

A core linkage map of the bumblebee *Bombus terrestris*

Lena Wilfert, Jürgen Gadau, and Paul Schmid-Hempel

Abstract: The bumblebee *Bombus terrestris* is an economically important pollinator and an emerging model species in quantitative and population genetics. We generated genetic linkage maps for 3 independent mapping populations of *B. terrestris*. The linkage map with the highest resolution had 21 linkage groups, which adequately represents the haploid chromosome number of *B. terrestris* ($n = 18$). This map can be considered saturated, with an average marker distance of 10.3 cM and an estimated genome coverage of 81%. Using flow cytometry, we have estimated the genome size of this species to be 625 Mb. With an estimated total recombination genome length of 2760 cM, this results in a ratio of 226 kb/cM between the physical and genetic genome sizes. A recurring set of microsatellites and amplified fragment length polymorphism (AFLP) markers allowed the alignment of 14 linkage groups between the 3 maps. We propose to adopt this core map as a reference tool for future genetic and molecular work in *B. terrestris*.

Key words: AFLP, microsatellite, RAPD, recombination frequency, genome mapping.

Résumé : Le bourdon *Bombus terrestris* est un pollinisateur de grande importance économique et une espèce modèle émergente en génétique quantitative et génétique des populations. Les auteurs ont produit des cartes de liaison génétique pour 3 populations indépendantes de *B. terrestris*. La carte avec la meilleure résolution comporte 21 groupes de liaison, ce qui représente bien le nombre haploïde des chromosomes de *B. terrestris* ($n = 18$). Cette carte peut être considéré comme saturée, étant caractérisée par une distance moyenne entre 2 marqueurs de 10,3 cM et une couverture génomique estimée à 81 %. A l'aide de la cytométrie en flux, nous avons estimé la longueur génétique de cette espèce à 625 MB. Avec une longueur génétique estimée à 2760 cM, cela résulte dans une relation entre estimation physique et génétique de 226 kb/cM. Une série de microsatellites et marqueurs AFLP communs a permis l'alignement de 14 groupes de liaison entre les trois cartes. Les auteurs proposent d'adopter cette carte de base comme outil de référence pour de futures études génétiques et moléculaires sur *B. terrestris*.

Introduction

Genetic linkage maps are the starting point for an in-depth understanding of a species' genomic make up. They offer insights into the recombination frequency and genetic genome size of a species. Furthermore, they allow the locations of genes underlying complex traits to be identified by using quantitative trait locus (QTL) analysis, thus providing an insight into the genetic architecture of fitness-relevant phenotypic variation. This knowledge is essential for many topics, from evolutionary genetics to molecular marker-assisted breeding. Research that stands to profit greatly from a genetic map is that dealing with the bumblebee *Bombus terrestris*, one of the most common native European species. *Bombus terrestris* is of economic importance as one of the most important natural pollinators of flowers, including many commercial crops, in cool and temperate regions. For this reason, the commercial produc-

tion of bumblebees has developed into a thriving branch of agribusiness (Ghazoul 2005).

Besides the considerable interest in bumblebees as commercial crop pollinators, they have also been used as a model species for research in ecology, behavior, physiology, foraging strategies, and pollination (Goulson 2003). *Bombus terrestris*, in particular, has received special attention in the field of ecology and evolution, including host-parasite interactions, ecological immunology, developmental biology, and social behavior (Baer and Schmid-Hempel 1999; Moret and Schmid-Hempel 2000; Lopez-Vaamonde et al. 2004). Even though bumblebees and honeybees are phylogenetically related, their social systems differ considerably and bumblebees can be very useful for comparative studies on the evolution of sociality (Mares et al. 2005).

In contrast to their widespread use in basic and applied biology, the genetic and genomic research on bumblebees is lagging behind. Although there have been recent efforts to study gene expression (Pereboom et al. 2005; Spaethe and Briscoe 2005), even basic genomic information is still very limited. As yet, only one rudimentary, low-coverage, genetic map based on random amplified polymorphic DNA (RAPD) markers has been published (Gadau et al. 2001).

As with all hymenoptera, *B. terrestris* is haplo-diploid, i.e., has a single-locus, complementary, sex-determination system (Cook and Crozier 1995), with females arising from diploid, fertilized eggs and males from haploid, unfertilized eggs. *Bombus terrestris* is normally singly mated (Schmid-Hempel and Schmid-Hempel 2000). A queen will found a

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colony in spring and eventually produces up to 1000 haploid males. Owing to the parthenogenetic origin of males, hymenopteran males are sometimes called “flying oocytes”. These features allow the direct measurement of recombination frequencies in haploid individuals. Consequently, the haplo-diploid system in combination with the large number of offspring derived from a single individual readily allows the construction of linkage maps in natural populations. Generally, such mapping in non-manipulated populations has relied on large numbers of sib-ships or on detailed pedigree information (Slate 2005). This information is rarely available for most wild populations, including those of bumblebees. However, we demonstrate in this article that pedigree information for the determination of true linkage phase is not necessary for the construction of accurate linkage maps in a haplo-diploid system. For that purpose, we compared linkage maps produced phase-known and phase-unknown based on the same mapping population (BBM-1). The phase of the markers in the mapping population BBM-1 was inferred indirectly by comparing the genotype of the queen of colony BBM-1 with those of her sisters.

To be of true value as a tool for basic and applied research, linkage maps have to be repeatable and comparable between labs and studies. We therefore used 3 independent, natural, mapping populations (BBM-1, BBM-2, and BBM-3) to develop a standardized core map for *B. terrestris* using markers that vary within natural populations. Our core map of *B. terrestris* comprises 14 linkage groups homologized via a set of recurring microsatellites and amplified fragment length polymorphism (AFLP) markers. This core map provides a starting point for more detailed and repeatable genetic and genomic studies on both applied and basic topics in *B. terrestris*. Beyond this, it allows for comparative genetic and genomic studies with the well-studied honeybee *Apis mellifera*.

Materials and methods

Mapping populations

Three independent mapping populations were established from individual queens. Populations BBM-2 and BBM-3 were raised directly from wild queens caught in northwestern Switzerland, near Basel, in 2003 (BBM-2) and 2000 (BBM-3), respectively. Young queens produced by wild-caught queens from a population in northeastern Switzerland, near Winterthur, were mated and hibernated in the laboratory in autumn 2003, allowing us to establish colony BBM-1 as a phase-known mapping population. All colonies were maintained at standard conditions (red light, 28 °C and 60% relative humidity (RH)) and fed ad libitum with sugar water and pollen (Gerloff and Schmid-Hempel 2005). Males were removed from their maternal colonies as callows. They were later freeze-killed and stored at -80 °C. All individuals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Genetic markers

AFLPs

For AFLP analysis, DNA from half the thorax muscle of individual males was isolated with a modified cetyltrimethyl-

lammonium bromide (CTAB) – phenol extraction method (Toonen 1997). Five hundred microlitres of 2× CTAB solution and 130 µg proteinase K were added to the dissected tissue and kept at 65 °C for 3 h. After adding 2 µg RNase A and thoroughly vortexing the samples, they were incubated for 10 min at 37 °C. This was followed by a standard phenol–chloroform extraction using 1/10 volume 3 mol/L sodium acetate as a precipitant.

AFLP markers (Vos et al. 1995) were generated using *EcoRI* and *MseI* (New England Biolabs NEB) as restriction enzymes. Genomic DNA was first digested with 3 U of *EcoRI* and 3 U of *MseI* for 1 h at 37 °C. Subsequently, ligation was carried out in a volume of 50 µL using 200 U T4 ligase (New England Biolabs, Ipswich, Mass.) at 37 °C over a period of 3 h. Pre-amplification was performed with C or G as pre-extensions to the standard *MseI* primer using 35 pmol of each primer and 2 µL of undiluted ligation reaction as a PCR template. All PCRs were carried out using 0.5 U *Taq* polymerase (MBI Fermentas, Burlington, Ont.), 10× PCR buffer with (NH₂)SO₄, 25 mmol/L MgCl₂, and 25 mmol/L dNTPs in a volume of 25 µL (pre-amplification) and 20 µL (selective amplification). For selective amplification, 5 µL of diluted pre-amplification reaction (1:20) and several combinations of selective *EcoRI* primers (1.7 pmol) with 3 selective base pairs and *MseI* primers (4.8 pmol) carrying 2–3 selective base pairs (see Table 1) were used. Primers were labeled with FAM, NED, VIC, and PET (population BBM-1); FAM, NED, and HEX (population BBM-2); or FAM, TAMRA, and JOE (population BBM-3). Pre-amplification PCRs and touch-down selective PCRs were carried out as described in Kaib et al. (2004).

AFLP-fragments were separated on an ABI PRISM 310C Genetic Analyzer (Applied Biosystems, Foster City, Calif.) using HI-DI-formamide as a carrier. Data were collected using the ABI PRISM GeneScan Analysis Software (Applied Biosystems), and samples were aligned using an internal size standard. We tested between 64 and 135 primer combinations per population; primer combinations were chosen according to the number of polymorphic bands and their size distribution. AFLP markers were designated with the letter “A”, followed by a numeric code for the *EcoRI* primer and a letter for the *MseI* primer, as well as the fragment length (see Table 1). For codominant AFLP markers, the fragment lengths of both alleles are indicated.

Microsatellites

DNA was extracted using a simple chelex extraction as described in Schmid-Hempel and Schmid-Hempel (2000). The reaction mixtures each contained 1–10 ng of total DNA; multiplex PCR of pairs of microsatellites (B10–11, 96, 100, 118, 119, 124, 126, and 132 (Estoup et al. 1993) and BL01–16, BT01–30 and Btern (Reber Funk et al. 2006)) were carried out according to Schmid-Hempel and Schmid-Hempel (2000). Gel electrophoresis was performed using Spreadex[®] gels (Elchrom Scientific, Cham, Switzerland) and SYBR[®] Gold staining according to the manual.

RAPDs

RAPD analysis in population BBM-3 was carried out as described in Gadau et al. (2001), following a standard phenol–chloroform DNA extraction (Gadau et al. 1996).

Table 1. Coding of AFLP markers.

<i>(a) EcoRI</i>													
Label	Extension added												
	01	02	03	04	05	06	07	08	09	10	11		
Sequence	AAC	AAG	ACA	ACC	ACG	ACT	AGC	AGG	AGT	ATC	ATG		
<i>(b) MseI</i>													
Label	Extension added												
	A	B	C	D	E	F	G	H	J	K	L	M	P
Sequence	CA	CAA	CAC	CAG	CAT	CC	CG	CT	CTT	GA	GC	GG	GT

Note: Two or 3 selective nucleotides were used for selective amplification; these are coded as numbers for the *EcoRI* primer and as a roman letter for *MseI* primers. For example, the code A06G_190/198 indicates that this is a codominant AFLP-marker (A) amplified with the primer combination *EcoRI*-ACT and *MseI*-CG; its 2 alleles are 190 and 198 bp.

Table 2. Phase determination in population BBM-1. All possible genotype combinations of the F₀ queen and her mate (paternal allele) are shown, given that the F₁ queen has to be heterozygous for a segregating locus.

F ₀ queen	5 F ₁ sisters	5 F ₂ progenies	Paternal allele
Co-dominant alleles (A, B, C)			
AC	AB, BC	n.a.	B
BC	AB, AC	n.a.	A
BB	AB	n.a.	A
AA	AB	n.a.	B
AB	AB, AA	n.a.	A
AB	AB, BB	n.a.	B
AB	AB	n.a.	Ambiguous ^a
Presence/absence alleles (A/B)			
BB	AB	100% A/B	A
AB ^b	AB or BB	50% A/B, 50% BB	B
AB	AA or AB	50% A/B, 50% A/A	A
AA or AB	AA or AB	100% A/B	Ambiguous ^c

Note: Analyzing the genotypes of the F₁ sisters allowed reconstruction of the genotype of the F₀ queen and her mate, and hence the phase, in most cases.

^aThe paternal allele could not be determined, see text.
^bUsing dominant markers, genotypes AA and AB could not be discriminated in diploid individuals.
^cThe paternal allele could not be determined unambiguously; although this segregation pattern would result if the F₀ queen was homozygous for the presence allele A and her mate carried allele B, it could also be the chance result of missing an F₁ queen with the diagnostic AA or BB genotype.

Genotyping

Worker-produced males

Using codominant microsatellite markers, worker-produced males could be removed from the mapping populations. In this singly mated haplo-diploid species, all workers inherit the single paternal allele. If the paternal allele differs from the maternal dominant alleles at a specific locus, an average of 50% of worker-produced males can be identified, because males will have a 50% chance to inherit the parental allele. Analyzing between 24 and 39 polymorphic microsatellite loci, worker-produced males could be effectively removed from the colonies even though the paternal genotype was not available.

Segregation distortion

We tested for segregation distortion, i.e., significant deviation from a 1:1 ratio of alleles, using a χ^2 test. Since linked

markers are not mutually independent, we did not perform a Bonferroni test. Instead, we used a threshold of $p < 0.01$ to account for the large number of tests.

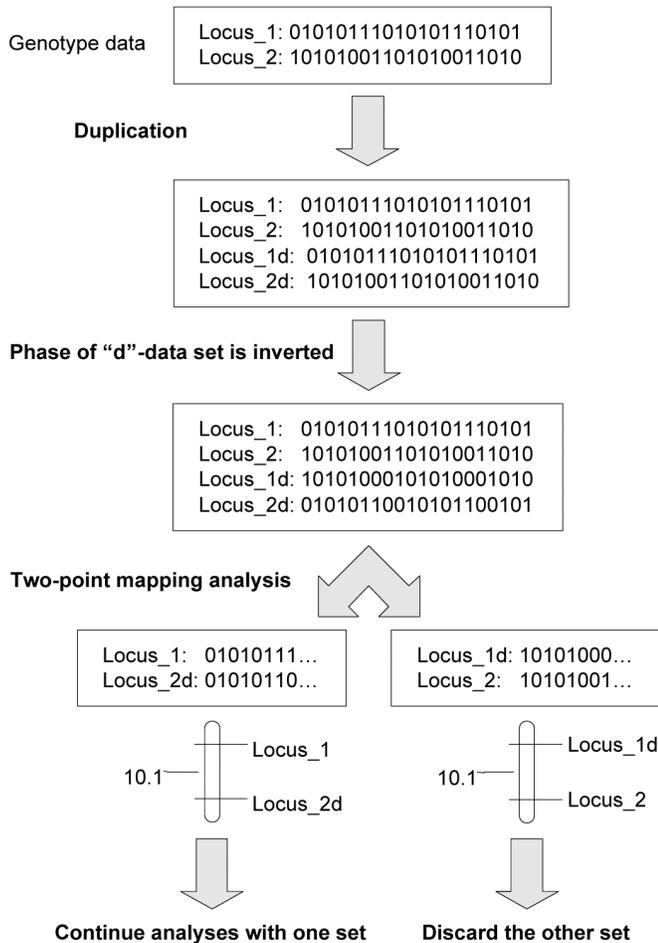
Marker control

To reduce errors in the scoring of genotype information, all poorly amplified bands and markers with more than 1/3 of marker information missing were rescored. Potentially codominant AFLPs were scored twice to confirm that the 2 alleles were indeed complementary. Additionally, markers were rearranged according to their linkage after initial mapping to check for errors in data entry that resulted in blocks of genotype information being shifted from their correct position.

Phase-determination

The haploid male population BBM-1 (F₂) was established from a laboratory-mated queen (F₁); the queen (F₁) and the

Fig. 1. Flow chart of the procedure for phase-unknown mapping based on a data set of 2 genetic loci genotyped for 20 individuals. This method results in a data set of markers with consistent, albeit not true, phase allocation. With this data set, mapping is then carried out as with a true phase-known data set.



drone were produced from unrelated colonies raised from 2 wild-caught queens (F_0) in 2003. This breeding scheme yielded the pedigree information necessary for phase-known mapping. The relevant genetic information is the phase of alleles in the F_0 generation, i.e., whether a particular allele was provided by the F_0 queen or by her mate. No direct genotype information of the field drone was available. Therefore, the paternal allele was inferred using the F_0 queen and 5 full sisters of the F_1 queen for codominant markers (see Table 2). For presence and (or) absence markers, the segregation patterns of 5 haploid F_2 progenies were additionally analyzed. This method can occasionally lead to ambiguous results; for example, the paternal allele cannot be determined if the F_0 queen and all F_1 queens show the same heterozygous genotype for a codominant marker. For a core of 177 markers, linkage phase could be unambiguously inferred in mapping population BBM-1; all other markers were scored as phase unknown. Every linkage group contained at least 2 unambiguous phase-known markers; this allowed the

Table 3. Summary of *B. terrestris* genome size.

	BBM-1	BBM-2	BBM-3
No. of markers (no. of unmapped markers)	246 (9)	124 (12)	118 (6)
No. of linkage groups	21	25	30
No. of linkage groups with >2 markers	20	19	21
Homologized vs. not homologized	15 vs. 6	17 vs. 8	18 vs. 12
Map size (cM)	2221.8	1223.1	1124.3
G_M (cM)	2352.3	1462.6	1510.7
G_E (cM)	2733.8	2785.5	2761.2
Physical recombination rate (kb/cM) ^a	229	224	226
Average marker spacing (cM)	10.3	12.5	12.8

^aCalculated as ratio of the estimated physical genome length of 625 Mb and the respective G_E .

true phase to be established for all markers in this population (see phase-unknown mapping).

Map construction

General procedure

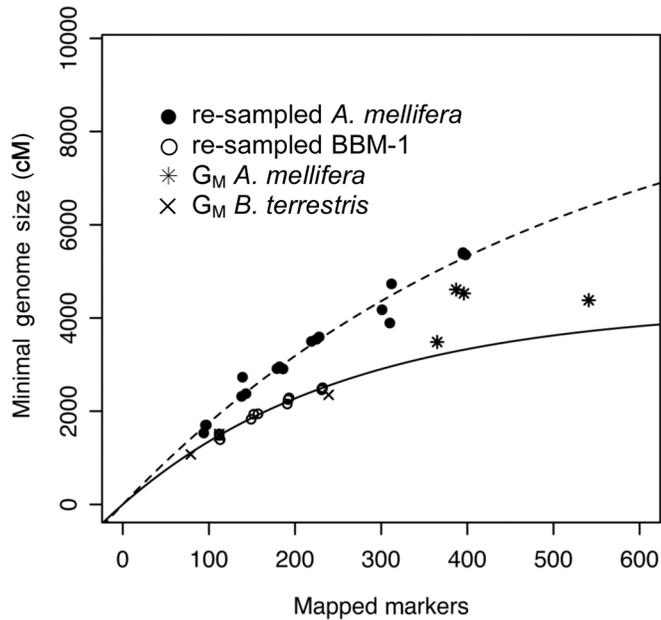
The mapping procedure in Mapmaker (Lander et al. 1987) followed a standard protocol. First, 2-point linkage analysis was carried out using the "GROUP" command (setting: LOD = 5.0; $\theta = 0.35$) to find a preliminary set of linkage groups. Secondly, multi-point analysis within all putative linkage groups generated in step 1 was carried out with the "FIRSTORDER" command (LOD = 5.0; $\theta = 0.35$). This analysis resulted in the most likely order of the markers in each linkage group. In the last step, the order found in step 2 was tested within each linkage group for all possible 3-point orders of consecutive markers using the "RIPPLE" command. The most likely order for every marker is shown.

All map distances (cM) were calculated from recombination frequencies (%) according to Kosambi's mapping function (Kosambi 1943). Kosambi's function was preferred over Haldane's function for 2 reasons: firstly, Kosambi's function resulted in less map extension when the "drop marker" command was used; secondly, Solignac et al. (2004) could show that for *A. mellifera*, the ideal mapping function is closest to Kosambi's function.

Phase-unknown mapping

For populations BBM-2 and BBM-3, being derived directly from wild-caught queens, no phase information was available. Therefore, phase-unknown mapping was carried out as described in Gadau et al. (2001). Except for the initial 2-point linkage analysis (step 1), this procedure is identical to the one described for phase-known mapping. Linkage group wide phase is determined using a doubled data set, in which each marker is represented in both possible phases (see Fig. 1). To achieve this, every marker allele was first arbitrarily assigned a phase ("1" for present or the shorter allele of a fragment length polymorphism, "0" for absent or the longer allele). Then, the genotype information was duplicated and added to the original data with the phase information being inverted (i.e., the "absent" or longer allele now

Fig. 2. Saturation curve for map BBM-1 and a RAPD map of *A. mellifera*. The curves were fitted to the model $G_n = G_{\text{total}} \times (1 - e^{-cn})$. Additionally, the published estimated minimal genome sizes of the *A. mellifera* (Hunt and Page 1995; Rueppell et al. 2004; Solignac et al. 2004) and *B. terrestris* (Table. 2; Gadau et al. 2001) maps are displayed.



being in phase “1”). This resulted in a data set in which each marker was represented twice with complementary phase information. This data set was used for 2-point linkage analysis. Since this was the doubled data set, every linkage group therefore had to be present twice. All of the linkage groups we found followed this prediction. Although this procedure cannot yield the true phase (maternal or paternal), it fixes the linkage phases of the markers within individual linkage groups. Once phase information was established, one set of each linkage group was arbitrarily discarded. This resulted in a data set in which each marker was represented only once in the correct phase. With this data set, multi-point analysis could be carried out as with a true phase-known data set.

A comparison of phase-known and -unknown mapping for the core group of phase-known markers had shown that there were no differences between these maps. Thus, 65 additional markers whose phase had not been directly determined were included for population BBM-1 by establishing phase information via a partially doubled data set in two-point linkage analysis. By joining the phase-known data set and the doubled phase-unknown data set, 2 sets of linkage groups were obtained—those containing only markers of unknown phase and those of established phase containing both phase-known and -unknown markers. Every linkage group contained at least 50% true phase-known markers. Thus, the correct phase for all markers could be established in population BBM-1.

Alignment of linkage groups

Linkage groups from different populations are considered to represent the same genetic area if they contain identical microsatellite or AFLP markers.

Genome map size estimation

To obtain an estimate for the minimum recombination genome length, G_M , the maximum distance between 2 markers (43.5, 34.7, and 32.2 cM in maps BBM-1, BBM-2, and BBM-3, respectively) were added for each linkage group exceeding the haploid karyotype of 18 chromosomes (Hoshiba et al. 1995). The total genome length was estimated using a method-of-moments approach with the estimated genome length, G_E being calculated as $G_E = N(N-1)X/K$ (Hulbert et al. 1988; Chakravarti et al. 1991). In this formula, N is the number of mapped markers, X the maximum distance between 2 markers at the threshold LOD score of 5, and K is the number of marker pairs at this minimum LOD score, which can be obtained via the “LODs” command in Mapmaker (Li et al. 2005). The achieved rate of genome coverage was obtained by dividing the total genome size, G , by G_E .

Saturation curve

The degree of saturation of the maps was addressed by fitting a first-order saturation model (Stadler et al. 2004). For this approach, the raw genotype data of population BBM-1 and an unpublished data set of 1021 RAPD markers in a population of 141 *A. mellifera* individuals (raw data provided by Greg Hunt) was randomly resampled (3 subsets each of 120, 160, 200, and 240 markers for BBM-1 and the *A. mellifera* data set and an additional 3 subsets of 320 and 400 markers for the latter). Linkage maps were constructed using MapMaker with a minimum LOD of 3 and a maximum θ of 0.34 to maximize the comparability with existing *A. mellifera* maps (Hunt and Page 1995). The minimum genome length estimate for these maps (G_M) and the number of mapped markers (n) were used to fit a first-order saturation model described by the function $G_n = G_{\text{total}} \times (1 - e^{-cn})$, with c being a kinetic constant, using the nls module of R Development Core Team (2005).

Flow cytometry

The physical genome size of *B. terrestris* was estimated by flow cytometry. Single-cell suspensions from worker flight muscles were obtained by applying a modification of the method of Lamatsch et al. (2000) for fish fin clips. The tissue was chopped in 2.1% citric acid – 0.5% Tween 20, and incubated at room temperature with gentle stirring for 10 min. For propidium iodide measurements, the cells were resuspended directly in staining buffer containing 154 mmol/L NaCl, 100 mmol/L Tris–Cl (pH 7.4), 1 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 0.2% bovine serum albumin (BSA), 0.1% NP-40, 25 U/mL RNaseA, and 50 µg/mL propidium iodide, and stained for approx. 1 h at 4 °C in the dark. Heparinized red blood cells from female chicken (*Gallus gallus*) were used as standard (crbc). Whole blood was diluted approximately 1:100 in minimal essential medium (MEM) containing a final concentration of 10% dimethyl sulfoxide, aliquoted and stored at –20 °C. After centrifugation, the cells were treated like the sample cells. The concentration of the samples was approximately 2×10^5 cells/mL, and the concentration of crbc slightly higher. Sample cells and reference cells were mixed at a ratio of 2/3 to 1/3 to obtain optimal results. Immediately before analysis, the samples were filtered through a 50 mm nylon

Fig. 3. The homologized core linkage groups LG01–LG14 are depicted; homologous markers are indicated in bold. The linkage groups from the high-resolution map BBM-1 are at the center of core linkage groups. The non-homologous linkage groups are displayed per population on the right-hand side. Map distances are given in Kosambi centimorgans (cM).

mesh to prevent obstruction of the flow chamber with chitin. DAPI measurements were performed on a Cell Analyzer CAII (Partec, Muenster, Germany). PI measurements were performed on a BD-L3R (Becton Dickinson, Franklin Lakes, N.J.) equipped with a 488 nm argon-ion laser with 20 mW power output. At least 10 000 cells were measured per sample. To determine the nuclear DNA content, the ratio of the channel numbers from the sample (bumblebee) and from chicken was multiplied by the known DNA content of crbc (2.5 pg/nucleus; Vinogradov 1998).

Results

Genotyping

The mapping populations BBM-2 and BBM-3 correspond to the males produced by single colonies founded in the laboratory by spring queens collected from natural populations in northwestern Switzerland. The males of the mapping population BBM-1 were produced by a daughter queen of a laboratory-reared colony originating from an area in north-eastern Switzerland, thus providing pedigree information.

A large number of informative meioses are necessary to achieve high resolution in genetic mapping. For the high-resolution mapping population BBM-1, all males produced by this colony were collected. Of these 577 males, 37 (6.4%) were excluded because microsatellite analysis proved them to be worker-derived. Although only mated queens can produce females in bumble bees, workers will frequently lay male eggs in large colonies (Alaux et al. 2005). Removing worker-derived males is important because they introduce random variation to the linkage information, potentially increasing the estimated recombination frequency between markers. The ratio of worker-derived males increased from an initial 3% at the onset of male production to 14% in the last batch of males produced by the colony BBM-1. This increase reflects heightened reproductive competition as the colony cycle unfolds (Duchateau and Velthuis 1988). The BBM-1 mapping population thus provided 540 individuals with a mean of 392 ± 136 (mean \pm SD) informative meioses per marker. This allowed reliable marker ordering down to less than 1 cM.

For the other 2 maps, the number of males in the mapping populations was 146 (colony BBM-2) and 182 (colony BBM-3), respectively. Both populations had an average of 119 informative meioses per marker. None of the males from population BBM-2 or BBM-3 proved to be worker produced. These 2 less-extensive mapping populations allow markers to be stringently arranged if they are linked at 2 cM (Hunt and Page 1995).

Three different types of genetic markers were used for mapping: microsatellites, AFLPs, and RAPDs. All 60 of the currently available microsatellites for *Bombus* spp. (Estoup et al. 1993; Estoup et al. 1995; Reber Funk et al. 2006) were tested for polymorphism in each mapping population. On average, 54% of the available microsatellites were polymorphic in any of our mapping populations (39 of 60 micro-

satellites in BBM-1, 34 in BBM-2, and 24 in BBM-3); any 2 populations share on average 21 ± 7.0 polymorphic microsatellites. For the development of AFLP markers, 32 primer combinations were chosen for population BBM-1, resulting in 219 reliably amplifying fragments. For populations BBM-2 and BBM-3, 12 and 8 combinations were tested, resulting in 103 and 50 polymorphic bands, respectively. Since the degree of polymorphism of different primer combinations was highly variable between mapping populations, for economic reasons, no particular emphasis was put on using identical primer combinations in different populations. However, populations BBM-1 and BBM-2 shared 6 primer combinations (A02E, A02G, A03F, A05B, A06G, and A07F), resulting in 14 potentially homologous markers (36% of markers per primer combination in population BBM-1, 25% in population BBM-2). Populations BBM-2 and BBM-3 had 3 combinations in common (A02H, A03D, and A07A), producing 3 potentially homologous markers. AFLPs are considered primarily dominant, but we discovered a considerable number of codominant AFLP markers (42 out of 207 in BBM-1, 15 of 102 in BBM-2, and 2 of 50 in BBM-3). RAPD markers were used only for population BBM-3; the selected Operon primers resulted in 63 reliably amplifying polymorphic bands.

Segregation distortion

To avoid biasing the maps, e.g., by including AFLP markers showing homoplasy (Vekemans et al. 2002), only markers that did not show significant segregation distortion were included in map construction. Therefore, 28 markers were removed in total (13 RAPDs, all in population BBM-3; 13 AFLPs, 5 in BBM-1, 1 in BBM-2, and 7 in BBM-3; 2 microsatellites, 1 each in BBM-1 and BBM-3).

Recovery rate

For the phase-known population BBM-1, the ratio of genotyped maternal and paternal alleles, the recovery rates of alleles, was investigated. We found that overall the chance of recovery, f_0 , is normally distributed (ratio of female to male alleles; mean \pm SD = 1.03 ± 0.12 , Kolmogoroff–Smirnov test, $p = 0.69$), but the recovery rate is slightly biased towards maternal alleles. The mean of $f_0 = 1.03 \pm 0.12$ significantly deviates from the expected ratio of 1 (1-sample t test, $t = 3.878$, $p < 0.001$). This recovery distortion is not caused by markers showing individual segregation distortion; if only markers that show no significant segregation distortion ($p > 0.05$) are included in the analysis, the result remains the same (1-sample t test, $t = 3.958$, $p < 0.001$). This bias towards the female f_0 allele could potentially be caused by them being more frequently the “absent” allele in AFLPs. With dominant markers, it is difficult to distinguish the absent allele from a mere accidental failure of amplification. Yet, this is not the case in this data set (χ^2 test, $\chi^2 = 0.824$, $p = 0.364$). The biased recovery rate towards the grand mother’s alleles in this population indicates the possibility of meiotic drive or some other biasing process in this

Table 4. Alignment of core linkage groups.

Linkage group	Naming locus	Additional microsatellites	Homologous AFLPs
LG01	B10	BT14, BT05, BT01	A05B_267
LG02	B96	BT10, B126	A06G_379/385
LG03	B100	BL06, BT12, BT17, BT30	
LG04	B118	BT07, BT08	
LG05	B119	BT03, BT09, BT11, BT21	
LG06	B124	BL16, BT04, BT19	A03F_189, A07F_064
LG07	B132	BL09, BL03, BT02, BT23, BTERN	
LG08	BL01	BL02, BL11, BT16, BT22	A05B_073
LG09	BL05	BT24	A02G_209/210
LG10	BL13	BT15	A02G_047/048, A02E_044
LG11	BT 18		
LG12	BT20		A02G_400/403, A02H_071
LG13	A03F_209/210		A03D_059, A05B_345
LG14	A02G_195		

haplo-diploid system. However, the present study cannot clarify this point further.

Phase-known and -unknown mapping

Two of the 3 linkage maps were constructed phase unknown (BBM-2, BBM-3), because the pedigrees of their field-caught founding queens were unknown. When constructing a traditional genetic linkage map, it is necessary to assign a linkage phase to every marker, i.e., alleles are coded according to whether they were contributed by the grandmother or the grandfather. To circumvent this problem and to test our phase-unknown mapping approach, the mapping population BBM-1 was generated from a first-generation laboratory-reared queen with known pedigree. To establish the reliability of the phase-unknown mapping approach, one third of all linkage groups of population BBM-1 were analyzed for the subset of markers whose phase had been determined by pedigree analysis. These results were compared with the results of mapping the same dataset as a phase-unknown population. There was no difference in the 2 sets of results, confirming that prior knowledge of linkage phase is not necessary for accurate genetic mapping in this system.

Genome size

The 246 markers of the high-resolution population BBM-1 mapped into 21 linkage groups, 20 of which consisted of at least 3 markers, while 9 markers remained unmapped. This map spans a total of 2222 cM. Since the karyotype of *B. terrestris* consists of 18 chromosomes (Hoshiya et al. 1995), the maximum distance between 2 linked markers of 43.5 cM has to be added for any excess linkage group; thus, the estimated G_M , for this population is 2352 cM. The reduced number of markers in populations BBM-2 and BBM-3 resulted in an inflated number of linkage groups and, consequently, a reduced genome length (see Table 3). G_E was then estimated by a method-of-moments approach (Hulbert et al. 1988; Chakravarti et al. 1991). This estimation yielded highly repeatable results with G_E values of 2734, 2786, and 2761 cM in maps BBM-1, BBM-2, and BBM-3, respectively. The 2 low-resolution maps therefore have an estimated genome coverage of 41% to 44%, whereas the high-resolution map achieves a coverage of 81%.

The physical genome size of *B. terrestris* has been determined as being 1.54 times larger than the *A. mellifera* genome, which, in prior calculations, lead to an estimate of 274 Mb (Gadau et al. 2001). Subsequently, the physical size of the honeybee genome has been revised from the originally published 178 Mb (Jordan and Brosemer 1974) to 265 Mb (J.S. Johnston, Texas A&M University, Texas, personal communication). Consequently, the estimate of the genome size of *B. terrestris* also required revision. Additionally, the initial estimate was biased as Gadau et al. (2001) used a method that preferentially stained the AT portion of the genome. Using propidium iodide staining, the *B. terrestris* genome has been estimated to be 2.71 times larger than the honey bee genome (= 575 Mb). However, owing to the current discrepancy in the estimated size of the *A. mellifera* genome, we provide here an estimate of the genome size independent of the honey bee genome size estimates (see Material and methods). Our revised estimate of the physical genome size of *B. terrestris* is 625 Mb. This results in a rate of genome-wide recombination of 226 Kb/cM, assuming a total recombination length of $G_E = 2760$ cM.

Saturation curve

The resampled data sets of both population BBM-1 and an *A. mellifera* population (RAPD genotype data provided by G.J. Hunt, Purdue University, Indiana) showed a highly significant fit to a first-order saturation model ($p < 0.001$). A comparison of both curves (Fig. 2) demonstrates that although the *Bombus* maps have not reached saturation, they are much closer to the saturated range of the curve compared with the resampled *Apis* maps and the published linkage maps of *A. mellifera* (Hunt and Page 1995; Rueppell et al. 2004; Solignac et al. 2004). This comparison adds further weight to the evidence of a higher recombination rate in *A. mellifera* (Gadau et al. 2000).

Marker distribution

Although the distances between markers for maps BBM-2 and BBM-3 are normally distributed (Kolmogoroff-Smirnoff test, $p_{BBM-2} = 0.07$, $p_{BBM-3} = 0.63$), BBM-1 showed a non-normal distribution ($p_{BBM-1} < 0.05$, mean = 10.3 ± 0.9). In the BBM-1 population, the distribution of marker distances

was skewed to the left, indicating a tendency for markers to be clustered (Krutovskii et al. 1998) in this map. Seven of the linkage groups for this map showed signs of clustering (2-tailed Kolmogoroff–Smirnov test against a uniform distribution), with $p < 0.001$ for LG09, LG10, and LG13 and $p < 0.05$ for LG06, LG08, LG12, and the largest non-homologized linkage group. These clusters might denote the chromosomes' centromeric region (Krutovskii et al. 1998; Gadau et al. 1999). The length of linkage groups overall was highly correlated with the number of markers per linkage group in all 3 maps (Pearson's correlation, $p < 0.001$, $R^2_{\text{BBM-1}} = 90.4\%$, $R^2_{\text{BBM-2}} = 79.7\%$, and $R^2_{\text{BBM-3}} = 86.1\%$). This indicates that there is no bias in marker allocation between linkage groups.

Core map

Using primarily microsatellites and additional homologous AFLPs, 14 core linkage groups could be homologized across the different maps (see Fig. 3). Three pairs of linkage groups were homologized merely based on single AFLP polymorphisms (indicated by a black star in Fig. 3) and an additional 2 pairs based on codominant AFLPs (indicated by a white star). Eight of the potentially homologous AFLPs were found to reliably cosegregate with microsatellites or other AFLPs, demonstrating that these were indeed homologous. Three potentially homologous markers remained unlinked in 1 population; however, this never led to the rejection of the hypothesis of homology as would be the case if a homologous marker were missing from an otherwise well-mapped area. In no case were potentially homologous markers mapped to different linkage groups. Although this is strong evidence for the homology of AFLP markers, homology can only be strictly assumed if it has been demonstrated by sequencing. The homologized core linkage groups have been named LG01–LG14 according to the name of mapped microsatellites (see Table 4).

Discussion

We present the first high-resolution standardized map of the bumblebee *B. terrestris*. With an average marker spacing of 10.3 cM and an estimated genome coverage of 81%, the map of population BBM-1 comes very close to being saturated. However, this map still contains 3 excess linkage groups as compared with the karyotype of *B. terrestris*. The number of excess linkage groups decreased with the number of markers per map (see Table 2). This is consistent with results from studies on *A. mellifera*. Solognac et al. (2004) found that the number of linkage groups dropped from 32 (with 297 markers) to 24 (with 541 markers). Nevertheless, their map still includes 8 excess linkage groups and the authors estimated that it would take hundreds of additional markers to saturate the *Apis* map. This caveat also applies to *B. terrestris*, although the low number of excess linkage groups indicates that the high-resolution *Bombus* map presented in this study is much closer to saturation. A relatively high degree of saturation is also demonstrated by a comparison of the saturation curves for BBM-1 and an *Apis mellifera* mapping population (see Fig. 2). This difference is probably due to the significant difference in recombination frequency between *A. mellifera* and *B. terrestris*.

Genetic linkage maps are ideally built with markers for which sequence information is available such as microsatellites, single-nucleotide polymorphism (SNPs), or sequence-tagged site (STS) markers. Since *B. terrestris* is not yet an extensively developed genetic model system, only a limited set of 60 polymorphic microsatellites has been developed so far (Estoup et al. 1993; Estoup et al. 1995; Reber Funk et al. 2006). To generate the large numbers of genetic markers necessary for linkage mapping, it was therefore necessary to resort to AFLPs and RAPDs. These markers provide the opportunity of rapidly generating hundreds of polymorphic markers at low cost.

Although AFLPs have been shown to be highly repeatable within populations (Jones et al. 1997), they are often considered to be inappropriate for comparisons between populations (Slate 2005). Although these concerns are valid in many systems, results from this study, as well as from the literature, show that AFLPs nevertheless can be valid markers not only for genetic mapping but also for interpopulation comparisons. An often-mentioned concern is the largely dominant nature of AFLP markers, which leads to a loss of information (Bensch and Akesson 2005). Yet, since *B. terrestris* is, like all hymenopterans, haplo-diploid, this problem is effectively circumvented by genotyping the haploid males where the absent allele is not masked. Indeed, AFLP analysis can also generate truly codominant markers (Wong et al. 2001). These can easily be identified in large mapping populations as cosegregating bands in repulsion phase, thus adding further valuable information. For populations BBM-1 and BBM-2, in which AFLPs were thoroughly assessed for codominance, the rate of codominant markers ranged between 20.3% and 14.7%, respectively. Although there is little information on codominant markers in mapping studies, rates close to 20% seem to be usual in plants and animals (Fishman et al. 2001; Parsons and Shaw 2002).

A more serious concern is whether fragments of the same size can be considered to be homologous or whether they merely show homoplasy. For interspecies comparisons, this question has been addressed experimentally by O'Hanlon and Peakall (2000). They conclude that AFLPs are suitable for phylogenetic analysis of closely related taxa. Parsons and Shaw (2002) confirmed the homology of same-size AFLP bands of the closely-related cricket species *Laupala paranigra* and *Laupala kohalensis* by sequencing the fragments. Homologous AFLP bands were used for aligning linkage groups in genetic maps, e.g., in barley (Waugh et al. 1997) and in crickets (Parsons and Shaw 2002). In this study, we constructed 3 genetic maps from unrelated mapping populations of *B. terrestris* using microsatellites, AFLPs, and (in population BBM-3) RAPDs. We show that there are recurring patterns of linked microsatellites and AFLP markers (Fig. 3; Table 4) and that there is no case in which the assumption of homology is violated by discrepancies in linkage group assignment. This is very strong evidence for the widespread homology of AFLP markers in *B. terrestris*.

To be valuable as a tool for basic and applied research, linkage maps have to be stable and comparable between laboratories and studies. The recurrence of markers between the genetic maps allowed a group of repeatable core linkage

groups to be defined (Fig. 3; Table 4). The considerable number of microsatellites and codominant AFLPs on the core linkage groups promises that genetic variability in most crosses or populations will be sufficient to anchor genetic information to the developed linkage maps. We propose using these homologized core linkage groups as a reference for future genetic work in *B. terrestris*. This will allow for independent genomic studies to be related to each other, and enable meta-analyses of the combined results, e.g., to study the variance of recombination frequency in natural populations.

Although bumblebees are also kept as semi-domesticated animals, there is both the potential and the need to study natural populations. Genetic mapping in applied or basic model species has become almost commonplace in the last 2 decades, but linkage maps of natural populations are still rare (Slate 2005). Most work on “wild” species involves either interspecies crosses, such as for the *Laupala* crickets (Parsons and Shaw 2002), or intricate crossing schemes involving several generations, such as in the butterfly *Heliconius melpomene* (Jiggins et al. 2005). Here, we show that this is not necessary in the haplo-diploid system and that accurate linkage maps can be constructed because large numbers of progeny are available from a single reproductive individual.

The availability of a genetic linkage map is the foundation of quantitative trait locus (QTL) analysis. A large proportion of QTL studies are concerned with applied agricultural research, as is demonstrated by 35% of all studies featuring “QTL” in their title belonging to the subject category “agronomy” (Web of Science, 25 January 2006). In this field, QTLs are of practical interest for marker-assisted selection, especially to select for disease resistance in livestock (Andersson 2001). In this approach, breeders aim at increasing and monitoring the breeding success by genotyping their stocks for markers associated with relevant QTLs, e.g., resistance to pathogens or product quality (see Collard et al. 2005). Although bumblebees have been commercially reared for much of the last half of the 20th century, little effort has gone into increasing their agricultural value, e.g. their pollinating efficiency, by selective breeding. Selecting bumble bees is currently prohibitively difficult and time intensive, not only because of the extensive monitoring of phenotypic traits but also because continued inbreeding results in a high proportion of colonies producing effectively sterile diploid males (Duchateau et al. 1994; Zayed 2004). These difficulties could be overcome with marker-assisted breeding. QTL studies are also of major importance in basic research, such as in the study of reproductive isolation (Orr 2001) or of fitness relevant traits and epistasis (Malmberg et al. 2005). The standardized core map presented in this paper serves as a basis for the comparative analysis of the bumblebee genome, and for further detailed studies in evolutionary and population genetics of social insects.

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