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Characterization of $(GT)_n$ and $(CT)_n$ microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*

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ABSTRACT

A set of 52 $(CT)_n$ and 23 $(GT)_n$ microsatellites in honey-bee, 24 $(CT)_n$ and 2 $(GT)_n$ microsatellites in bumble-bee ($n>6$) have been isolated from partial genomic librairies and sequenced. On average, $(CT)_n$ and $(GT)_n$ microsatellites occur every 15 kb and 34 kb in honey-bee and every 40 kb and 500 kb in bumble-bee, respectively. The prevailing categories are imperfect repeats for $(CT)_n$ microsatellites in bumble-bee, and perfect repeats for both $(CT)_n$ and $(GT)_n$ microsatellites in honey-bee. Comparisons with data available in vertebrates indicate a lower proportion of perfect repeats in bees but length distributions are very similar regardless the phylum. This result extends to insects the concept of an evolutionary conservation for quantitative and qualitative characteristics of $(CT)_n$ and $(GT)_n$ microsatellites. Many $(CT)_n$ and $(GT)_n$ repeats are surrounded with various types of microsatellites, revealing an associative distribution of short repeat sequences. As expected, a high level of intrapopulational polymorphism has been found with one tested honeybee microsatellite. Also, flanking regions of this microsatellite are similar enough to allow PCR amplification in several other species of *Apis* and *Bombus*.

INTRODUCTION

Eukaryotic and, to a lesser extent, prokaryotic genomes, are densely interspersed with tandem repeats commonly termed microsatellites (1, 2). Most of simple DNA motives composed of one to five nucleotides can be found in eukaryotic genomes, in transcribed as well as non transcribed sequences (3). $(GT)_n$ repeats seem to be the most abundant microsatellites in higher vertebrates and occur every 30kb, 21kb and 18kb, on average, in human, rat and mouse, respectively (4). $(CT)_n$ repeats form another abundant class of microsatellites in mammalian species (5). Both classes of repeat sequences are widely distributed throughout the genome but are under-represented in the centromeric and telomeric regions of chromosomes (6, 7). All types of microsatellites have shown variation in the number of repeats and display a level of polymorphism generally greater than that of standard unique-sequence probe and as high as that of many minisatellites (review in 8). Therefore, microsatellite loci constitute powerful nuclear markers, increasingly used for identity testing, population studies, linkage analysis and genetic mapping (9, 10, 11, 12).

Most microsatellite loci investigated so far have been taken from published sequences. This procedure is possible only for a few species (mainly human and mouse) and explain, at least in part, the advancement in microsatellite characterization for these species. In contrast, very little is known about microsatellites in non mammalian vertebrates and in insect species. The few studies devoted to this subject concerned essentially *Drosophila* species. *In situ* hybridization on polytene chromosomes have shown the presence of $(GT)_n$ and $(CT)_n$ repeats, which were similarly distributed in distantly related species (13). The relative proportions of $(GT)_n$, $(CT)_n$, T_n, C_n and $(GTC)_n$ repeats were estimated in *Drosophila* (14). Length polymorphism was also described for a $(CAG)_n$ microsatellite located in the Notch gene of *Drosophila melanogaster* (15). Inheritance and mutation in loci with long $(GATA)_n$ tracts were recently studied in a moth (16). This fragmentary knowledge of microsatellites in invertebrates has prompted us to characterize $(GT)_n$ and $(CT)_n$ repetitive sequences in two hymenopteran species, the honey-bee (*Apis mellifera*) and the bumble-bee (*Bombus terrestris*).

In the present study, partial genomic libraries of *A.mellifera* and of *B.terrestris* were constructed in plasmid vector and screened with $(GT)_n$ and $(CT)_n$ synthetic oligonucleotides. The quantitative and qualitative characteristics of the microsatellites cloned this way have been analyzed and compared to data already available for vertebrate genomes (4, 5, 6, 17).

MATERIALS AND METHODS

Construction of the two partial genomic libraries

Total DNA from 12 workers from one hive of *A.mellifera* and from 25 males from one nest of *B.terrestris* was extracted according to (18) with slight modifications (19). The DNA extract was digested to completion with *Sau*3A. Restriction fragments between 200–600 bp for *A.mellifera* and between 200–370 bp and 480–600 bp for *B.terrestris* were selected with DEAE paper (20). The size of the fragments selected for *B.terrestris* was justified by the presence of satellite bands located between 380 and 460 bp. The fragments extracted from the gel were ligated

with pTZ18 vector (Stratagene) and amplified after transformation into competent XL1 blue cells (Stratagene), according to standard protocols (20).

Screening of libraries and sequencing

About 3,000 recombinant clones for *B.terrrestris* and 2,000 for *A.mellifera* were transferred manually on solid LB medium plates over a squared matrix, in order to facilitate the identification of double positive clones. After a double transfer on Hybond-N nylon membranes (Amersham), the screening procedure was carried out using an equal mix of $(CT)_{10}$ and $(GT)_{10}$ oligonucleotides labelled with the DIG oligonucleotide tailing kit (Boehringer); the DIG nucleic acid detection kit (Boehringer) was used for detection. A relatively low hybridization and washing temperature (50°C) was applied in order to detect also short repeat sequences. Proteinase treatment, washing and detection steps were performed according to the protocols of Boehringer kits except that the washing and blocking steps, occurring before the incubation with the DIG-AP conjugate, were performed at hybridization temperature.

Double positive clones were directly analyzed by sequencing reactions on alkaline denatured plasmid DNA (21) using the dideoxynucleotide chain termination method (22) with T7 polymerase (Pharmacia).

PCR amplifications

Standard polymerase chain reactions were carried out in 25 µl of a mixture containing 15–25 ng of total DNA template, 30 pmol of each primer, 75 µM each dGTP, dCTP and dTTP, 3 µM dATP, 0.2µl α^{35}S-dATP at 1000 Ci/mmol, 1.7 mM $MgCl_2$, 1×Promega reaction buffer and 1.2 unit of Promega *Taq* polymerase. After one denaturing step of 5 min at 94°C, samples were processed through 30 cycles consisting of 30 sec at 94°C, 30 sec at 58°C and 30 sec at 72°C. The last elongation step was lengthened to 10 min. Aliquots of 12 µl of amplified DNA were mixed with 9 µl of formamide stop solution (T7 sequencing kit, Pharmacia). 4 µl of the mixture were heated 5 min at 85°C and electrophoresed on standard DNA sequencing gel (5M urea, 6% acrylamide).

Computer treatment of sequences

Personal programs were used to compare sequences of the flanking regions of the cloned microsatellites, in order to detect loci which could have been isolated several times in each species, and to find some possibly conserved microsatellite loci between *A.mellifera* and *B.terrestris*.

RESULTS

Density and copy number of $(CT)_n$ and $(GT)_n$ microsatellites

For the two types of dinucleotide motives, a sequence was counted as a microsatellite if the total number of repeats (n) was greater than six (4). A total of 75 microsatellites [52 $(CT)_n$ and 23 $(GT)_n$ blocks] were isolated from honey-bee DNA and 26 microsatellites [24 $(CT)_n$ and only 2 $(GT)_n$ blocks] from bumble-bee DNA. The partial genomic libraries of honey-bee and bumble-bee were composed of 2,150 and 3,130 clones, respectively. The average size of the cloned inserts determined after digestion and electrophoresis on 5% acrylamide gel was 360 bp for honey-bee and 310 bp for bumble-bee; consequently, the total number of base pairs analyzed was 360×2150 = 774,000 bp for honey-bee and 310×3130 = 970,000 bp for bumble-bee. The average distance between neighbouring microsatellites can be estimated by dividing the total length of screened DNA by the number of isolated microsatellites. This estimate makes sense essentially if these microsatellites are fairly evenly distributed, like in all other species studied (6, 12, 13). Thus, in the cloned fraction of the honey-bee genome, $(CT)_n$ microsatellites occur on average every 15 kb and $(GT)_n$ microsatellites every 34 kb. In the bumble-bee, $(CT)_n$ microsatellites occur every 40 kb and $(GT)_n$ microsatellites every 500 kb.

Our partial genomic librairies represent a large part of the genomes (i. e. 40% and 30% for honey-bee and bumble-bee respectively, according to densitometric measurements). We will

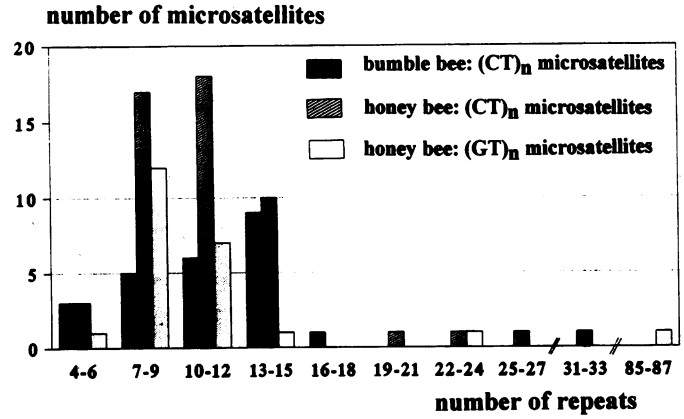

Figure 1. Length distribution of $(CT)_n$ microsatellites in honey-bee and bumble-bee and $(GT)_n$ microsatellites in honey bee. The three categories of microsatellites (perfect, imperfect, and compound repeat sequence) are pooled. Abscissa values represent the number of repeats within the longest run of uninterrupted repeats.

Table 1. Average distance in kb between $(GT)_n$ and between $(CT)_n$ microsatellites in bumble-bee, honey-bee and several vertebrate species: salmon (1), pigs (6), mouse (4), rat and human (4, 5)

	bumble-bee	honey-bee	salmon	pigs	mouse	rat	human
$(GT)_n$	(500)	34	12	47	18	15	30
$(CT)_n$	40	15	N.D.	N.D.	N.D.	50	113

N.D.: not determined. Because only two $(GT)_n$ microsatellites have been isolated in bumble-bee, the corresponding estimate is certainly not accurate and thus noted between parentheses.

Table 2. Percentages of different categories for $(CT)_n$ microsatellites (perfect, imperfect, and compound, see text) in bumble-bee and honey-bee, and for $(GT)_n$ microsatellites in honey-bee, pigs (5) and human (17)

	bumble-bee	honey-bee		pigs	human
motif	$CT^{(24)}$	$CT^{(52)}$	$GT^{(23)}$	$GT^{(108)}$	$GT^{(57)}$
perfect	21	46	48	71	61
imperfect	62	31	22	19	26
compound	17	23	30	10	12
$n \geq 0$	67	62	44	75	–

Percentages of microsatellites (all categories pooled) with a number of uninterrupted repeats within the longest run greater than or equal to 10 ($n \geq 10$) are also listed. Numbers between parentheses indicate the number of $(CT)_n$ or $(GT)_n$ microsatellites analyzed. Due to the low number of $(GT)_n$ microsatellites isolated in bumble-bee, no percentage are mentioned in this table.

Table 3. Repeat sequences associated in an adjacent or a proximal way with $(GT)_n$ and $(CT)_n$ microsatellites of honey-bee and bumble-bee

	type of associated repeat sequence	distance in nucleotides (d) d = 0	d < 20	d ≥ 20
honey-bee $(CT)_n$ microsatellites	mononucleotide	T_{13} T_{10} T_{29}	T_{10}	G_{12} T_{13} T_{18} T_{12} T_{10}
	dinucleotide	$(GT)_8$ $(GT)_{86}$		$(CT)_7$ $(CT)_{10}$ $(AT)_7$ $(AT)_9$ $(AT)_{10}$
	trinucleotide	$(CCT)_9$ $(CCT)_9$ $(ATA)_4$	$(GGT)_4$ $(GCC)_9$ $(GGT)_7$	$(CCT)_8$ $(GGT)_9$ $(GCC)_4$
	tetranucleotide	$(ATCT)_3$ $(ATCT)_3$ $(TAAA)_3$	$(CTTT)_4$	$(GGGA)_5$ $(TACA)_5$ $(GCAC)_4$
	pentanucleotide	$(GCAC)_4$		$(CCGTG)_5$
honey-bee $(GT)_n$ microsatellites	mononucleotide		T_{10}	T_{10}
	dinucleotide	$(CT)_{10}$ $(CT)_{11}$ $(AT)_6$ $(AT)_7$ $(AT)_{10}$	$(GT)_{10}$ $(GT)_7$	$(GT)_7$ $(GT)_{11}$ $(GT)_9$ $(GT)_9$
	tetranucleotide	$(CATA)_5$ $(CATA)_5$		
bumble-bee $(CT)_n$ microsatellites	monunucleotide	T_{10} C_{11}		
	dinucleotide			$(CT)_7$ $(CT)_{15}$ $(GT)_7$
	trinucleotide	$(CCT)_4$		$(CCT)_6$
	tetranucleotide	$(CTAT)_9$		$(GGCT)_6$

To be scored as an associated repeat, the number of repeats must be ≥10 for mononucleotide motives, ≥6 for dinucleotide motives, ≥4 for trinucleotide motives and ≥3 for tetra and pentanucleotide motives. The distance (d) between the $(CT)_n$ or $(GT)_n$ microsatellite and the repeat sequence associated has been classified in three categories: adjacent association (d=0 nucleotide), and proximal association (d<20 and d≥20 nucleotides). Proximally associated repeats localized at less than 20 nucleotides are scored separately because the selection of primers that would permit independent PCR amplifications of the two associated microsatellites becomes very difficult.

consider below that these partial libraries are representative of the whole genome, at least of the 90% which are single copy in honey-bee (23). This implies that *Sau*3A restriction sites and $(CT)_n$ or $(GT)_n$ microsatellites have independant distributions. Thus, $(CT)_n$ microsatellites are more common than $(GT)_n$ blocks in the genomes of both species, with 2.3 and 12.5 more $(CT)_n$ microsatellites for honey-bee and for bumble-bee, respectively. The average distances between microsatellites in the genomes of honey-bee, bumble-bee, and several vertebrate species for comparison, are listed in table 1.

Considering that the haploid genome of honey-bee consits of 180 Mb (23), rough estimates of the total number of $(CT)_n$ and $(GT)_n$ microsatellites amount to 11,700 and 5,300 loci respectively in this species.

Characteristics of $(CT)_n$ and $(GT)_n$ microsatellites

According to Weber (17), $(CT)_n$ and $(GT)_n$ microsatellites were classified in three categories: perfect (no interruption in the run of dinucleotide repeats), imperfect (one or more interruptions in the run of repeats), or compound (a run of perfect or imperfect repeats adjacent to a run of another simple sequence repeat). Proportions of each category are listed in table 2 for honey-bee, bumble-bee, pigs and human. The predominant categories are imperfect repeats for $(CT)_n$ microsatellites in bumble-bee and perfect repeats for $(CT)_n$ and $(GT)_n$ microsatellites for honey-bee. However, $(GT)_n$ perfect repeats are less frequent and $(GT)_n$ compound repeats are more frequent in honey-bee than in pigs and human.

Regardless of the repeat sequence category, the length of the longest run of uninterrupted repeats was found to be the best predictor of informativeness of polymorphism for $(GT)_n$ microsatellite in human (17). Therefore, we built the length distributions of uninterrupted repeats for $(GT)_n$ and $(CT)_n$ microsatellites in honey-bee and $(GT)_n$ microsatellites in bumble-bee (figure 1). The three distributions are similar except that the average length of the longest $(GT)_n$ uninterrupted run is slightly shorter in honey-bee. Since $(GT)_n$ microsatellites in human with a run of uninterrupted repeats of 20 bp or longer (n≥0) turned out to be polymorphic enough for mapping purpose

5'GTGTCGCAATCGACGTAACCGATTTTCCCCGCGATGAATCAACGCTGTCTCTG

CAAAGCACGCGGCGATCGTAAAAATTCGCGGTGGGGTACCACTTTT**(CT)$_{13}$**GTC

TCCCCTTTTTCTTTTCACGCTTATCCACGCGTCATCGCAC**(GGT)$_9$**GGCCGCAACC

TCCTTTCGTCACGATCGGTAATCGAC3'

3'GCAGTGCTAGCCATTAGCTG5'

Figure 2. Sequence of the microsatellite A14 of *A.mellifera*. The underlined 20 bp sequences correspond to the primers selected for PCR reactions.

(17), the proportion of $(GT)_n$ and $(CT)_n$ microsatellites (all categories pooled) with these characteristics was determined in bees and compared in table 2 with data on pigs.

Associations between microsatellites

Repeat sequences adjacent (compound repeats) or close to $(GT)_n$ and $(CT)_n$ microsatellites have been scored. There appear to be many associated microsatellites: adjacent and proximal associations concern 56% and 52% of $(CT)_n$ and $(GT)_n$ microsatellites respectively in honey-bee and 34% of $(CT)_n$ microsatellites in bumble-bee (table 3). For comparison, 43% (6/14) of *Apis* and 12% (3/26) of *Bombus* false positive clones contain repeat sequences. The overall diversity of the motives associated with $(CT)_n$ or $(GT)_n$ microsatellites is also quite large: the two possible mononucleotidic motives, three of the four dinucleotides, four of the ten trinucleotides, eight different tetranucleotides and even one pentanucleotide have been found (table 3). This contrasts with the uniformity of microsatellites observed in the false positive clones which are all $(T)_n$ except for one $(GCTC)_6$ found in *Bombus*.

Assuming that the false positive clones are representative of the genomic sequences free of $(GT)_n$ or $(CT)_n$ repeats, non-mononucleotide microsatellites are significantly more frequent in inserts containing $(CT)_n$ or $(GT)_n$ microsatellites than in inserts deprived of these sequences (25/69 versus 0/24 for the honey-bee and 9/24 versus 1/26 for the bumble-bee). This implies clearly that non-mononucleotide microsatellites do not have independant distributions but form clusters. Also, but only in the

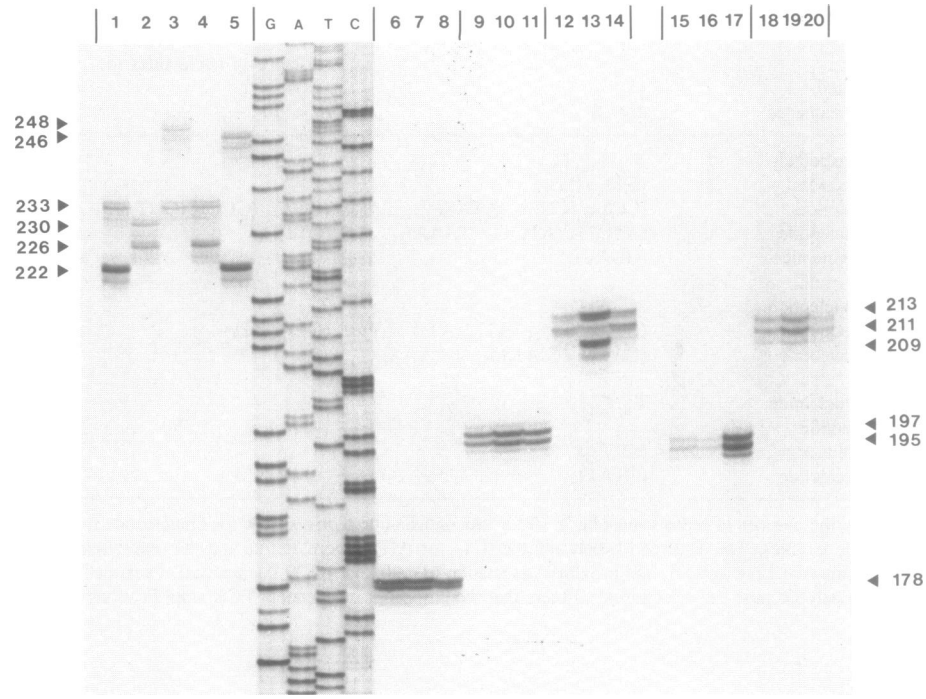

Figure 3. Intrapopulational hypervariability of the core sequence and interspecific conservation of the flanking regions of the microsatellite A14 cloned in *A.mellifera*. PCR reactions were performed on 5 workers of *A.mellifera* (1, 2, 3, 4, 5), 3 workers of *A.cerana* (6, 7, 8), *A.florea* (9, 10, 11), *A.dorsata* (12, 13, 14), *B.pascuorum* (15, 16, 17) and *B.terrestris* (18, 19, 20). A sequencing reaction was used as a size marker. Numbers on the sides of the figure refer to the length of allelic forms. The association of di and trinucleotides repeats in this microsatellite probably accounts for length variations between alleles of even or odd numbers of bp.

honey-bee, mononucleotides stretches are significantly less frequent in positive clones than elsewhere.

Intrapopulational polymorphism and cross priming with other species

To test the variability of Apoid microsatellites, we have chosen one microsatellite of *A.mellifera* (microsatellite A14) composed of 13 CT uninterrupted tandem repeats associated in a proximal way with 9 trinucleotide (GGT) tandem repeats. The amplification of the cloned sequence with our primers would produce a 231 bp fragment (figure 2). PCR amplifications with α^{35}S-dATP were performed on genomic DNA of 29 workers from *A.mellifera* unrelated colonies (1 worker per colony) from Montfavet (France). This microsatellite was found to be highly polymorphic, showing 13 allelic forms the lengths of which range from 216 to 248 bp. The phenotypes of 5 workers are shown in figure 3.

The interspecific similarity of microsatellite flanking sequences was first studied by comparing sequences of the flanking regions of the microsatellites cloned in *A.mellifera* and *B.terrestris*. Allowing a sequence similarity of 80%, we failed to find any microsatellite locus present in both species. This result was expected given the large number of $(CT)_n$ and $(GT)_n$ microsatellites in both genomes.

A direct experimental approach has consisted in testing PCR amplifications with primers of the *A.mellifera* microsatellite A14 on three other species of *Apis*, *A.cerana*, *A.florea* and *A.dorsata*, (three workers per colony, one colony per species) and two species of *Bombus*, *B.pascuorum* and *B.terrestris* (three unrelated workers per species). We obtained PCR amplifications for all these 5 species without changing PCR conditions (figure 3). Except for *A.dorsata* which showed one heterozygous individual, all other species were monomorphic. But the alleles encountered in these species were all different and characterized by a length less than that of alleles of the afore-mentioned population of *A.mellifera*.

DISCUSSION

The general characteristics of $(GT)_n$ and $(CT)_n$ microsatellites in honey-bee and bumble-bee, that is the high density in the genome, the proportion of the three categories (perfect, imperfect and compound repeat sequence) and the length distribution, are similar to those of $(GT)_n$ and $(CT)_n$ microsatellites in mammalian genomes. This result enlarges to insect the concept of an evolutionary conservation for quantitative and qualitative characteristics of these microsatellites.

One of the principal differences concerns the relative frequency of $(CT)_n$ and $(GT)_n$ microsatellites: $(CT)_n$ repeats in honey-bee and bumble-bee are more frequent than $(GT)_n$ ones whereas the opposite has been found in mamalian species (human, rat, pigs) and in *Drosophila* (14).

Another point worth mentioning is the significantly lower proportion of $(GT)_n$ microsatellites with more than 10 uninterrupted repeats in honey-bee than in a genomic library of pigs prepared in the same way (table 2). Since longer uninterrupted runs tend to be the most informative, this characteristics might represent a handicap for honey-bee geneticists, at least when hypervariability is needed. This is somewhat compensated by the fact that the above proportion for $(CT)_n$ microsatellites in honey-bee is not significantly different from that found for $(GT)_n$ microsatellites in pigs. In addition, the proportion of $(GT)_n$ compound microsatellites is larger in honey-bee than in pigs and human (table 2). Compound microsatellites probably have a higher polymorphism potential since they are composed of two repeat sequences, which both are likely to vary in length.

In honey-bee and bumble-bee, microsatellites other than T_n and C_n appear to be associated in clusters. Inserts containing two $(CT)_n$, two $(GT)_n$ or a mixture of both sequences could have be preferentially selected by our probes. Even if these types of associations are excluded from the computations, a significant association between $(CT)_n$ or $(GT)_n$ and other types of non-mononucleotide microsatellites is still observed. In species such as human and mouse, the large amount of data available show that compound repeats represent about 10% of the microsatellites (table 2). However, there seems to be no mention of an association in clusters of microsatellites in the literature. In any case, this brings a new insight upon the structure of the genome of bees.

The study of the repeat sequences associated to bumble-bee and honey-bee $(GT)_n$ and $(CT)_n$ microsatellites provides indications on the type of microsatellite composed of tri and tetranucleotidic motives that should be investigated preferentially in honey-bee and bumble-bee in the future. These motives deserve special attention because they are easier to type than dinuclotide microsatellites. They are however less frequent than $(GT)_n$ and $(CT)_n$ microsatellites: e. g. they occur only every 300–500 kb on the human chromosome X (24). It is thus important to know which type of tri and tetranucleotidic motives are the most frequent in a genome. According to our data on associated microsatellites, GGT, ATCT, GCAC and especially CCT motives are presumably the most suitable motives for further analysis in honey-bee, bumble-bee and perhaps many other insect species.

Besides genetic mapping, the microsatellites isolated in honey-bee and bumble-bee are likely to be of general utility in a large scope of genetic studies. As for other hymenopteran species, few nuclear markers, including allozymes, are available in honey-bee (25, 26, 27) and bumble-bee (28). Mitochondrial DNA provided a good discrimination between some honey-bee races but failed to exhibit noticeable variability within races (29).

The flanking sequences of one microsatellite locus turned out to be similar enough to allow PCR amplification in species of the same genus (*Apis*) and of a related genus (*Bombus*). This can be paralleled with the interspecific conservation of microsatellite loci which has been recently shown for several mammalian species, i.e. human and other primates and, to a lesser extent, between species of different orders, i.e. primates and rodents (4). Schlötterer *et al.* (30) also found a high level of conservation of microsatellite loci in eleven cetacean species belonging to eight different genera. In that respect, it would be interesting to verify how conserved are microsatellite loci in bees species and to determine the limits of this conservation over related taxa.

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