblebees.

Chapter VI

Detection of diploid and haploid drones in a bumblebee mass-breeding

Redrafted after:

Maebe, K., Meeus, I., Wäckers, F., Smagghe, G. (2013) Scientific note on microsatellite DNA analyses revealing diploid and haploid drones in bumblebee mass-breeding. Apidologie 45, 189-191.

6.1 Introduction

Bumblebees as *Bombus terrestris* (L.) are used worldwide in greenhouses for the pollination of different crops such as tomatoes and sweet pepper (Velthuis & van Doorn, 2006). The commercial breeding of bumblebees was already in 2006 estimated to represent a yearly turnover of €55 million, and the pollinated greenhouse tomatoes had an estimated value of €12,000 million per year (Velthuis & van Doorn, 2006) and have increased since then.

The breeding of bumblebees occurs in-house with strict procedures in place to exclude contact with the outside environment and to prevent inbreeding. For *B. terrestris* this in-house production has been extensively optimized (Velthuis & van Doorn, 2006). However, not all queens will start up typical worker-producing colonies, which switch into sexual producing colonies after the 'switching point' (i.e., the moment that a queen switches from laying diploid workers into laying only haploid drones and/or diploid daughter queens). In a small number of colonies, the first offspring already contains drones instead of only female workers. This early production of drones, at a worker/drone sex ratio of 1:1, has already been reported in a range of haplo-diploid insects. These drones are typically diploid, and could be a consequence of inbreeding or homozygote alleles at the sex determination loci (Duchateau *et al.*, 1994; Whitehorn *et al.*, 2009). As in bumblebees, sex is determined by the presence of complementary alleles at a single sex determination locus (SDL) where heterozygotes at this locus will develop into diploid females and hemizygotes into haploid drones, while homozygotes develop into diploid drones (Duchateau *et al.*, 1994; Whitehorn *et al.*, 2009).

Quality assurance (QA) within the breeding facility eliminates early drone-producing colonies; such colonies are disapproved for sales into the market. This phenomenon of early diploid drone producing can be easily scored by sexing the first batch of offspring. Here, we received 6 QA failed colonies (i.e., early drone-producing colonies) from a commercial mass rearing facility in order to investigate why these colonies produced males so early.

6.2 Material and methods

All specimens from the 6 QA failed colonies of a commercial mass rearing facility were killed and sex determination of each specimen was done with the use of a microscope (Kyowa optical SDZ-P, Kyoto). We separated workers and drones based on the presence or

absence of a sting and male genitalia (Figure 6.1).

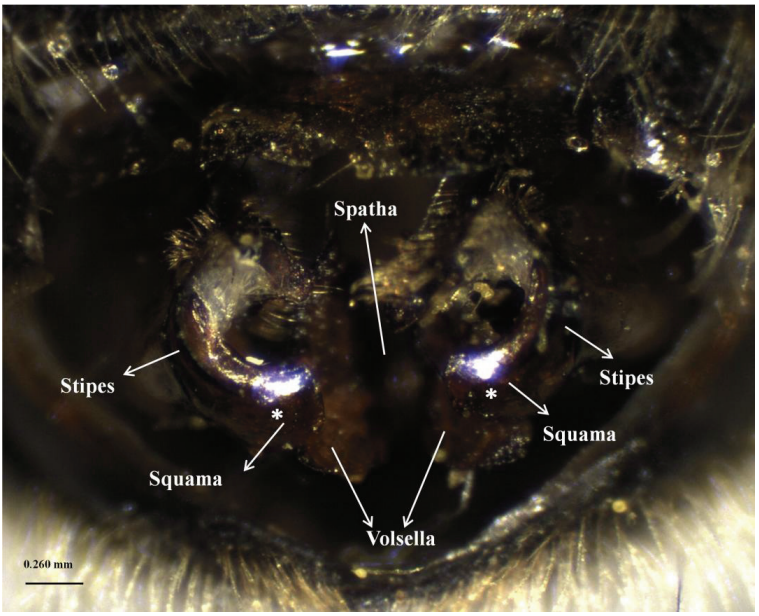

Figure 6.1 Micrograph of the male genitalia (white stars) of a diploid drone.

Based on the frequency of drones in each colony, we divided these colonies in two groups: colonies which seemed to have a biased 1:1, worker:drone ratio (group 1), and colonies which consisted of almost only drones (group 2) (Table 6.1). Subsequently, we investigated what could be reason for the early drone production in these colonies. Is it the typical diploid drones production with a biased sex-ratio towards more drones or does the queen produces haploid drones, because the mated queens have problems to fertilize her eggs?

To investigate if the colonies produce diploid or haploid drones, we used microsatellites. From each colony, we genotyped the queen and 5 other specimens: 1 or 2 worker(s) if present, and 3 to 4 drones (Table 6.1). Bumblebee DNA was extracted from one middle leg of each bumblebee specimen with the same method as described in chapter 2. Workers were genotyped at 10 microsatellite loci: B11, B100, B121, B126 and B132 (Estoup *et al.*, 1993) and BT04, BT08, BT10, BT11 (Reber-Funk *et al.*, 2006) as originally developed from *B. terrestris*, and BL02 (Reber-Funk *et al.* 2006) as derived from *B. lucorum*. Microsatellites

were then amplified by PCR and visualized with capillary electrophoreses as described in chapter 2.

Table 6.1 Overview of the morphological and genetic data for each of the colonies, divided in two groups based on the sex ratio: group 1 contains 3 colonies with a biased worker:drone sex ratio of 2:3, while group 2 contains 3 colonies which consisted out of almost only drones. Data present the numbers of drones and workers within each colony, worker:drone sex ratio, presence of workers laying eggs and a queen helper, and ploidy of the drones (diploid/haploid) as determined with microsatellite analysis.

		Numbers of		Worker:drone	Worker	Queen	
Group	Colony	drones	workers	sex ratio	laying eggs	helper	Ploidy of drones
Group 1	Colony A	17	9	2:3	No	-	Diploid
	Colony B	11	7	2:3	No	-	Diploid
	Colony C	9	5	2:3	No	Present	Diploid
Group 2	Colony D	16	1	9:10	No	Present	Haploid
	Colony E	8	1	9:10	Yes	Present	Haploid
	Colony F	21	3	9:10	Yes	Present	Haploid

As shown in Table 6.1, queens, workers and drones were identified being diploid when they scored being heterozygous at minimum one loci, while bumblebees that scored homozygous for each microsatellite were scored as haploid. Based on 10 microsatellite loci, the probability of scoring a true haploid drone as a haploid and not as a homozygote diploid drone is high. Even when using a high allele frequency (f) in all microsatellite loci of 0.5, the probability (P) is still 99.9% with $P = (1-(f)^{10})*100$.

6.3 Results and discussion

All drones produced by the 3 colonies belonging to group 1 (colony A, B and C), were diploid and were all offspring of their founding queen (Table 6.1). Furthermore, we tested if the sex ratios in those colonies were biased from the expected 1:1, worker:diploid male ratio. Based on a χ^2 test (with Yates correction) the three colonies A, B and C showed no significant deviation from the 1:1 sex ratio (Yates $\chi^2 = 1.885$, $P = 0.170$; Yates $\chi^2 = 0.500$, $P = 0.480$ and Yates $\chi^2 = 0.643$, $P = 0.423$; respectively). However, we found a slight but significant bias from the normal 1:1 sex ratio when we pooled the data over all three colonies (Yates $\chi^2 = 3.879$, $P < 0.05$). This was surprising as one might expect the opposite because diploid males have a reduced viability in comparison to workers. However, it should be remarked here that the breeding facility made the selection of the colonies based on the

number of drones in the first brood. We therefore speculate that the unexpected 2:3 ratio can be explained by a sampling bias for colonies with larger numbers of drones. The colonies tested can thus be considered as typical examples of diploid drone-producing colonies explained by inbreeding or mating of non-related specimens with the same alleles for the sex loci (Table 6.1).

The colonies belonging to group 2 produced only haploid drones (Table 6.1). However, not all of these haploid drones originated from the founding queen. Some of those haploid drones were offspring produced by the queen helper, that is a bumblebee worker placed together with the queen to induce egg laying (Table 6.1). This indicates that the queen of those colonies had problems to fertilize her eggs and could lay only unfertilized eggs which will develop into haploid drones (Colony D, E and F; Table 6.1). Furthermore, it also indicates that the queen helpers started producing haploid drones themselves, probably induced by the inferior egg laying capacity of the queen (Colony E and F; Table 6.1).

In conclusion, the data of this chapter showed that early drone-producing colonies from mass producing facilities can produce diploid or haploid drones. The early presence of haploid drones indicates that the queen had problems in the fertilization of her eggs, while the presence of diploid drones confirms some level of inbreeding. In addition, we remark that morphometrics and wing landmarks are interesting for future research to evaluate if these can be used for separating diploid drones from haploid ones.

Chapter VII

General conclusions and future perspectives

7.1 Impact of measuring genetic diversity: conclusions and future perspectives

Bumblebees species are important pollinators in natural and managed ecosystems. Here, in this dissertation microsatellite DNA technology was employed to measure population genetic parameters of endangered and more stable bumblebee populations, to assess and to identify genetic loci linked with the commercial pollination service.

7.1.1 *Genetic diversity of historical bumblebee populations*

In chapter 2 and 3, we examined the role and impact of genetic parameters on the observed bumblebee declines in natural populations. Therefore, we genotyped pin-mounted bumblebee specimens sampled from extensive historical bumblebee collections. This provided a unique opportunity to examine genetic parameters of past populations and compare these parameters with those of recent bumblebee populations presented in the literature. This approach allowed for the unique investigation of the role of these genetic parameters in bumblebee decline. In the case-study of *B. veteranus* (chapter 2) and the comparison between populations of declining and more stable bumblebee species (chapter 3), we detected low levels of genetic diversity in the historical populations of the declined bumblebee species. These levels of genetic variation are lower than the observed levels of genetic diversity within the populations of the more stable species, but are comparable with those found in the contemporary populations of these declining species. Furthermore, the historical populations of *B. veteranus* showed indications of inbreeding. These results indicate that inbreeding and low levels of genetic variation were already present several decades before the general drivers of bumblebee decline (around 1950) are believed to have acted on these bumblebee populations (Rasmont & Mersch, 1988, Rasmont *et al.,* 1993; Goulson *et al.,* 2008). As a consequence, we believe that: (i) inbreeding does not directly result in the collapse of populations, (ii) that there was no major drop in genetic diversity caused by the general drivers of bumblebee decline in the populations of the declined bumblebee species, and (iii) that bumblebee species with low levels of genetic diversity were the first to decline.

At first sight, the conclusion of the first and last point, saying that inbreeding does not directly result in the collapse of the populations of *B. veteranus* in Belgium and that low levels of genetic diversity will lead to the collapse of bumblebee populations, seems contradictory as inbreeding populations generally have a low genetic diversity. However,

these conclusions are not contradictory. The case-study of *B. veteranus* merely presents the fact that inbreeding and thus low genetic diversity on itself must not lead towards decline, while the third conclusion predisposes species with a low genetic diversity to decline when the environment is less suited for these low H_E or inbred species.

That inbreeding does not directly result in the collapse of a population in Hymenoptera has already been demonstrated in the fire ant *Solenopsis invicta* (Ross & Fletcher, 1986), in the solitary bee *Lasioglossum leucozonium* (Zayed *et al.*, 2007), in the stingless bee *Melipona scutellaris* (Alves *et al.*, 2011), and in the bumblebee *B. terrestris* (Schmid-Hempel *et al.*, 2007). The latter study demonstrated that *B. terrestris* could successfully invade and colonize Tasmania despite a drastic genetic bottleneck. The success is due to the very favourable environment with no direct inter-species competition and no pathogens (Schmid-Hempel *et al.*, 2007). The results of these studies combined with our data, indicates that under optimal or good environmental conditions, high levels of inbreeding does not necessary restrict bumblebees (Hymenoptera) to become locally abundant. That no direct negative fitness effects occur within these populations could be due to the strong effect of purging selection against recessive deleterious alleles in the haploid males (Sorati *et al.,* 1996; Packer & Owen, 2001).

Furthermore, our results showed that the historical populations of the declining bumblebee species had lower levels of genetic diversity than found within the historical populations of co-distributed more stable species. Following conclusion one, this result should not be a major problem when the environment is favourable. However, this result was before the general drivers of bumblebee decline are believed to have acted (Rasmont & Mersch, 1988, Rasmont *et al.,* 1993; Goulson *et al.,* 2008). Due to these stressors, the environment changed rapidly which had a major impact on bumblebee populations. Especially on species with lower genetic diversity as they will decline first in comparison with species which have higher levels of genetic variation in their populations. Indeed, it is known that populations with a lower genetic variation will be at risk of decline as they will be more vulnerable to changes and stressors in the environment (Reed & Frankham, 2003; Spielman *et al.,* 2004; Frankham, 2005; Whitehorn *et al*., 2009; Zayed, 2009; Goulson *et al.,* 2011). For instance, populations which have lower levels of genetic variation on the genes responsible for light sensitivity, body size or the eye parameters will be lesser adapted to an environment with

lower light intensities. When these populations would be exposed to repeated long periods of bad weather, or undergo large shifts in their geographic distribution range to the North due to climatic change, or other shifts in their daily rhythm, this could lead to an increased food pressure. Indeed, as many flowers accumulate nectar and pollen overnight (Corbet *et al.,* 1995), a colony which is not able to forage at dawn will have less access to high quality food resources.

7.1.2 *Implications for conservation of natural bumblebee populations*

What do these results imply for the conservation of bumblebee populations? Goulson *et al.* (2008) and Lozier *et al.* (2011) stated before that populations at risk of decline could be detected by comparing the intra-population genetic diversity levels between different bumblebee species. However, from which level of genetic diversity do we say that a population is threatened to decline? This critical level of genetic diversity or cut-off value of H_E above which a population is viable, is not (yet) known. Finding this value is one goal within population genetic studies (as discussed in Markert *et al.*, 2010). Although this value will again highly depend on the suitability of the environment, our results in chapter 3 show that the level of genetic diversity of populations, measured with H_E and A_R, can give a pretty good prediction for which population would crash and deserves the most attention for conservation.

Indeed, if one would have performed the same genetic analysis as presented in chapter 3 around the year 1930, and had made then a prediction of which bumblebee species will be more vulnerable for decline and which not, based on the detected levels of both genetic diversity parameters (H_E and A_R), one would have made a good prediction (Figure 7.1). Of the five species with low historically genetic diversity levels, meaning H_E lower than 0.550 and A_R lower than 3.5, all showed more severe declines after 1950 (Figure 7.1). Of the 6 species, which one would have predicted to be able to be more resistant to possible future declines based on their higher levels of genetic diversity (H_E higher than 0.550 and a A_R higher than 3.5; Figure 7.1), 4 species belong now to the more stable *Bombus s*pecies. Thus, based on the estimated genetic diversity levels one should have made a prediction in 1930 which determined the fate of 9 out of 11 bumblebee species or 82% correctly (Figure 7.1). In general, this result suggests that determination of the genetic diversity is a very good tool to predict bumblebee decline.

However, knowing the genetic diversity will not always identify which population is threatened. Indeed, although not as severe as the declining group, also the more stable species underwent distribution declines. Thus, even species with a high genetic diversity could still be at risk for extinction.

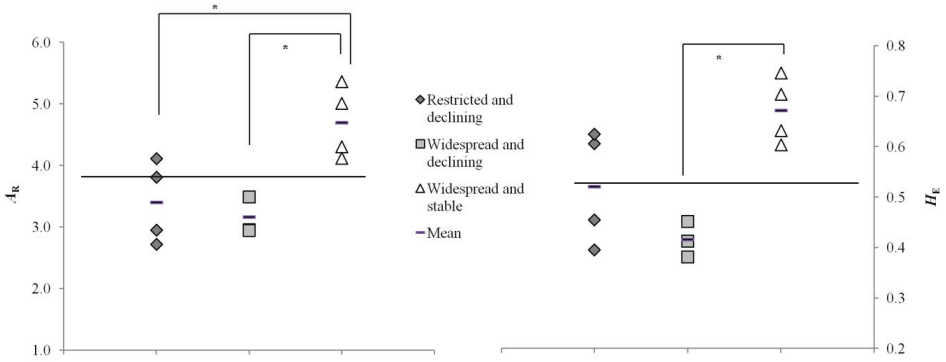

Figure 7.1 Comparison of the mean allelic richness (A_R) and expected heterozygosity (H_E) averaged across loci between the populations of the declining and more stable *Bombus* species within the time period 1918-1926. With indication of the significance levels, * = $P < 0.05$ and ** = $P < 0.01$.

Bumblebee populations can be restored by enhancing the size and connectivity of neighbouring populations as this will result in an increased genetic diversity. The scale on which these conservation measures need to be applied depends heavily on the species dispersal ability (Goulson *et al.*, 2011). Populations of bumblebee species with a more limited dispersal rate will have less chance of successful colonize a neighbouring patch than species with higher dispersal ability. This is why we consider that this species dependent dispersal range is essential for a correct implementation of mitigation measures. Although some studies have indications of different dispersal abilities for species from different subgenera (see chapter 1.2.3; Darvill *et al.*, 2010; Goulson *et al.*, 2011), the actual dispersal abilities of the reproductive's of many bumblebee species are not well known. The male or queen dispersal range is very difficult to determine. Many attempts of a theoretical calculation of the dispersal range are made, and this based on bumblebee nest density, foraging range and sibship reconstruction methods (Kraus *et al.*, 2009; Lepais *et al.*, 2010). However, only by successfully following a bumblebee one could reliable determine its dispersal range. A

harmonic radar system for tracking insect movements is already developed (Osborne *et al.*, 1997; 1999). With this technique foraging distances of workers can be obtained (Osborne *et al.*, 1999). However, the range of the radar is limited and can easily be disrupted by landscape or other features. Thus, although the harmonic radar is an unique method to measure foraging paths and distances of bumblebees, it cannot give the maximum foraging ranges or the dispersion distances of reproductive's (Goulson, 2010). In our opinion this could only be accomplished by the use of very small transmitters with gps-technology. Once developed for their use in bumblebees, one would gain also much information of bumblebee biology, especially of their dispersal, mating and nest behaviour. Thus, it is clear that this kind of technology will, aside from dispersal rate, reveal a vast variety of data, important for the conservation biologist, to setup effective conservation measures for particular species and within certain environments. Thus although improving the genetic diversity of the populations of restricted bumblebee species is still a valuable strategy, as bumblebee populations with high genetic diversity are less likely to decline or to go locally extinct, in our opinion, restoration of genetic diversity should not be the primary goal in conservation.

Indeed, we recommend that future conservation strategies primarily focus on creating more suitable habitat for sustaining bumblebee populations. It is often argued that low genetic diversity could lead towards an extinction vortex. As described in chapter 1.2.3 small bumblebee populations will have a reduced genetic diversity and go extinct, despite the presence of an apparent suitable habitat (Reed & Frankham, 2003; Spielman *et al.,* 2004; Frankam, 2005; Zayed, 2009; Goulson, 2010). That bumblebee populations can become increasingly small and isolated is due to the different drivers of bumblebee decline such as: land-use change, use of pesticides, the spread of pathogens, and climate change (as discussed in chapter 1.2.2; reviewed in Potts *et al.*, 2010). So, to preserve bumblebee genetic diversity and to ensure that both bumblebees species with low and high levels of genetic diversity will not go extinct, one must tackle the current drivers of bumblebee decline.

7.1.3 *Future perspective: from population genetics to population genomics*

When we screened the historical populations of bumblebee species which exhibit dramatic loss of their distribution range, no apparent effect on their level of genetic diversity was noticed. This is actually supported by simulation studies (e.g. Lozier & Cameron, 2009). However, no loss of alleles at a few microsatellite loci (n = 10) does not actually mean that

selection which happened the latest century did not leave traces in the genome of these insects. Indeed, the low amount of genetic neutral markers are too scattered and have too few power to detect any selection on different QTLs (Ouborg *et al.*, 2010). And thus will also not be able to detect any selection on the QTLs which are possible associated with the survival of bumblebees after the introduction of the stressors inflicted on bumblebees after 1950. However, recent developments in genomic techniques, such as next generation sequencing (NGS) and whole genome scans, made genome-wide estimates of functional genetic variation possible (Ouborg *et al.*, 2010). This transition of conservation genetics to conservation genomics allows the investigation of genes under selection and their interaction with environmental conditions. In human genetics, the integration of NGS and automatic SNP analysis has revolutionized the search for genes under selection pressure (Oleksyk *et al.*, 2010; Sturm & Duffy, 2012). In insects, and more specially in bumblebees, these technologies have so far been untouched to study population dynamics. The publication of the bumblebee genome (expected end 2014) will allow the use of the same innovative approaches to investigate how genetic variation on QTLs interacts with the sustainability of a species towards different stressors and how this is implicated in decline and extinction of bumblebees.

7.2 Selection of markers for MAS: conclusions and future perspectives

7.2.1 *Microsatellites to improve bumblebee populations within a mass-rearing facility*

The microsatellite DNA technology was also used to selectively validate and improve the mass-breeding of bumblebees for biological pollination. In order to be able to perform the ultimate goal: MAS to improve the pollination service of managed bumblebees, different criteria need to be met. Firstly, a trait needs to be selected which has the potential to improve the pollination service. In this dissertation two phenotypes associated with the commercial potential of this service were chosen: light sensitivity and body size.

The choice of light sensitivity was made because some studies showed that bumblebees also have troubles finding their way back to the colony and have a decreased foraging activity within the artificial light environment of the greenhouse (Morandin *et al.,* 2001, Blacquière *et al.,* 2006; 2007; Roman & Szczęsna, 2008, Johansen *et al.,* 2011). Furthermore, the selection of this trait was chosen based on its usefulness in greenhouses. When managed bumblebees

are used, there is a chance that some specimens will escape from the greenhouse and interact with the native population, which in turn could cause pathogen spillover (Colla *et al.*, 2006; Otterstatter & Thomson, 2008). When interbreeding of managed species with wild species is still possible, which is the case here with bumblebees, the application of MAS is somewhat more difficult. Indeed, escaped reproductives could mate with the reproductive castes of neighbouring populations, resulting in (i) the accumulation of an allel or alleles which could imply negative fitness effects for the native population on a longer term or (ii) eroding genetic diversity of the native population (Potts *et al.*, 2010). Although bumblebee queens can be prevented to escape from the greenhouse with the use of a queen lock or a queen excluder opening (= a smaller opening of the bumblebee nest which prevent new queens from escaping the nest), drones are still able to disperse freely. However, within a greenhouse, the use of nets can prevent that a majority of drones are able to escape into the wild (Koide *et al.*, 2008). These measures are not yet obliged in Europe but could be implemented in a greenhouse, like for example in Japan where nets are obliged by law (Koide *et al.*, 2008). These nets could also help to keep the with MAS selected bumblebees in the greenhouse. Even if some reproductives are still able to escape the greenhouse, the by MAS selected allel(es) would give only a selective advantage within the artificial light conditions of a greenhouse. Thus, selection for this trait will normally cause no extra problems for the native bumblebee populations.

We identified that different bumblebee colonies indeed respond differently in changing light conditions and saw that these differences were linked with the critical light sensitivity (CLS) of these bees. Therefore, improving CLS of bees could be a good strategy to enhance foraging in an artificial light environment or in weak light conditions, and in turn may improve the pollination service of bumblebees within greenhouses.

Selection towards an improved CLS is not an easy task. As Kapustjanskij *et al.* (2007) suggested bigger is better, one could select for bigger bees, having better light perception. However, a simple morphology-based selection strategy towards bigger bumblebees will not necessarily result in more light sensitive bumblebees or better foragers in weaker light conditions. Indeed, although we confirmed in chapter 4 that bigger bees had bigger eyes within colonies, between colonies this correlation was lost. Colonies containing small bumblebees had a better light perception compared to colonies with bigger specimens. Thus,

although body size is an important parameter for better light perception, as bigger bumblebee workers will be better equipped to capture light, improved vision is not only a consequence of improved light perception. Other genetic characteristics like signal transduction will disrupt the result of the bumblebee body size based selection for an improved CLS. Indeed, as hypothesized in chapter 4, other morphological parameters such as larger photoreceptors (rhabdomeres) or genetic parameters like the molecular capturing of photons, signal transduction and neuron composition could play a more important role between bumblebee families. Thus, a morphological based breeding program selecting for light sensitive bees is impossible to perform. For MAS, we can first identify the QTL(s) linked with CLS, which actually could lead to an improved CLS. In chapter 5, we identified the *Phosrestin-1-like* gene as the major candidate gene for an improved CLS due to the known phototransduction function of *Phosrestin-1* (Xiong & Bellen, 2013). By implementing the SSR genetic marker linked with this QTL in the breeding program, one can make a more controlled selection towards bumblebees with improved CLS. In addition, this result indicates that signal transduction could be the factor causing the differences in light sensitivity between the bumblebee families observed in chapter 4.

7.2.2 *Future perspective: validation of the selected markers for their use in MAS*

The SSR marker that is linked with the *Phosrestin-1-like* gene could be used in marker-assisted breeding towards the breeding of bumblebees with an improved CLS. Furthermore, also the identified markers at the unique QTLs for drone body mass and body size could be used in MAS towards bigger bumblebees. However, before these QTLs could actually be used to breed bigger bumblebees or even bigger insects, with the idea to create a possible higher product for the food market, these QTLs need to be validated. First, in a more broad genetic background, using multiple bumblebee populations and secondly, in populations of other insects for their more general use.

7.3 Inbreeding detection within a bumblebee mass-rearing facility: conclusion and future perspective

Furthermore, the microsatellite technology could be used as an additional validation step within a mass-rearing facility. The detection of diploid males and their abundance could be a very helpful and may even be an essential validation step of the production process within

these mass-rearing facilities. Indeed, by detecting for the presence of diploid drones the level of inbreeding within this selection system can be monitored. By early detection of inbreeding, the outbreak of negative effects can be avoided. However, other techniques such as morphometrics and wing landmarks could be an easier, more direct and even cheaper way for separating diploid drones from haploid ones, and thus to evaluate the production process of a mass-rearing facility. Indeed, an smartphone app which is capable of the identification of bumblebee species and male ploidy, based on recognizing wing landmarks on a photo, could help in monitoring bumblebees. The data obtained by application of this tool would create a huge database which would become valuable for further bumblebee conservation strategies. Currently, Dr. De Meulemeester of the Naturalis Center in Leiden (The Netherlands) is under supervisor of Prof. Biesmeijer performing this research in two projects: “Better tools for identification and monitoring of bees” and “Monitoring trends in wild bee populations based on wing shape morphometric”.

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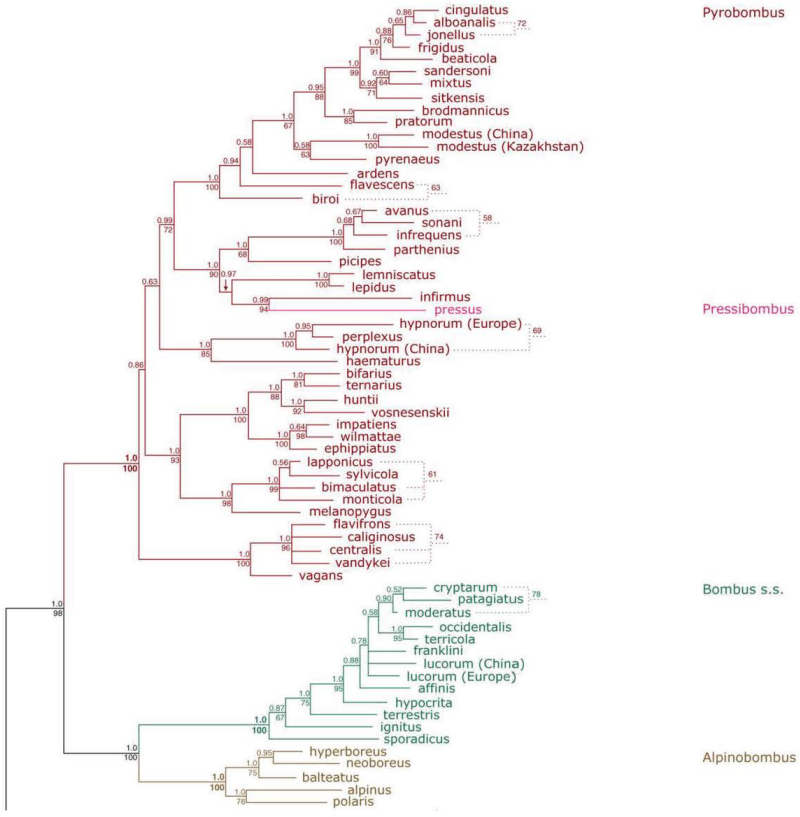
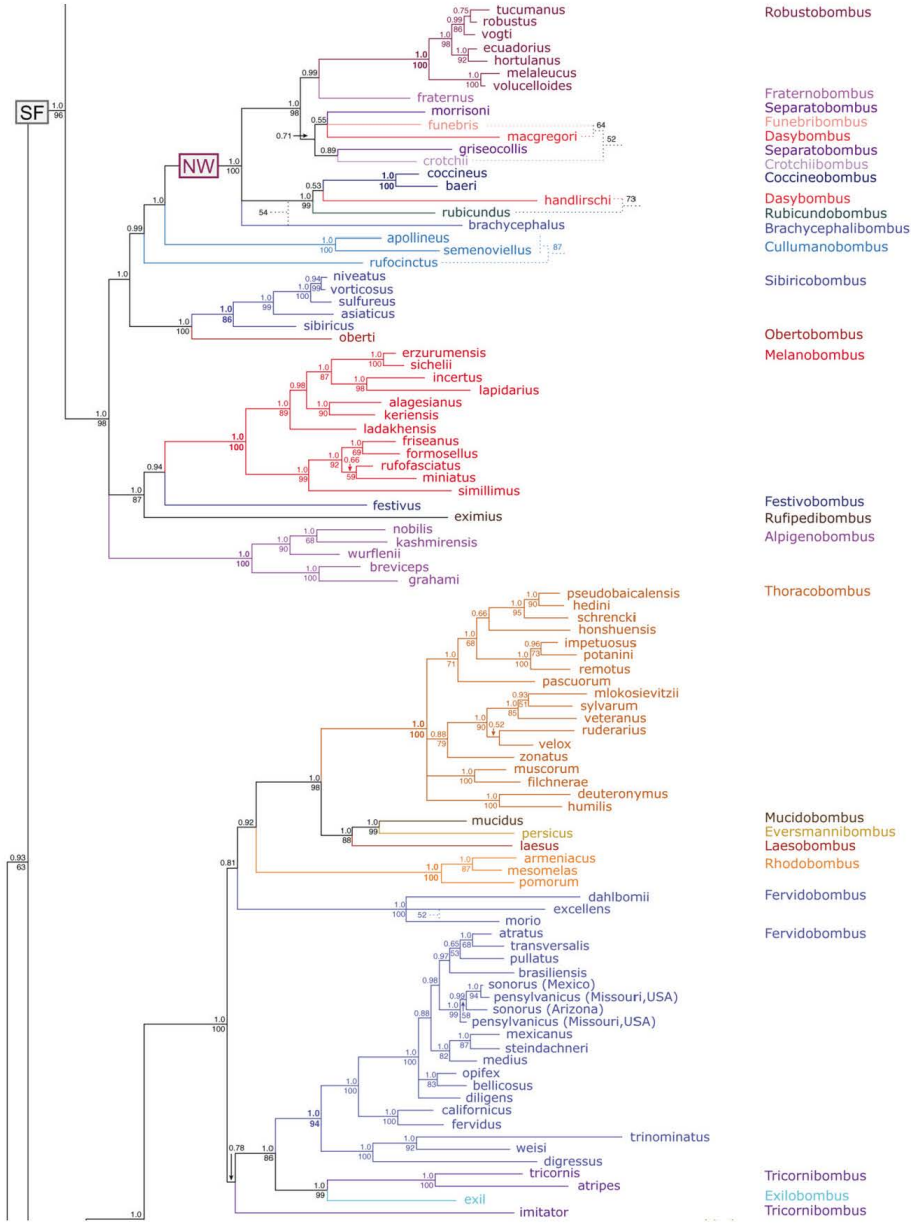
Supplementary data

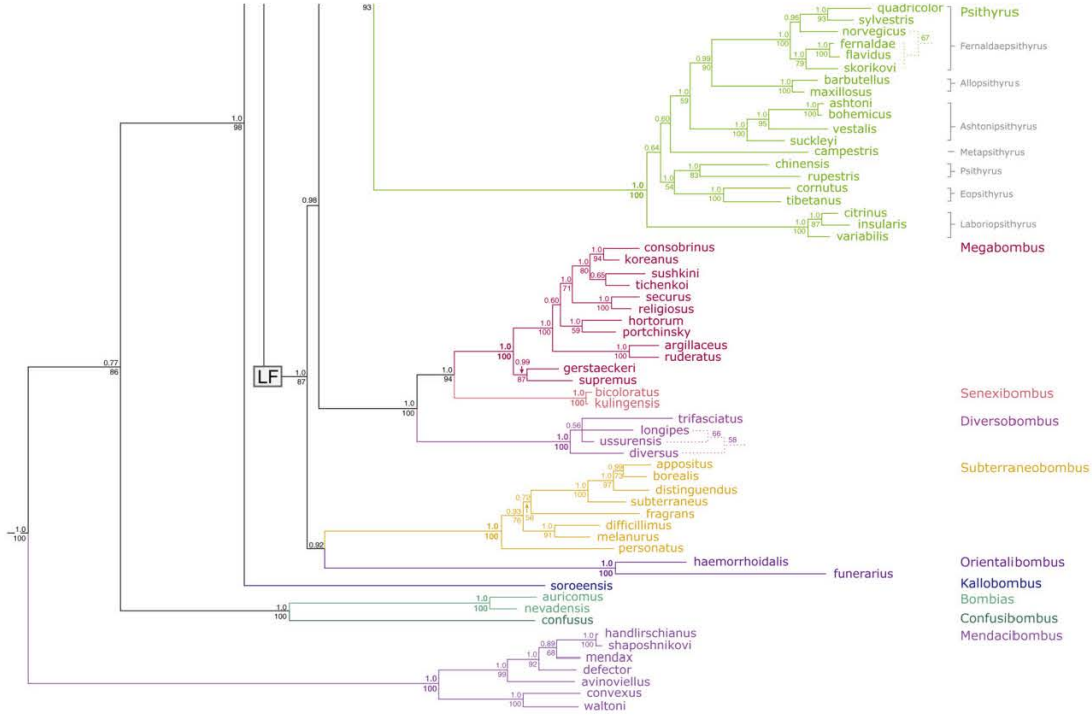
Supplementary File S1. Phylogenetic tree of 218 species from the genus *Bombus*, estimated from Bayesian analysis of combined sequence data from five gene fragments (16S rRNA, opsin, ArgK, EF-1α, and PEPCK). The subgenera are individually colour-coded and labelled. Values above and below the branches are Bayesian posterior probabilities and parsimony bootstrap values, respectively. From Cameron *et al.* (2007).



Supplementary File S2. Phylogenetic tree of 218 species from the genus *Bombus*. Values above and below the branches are Bayesian posterior probabilities and parsimony bootstrap values, respectively. NW stands for New World clade and SF for short-faced clade. From Cameron *et al*. (2007).



Supplementary File S3. Phylogenetic tree of 218 species from the genus *Bombus*. Values above and below the branches are Bayesian posterior probabilities and parsimony bootstrap values, respectively. The outgroups are represented as dashed lines and have been shortened for visual purposes. LF stands for long-faced clade. From Cameron *et al.* (2007).



Supplementary File S4. Summary of collection information of the specimens used after removal of the identified sisters.

Time period	Provence	Collection site	Latitude[1]	Longitude[1]	Date[2]	Determinated by	Collection*	Reference bib[3]	Sample Code
1895	Luxembourg	Botassart	49.858	5.0161	15-May	Rasmont P.	RBINS	Rasmont, 1988	BV01
	Luxembourg	Botassart	49.858	5.0161	8-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV03
	Hainaut	Trivières	50.409	4.1949	9-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV04
	Luxembourg	Botassart	49.858	5.0161	19-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV06
	West Flanders	Blankenberge	51.3144	3.0704	15-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV07
	West Flanders	Blankenberge	51.3144	3.0704	5-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV11
1915	Flemish Brabant	Halle	50.77	4.2	00/00	Rasmont P.	RBINS	Rasmont, 1988	BV13
	East Flanders	Moorsel	50.9499	4.0664	9-May	Rasmont P.	RBINS	Rasmont, 1988	BV14
	Flemish Brabant	Tervuren	50.8553	4.4904	13-Jun	Rasmont P.	RBINS	Rasmont, 1988	BV16
	Hainaut	La Louvière	50.4989	4.1972	Jul-00	Rasmont P.	RBINS	Rasmont, 1988	BV18
	East Flanders	Moorsel	50.9499	4.0664	3-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV20
	East Flanders	Moorsel	50.9499	4.0664	22-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV24
	Flemish Brabant	Buizingen	50.7687	4.2041	31-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV25
	East Flanders	Moorsel	50.9499	4.0664	8-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV26
	East Flanders	Moorsel	50.9499	4.0664	22-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV27
	Brussels	Petite Espinette	50.7712	4.3814	29-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV28
	East Flanders	Overmere	51.041	3.9258	31-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV29
	Hainaut	Le Roeulx	50.5003	4.0562	6-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV31
	Liège	Francorchamps	50.4693	5.8876	12-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV33
	Limburg	Helchteren	51.1108	5.3559	18-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV34
	Liège	Hockai	50.4657	6.0283	24-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV35
	Flemish Brabant	Halle	50.7687	4.2041	5-May	Rasmont P.	RBINS	Rasmont, 1988	BV65
	Flemish Brabant	Halle	50.7687	4.2041	2-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV66
	Flemish Brabant	Halle	50.7687	4.2041	10-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV68

Time period	Provence	Collection site	Latitude[1]	Longitude[1]	Date[2]	Determinated by	Collection*	Reference bib[3]	Sample Code
	Flemish Brabant	Halle	50.7687	4.2041	10-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV69
	Flemish Brabant	Halle	50.7687	4.2041	21-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV71
	Limburg	Helchteren	51.1108	5.3559	9-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV73
	Limburg	Helchteren	51.1108	5.3559	13-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV75
	Limburg	Helchteren	51.1108	5.3559	13-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV76
	East Flanders	Moorsel	50.9499	4.0664	11-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV78
	East Flanders	Moorsel	50.9499	4.0664	7-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV79
	East Flanders	Moorsel	50.9499	4.0664	12-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV80
	Brussels	Petite Espinette	50.7712	4.3814	815	Rasmont P.	RBINS	Rasmont, 1988	BV82
	Brussels	Petite Espinette	50.7712	4.3814	29-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV84
	Brussels	Petite Espinette	50.7712	4.3814	29-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV85
	Hainaut	Trivières	50.409	4.1949	4-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV86
	Hainaut	Trivières	50.409	4.1949	18-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV88
	Hainaut	Trivières	50.409	4.1949	24-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV89
	Hainaut	Trivières	50.409	4.1949	12-Oct	Rasmont P.	RBINS	Rasmont, 1988	BV91
	Hainaut	Trivières	50.409	4.1949	14-Oct	Rasmont P.	RBINS	Rasmont, 1988	BV92
1923	Walloon Brabant	Archennes	50.7635	4.6293	17-Jul	Rasmont P.	RBINS	Rasmont, 1988	BV42
	Liège	Buellingen	50.3828	6.2559	3-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV43
	Liège	Weverce	50.4727	6.2507	4-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV44
	Liège	Vaux	50.5625	5.752	20-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV45
	Hainaut	Lesdain	50.5046	3.3512	9-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV46
	Hainaut	Lesdain	50.5046	3.3512	9-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV47
	Hainaut	Lesdain	50.5046	3.3512	9-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV50
	Hainaut	Lesdain	50.5046	3.3512	9-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV51
	Hainaut	Lesdain	50.5046	3.3512	9-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV52
	Liège	Buellingen	50.3828	6.2559	Sep-00	Rasmont P.	RBINS	Rasmont, 1988	BV98

Time period	Provence	Collection site	Latitude[1]	Longitude[1]	Date[2]	Determinated by	Collection*	Reference bib[3]	Sample Code
	Liège	Buellingen	50.3828	6.2559	3-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV100
	Liège	Buellingen	50.3828	6.2559	3-Aug	Rasmont P.	RBINS	Rasmont, 1988	BV101
	Liège	Buellingen	50.3828	6.2559	Sep-00	Rasmont P.	RBINS	Rasmont, 1988	BV102
	Liège	Buellingen	50.3828	6.2559	Sep-00	Rasmont P.	RBINS	Rasmont, 1988	BV103
	Hainaut	Kain	50.5945	3.3519	11-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV106
	Hainaut	Kain	50.5945	3.3519	11-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV108
	Hainaut	Kain	50.5945	3.3519	11-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV110
	Hainaut	Kain	50.5945	3.3519	11-Sep	Rasmont P.	RBINS	Rasmont, 1988	BV111

[1]Following the World Geodetic System

[2]Month/Day

[3]Reference bib, specimens determinated and used in Rasmont & Mersch (1998)

*RBINS = Royal Belgian Institute of Natural Sciences

Supplementary File S5. Distribution, trend of decline and red list status of the different *Bombus* spp. In this table we presented, the distribution before and after 1970, trend of decline and red list status of the different *Bombus* spp. following Peeters and Reemer (2003). Species distribution is calculated as the relative areal size = (amount of hour blocks a species is found / the total amount of hour blocks checked) *100%, with an hour block = 5 x 5 km block. The decline in distribution or trend is calculated by Peeters and Reemer (2003) as: (the relative areal size of after 1970 - relative areal size before 1970) / relative areal size before 1970 * 100%).

	Distribution in the Netherlands					
	Before 1970		1970-2001			Red list status
Species	Area size	Hour block	Area size	Hour block	Trend	*1970-2003*
Widespread / stable						
B. pascuorum	42.2%	373	31.2%	343	-26.2%	
B. hortorum	20.3%	179	16.0%	176	-21.0%	
B. pratorum	23.5%	208	21.6%	238	-8.1%	
B. lapidarius	25.9%	229	16.1%	177	-37.9%	
Widespread / declining						
B. ruderarius	16.4%	145	5.5%	61	-66.2%	vulnerable
B. muscorum	21.0%	186	3.6%	40	-82.8%	endangered
B. veteranus	19.9%	176	1.2%	13	-94.1%	endangered
Restricted /declining						
B. humilis	7.8%	69	1.9%	21	-75.5%	endangered
B. sylvarum	5.3%	47	0.1%	1	-98.2%	critically endangered
B. ruderatus	8.7%	77	0.5%	6	-93.8%	critically endangered
B. subterraneus	2.5%	22	0.0%	0	-100.0%	Disappeared

Supplementary File S6. Scoring efficiency of the microsatellite loci for each Bombus spp. in time period 1918-1926. With n = the number of workers and between brackets the number of workers used in all further analysis, *NA* = the number of specimens that were not amplifiable, *FS* = the number of full sibs, and *PUA* = the proportion of unsuccessfully amplified individuals per locus. Microsatellite loci not used for further analysis are underlined with a full line, loci that were not used in only one population of a certain species are underlined with a dotted line, *L* = the maximum number of loci used in further analysis, *ANL* = the average numbers of loci successfully amplified per individual per species, and * = workers of *B. pascuorum* from two additional time points: 1942-1960 and 1975-1995.

Group	*Species*	*N*	*NA*	*FS*	PUA for each microsatellite loci										*L*	*ANL*
					B11	**B121**	**B132**	**B100**	**B126**	**BT11**	**BL02**	**BT04**	**BT08**	**BT10**		
Declining *spp.*	*B. humilis*	20 (16)	0	4	0.0%	0.0%	25.0%	0.0%	31.3%	0.0%	0.0%	0.0%	18.8%	0.0%	10	9.25
	B. muscorum	20 (15)	3	2	6.7%	6.7%	46.7%	6.7%	6.7%	0.0%	0.0%	0.0%	33.3%	0.0%	10	8.93
	B. ruderarius	28 (18)	3	7	61.1%	0.0%	27.8%	0.0%	0.0%	0.0%	0.0%	0.0%	27.8%	27.8%	10	8.39
	B. ruderatus	17 (12)	2	3	100.0%	0.0%	41.7%	0.0%	0.0%	0.0%	58.3%	41.7%	16.7%	0.0%	9	7.42
	B. subterraneus	7 (7)	0	2	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	100.0%	0.0%	8	8.00
	B. sylvarum	16 (11)	3	2	9.1%	0.0%	54.5%	0.0%	54.5%	0.0%	0.0%	0.0%	45.5%	0.0%	10	8.36
	B. veteranus	8 (7)	0	1	0.0%	0.0%	14.3%	0.0%	14.3%	0.0%	14.3%	0.0%	14.3%	0.0%	10	9.43
Stable *spp.*	*B. hortorum*	30 (22)	3	5	0.0%	0.0%	25.0%	0.0%	31.3%	0.0%	70.0%	0.0%	18.8%	0.0%	10	7.05
	B. lapidarius	19 (12)	6	1	0.0%	0.0%	41.7%	41.7%	100.0%	0.0%	58.3%	0.0%	58.3%	0.0%	9	7.00
	B. pratorum	10 (8)	1	1	0.0%	0.0%	12.5%	12.5%	12.5%	0.0%	0.0%	25.0%	37.5%	0.0%	10	9.00
	B. pascuorum	127(106)*	6	15	2.8%	0.0%	10.4%	100.0%	1.9%	3.8%	100.0%	14.2%	3.8%	0.0%	8	7.67
	Total	302(234)*	27	41											9.5	8.23

Supplementary File S7. Sensitivity analysis of genetic diversity. After removal of identified sisters, we conducted a sensitivity analysis of the calculated mean expected heterozygosity (H_E) for each population of the different *Bombus* spp. in the time period 1918-1926, based on more stringent exclusion policies for missing data. From a maximum of 5 microsatellite loci with missing values within one specimen towards only one locus with missing data. With n = the total number of workers in each exclusion step and * = too low number of specimens.

			Maximum microsatellite loci with missing values				
Species	**Location**	**Year**	***5***	**4**	**3**	**2**	**1**
		n	**161**	**159**	**154**	**139**	**112**
Widespread / stable							
B. hortorum	Gelderland	1918	0.697	0.697	0.708	0.688	- *
	Overijsssel	1918	0.763	0.763	0.763	0.767	0.767
	Z-Holland	1923	0.778	0.778	0.765	0.773	0.703
B. lapidarius	Limburg	1918	0.553	0.553	0.553	0.644	0.622
	Overijssel	1918	0.710	0.710	0.710	0.710	0.710
B. pratorum	Overijssel	1918	0.604	0.604	0.604	0.613	0.613
B. pascuorum	Limburg	1918	0.694	0.694	0.694	0.691	0.691
	N-Holland	1924	0.702	0.702	0.659	0.608	0.611
	Overijssel	1918	0.685	0.685	0.685	0.685	0.685
	Gelderland	1925	0.733	0.733	0.733	0.734	0.690
	Total		**0.692**	**0.692**	**0.687**	**0.691**	**0.677**
Restricted / declining							
B. humilis	Gelderland	1926	0.425	0.425	0.425	0.425	0.372
	Limburg	1918	0.366	0.366	0.366	0.366	0.299
B. ruderatus	Z-Holland	1923	0.543	0.543	0.543	0.509	0.493
	Overijssel	1918	0.669	0.685	0.685	0.594	0.525
B. subterraneus	Overijssel	1925	0.625	0.625	0.625	0.625	0.605
B. sylvarum	Limburg	1918	0.451	0.451	0.451	0.470	0.484
	Limburg	1920	0.458	0.458	0.458	0.458	0.458
	Subtotal		***0.508***	***0.508***	***0.508***	***0.492***	***0.462***
Widespread / declining							
B. muscorum	Limburg	1918	0.401	0.383	0.383	0.383	0.389
	Overijssel	1918	0.503	0.498	0.498	0.498	0.498
B. ruderarius	Limburg	1918	0.496	0.496	0.496	0.570	0.570
	N-Holland	1924	0.490	0.490	0.490	0.496	0.458
	Overijssel	1918	0.252	0.252	0.252	0.250	0.289
B. veteranus	Limburg	1918	0.382	0.382	0.382	0.364	0.364
	Subtotal		***0.421***	***0.417***	***0.417***	***0.430***	***0.428***
	Total		**0.466**	**0.466**	**0.466**	**0.464**	**0.446**

Supplementary File S8. Estimation of genetic diversity after extra data exclusion steps. Recalculations of the genetic diversity after removal of three species (*B. subterraneus*, *B. ruderatus* and *B. lapidarius*) and populations with non-amplifications and based on the same eight microsatellite loci in each species.

				A_R		H_E	
Species	**Location**	**Year**	***n***	**Mean**	**SE**	**Mean**	**SE**
Widespread / stable							
B. hortorum	Gelderland	1918	8	5.428	0.845	0.720	0.081
	Overijsssel	1918	7	5.515	0.429	0.779	0.025
	Z-Holland	1923	7	5.648	0.468	0.787	0.023
B. pratorum	Overijssel	1918	8	4.945	0.589	0.727	0.044
B. pascuorum	Limburg	1918	9	4.962	0.582	0.694	0.085
	N-Holland	1924	9	4.777	0.692	0.702	0.072
	Overijssel	1918	8	5.035	0.704	0.685	0.089
	Gelderland	1925	7	5.250	0.457	0.733	0.041
	Total		**63**	**5.195**	**0.118**	**0.728**	**0.014**
Restricted / declining							
B. humilis	Gelderland	1926	8	3.546	0.410	0.574	0.078
	Limburg	1918	8	3.182	0.363	0.522	0.072
B. sylvarum	Limburg	1918	6	3.821	0.610	0.601	0.089
	Limburg	1920	5	3.286	0.565	0.589	0.069
	Subtotal		***27***	***3.459***	***0.143***	***0.572***	***0.017***
Widespread / declining							
B. muscorum	Limburg	1918	7	3.603	0.640	0.516	0.109
	Overijssel	1918	8	4.360	0.517	0.613	0.078
B. ruderarius	Limburg	1918	7	4.149	0.594	0.620	0.102
	N-Holland	1924	5	3.750	0.697	0.610	0.089
	Overijssel	1918	6	3.663	0.792	0.566	0.107
B. veteranus	Limburg	1918	7	4.153	0.423	0.619	0.064
	Subtotal		***40***	***3.946***	***0.128***	***0.591***	***0.017***
	Total		**67**	**3.751**	**0.144**	**0.583**	**0.014**

Supplementary File S9. Population structuring of the *B. pascuorum* populations. Pairwise F_{ST} (with ENA correction) for the different populations of *B. pascuorum* under the diagonal and the harmonic mean of *D*est across loci above the diagonal, a) between locations within a time period and b) within a location between time periods. With indication of the significance level, ** = $P < 0.001$ and * = $P < 0.005$.

a)

Limburg	1918	1949	1989
1918	-	0.017	0.073
1949	0.018	-	0.079
1989	0.053**	0.084*	-

N-Holland	1924	1955	1980
1924	-	0.057	0.045
1955	0.012	-	0.001
1980	0.032	-0.010	-

Overijssel	1918	1990
1918	-	0.032
1990	0.045	-

Gelderland	1925	1951	1980	1975
1925	-	0.006	0	0.030
1951	0.002	-	-0.010	0
1975	-0.001	0.002	0.013	-

b)

1918-1925	N-Holland	Limburg	Overijssel	Gelderland
N-Holland	-	0.088	0.002	0.018
Limburg	0.039	-	0.012	0.059
Overijssel	0.023	0.011	-	0.008
Gelderland	0.013	0.040	0.032	-

1944-1955	N-Holland	Limburg	Drenthe	Gelderland
N-Holland	-	0.060	0.022	0.011
Limburg	0.036	-	0.058	0.057
Drenthe	0.070	0.050	-	0.068
Gelderland	0.022	0.075*	0.033	-

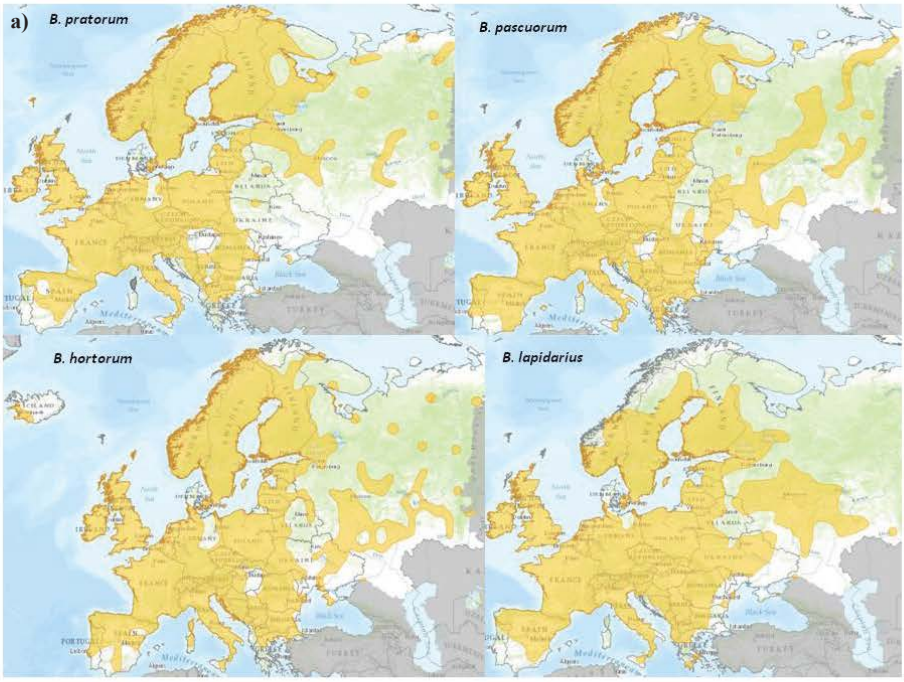
1975-1990	N-Holland	Limburg	Overijssel	Gelderland
N-Holland	-	0	0.001	-0.001
Limburg	-0.009	-	0.042	0.003
Overijssel	-0.014	0.012	-	0.014
Gelderland	0.013	0.001	0.004	-

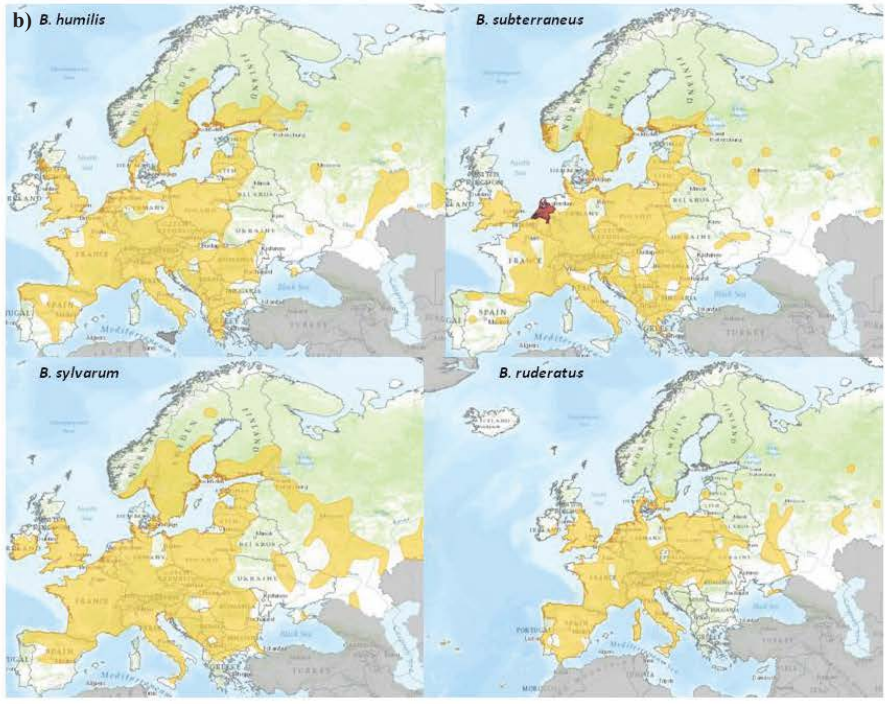
Supplementary File S10. Comparison of the genetic diversity in historical and recent populations of declining and more stable bumblebee species. The data was obtained from our study and from the available data on recent populations found in the literature. With time periods: 'historical' = 1895-1930; and 'recent '= 1975-2010'.

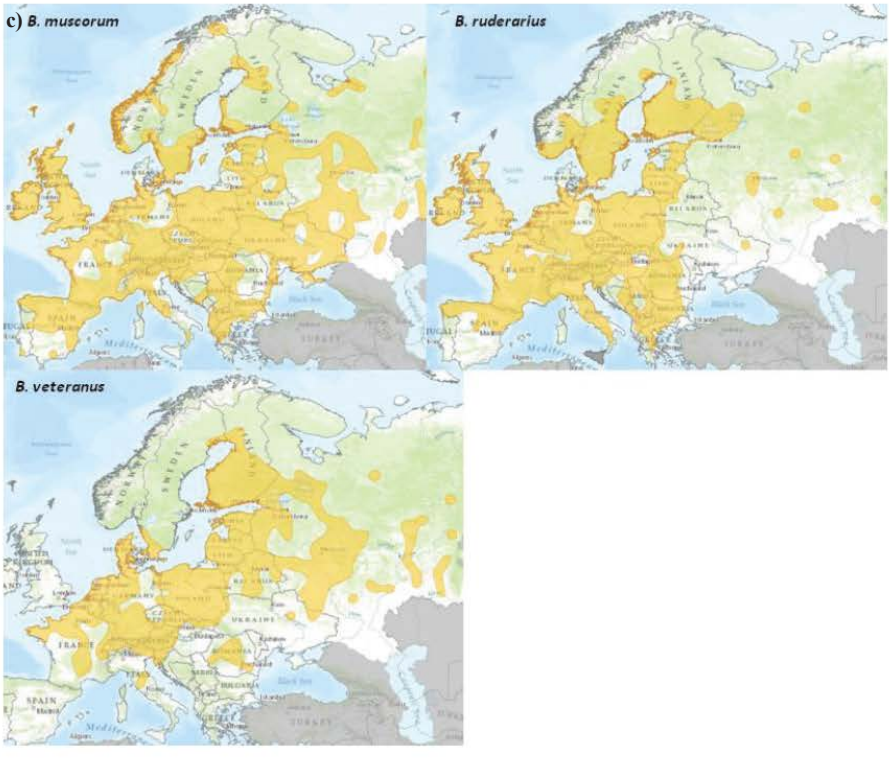
Bombus species	Country	Sample size	Collection time	H_E	SE	A_R	SE	Reference
Declining *spp*.								
B. humilis	Netherlands	16	1918-1926	0.396	0.096	2.717	0.435	This chapter
B. humilis	UK	150	2005	0.460	0.070	4.000	0.340	Connop *et al.*, 2010
B. muscorum	Netherlands	15	1918-1926	0.477	0.107	3.514	0.620	This chapter
B. muscorum	UK	35.5	2003-2005	0.509	0.013	4.010	0.060	Darvill *et al.*, 2010
B. ruderarius	Netherlands	19	1918-1926	0.413	0.106	2.957	0.600	This chapter
B. ruderatus	Netherlands	12	1918-1926	0.606	0.067	3.808	0.475	This chapter
B. subterraneus	Netherlands	5	1918-1926	0.625	0.078	4.111	0.526	This chapter
B. sylvarum	Netherlands	11	1918-1926	0.455	0.110	2.947	0.595	This chapter
B. sylvarum	France	18	2004	0.530	0.090	4.000	0.850	Ellis *et al.*, 2006
B. sylvarum	UK	173	2003-2004	0.390	0.020	3.120	0.100	Ellis *et al.*, 2006
B. sylvarum	UK	150	2005	0.520	0.110	5.570	1.590	Connop *et al.*, 2010
B. veteranus	Belgium	6	1895	0.607	0.062	3.470	0.345	Chapter 2
B. veteranus	Belgium	34	1915	0.577	0.117	3.680	0.626	Chapter 2
B. veteranus	Belgium	18	1923	0.578	0.118	3.710	0.645	Chapter 2
B. veteranus	Netherlands	7	1918-1926	0.636	0.060	4.236	0.388	This chapter
Stable *spp.*								
B. hortorum	Netherlands	21	1918-1926	0.746	0.045	5.362	0.593	This chapter
B. hortorum	UK	86	2003-2005	0.890		5.700		Goulson *et al.*, 2011
B. lapidarius	Netherlands	12	1918-1926	0.632	0.083	4.302	0.786	This chapter
B. pascuorum	Netherlands	33	1918-1926	0.704	0.036	5.013	0.292	This chapter
B. pascuorum	Netherlands	30.5	1975-1995	0.692	0.036	5.148	0.344	This chapter
B. pascuorum	UK	32	2003-2004	0.520	0.110	7.070	1.240	Ellis *et al.*, 2006
B. pratorum	Netherlands	8	1918-1926	0.671	0.057	4.46	0.603	This chapter
B. terrestris	Poland	238	2008-2009	0.720	0.072*	7.933	2.517*	Kraus *et al.*, 2011
B. terrestris	UK	24	1998-2000	0.826	0.019	5.079	0.700	Schmid-Hempel *et al.*, 2007
B. terrestris	Spain	53	2003	0.600	0.080*	4.200	1.600*	Kraus *et al.*, 2009
B. terrestris	Germany	337	2004-2005	0.730	0.100*	7.150	2.200*	Kraus *et al.*, 2009
B. jonellus	UK	42	2003-2005	0.755	0.071	10.02	1.980	Darvill *et al.*, 2010

*** = SD used instead of SE**

Supplementary File S11. Distribution maps of the different *Bombus* species used in chapter 4. Distribution maps adapted from IUCN, (2014). In orange = resident; and red = extinct. With a) the distribution maps of the widespread more stable species; b) the distribution maps of the widespread declining species; and c) the distribution maps of the restricted declining species.




b) B. humilis
B. subterraneus
B. sylvarum
B. ruderatus


c) B. muscorum
B. ruderarius
B. veteranus

Supplementary File S12. Characteristics of the microsatellite markers used. From each SSR marker we present the forward and reverse primer sequences, GenBank accession number, annealing temperature (*Ta*), the observed size range of the PCR product, the location (LG) and the original reference.

Marker	Accession number (GenBank)	LG *B.t.*	Forward primer sequence	Reverse primer sequence	*Ta*	Range	Source
0007_47n22	FQ377670	B11	GGTTATGATTGCACACTGTTT	GCACATTAAATTATTGCGTACA	60	152-158	Stolle et al., 2011
0043_71h15	FQ377672	B03	AATTTATGCGAAGATGATGTTA	TTAGTAACTGACTGCTGCTACG	60	164	Stolle et al., 2011
0053_71f10	FQ377673	B02	GTAAAGCGGAGAAACAAGATAG	CTTCTTCCACCTCTCATTTG	60	168	Stolle et al., 2011
0062_62f17	FQ377675	B02	TAAAATTGCTGGCTGGAAGCAT	CAGAGAACAACATCGTGGCAAA	60	197-213	Stolle et al., 2011
0071_59g6	FQ377677	B13	TACGATTCACCGATCTTAAATA	TTAATCGGAAGACACTGGAC	60	187-191	Stolle et al., 2011
0078_59o4	FQ377680	B14	AATCTCGTAATTCCAAGCTTAC	GATTTACTTGGGCAGACTTTAG	60	150-156	Stolle et al., 2011
0083_47g5	FQ377681	B13	TCTTAATCGATTCAAACATCCT	GATGAGTGTCATCCTTCTGAAT	60	136-148	Stolle et al., 2011
0103_38m23	FQ377683	B05	GTATCGCGATTGGTAATTATG	ACATCTTTGTATCTTCGAATCC	60	187	Stolle et al., 2011
0141_44j1	FQ377689	B02	CTAGGCCAGAATAGAGTCGTC	AGATTCGAGTGCTTTCCTCT	60	137-157	Stolle et al., 2011
0152_56e6	FQ377691	B09	GAACCTGTGTTCCTCTCGTA	TCTACTACACTTTGTCCGTTGA	60	146-148	Stolle et al., 2011
0162_69a8	FQ377693	B12	GAAGGAGTTGAATCATTAGGTC	TTCGTAGGGTGATAGAGGTG	60	155-157	Stolle et al., 2011
0172_44e21	FQ377695	B04	ATAATGCAGTTCCTCGAGTCT	GCTGTATTGGGTAGAAGAAAGA	60	148-150	Stolle et al., 2011
0177_44p18	FQ377696	B03	TTGACGATATTCTCTCACGATA	GCGTTTCTATCAGAAGCTACAC	60	169-172	Stolle et al., 2011
0180_50k19	FQ377697	B17	CCTTCCTGGAGGTAACCTTCTT	TTCATACGCGAGGTATGTGGAG	60	216	Stolle et al., 2011
0187_69g1	FQ377698	B18	TCTTGTATTAACCCAACGTACA	GCAGCTAACGGATCTTATTCTA	60	161-169	Stolle et al., 2011
0195_69j13	FQ377699	B01	CTGAACAATAATTACCGACAGA	GACAATTTCGATTACGAGACTT	60	150-154	Stolle et al., 2011
0196_69p16	FQ377700	B01	CGCTGAATCTAGACGCTATAA	ATCAGTGGCAATACATGTAAAC	60	188-194	Stolle et al., 2011
0198_69f24	FQ377701	B01	AAATAGCTCGACACTGAGAGAC	ATCCATAAGCGTGTAAGAAAGT	60	164-168	Stolle et al., 2011
0207_63e15	FQ377704	B03	TGTCTTTACGTCCATGTTACAC	CGTTCTCTATATACGGCAAGTT	60	193-197	Stolle et al., 2011
0216_63a9	FQ377705	B05	TCATAACGTTTCACATCTTGAC	GTCTAAAGTTCTATGCCACGTT	55	175-177	Stolle et al., 2011
0221_63h9	FQ377706	B03	GTTATCGTATTTACACCGGAAC	TTTCTTCGCAAGATAGAGAGAG	55	154-158	Stolle et al., 2011
0222_63d21	FQ377707	B15	TCAATCTTCGATCTACGTAACA	AAATACGTGGCATTAACTCG	60	165-169	Stolle et al., 2011
0232_81d20	FQ377710	B13	GCGAGTCTGTACAATGAATATG	ACGGAACAACGAACAACTTA	60	171-177	Stolle et al., 2011
0242_81d21	FQ377711	B09	CCTCGATATCACCATAGGAA	ACAGATGTATCCGTGCAGTT	60	183-187	Stolle et al., 2011

Marker	Accession number (GenBank)	LG *B.t.*	Forward primer sequence	Reverse primer sequence	*Ta*	Range	Source
0244_8l18	FQ377712	B13	AGAAGCTACAGTTGATGAGGAT	GAGTTCTCTGCTTGTCTGATG	60	142-150	Stolle et al., 2011
0255_16m20	FQ377713	B16	TCGTAAAGCTGAGAGCTATAAA	AAGATCGAGAGAACAGGATTAG	60	155-161	Stolle et al., 2011
0266_60m24	FQ377714	B10	AACGTTTCGAGAATAATAAAGC	AGGTAAAATGAACGAGACAAAG	60	213	Stolle et al., 2011
0268_60h13	FQ377715	B05	TCTGTAAACACAAGAGAAGTTCA	TCTGACGGTAGGACAATACTAA	60	210-203	Stolle et al., 2011
0275_90o12	FQ377716	B04	AGGACATCTGGTCTTAATGAAA	TACGCAGATCGTTGTTATACAT	60	163-165	Stolle et al., 2011
0281_20d1	FQ377719	B06	GTAGCCTTCTCTATGCCATTT	AGAACGGGTACGTGTAAATAGT	60	131-135	Stolle et al., 2011
0289_60l4	FQ377722	B10	TGTTGTATTGTGAATGTACACG	GCATGTAAACCAGACTTAATTG	54	162-164	Stolle et al., 2011
0291_60p14	FQ377723	B03	TCTGCTACGTTTAATTACTGGA	ACCCTCTTACCTATTTGGTGTA	54	196	Stolle et al., 2011
0292_60b14	FQ377724	B03	GCGTACGATATAAGGAAAGAGA	GTGAGTTCGAGCAATAATCC	55	153-167	Stolle et al., 2011
0294_10o4	FQ377725	B09	AGTACGATAAAGCCAGGAAAG	TGTATGCCTATTGTACGAGTGT	55	169-177	Stolle et al., 2011
0303_10b14	FQ377727	B02	AAAGTGTCATCGACCAGAAG	CTCGTTCGTTTAATTAGTCGTC	60	164	Stolle et al., 2011
0304_9i13	FQ377728	B04	GTATGAGTGAGTGATGTGCAAG	CCCTTCATCTCTGAACAATATC	55	154-160	Stolle et al., 2011
0320_15c24	FQ377729	B12	TTTCATTGTTTCCCTATTTCA	CCTTACGTACTTCCGTTATCTC	55	162	Stolle et al., 2011
0321_15f5	FQ377730	B17	ATGACAAAATATAGCACTGTATGTGT	GCAGAACGAACAAGATGTTCAA	60	210-212	Stolle et al., 2011
0336_1l24	FQ377734	B13	ACTTAGACACGCCTCAATTATC	GTTGAACCTTTGTTGAGAAGAT	60	114-136	Stolle et al., 2011
0338_2i5	FQ377735	B07	TCGTACTTCGTTCATCTAATCA	GGAATTTGTAATTTCGTTTGTT	54	172-174	Stolle et al., 2011
0357_2o10	FQ377736	B05	CGACAGTTGTTATTACGATGAA	CCCTTTAAGCAGACGTATTTAG	60	163-171	Stolle et al., 2011
0360_2n11	FQ377737	B01	ATAATTCCCGAACGAATGTC	TAACGTTATCCGGGTACAAA	55	231-237	Stolle et al., 2011
0365_7n6	FQ377738	B03	GCCATCAATAGATCAAAGAAAT	CTCCTCGGTCTGTTGTTTAT	60	138-140	Stolle et al., 2011
0370_12o14	FQ377739	B11	GGAACACGTATTACACAAAGTCT	CGAGAGACAGAGAGAAAGAAAG	54	221-225	Stolle et al., 2011
0374_12n22	FQ377741	B09	CCAGAGTGAGAAAGAGAGAGAG	CATAAATGTCCCACCTACATC	60	133	Stolle et al., 2011
0379_5i10	FQ377743	B07	AGAGAGAAAATCGAGGAAAAG	CGCAAAGTATTGCATAAATAAG	54	201-205	Stolle et al., 2011
0382_5e22	FQ377744	B11	GATAGAAACGACCAGGCTTAT	GGAACGAGTAACAAGGTAGAGA	60	167-169	Stolle et al., 2011
0392_16e2	FQ377746	B09	AATTATCATCGCGTAGGTACA	GTAAGCGTTTACACGAACAAC	60	183	Stolle et al., 2011
0403_13g6	FQ377748	B07	ATTTACTGCTCGATACTTTCGT	ATCGAGTTCTTATTTCATCCTG	60	175-179	Stolle et al., 2011
0428_13l21	FQ377750	B02	AAGGTAACAGAAGAGACGATTG	GTCATTGTCAAGAGGTGGAG	60	154-156	Stolle et al., 2011
0466_5f11	FQ377753	B14	GTGTGTGCAAATAGCTACAGAT	GTCCCTTTACCCTTTAGATACC	60	163-171	Stolle et al., 2011
0482_3k3	FQ377756	B13	ATGGCAAGTGTTCTCGTACT	ATCTATTTACCAGCGAAGCTC	54	204	Stolle et al., 2011

Marker	Accession number (GenBank)	LG *B.t.*	Forward primer sequence	Reverse primer sequence	*Ta*	Range	Source
0487_3f11	FQ377758	B12	CACCTTACAATATAGGTCAGTTGT	GTCTAGGTGCTCAATGGATATT	55	144-152	Stolle et al., 2011
0503_3m14	FQ377760	B13	ACAACTAATTTGCTGCCTCTAC	TAGGATCATTAAACGAGTCTCC	60	167-183	Stolle et al., 2011
0526_4c10	FQ377761	B07	TCACGTTGTGTCAACTGTAAA	AGATTCAAGACGAAAGAATTTG	55	162-176	Stolle et al., 2011
0533_15c9	FQ377762	B07	CGAAGAACATAAGCAGAGGTAG	CTTCCTCTTCGGTTCTCATAC	60	160	Stolle et al., 2011
0535_15i17	FQ377763	B05	GTCGCATTAAATACAAGCTACA	TTTCAAAGTGATATACAGGGAAG	55	139-157	Stolle et al., 2011
0543_6o7	FQ377764	B12	AGCTAAATTAACCAACACCAAT	GGCAGAGGAATATGATACAAGT	55	163-181	Stolle et al., 2011
0553_18c8	FQ377768	B09	AGGATTCCATTTCGAGAATAA	CAATGCACTACAAAGTTAGTTCC	54	236-242	Stolle et al., 2011
0566_20o5	FQ377772	B03	TGTTAATCGTCTGTCACCTTT	GTAGCAAGAAGTAGGCAAATG	55	180	Stolle et al., 2011
0576_20n23	FQ377773	B08	CCGTGCTATACTCACATTTCTA	ACGATCTATGTACCACGATTCT	55	166-168	Stolle et al., 2011
0579_22m16	FQ377774	B10	GCCAGGTACATATATCCCTATT	TTCCATATTTGCTGTCACTTT	60	186-202	Stolle et al., 2011
0583_22l4	FQ377776	B15	CGAATGAAATTAGCTCCACTAC	CAATTTCTTTCTCTTACGAAGC	60	126-140	Stolle et al., 2011
0594_19n18	FQ377778	B15	TTCAGAAGCATTCTCGAATTA	ATACGAAGAGAAATAGGGTACG	60	208-212	Stolle et al., 2011
0606_19m4	FQ377781	B02	ATAACGAGGAGAGTGGTAACTG	GTCTCCTAGCATCTTCTTTGTAA	60	235	Stolle et al., 2011
0607_19k14	FQ377782	B07	TCCATATGAAGATCACAGAGAA	TTAATCAGTGCATGCTTAGTGT	60	154-160	Stolle et al., 2011
0608_19h1	FQ377783	B17	GATCGATAAACGTCCAACTTAC	ATGGATTCTATCATCAATTCGT	60	209-211	Stolle et al., 2011
0613_19h23	FQ377784	B12	TTTATTCTACGCAAATGGTG	TATCAATATCAGTATCGGCATC	60	190-222	Stolle et al., 2011
0614_19d6	FQ377785	B08	AAGTAGAACGGATACAGAAACG	ACTCCAGTATGAGATGGAAGTC	60	186-196	Stolle et al., 2011
0627_20n22	FQ377789	B08	CGTGTAAACACACATAAAGAGC	GTTTCGTCTTCGCTCTAGATAC	60	176-192	Stolle et al., 2011
0631_34k4	FQ377790	B05	ATAACCGAAAGACAAAGTTCAC	GCTCTTGCTCTTTCTTTATCTT	60	160	Stolle et al., 2011
0632_34i8	FQ377791	B03	TTCCCGTATTATGTAACTCAGA	GCTTGGAGAAGATAGTTAAACG	60	189	Stolle et al., 2011
0636_34m4	FQ377792	B14	AGTGAAAGTTGACGAAGAACA	CCGAGATCTCTCTCTGTACTGT	60	145-151	Stolle et al., 2011
0644_83i19	FQ377796	B06	CATTGTCGAGTGAATATCGAG	TAGAAATCATTGCAACAGAGAA	60	166-168	Stolle et al., 2011
0646_83e8	FQ377798	B04	GTTTCTCTCTTCCCTCTTTCC	AAGATGCAGAGGAAAGTAAATG	60	155-165	Stolle et al., 2011
0655_82m17	FQ377800	B14	TACATCTACTCGTCTCCCTCTC	ACGGATAGACAAACAGAGAATC	60	134-136	Stolle et al., 2011
0669_84l15	FQ377804	B09	TGCTTGACGAATATGAAATG	AAACAGATCGAGAGAAAGAGAG	60	170-180	Stolle et al., 2011
0686_86i9	FQ377807	B14	AAAGATAGAAGAAGGAAGCACA	TCGAACTATCCACCAGCTATAC	60	130-136	Stolle et al., 2011
0712_84o11	FQ377811	B06	TTTCGATGGTGGTTTGTACT	CTTGCCGATATATTACCTCTTC	60	198-210	Stolle et al., 2011
0725_82m14	FQ377814	B06	TCTATCAAACACGTAAAGCGTA	ATTTATGACCTCTCCTCTCACA	60	161-195	Stolle et al., 2011

Marker	Accession number (GenBank)	LG *B.t.*	Forward primer sequence	Reverse primer sequence	*Ta*	Range	Source
0731_75c7	FQ377815	B09	GTGTACAGGCATACAGAAAGTG	GAAAGAGGAAGAGAGAAATCAA	60	181-185	Stolle et al., 2011
0742_75d18	FQ377818	B05	ACAAAGTGGTGCCATATTTATT	AGATACTGTGACCTAAGGGAAA	60	170-174	Stolle et al., 2011
0745_75i17	FQ377819	B13	GAATACACATGTCTCTGGTTCA	GAGAGTCGATCTTGTGAGAGAT	60	168	Stolle et al., 2011
0751_11c2	FQ377820	B16	CTTCAACGTCCATCCTGTATC	CCATCTTTTTCACCCTTGTATATAC	60	193-199	Stolle et al., 2011
0752_11m6	FQ377821	B10	AAATTAACACCAGCGGTTCT	AACTTTCTAAGCGTGTGCAG	60	163-175	Stolle et al., 2011
0774_1a20	FQ377826	B14	CTCCCTCTCTCTCTTTCTCTTT	CCCATATCAGTGACAAAGAAC	54	175-189	Stolle et al., 2011
0777_1j15	FQ377827	B10	TGCACTCATAGAATGAGAAAGA	GGATTTGTGGACGTTAATTG	55	152-166	Stolle et al., 2011
0795_67k24	FQ377830	B03	GAATTCCCAGAGAACAATTTC	TAAATTTACGAGTTTGCACAAG	60	161-169	Stolle et al., 2011
0801_67f8	FQ377832	B01	ATACTGTACGCGCATGTAATAA	TAATTTCTTCTCCTCGTTTCTC	55	167-195	Stolle et al., 2011
0803_67i16	FQ377833	B10	CCAGGTAAAGGTAACAAATCAC	GTGTTAGGGACACGTCAAGT	60	172	Stolle et al., 2011
0810_65a23	FQ377835	B06	TTAACAAATCCGAATTTAAAGG	GATAGTGGTTGCTTGTCATCTT	55	136-140	Stolle et al., 2011
0811_65m2	FQ377836	B08	TACAACTTGACGAGGAAATAGG	TTAAGCGAGCCCTATACTTATG	60	180-188	Stolle et al., 2011
0867_70k14	FQ377846	B12	ATATTACATTCCTGGTGACCTC	CTACATTCTTTCTGTTCCCTGT	60	177-181	Stolle et al., 2011
0869_70d5	FQ377847	B08	ATCTGATATCTATGCGCTCTTT	AAGCAGATGGGTTAAGTGTAGT	60	163-175	Stolle et al., 2011
0885_52p13	FQ377850	B02	TTCATACTCTTTCACAGCCTCT	AATGACGAGATGAGACTGAAAT	60	160-164	Stolle et al., 2011
0887_52l8	FQ377851	B09	GGCGAGTGTAACGTTGTATTT	GATATTACGCTCTGGAACCAA	60	187-191	Stolle et al., 2011
0904_31d21	FQ377854	B14	TTAAACCGAGGAGAGAGATTAC	GAGAAGAGACGTTTGAGAGAAC	60	204-212	Stolle et al., 2011
0916_31f17	FQ377858	B06	CCCATCAAATTTAACTGTTCTT	GCGAGTCATTACTGTCTCTCTT	60	170	Stolle et al., 2011
0917_31j16	FQ377859	B16	GTGTGGAAGAGACGAGATAGAT	CTTCTTCGTCACGTTTACTCTC	60	188-204	Stolle et al., 2011
0919_66k13	FQ377861	B01	TAGACCGATTTGTTACTGATTG	CATGCTGTTATGGTATTTCTGA	60	164	Stolle et al., 2011
0930_40o1	FQ377864	B11	GCTGAAAGCTCGACTTCTAC	AAATTTCTCACTGCTAAGAGGA	60	157-177	Stolle et al., 2011
0939_33h17	FQ377865	B01	GAACACAGCGAGAAAGAGAG	CATTATCGTGTGAACTTGGAC	60	152-174	Stolle et al., 2011
0940_33f14	FQ377866	B02	AGTGGAAATCTCACACATGC	AGGAGTTTCGTCGTTTCTTT	60	175-177	Stolle et al., 2011
0942_23k3	FQ377867	B06	TCTCATTTCTCTCCTTCTTCC	ATACAAGAAACGAGCCAGATAC	60	223-225	Stolle et al., 2011
0950_23a2	FQ377870	B18	CGTACTAAACGGTGTATCGTC	GTAATTGAGCTCTCCTGTGG	60	182	Stolle et al., 2011
0956_26c17	FQ377871	B02	TCTCTTGCTTCTCGTTCTACTT	GGCTCTTAAACCAGACAGTTT	60	173-175	Stolle et al., 2011
Apis_UN075	AADG05004561	B06	GTCGTCGCATGAAAGGCC	GCAACCTCGTGCCCAGAT	0	0	Solignac et al., 2007
B118		B03	CCTAAGTCGCTATATCTTCG	GAAACACGTATCTACATCTACAG	57	240	Estoup et al., (1993; 1995)

Marker	Accession number (GenBank)	LG *B.t.*	Forward primer sequence	Reverse primer sequence	*Ta*	Range	Source
BL02		B01	GAACAGTGAGAGCGAGGAACAGAG	TTGCCACGTATATCCGAGCGAACC	52	163-171	Reber-Funk et al., (2006)
BL05		B07	CGTTCAACATTAGATGTAGAGTACC	CGGACACAAGTAATAAGATAGG	50	176-178	Reber-Funk et al., (2006)
BL13		B15	CGAATGTTGGGATTTTCGTG	GCGAGTACGTGTACGTGTTCTATG	53	205-217	Reber-Funk et al., (2006)
BL16		B13	CGTCCTCTCCAATGTGTGACTC	GGATCGGTTTAACAACGAAGTC	48	124-140	Reber-Funk et al., (2006)
Boro115	HQ682231	B06	AGGAACCGAGCGATAGAACCAC	GCTTTGCCTTTCCATCTTGCTG	47	175-183	Stolle et al., 2011
BT02		B11	AGGAACCGAGCGATAGAACCAC	GCTTTGCCTTTCCATCTTGCTG	53	175-183	Reber-Funk et al., (2006)
BT05		B02	TTTCCTATGCCGAACGTCACC	CCCAGATAAAAGACCGCCTCTAGTC	53	194-220	Reber-Funk et al., (2006)
BT08		B03	AGAACCTCCGTATCCCTTCG	AGCCTACCCAGTGCTGAAAC	52	208-230	Reber-Funk et al., (2006)
BT10		B08	TCTTGCTATCCACCACCCGC	GGACAGAAGCATAGACGCACCG	53	178-188	Reber-Funk et al., (2006)
BT11		B09	AAGAGAGAGACAGAGAGAGATAGGG	GCGTTTTGACGATTAGATTAGAGCC	52	153-177	Reber-Funk et al., (2006)
BT15		B15	ACTTAGCCAGCCATCGCTAC	CTCTCTCTTTCTCTCTCTTATACGC	53	182-214	Reber-Funk et al., (2006)
BT20		B10	TTCCACAGCGTTTTCTTAAGTC	ATGGACGGCGAGATCGTGAG	52	157-165	Reber-Funk et al., (2006)
BT23		B11	GCAACAGAAAATCGTCGGTAGTG	GCGGCAATAAAGCAATCGG	54	198-216	Reber-Funk et al., (2006)
BT24		B07	TCTTTCCGTTTTCCCCCTG	CACCCACTTACATACATACACGCTC	52	227-257	Reber-Funk et al., (2006)
BT30		B12	ATCGTATTATTGCCACCAACCG	CAGCAACAGTCACAACAAACGC	53	201-203	Reber-Funk et al., (2006)
BTERN01		B11	CGTGTTTAGGGTACTGGTGGTC	GGAGCAAGAGGGCTAGACAAAAG	49	120-122	Reber-Funk et al., (2006)
BTMS0071	FJ616203	B15	CGCGTAAATTATTCCCCTCCC	CCATCTCGCGCAGAATGTTT	57	237-239	Stolle et al., (2009)
BTMS0081	FJ616212	B06	ACGCGCGCCTTCTACTATC	AGGGACACGCGAACAGAC	60	321-327	Stolle et al., (2009)
BTMS0087	FJ616218	B10	CGCGACGTATAGACAGAGGA	AGCTGCCAGCGCTAAAGTAT	60	202-210	Stolle et al., (2009)
BTMS0093	FJ616224	B09	AGATTGCGATGGCTAAAGTCG	AAAGTCTCTACTGTCGCGCT	51	316-320	Stolle et al., (2009)
BTMS0099	FJ616230	B06	TGTCGGTGTTTCAACACTTTGT	AAAGAGGCGACTACGGTCAA	51	192-196	Stolle et al., (2009)
BTMS0102	FJ616233	B12	AATCGCAAGGGAAAACGTCC	TCTTCTCCGGTGTTTTCGGA	60	219-225	Stolle et al., (2009)
BTMS0103	FJ616234	B15	CAGGTGTTGCCGGCTAGATA	CTCAACGGATCTGGGACAGT	55	314-343	Stolle et al., (2009)
BTMS0124	FJ616254	B06	CGCCGTAATGTTAACTCC	ACTCAATCCAAACGCCACC	54	270	Stolle et al., (2009)
BTMS0129	FJ616259	B10	CCTCGCGAATAGATAAAT	AGCTACCGTGCCTGTCC	55	154-160	Stolle et al., (2009)
BTMS0130	FJ616260	B09	AGACAAAGGGAGATGGTG	TTTCGTTCCTCGTGCTAC	52	302-306	Stolle et al., (2009)
BTMS0131	FJ616261	B10	TACAAACGATGCGTGAGG	AGTCAAGTAAGTCCTACCG	48	331-335	Stolle et al., (2009)
BTMS0147	FJ616276	B15	TTGAGAAAGTAGAAAAATGGA	TCTGTTTATCGATCCTCTTC	51	170-174	Stolle et al., (2009)

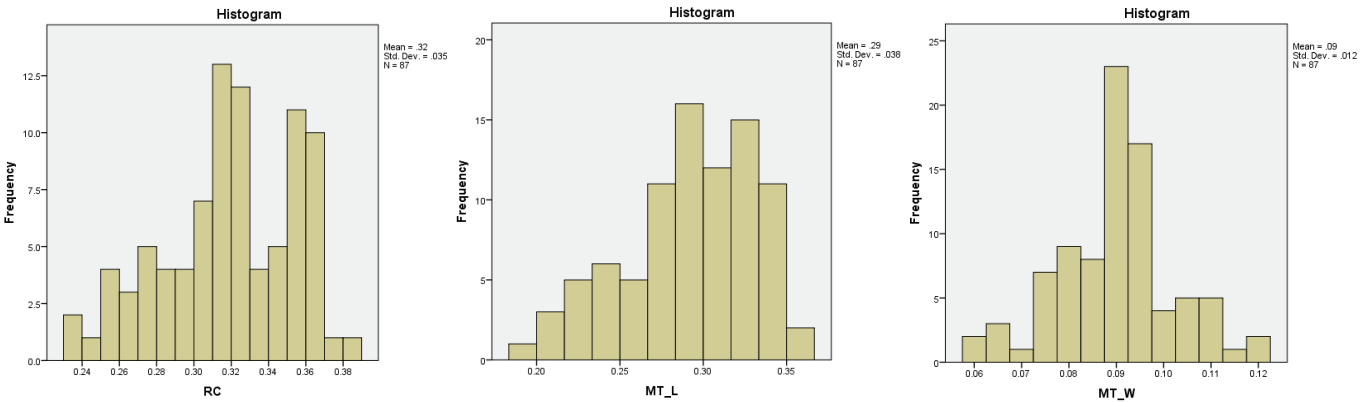
Supplementary File S13. Distribution information of the 100 markers used for preliminary linkage mapping. The number of markers on each linkage group (n), the size of this linkage group (size LG), and the minimum (Min. d) and maximum (Max. d) distances between two markers on each linkage group.

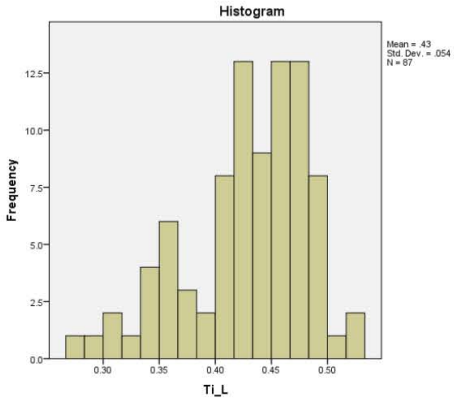
	size LG (cM)	n	Min. d (cM)	Max. d (cM)
LG01	121.01	6	6.95	22.29
LG02	125.20	6	8.23	26.05
LG03	96.35	7	7.18	18.26
LG04	80.66	4	3.68	25.33
LG05	102.84	5	12.93	22.95
LG06	171.70	9	2.72	65.56
LG07	161.43	8	5.26	49.87
LG08	91.64	6	2.58	17.91
LG09	109.48	6	8.30	23.70
LG10	126.46	7	10.42	28.13
LG11	116.30	7	12.09	28.72
LG12	111.39	7	9.78	20.00
LG13	105.74	5	11.97	22.53
LG14	73.44	4	7.12	26.03
LG15	96.55	5	13.22	36.44
LG16	77.87	3	9.97	40.38
LG17	83.14	3	17.98	40.55
LG18	51.01	2	6.01	45.00
Mean	*105.68*	*5.56*	*8.69*	*31.09*

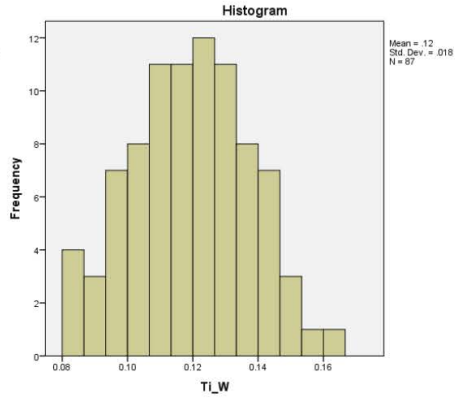
Supplementary File S14. Kolmogorov-Smirnov test of normality for each trait.

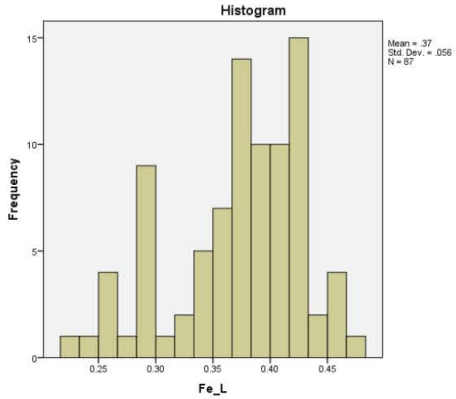
	Kolmogorov-Smirnov[a]		
Trait	**Statistic**	**df**	**Sig.**
RC	0.091	87	0.071
MT_L	0.088	87	0.095
MT_B	0.102	87	0.026
Ti_L	0.094	87	0.057
TI_B	0.053	87	0.200
Fe_L	0.119	87	0.004
Fe_B	0.300	87	0.000
Tr_L	0.074	87	0.200
Tr_B	0.120	87	0.004
Tarsus	0.092	87	0.066
Poot	0.104	87	0.021
E_L	0.124	87	0.002
E_B	0.186	87	0.000
Facet	0.135	87	0.000
Ocel	0.077	87	0.200
E_S	0.147	87	0.000
Omma	0.102	87	0.027
BLUE	0.271	87	0.000
UV	0.177	87	0.000
Weight	0.060	87	0.200
log_blue	0.174	87	0.000
log_uv	0.166	87	0.000
Pc1_E	0.134	87	0.001
Pc2_E	0.152	87	0.000
Pc3_E	0.066	87	0.200
Pc1_S	0.081	87	0.200
Pc2_S	0.131	87	0.001
Pc3_S	0.176	87	0.000
Pc4_S	0.066	87	0.200
Pc5_S	0.150	87	0.000

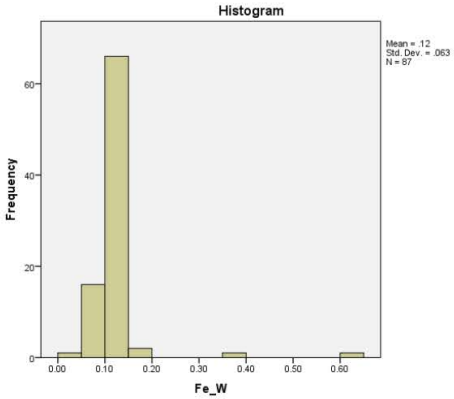
Supplementary File S15. Histogram of all investigated morphological traits: forewing radial cell length (RC), metatarsus length (MT_L), metatarsus width (MT_W), tibia length (Ti_L), tibia width (Ti_W), femur length (Fm_L), femur width (Fm_W), trochanter length (Tr_L), trochanter width (Tr_W), tarsus length (tarsus), length of hind leg (Leg), length of compound eye (E_L), width of compound eye (E_W), total surface of compound eye (E_S), diameter of facet (Facet), total numbers of ommatidia (Om), diameter of median ocellus (MOc), body mass (weight), and the transformed critical light sensitivity in blue and UV light conditions (log_blue and log_UV, respectively).

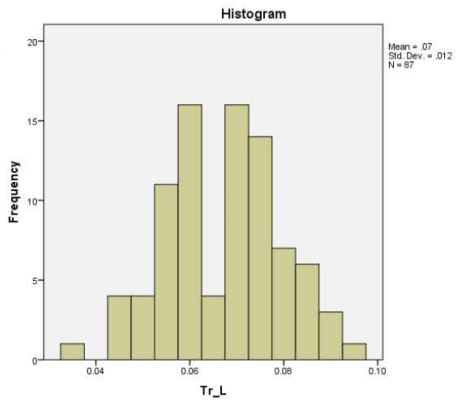


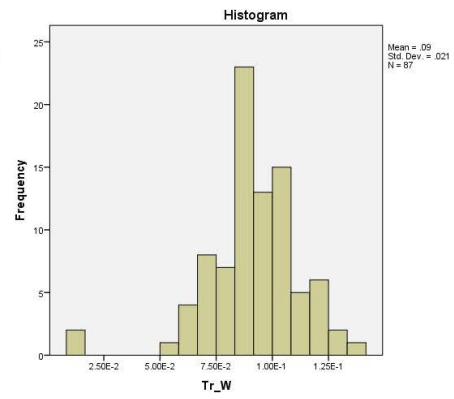

Histogram
Mean = .43
Std. Dev. = .054
N = 87
Frequency
Ti_L

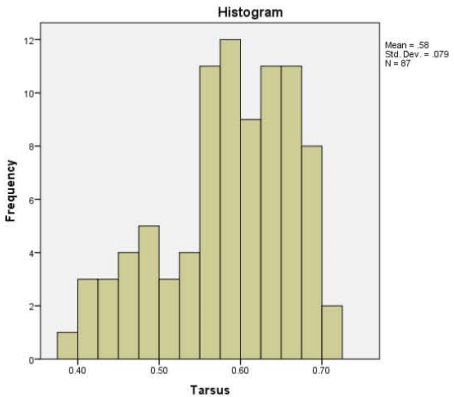

Histogram
Mean = .12
Std. Dev. = .018
N = 87
Frequency
Ti_W

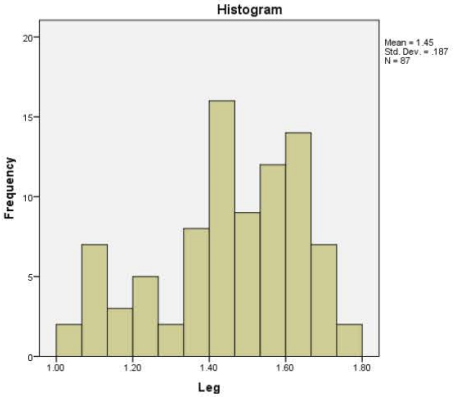

Histogram
Mean = .37
Std. Dev. = .056
N = 87
Frequency
Fe_L

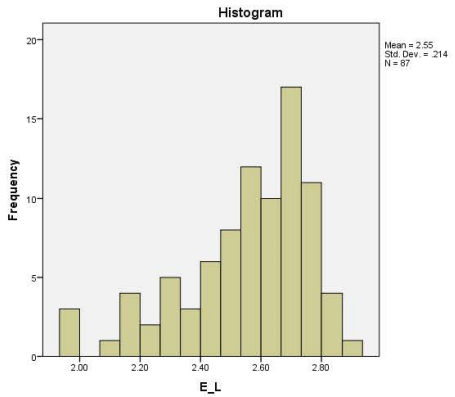

Histogram
Mean = .12
Std. Dev. = .063
N = 87
Frequency
Fe_W

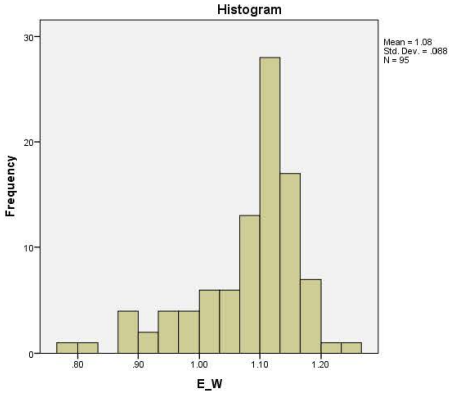

Histogram
Mean = .07
Std. Dev. = .012
N = 87
Frequency
Tr_L

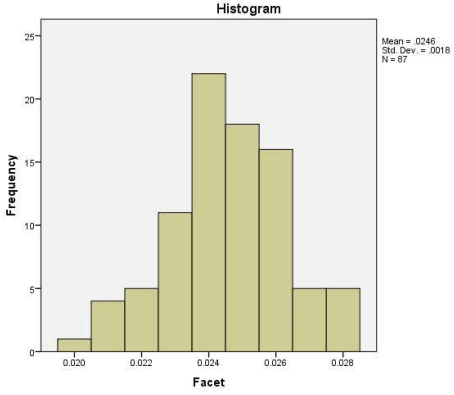

Histogram
Mean = .09
Std. Dev. = .021
N = 87
Frequency
Tr_W

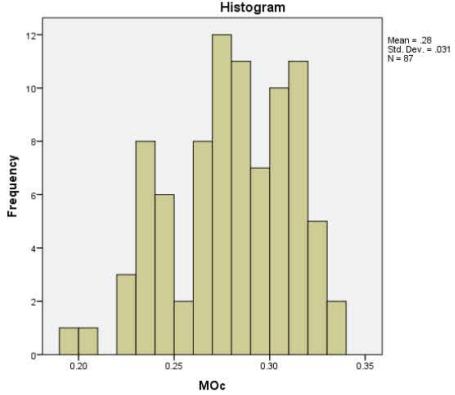

Histogram
Mean = .58
Std. Dev. = .079
N = 87
Frequency
Tarsus

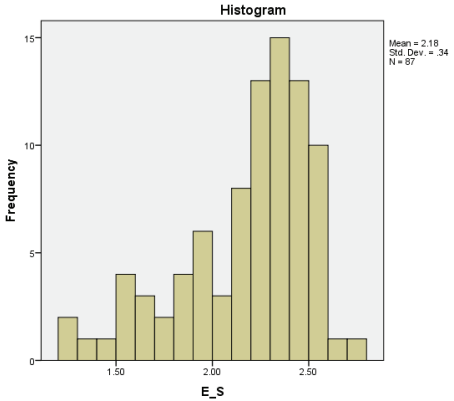

Histogram
Mean = 1.45
Std. Dev. = .187
N = 87
Frequency
Leg

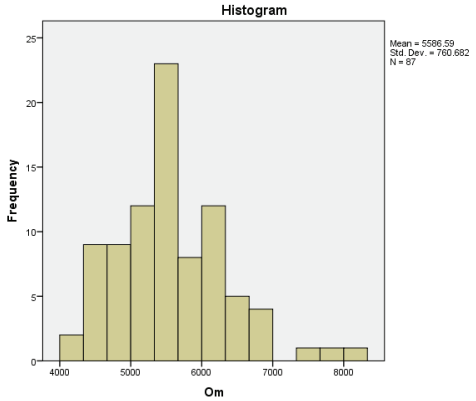

Histogram
Mean = 2.55
Std. Dev. = .214
N = 87
Frequency
E_L

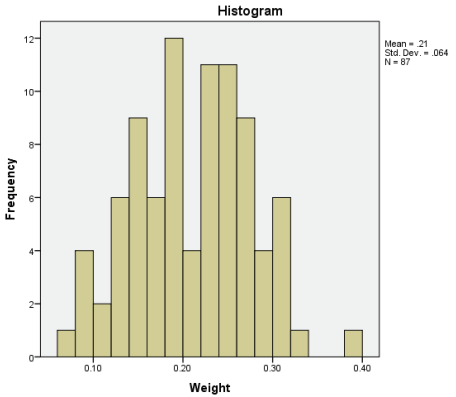

Histogram
Mean = 1.08
Std. Dev. = .088
N = 95
Frequency
E_W

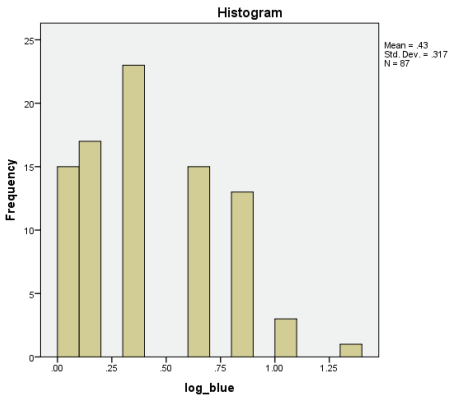

Histogram
Mean = .0246
Std. Dev. = .0018
N = 87
Frequency
Facet


Histogram
Mean = .28
Std. Dev. = .031
N = 87
Frequency
MOc


Histogram
Mean = 2.18
Std. Dev. = .34
N = 87
Frequency
E_S


Histogram
Mean = 5586.59
Std. Dev. = 760.682
N = 87
Frequency
Om


Histogram
Mean = .21
Std. Dev. = .064
N = 87
Frequency
Weight


Histogram
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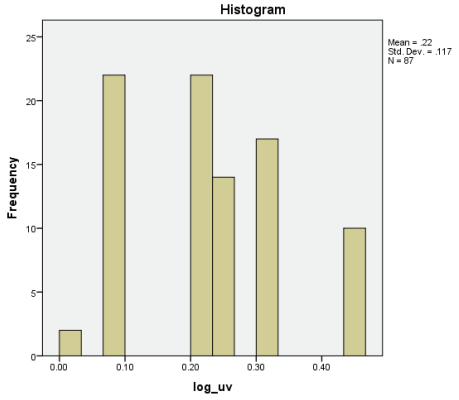
Std. Dev. = .317
N = 87
Frequency
log_blue


Histogram
Mean = .22
Std. Dev. = .117
N = 87
Frequency
log_uv

Supplementary File S16. Principal Component Analysis (PCA) of the different body size traits and eye parameters.The eigenvalues and eigenvectors of the PCA are given for: (i) the different body size traits and (ii) the eye parameters.

(i) Principal Component Analysis (PCA): *body size parameters*

Eigenvalues

PC	Eigenvalues	%Variation	Cum.%Variation
1	5.91	53.8	53.8
2	1.57	14.2	68.0
3	0.996	9.1	77.1
4	0.716	6.5	83.6
5	0.656	6.0	89.5

Eigenvectors
(Coefficients in the linear combinations of variables making up PC's)

Variable	PC1	PC2	PC3	PC4	PC5
Radial cell	-0.239	-0.179	-0.129	0.783	-0.395
Mt_L	-0.373	-0.085	-0.189	0.053	0.055
Mt_W	-0.362	-0.092	-0.173	-0.205	0.126
Ti_L	-0.395	-0.044	-0.153	-0.043	0.070
Ti_W	-0.346	-0.101	-0.179	-0.313	-0.081
Fe_L	-0.356	-0.049	-0.311	-0.113	-0.055
Fe_W	-0.210	-0.130	0.575	-0.218	-0.681
Tr_L	-0.225	-0.319	0.461	-0.162	0.285
Tr_W	-0.250	-0.104	0.430	0.391	0.516
Tarsus	-0.239	0.635	0.135	0.045	0.005
Leg	-0.237	0.636	0.136	0.029	-0.015

(ii) Principal Component Analysis (PCA): *eye parameters*

Eigenvalues

PC	Eigenvalues	%Variation	Cum.%Variation
1	4.45	74.1	74.1
2	0.654	10.9	85.0
3	0.488	8.1	93.2

Eigenvectors
(Coefficients in the linear combinations of variables making up PC's)

Variable	PC1	PC2	PC3
E_L	-0.458	-0.013	0.118
E_W	-0.456	-0.021	0.113
Facet	-0.325	0.829	-0.319
MOc	-0.388	-0.235	0.521
E_S	-0.453	0.030	0.134
Om	-0.347	-0.506	-0.763

Supplementary File S17. List of candidate genes for critical light sensitivity of bumblebee drones in blue light. List of the place, accession number, name and annotation information of all genes, at QTL qBLU3 on LG 3, which can all be linked with the critical light sensitivity of bumblebee drones in blue light.

Start	stop	Accession	Locus	Description
1379268	1440568	XM_003394234.1	LOC100651751	sex-regulated protein janus-A-like (LOC100651751), mRNA
1380872	1425353	XM_003394227.1	LOC100650954	phosrestin-1-like (LOC100650954), mRNA
1444315	1449818	XM_003394350.1	LOC100646286	hypothetical protein LOC100646286 (LOC100646286), mRNA
1450487	1452349	XM_003394349.1	LOC100646173	hypothetical protein LOC100646173 (LOC100646173), mRNA
1500499	1526455	XM_003394228.1	LOC100651071	hypothetical protein LOC100651071 (LOC100651071), mRNA
1563777	1575057	XM_003394224.1	LOC100650594	probable ATP-dependent RNA helicase CG8611-like (LOC100650594), mRNA
1574645	1588141	XM_003394225.1	LOC100650718	hypothetical protein LOC100650718 (LOC100650718), mRNA
1592463	1595546	XM_003394223.1	LOC100650476	integrator complex subunit 10-like (LOC100650476), mRNA
1594866	1601254	XM_003394221.1	LOC100650237	protein transport protein Sec31A-like (LOC100650237), mRNA
1601991	1605404	XM_003394220.1	LOC100650112	polypeptide N-acetylgalactosaminyltransferase 3-like (LOC100650112), mRNA
1612411	1629559	XM_003394348.1	LOC100646056	hypothetical protein LOC100646056 (LOC100646056), mRNA
1740947	1796898	XM_003394219.1	LOC100649991	transcription factor hamlet-like (LOC100649991), mRNA
1965450	1986889	XM_003394347.1	LOC100645939	SPRY domain-containing SOCS box protein 1-like (LOC100645939), mRNA
1994868	1996819	XM_003394346.1	LOC100645823	hypothetical protein LOC100645823 (LOC100645823), mRNA
2007601	2024300	XM_003394218.1	LOC100649874	cyclin-dependent kinase 5 activator 1-like (LOC100649874), mRNA
2028512	2029978	XM_003394217.1	LOC100649763	protein transport protein SFT2-like (LOC100649763), mRNA
2029943	2031725	XR_131866.1	LOC100649651	hypothetical LOC100649651 (LOC100649651), miscRNA
2033121	2041004	XM_003394216.1	LOC100649528	hypothetical protein LOC100649528 (LOC100649528), mRNA
2048620	2057193	XM_003394215.1	LOC100649406	serine/threonine-protein phosphatase 4 regulatory subunit 4-like (LOC100649406), mRNA
2058202	2237814	XM_003394213.1	LOC100649165	hypothetical protein LOC100649165 (LOC100649165), mRNA
2154619	2155506	XM_003394214.1	LOC100649286	hypothetical protein LOC100649286 (LOC100649286), mRNA
2239904	2242915	XM_003394344.1	LOC100645588	hypothetical protein LOC100645588 (LOC100645588), mRNA
2242543	2245756	XM_003394212.1	LOC100649049	kelch domain-containing protein 10-like (LOC100649049), mRNA
2250046	2266693	XR_131865.1	LOC100648815	hypothetical LOC100648815 (LOC100648815), miscRNA
2265285	2270449	XM_003394211.1	LOC100648931	hypothetical protein LOC100648931 (LOC100648931), mRNA
2270494	2271893	XM_003394210.1	LOC100648703	DNA repair protein RAD51 homolog 4-like (LOC100648703), mRNA
2275283	2295240	XM_003394209.1	LOC100648591	hypothetical protein LOC100648591 (LOC100648591), mRNA
2297014	2299967	XM_003394208.1	LOC100648391	cytochrome P450 6k1-like (LOC100648391), mRNA

Start	stop	Accession	Locus	Description
2299671	2305966	XM_003394207.1	LOC100648275	DNA topoisomerase 2-binding protein 1-like (LOC100648275), mRNA
2306495	2489235	XM_003394206.1	LOC100648160	protein-tyrosine sulfotransferase-like (LOC100648160), mRNA
2490288	2494623	XM_003394205.1	LOC100648049	protein MTO1 homolog, mitochondrial-like (LOC100648049), mRNA
2499310	2650532	XM_003394204.1	LOC100647927	probable G-protein coupled receptor Mth-like 1-like (LOC100647927), mRNA
2668080	2681265	XM_003394203.1	LOC100647804	hypothetical protein LOC100647804 (LOC100647804), mRNA
2720952	2732569	XM_003394202.1	LOC100647689	GTP-binding protein Rhes-like (LOC100647689), mRNA
2733853	2735909	XM_003394201.1	LOC100647569	hypothetical protein LOC100647569 (LOC100647569), mRNA
2811892	2813218	XM_003394343.1	LOC100645483	somatostatin receptor type 4-like (LOC100645483), mRNA
2871628	2940085	XM_003394199.1	LOC100647330	guanylate cyclase 32E-like (LOC100647330), mRNA
2939790	2940990	XM_003394200.1	LOC100647452	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6-like (LOC100647452), mRNA
2941318	2944004	XM_003394198.1	LOC100647213	tRNA-splicing ligase RtcB homolog (LOC100647213), mRNA
2944108	3059184	XR_131874.1	LOC100645366	hypothetical LOC100645366 (LOC100645366), miscRNA
3062781	3065425	XM_003394195.1	LOC100646894	pescadillo homolog (LOC100646894), mRNA
3065480	3070181	XM_003394194.1	LOC100646771	protein pelota-like (LOC100646771), mRNA
3070000	3073295	XM_003394196.1	LOC100647012	probable serine hydrolase-like, transcript variant 1 (LOC100647012), mRNA
3070000	3074784	XM_003394197.1	LOC100647012	probable serine hydrolase-like, transcript variant 2 (LOC100647012), mRNA
3075349	3076615	XM_003394193.1	LOC100646650	hypothetical protein LOC100646650 (LOC100646650), mRNA
3076597	3081931	XM_003394192.1	LOC100646529	UPF0636 protein C4orf41 homolog (LOC100646529), mRNA
3082052	3084507	XM_003394191.1	LOC100646403	probable G-protein coupled receptor AH9.1-like (LOC100646403), mRNA
3086097	3087571	XM_003394342.1	LOC100645238	lymphokine-activated killer T-cell-originated protein kinase-like (LOC100645238), mRNA
3087641	3094415	XM_003394190.1	LOC100646285	syntaxin-1A homolog (LOC100646285), mRNA
3088939	3095239	XM_003394189.1	LOC100646172	condensin-2 complex subunit D3-like (LOC100646172), mRNA
3097469	3108358	XM_003394188.1	LOC100646055	ankyrin repeat domain-containing protein 29-like (LOC100646055), mRNA
3108776	3118908	XM_003394187.1	LOC100645904	acyl carrier protein, mitochondrial-like (LOC100645904), mRNA
3109468	3117237	XM_003394185.1	LOC100645705	TBC1 domain family member 9-like, transcript variant 1 (LOC100645705), mRNA
3109535	3116937	XM_003394186.1	LOC100645705	TBC1 domain family member 9-like, transcript variant 2 (LOC100645705), mRNA
3119005	3122275	XM_003394182.1	LOC100645365	mps one binder kinase activator-like 4-like (LOC100645365), mRNA
3121758	3138923	XM_003394183.1	LOC100645482	alba-like protein C9orf23 homolog (LOC100645482), mRNA
3127235	3130577	XM_003394180.1	LOC100644997	aromatic-L-amino-acid decarboxylase-like (LOC100644997), mRNA
3133043	3137030	XM_003394181.1	LOC100645237	histidine decarboxylase-like (LOC100645237), mRNA
3137421	3138388	XM_003394184.1	LOC100645587	peptidyl-prolyl cis-trans isomerase H-like (LOC100645587), mRNA

Start	stop	Accession	Locus	Description
3139162	3142194	XR_131864.1	LOC100645116	zinc finger protein 595-like (LOC100645116), miscRNA
3142893	3146252	XM_003394179.1	LOC100644877	cGMP-dependent protein kinase, isozyme 1-like (LOC100644877), mRNA
3146544	3170527	XR_131863.1	LOC100644751	ATP-binding cassette sub-family C member 9-like (LOC100644751), miscRNA
3178322	3510902	XM_003394178.1	LOC100644512	RNA binding protein fox-1 homolog 2-like (LOC100644512), mRNA
3178340	3363539	XM_003394177.1	LOC100644512	RNA binding protein fox-1 homolog 2-like (LOC100644512), mRNA

Summary

SUMMARY

The decline of pollinator species is an emerging threat that is gaining attention worldwide and is instigating both ecological and economic concerns. Bumblebees are, as generalist foragers, essential pollinators in natural and managed ecosystems. Several hypotheses have been proposed to explain the observed declines in bee populations, including pathogen infections, pesticides and landscape modifications. Also population genetic aspects will play a role in bee declines with genetic threats such as inbreeding and loss of genetic diversity. In order to secure pollination services and improve conservation strategies a better understanding of genetic factors influencing bumblebee populations is vital.

In this dissertation, we first studied the loss of the pollination service of natural populations (in chapter 2 and 3) by focussing on the genetic parameters associated with bumblebee decline. To do this, we examined microsatellite data of pin-mounted bumblebee specimens sampled from extensive bumblebee collections. Museum collections provided a unique opportunity to examine the population structure and the genetic diversity of past populations. The use of historical specimens allowed for unique analyses of comparison between genetic parameters of past populations and recent populations. Our goals were to examine how genetic diversity and inbreeding are correlated with species extinction. In the case-study of *B. veteranus* (chapter 2), we detected low levels of genetic diversity and inbreeding in all populations in a time period of three decades (1895-1923) in Belgium. Furthermore, in chapter 3, we further investigated the genetic diversity levels of historical bumblebee populations. In this case study, we compared the level of genetic diversity of historical populations from seven declining *Bombus* species and four more stable species collected between 1918 and 1926 from 6 provinces of the Netherlands. Historical populations of declining bumblebee species showed significantly lower genetic diversity than co-distributed stable species. These results indicate that inbreeding and low levels of genetic variation were already present several decades before the general drivers of bumblebee decline are believed to have acted on these bumblebee populations. As a consequence we believe that: (i) inbreeding does not directly result in the collapse of populations, (ii) that there was no major drop in genetic diversity caused by the general drivers of bumblebee decline in the populations of declined bumblebee species, and (iii) that bumblebee species with a low levels of genetic diversity were the first to decline.

Aside from describing the genetic viability of natural populations, microsatellite analyses were also performed in this dissertation to search for genetic markers associated with a specific interesting commercial characteristic of bumblebees. In chapter 4 and 5, we used the microsatellite technology to identify genes correlated with two phenotypes: the impact of light intensity and body size. Before we were able to achieve this, we needed to develop bioassays that could distinguish light sensitivity differences between colonies (colony level) and between individuals (individual level). In chapter 4 we described the developed bioassays and investigated the connection between light sensitivity and foraging behavior. Furthermore, we tested if bumblebee body size, weight and morphological parameters of the eye correlated with the measured light sensitivity of the workers. We found that the recruitment to forage in artificial low light is less impaired in light sensitive colonies and that not only the external morphology parameters determine the light sensitivity of bumblebees and their eagerness to forage in weak light conditions. Although we confirmed that bigger bees had bigger eyes within colonies, between colonies this correlation was lost. Colonies containing small bumblebees had a better light perception compared to colonies with bigger specimens. Thus, although body size is an important parameter for better light perception, as bigger bumblebee workers will be better equipped to capture light, improved vision is not only a consequence of improved light perception. Other physiologic-genetic characteristics like signal transduction will disrupt the result of the bumblebee body size based selection for an improved critical light sensitivity. In chapter 5, we performed a quantitative trait loci (QTL) analysis to search for one or more microsatellite marker(s) linked with light sensitivity and body size. By both composite interval mapping and multiple QTL model mapping using 135 microsatellite DNA markers we identified several QTLs for 19 of the 20 investigated traits in *B. terrestris* drones. Multivariate principal components analysis confirmed these univariate QTLs. For light sensitivity, we also identified several candidate genes, with the *Phosrestin-1-like* gene as a primary candidate for its phototransduction function. The QTLs and markers we identified here, could be used in marker-assisted breeding to improve selection towards light sensitive bumblebees.

Finally, in chapter 6, we show a direct application of the microsatellite technology in bumblebee breeding facilities. Microsatellites can be integrated within a bumblebee mass-breeding to detect diploid drones. The presence of diploid drones can be used as a validation of their production process.

Samenvatting

De achteruitgang van bestuivers is een bedreiging voor het ecosysteem, dat wereldwijd steeds meer aandacht krijgt en waarbij de bezorgdheid zowel op ecologisch als op economisch vlak toeneemt. Hommels zijn als generalistische bestuivers essentieel in enerzijds de natuurlijke en anderzijds ook de antropogene ecosystemen. Verschillende hypotheses zijn vooropgesteld om de waargenomen achteruitgang van hommelpopulaties te verklaren, waaronder: pathogeen infecties, gebruik van pesticiden en veranderingen in het landschap. Ook populatie genetische aspecten spelen een rol in de waargenomen achteruitgang van hommels. Inteelt en verlies aan genetische diversiteit zijn de grootste genetische bedreigingen. Met het oog op het behoud van de natuurlijke en commerciële bestuivingdiensten en het verbeteren van de huidige conserveringsstrategieën is het beter begrijpen van de impact van genetische factoren op hommelpopulaties van levensbelang.

Als eerste, in hoofdstuk 2 en 3 van dit proefschrift, werd het verlies aan natuurlijke bestuiving van hommelpopulaties onderzocht door de focus te leggen op de hommel achteruitgang en genetische parameters die hiermee verbonden zijn. Dit werd onderzocht via het bemonsteren van opgepinde hommelspecimens uit uitgebreide historische hommelcollecties en het genotyperen ervan met behulp van microsatelliet DNA merkers. Deze museum collecties bieden een unieke gelegenheid om de populatie structuur en de genetische diversiteit van oude hommel populaties te onderzoeken. Door middel van deze historische stalen is het nu mogelijk om de genetische parameters van oude populaties te vergelijken met deze verkregen uit meer recente populatie teruggevonden in de literatuur. Onze doelstellingen hierbij waren: onderzoeken hoe de genetische diversiteit en inteelt gecorreleerd zijn met het uitsterven van hommels. In de studie *van B. veteranus* in België (hoofdstuk 2), detecteerden we lage niveaus aan genetische diversiteit en inteelt in alle populaties over een periode van dertig jaar (1895-1923). In hoofdstuk 3 zette het onderzoek zich verder door de genetische diversiteit binnen historische hommelpopulaties na te gaan. In dit hoofdstuk, vergeleken we de genetische diversiteit van de historische populaties van zeven achteruitgaande *Bombus* soorten en vier stabieler soorten verzameld tussen 1918 en 1926 in 6 provincies van Nederland. De historische populaties van achteruitgaande hommelsoorten vertoonden een significant lagere genetische diversiteit dan stabiele soorten met eenzelfde distributie. Deze resultaten geven aan dat inteelt en lage genetische variatie reeds aanwezig waren enkele decennia voordat de algemene oorzaken van hommelachteruitgang ook maar konden gehandeld hebben op deze hommelpopulaties.

Bijgevolg besluiten we dat: (i) inteelt niet direct leidt tot de ineenstorting van populaties, (ii) er geen grote daling in genetische diversiteit veroorzaakt werd in populaties van dalende hommelsoorten door de algemene oorzaken van hommel achteruitgang, en (iii) hommelsoorten met lage niveaus aan genetische diversiteit als eerste een achteruitgang vertonen.

Naast het beschrijven van de genetische levensvatbaarheid van natuurlijke populaties, werden als tweede aspect in dit doctoraat ook microsatelliet analyses uitgevoerd voor het identificeren van genetische merkers geassocieerd met een specifiek commercieel interessante eigenschap van hommels. In hoofdstuk 4 en 5, hebben we gebruik gemaakt van de microsatelliet technologie om genen gecorreleerd met twee fenotypes te identificeren: de invloed van lichtintensiteit en lichaamsgrootte. Hiervoor werden ten eerste bioassays ontwikkeld die verschillen in lichtgevoeligheid tussen kolonies (kolonie-niveau) en tussen individuen (individueel niveau) kunnen onderscheiden. In hoofdstuk 4 beschreven we de ontwikkelde bioassays en onderzochten we het verband tussen lichtgevoeligheid en foerageergedrag. Verder werd getest of de hommel lichaamslengte, gewicht en morfologische parameters van het oog correleerden met de gemeten lichtgevoeligheid van de foerageerders. In dit hoofdstuk hebben we vastgesteld dat het uitsturen van werksters om te gaan foerageren in kunstmatig lage licht condities minder wordt aangetast in lichtgevoelige kolonies, en dat niet alleen de externe morfologische parameters de lichtgevoeligheid van hommels en hun gretigheid om te foerageren in zwakke lichtomstandigheden bepalen. Inderdaad, binnen kolonies bevestigden we dat grotere hommels grotere ogen hadden, maar tussen kolonies was deze correlatie verdwenen. Sommige kolonies met kleinere hommels hadden een betere licht perceptie dan kolonies met grotere exemplaren. Desondanks lichaamslengte een belangrijke parameter voor een betere licht perceptie is, doordat grotere hommels beter uitgerust zijn om licht op te vangen, is een verbeterd zicht niet alleen een gevolg van een verbeterde lichtperceptie. Ook andere fysiologisch-genetische processen, zoals signaaltransductie, zullen het resultaat van een op lichaamsgrootte gebaseerde selectie voor een verbeterde kritische lichtgevoelig verstoren. In hoofdstuk 5 werd een ‘quantitative trait loci’ (QTL) analyse uitgevoerd waarbij één of meer microsatelliet merker(s) gekoppeld aan lichtgevoeligheid en lichaamsgrootte werden gezocht. Met gebruik van 135 microsatelliet DNA merkers in *B. terrestris* darren werden zowel door de ‘composite interval mapping’ en de ‘multiple QTL model mapping’ meerdere QTLs voor 19 van de 20 onderzochte kenmerken geïdentificeerd.

Multivariate principale-componentenanalyse bevestigde deze univariate QTLs. Voor lichtgevoeligheid werden ook verscheidene kandidaat-genen geïdentificeerd, met de '*Phosrestin-1-like'* gen als primaire kandidaat door haar fototransductie functie. Ook andere QTLs en merkers die hier geïdentificeerd werden, kunnen worden gebruikt in de selectie naar lichtgevoelige hommels via 'marker-assited breeding'.

Tenslotte beschrijven we in hoofdstuk 6 een directe toepassing van de microsatelliet technologie in hommelkwekerijen. De microsatelliet technologie kan worden geïntegreerd binnen een hommel massakwekerij voor de detectie van diploïde darren. De aanwezigheid van deze diploïde darren kan gebruikt worden ter validatie van het productieproces.

Curriculum vitae

PERSONAL DETAILS

Name	Kevin Maebe
Gender	Male
Nationality	Belgian
Place of birth	Ghent, Belgium
Date of birth	November 1th 1982

EDUCATION

2000-2007 Master in the Biology, with distinction at Ghent University

Master thesis: Morfologische en genetische differentiatie bij bosmieren van het *Formica rufa* complex

Promotor: Prof. Jean-Pierre Maelfait; co-promoter: Dr. Wouter Dekoninck

WORK EXPERIENCE

2011-2015 Ghent University

Subject: Microsatellites to identify the impact of genetic parameters on bumblebee decline and genes associated with foraging.

Place of research: Laboratory of Agrozoology, Department of Crop protection, Faculty of Bioscience Engineering, Ghent University. Coupure Links, 653, 9000 Ghent.

2010-2011 Ghent University

Subject: Studie van het marktaandeel van de Cannabis die in België wordt geteeld op de Belgische Cannabismarkt (GEOCAN); Detectie van sporen van de productie van synthetische drugs in oppervlaktewater (GEOAMP).

Place of research: Laboratory of Toxicology, Department of Clinical Chemistry, Microbiology and Immunology, Ghent University. De Pintelaan 185, 9000 Ghent.

2008-2009 Royal Belgian Institute of natural Sciences

Subject: Loss of genetic diversity and increased genetic structuring in response to forest area reduction in a ground dwelling insects.

Place of research: Department Entomology, Royal Belgian Institute of natural Sciences, Vautierstraat 29, 1000 Brussels

SCIENTIFIC OUTPUT

A1 peer reviewed publications

1. Maebe, K., Meeus, I., De Riek, J., Smagghe, G. (2015) Quantitative trait loci for light sensitivity, body weight, body size, and morphological eye parameters in the bumblebee, *Bombus terrestris*. PLoS ONE. Submitted, under review.

2. Lecocq, T., Gérard, M., Maebe, K., Brasero, N., Dehon, L., Smagghe, G., Valterova, I., De Meulemeester, T., Rasmont, P., Denis Michez (2015) Chemical reproductive traits of diploid *Bombus terrestris* males: consequences on bumblebee conservation. J. Insect Conserv. Submitted: under review.

3. Maebe, K., Meeus, I., Ganne, M., De Meulenmeester, T., Biesmeijer, K., Smagghe, G. (2015) Microsatellite analysis of museum specimens reveals historical differences in genetic diversity between declining versus stable *Bombus* species. PLoS ONE. Submitted, under review.

4. Meeus, I., Parmentier, L., Billiet, A., Maebe, K., Van Nieuwerburgh, F., Deforce, D., Vandamme, P., Wäckers, F., Smagghe, G. (2015) MiSeq 16S rRNA amplicon sequencing demonstrates that indoorreared bumblebees (*Bombus terrestris*) harbor a core subset of bacteria normally associated with the wild host. PLoS ONE. Submitted, under review.

5. Gerard, M., Michez, D., Fournier, D., Maebe, K., Smagghe, G., Biesmeijer, J.C., De Meulemeester, T. (2015) Discrimination of haploid and diploid males of *Bombus terrestris* (Hymenoptera: Apidae) based on wing shape. Apidologie. Accepted.

6. Palma, J.*, Maebe, K.*, Guedes, J.V.C., Smagghe, G. (2015) Molecular variability and genetic structure of Chrysodeisis includes (Lepidoptera: Noctuidae), an important soybean defoliator in Brazil. PLoS ONE. In press.
   * co-first authors

7. Dekoninck, W., Maebe, K., Breyne, P., Hendrickx, F. (2014) Polygyny and strong genetic sructuring within an isolated population of the wood ant *Formica rufa*. J. Hymenopt. Res. 41, 95-111.

8. Maharramov, J., Meeus, I., Maebe, K., Arbetman, M., Morales, C., Graystock, P., Hughes, W.O.H., Plischuk, S., Lange, C.E., de Graaf, D.C., Zapata, N., de la Rosa, J.J.P., Murray, T.E., Brown, M.J.F., Smagghe, G. (2013) Genetic variability of the neogregarine *Apicystis bombi*, an etiological agent of an emergent bumblebee disease, PLoS ONE Vol. 8, Issue 12.

9. Maebe, K., Meeus, I., Wäckers, F., Smagghe, G. (2013) Scientific note on microsatellite DNA analyses revealing diploid and haploid drones in bumblebee mass-breeding. Apidologie 45, 189-191.

10. Maebe, K., Meeus, I., Smagghe, G. (2013) Recruitment to forage of bumblebees in artificial low light is less impaired in light sensitive colonies, and not only determined by external morphological parameters. J. Insect Physiol. 59, 913-918.

11. Maebe, K., Meeus, I., Maharramov, J., Grootaert, P., Michez, D., Rasmont, P., Smagghe, G. (2013) Microsatellite analysis in museum samples reveals inbreeding before the regression of *Bombus veteranus*. Apidologie 44(2), 188-197.

12. Gaublomme, E., Maebe, K., Van Doninck, K., Dhuyvetter, H., Li X., Desender, K., Hendrickx, F. (2013) Loss of genetic diversity and increased genetic structuring in response to forest size reduction in the flightless carabid beetle *Carabus problematicus* (Coleopera, Carabidae). Insect Conserv. Diver. 6(4), 473-482.

13. De Backer, B., Maebe, K., Verstraete, A.G., Charlier, C. (2012) Evolution of the content of THC and other major cannabinoids in drug-type cannabis cuttings and seedlings during growth of plants. J. Forensic Sci 57(4), 918-922.

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2. De Backer, B., Maebe, K., Legrand, S.-A., Colman, C., Theunis, L., Charlier, C., De Ruyver, B., Verstraete, A.G. (2011) Studie van het marktaandeel van cannabis die in België wordt geteeld op de Belgische cannabismarkt – GEOCAN. *Wetenschap En Maatschappij*. Gent: Academia Press.

3. Vandenplas, S., Dekoninck, W., Maebe, K. & Hendrickx, F. (2010) Areaaluitbreiding en genetische verwantschapsanalyse bij de thermofiele mierensoort, *Lasius emarginatus* (OLIVIER, 1792). Bulletin S.R.B.E./K.B.V.E. 146, 90-94.

4. Maebe, K., Dekoninck, W., Maelfait, J.-P. (2008) Op zoek naar *Formica* mieren en hun myrmecofielen in het Dijleland . Boomklever 2**,** 2-13.

Participation at international conferences

6th European Conference of Apidology, Murcia, Spain, 9-11 September 2014.

Maebe, K., Meeus, I., Ganne, M., De Meulenmeester, T., Biesmeijer, K., Smagghe, G. (2014) "Microsatellite analysis of museum specimens reveals historical differences in genetic diversity between declining and stable *Bombus* species". (oral)

Parmentier, L., Meeus, I., Louwye, S., Maebe, K., Smagghe, G. (2014) "Commercial bumblebee hives to assess an anthropogenic environment for pollinator support: a case study around Ghent (Belgium)". (poster)

Meeus, I., Piot, N., Jinzhi, N., Maebe, K., Smagghe, G. (2014) "Different measures to prevent virus prevalence in reared bumblebees". (poster)

2nd International Conference on Pollinator Biology, Health and Policy, State College, USA, 14-17 August 2013.

Maebe, K., Meeus, I., Ganne, M., De Meulenmeester, T., Biesmeijer, K. & Smagghe, G. (2013) "Microsatellite analysis of museum specimens reveals historical differences in genetic diversity in declining versus stable *Bombus* species". (poster)

5th European Conference of Apidology, Halle an der Saale, Germany, 3-7 September 2012.

Maebe, K., Meeus, I., Maharramov, J., Grootaert, P., Michez, D., Rasmont, P., Smagghe, G. "Microsatellite analysis in museum samples reveals inbreeding before the regression of *Bombus veteranus*". (oral)

Maebe, K., Meeus, I., Smagghe, G. "Impact of different light conditions on the foraging behaviour of bumblebees" (poster).

Bee-together Meeting, Ghent, Belgium, 21 December 2010.

Maebe, K., Meeus, I., Smagghe, G. 'Development of multiplex PCR technology with microsatellite primers to study the genetic structure of bumblebee populations'. (poster)

Symposium van The Royal Belgian Entomological Society, Brussels, Belgium, 2008.

Maebe, K., Dekoninck, W., Hendrickx, F., Maelfait, J.P. 'Evolutionary ecology of insects and spiders'. (poster)