Genetics of arrhenotokous and thelytokous reproduction in Venturia canescens (Hymenoptera)

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CHAPTER 4

A genetic linkage map of the parasitic wasp *Venturia canescens* (Hymenoptera)

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Abstract
We present the first genetic linkage map for the parasitic wasp *Venturia canescens* containing 29 microsatellites and 19 amplified fragment length polymorphism (AFLP) markers. The linkage map has a resolution of 11 linkage groups, which is the haploid complement of *V. canescens* (N = 11). The map covers 1040 cM and an average marker spacing of 21.6 cM. Using flow cytometry we have estimated the genome size of *V. canescens* to be between 245 Mb and 279 Mb. We map a Virus Like Particle gene onto linkage group II and the complementary sex determining locus on linkage group IV. The linkage map provides a basis for future genetic studies in *V. canescens*.

Introduction

Insects of the order Hymenoptera, including sawflies, ants, bees and wasps are of great economical importance. The honey bee (*Apis mellifera*) has a long tradition of domestication for honey production. Bumblebees are used as pollinators in green houses. Parasitoid wasps are widely exploited as biocontrol agents of insect pests, and some species are of use in forensic sciences. Surprisingly, unlike many species of cattle and crops, the genetics of these beneficial insects has been very little studied. Whereas complete genome sequences are nowadays available in agricultural sciences for many animals and plants, such as for example the chicken, cow and pig (International Chicken Genome Sequencing Consortium, 2004; http://www.hgsc.bcm.tmc.edu/projects/bovine; http://www.piggenome.org) and wheat, tomato and potato (http://wheat.pw.usda.gov; http://mips.gsf.de/proj/ plant/jsf/tomato/index.jsp; www.potatogenome.net), only the genomes of the honey bee and the parasitoid wasp *Nasonia* have been fully sequenced (The Honeybee Genome Sequencing Consortium, 2006; The *Nasonia* Genome Sequence Consortium, in press). As a consequence, detailed quantitative trait locus (QTL) studies for traits of economic importance are unavailable for any Hymenoptera except foraging behaviour in *A. mellifera* (Table 4.1). The few mapped genes in Hymenoptera include colour mutations, allozymes and the sex determining locus (*csd*) (e.g. Antolin et al., 1996; Hunt & Page, 1995; Gadau *et al.*, 2001; Solignac *et al.*, 2004).

Construction of a genetic linkage map is a first step towards genomic localization and characterization of traits of interest. Detailed linkage maps are essential for QTL mapping and efficient identification of genes that underlie the QTLs. In addition, linkage maps are an essential tool for a genome project, e.g. to serve as a framework for assembling the full genome sequence from a set of scaffolds. Hymenopteran insects are particularly well suited for genomic analysis because of their haplodiploid reproduction; males are haploid and develop from unfertilised eggs, whereas females are diploid and develop from fertilised eggs. Haploidy of males can be used efficiently to construct linkage maps and to map traits of interest, because there are no dominant interactions among markers and loci.

Venturia canescens (Gravenhorst) is an endoparasitoid of pyralid moth larvae (Beling, 1932; Salt, 1976). The main mode of haplodiploid reproduction in *V. canescens* is arrhenotoky, where haploid males develop from unfertilised eggs and diploid females from fertilised eggs. A less common mode of reproduction is thelytoky where diploid females develop from unfertilised eggs and males do not occur. *V. canescens* has been widely used as a biological model in behavioural, population dynamic, genetical and physiological studies, but the genetic basis of studied traits have hardly been considered. A commonly used locus in *V. canescens* for genetic studies is the Virus Like Particle locus (VLP). VLPs cover the egg surface preventing the egg to be detected by the immune system of the host (Feddersen, *et al.*, 1986; Hellers *et al.*, 1996). The genomic location of the VLP locus remains unknown.

Table 4.1. Overview of genetic maps in the Hymenoptera with mapped loci and QTLs.

Species	Family	Markers	Traits	Reference
Athalia rosae	Tenthredinidae	RAPD	Yellow fat body (yfb)	Nishimori *et al.*, 2000
Acromyrmex echinator	Formicidae	AFLP		Sirviö *et al.*, 2006
Apis mellifera	Apidae	RAPD	Major sex determination locus (X locus, *csd* locus)	Hunt *et al.*, 1995
			Malate dehydrogenase (*MDH-1*)	
			Black body colour (*blk*)	
			Foraging behaviour	
			Defensive behaviour	Breed *et al.*, 2004
			Alarm pheromone levels	Hunt *et al.*, 1999
			Hygienic behaviour	Lapidge *et al.*, 2002
			Stinging behaviour and body size	Hunt *et al.*, 1998
			Sucrose responsiveness	Scheiner *et al.*, 2004
		AFLP	Foraging behaviour	Rueppell *et al.*, 2004
		Microsatellites	Malate dehydrogenase (*MDH*)	Solignac *et al.*, 2004
			3 sex-linked markers	
			Thelytoky	Lattorff *et al.*, 2007
		Microsatellites		Solignac *et al.*, 2007
Bombus terrestris	Apidae	Microsatellites	Sex locus (*csd locus*)	Gadau *et al.*, 2001
		RAPD	Fitness trait associated with male reproduction and immune defence	Wilfert *et al.*, 2007c
			Host-parasite interactions	Wilfert *et al.*, 2007b
		Microsatellites		Wilfert *et al.*, 2006
		RAPD		
		AFLP		
Melipona quadrifasciata	Apidae	RAPD		Garcia Tavares *et al.*, 2002
Bracon hebetor	Braconidae	RAPD	*Black body*	Antolin *et al.*, 1996
			Cantaloupe	
			Honey	
			X-sex gene (csd locus)	

Table 4.1. Continued

Species	Family	Markers	Traits	Reference
Bracon sp. near *hebetor*	Braconidae	RAPD	Sex locus (*csd locus*) Phenotypic marker *blonde* Sex-related traits	Holloway *et al.*, 2000
Nasonia spp.	Pteromalidae		Visible mutant markers	Saul *et al.*, 1993
		RAPD	Visible mutant markers Hybrid incompatibility	Gadau *et al.*, 1999
			Wing and head size	Gadau *et al.*, 2002
		microsatellites RAPD STS	Wing and head size	Rütten *et al.*, 2004
		microsatellites AFLP STS	Male courtship behaviour	Pietsch, 2005
		microsatellites	Transmission ratio distorting loci (TRDL) (Hybrid inviability)	Niehuis *et al.* 2008
		EST AFLP microsatellites	Mate discrimination	Velthuis *et al.* 2005 Peire, 2007
		AFLP	Visible mutant markers	Gadau *et al.*, 2008
Trichogramma brassica	Trichogrammatidae	RAPD		Laurent *et al.*, 1998
Aphelinus asychis	Aphelinidae	RAPD		Kazmer *et al.*, 1995
Venturia canescens	Ichneumonidae	microsatellites AFLP	*VLP* *csd* locus	This study

The sex determining mechanism in *V. canescens* is single locus complementary sex determination (sl-CSD), which was originally clarified by comparing sex ratios between crosses of related and unrelated individuals using an arrhenotokous strain (Beukeboom, 2001). Recently, generation of diploid males under inbreeding was confirmed by means of flow cytometry (Mateo Leach *et al.*, this study). Under CSD, individuals which are hemi- or homozygous at the sex locus will develop into males whereas heterozygous individuals become females (Whiting, 1943). The sex locus (*csd*) has been mapped in a number of species (Table 4.1) and characterized in the honey bee (Beye, 2004). Interestingly, CSD appears incompatible with automictic parthenogenesis, but can operate as long as the *csd* locus is located in a chromosomal region where heterozygosity is maintained, e.g. close to a centromere on one of the eleven chromosomes of *V. canescens*, or in a region in which recombination is prevented by inversions (Beukeboom & Pijnacker, 2000).

Here we present the first genetic map for *Venturia canescens* based on 29 microsatellite and 19 AFLP markers spread over 11 linkage groups and a total map size of 1040 cM. We use microsatellites because they are codominant and AFLPs because they are easy and inexpensive to produce. Moreover, the disadvantage of AFLPs as dominant markers is avoided in Hymenoptera by genotyping haploid males, i.e. both presence and absence of bands is informative. The microsatellites are a combination of an already published set by Butcher *et al.* in GenBank and newly developed ones (see Chapter III). In this study we map the *csd* locus on linkage group IV and one of the *VLP* genes on linkage group V.

Materials and Methods

Insects

Wasps used to construct the map originated from three laboratory populations (Antibes, Mont Boron-Valbonne and Valence) collected in Southern and Central France in 2003 and 2004. The Mont Boron-Valbonne strain is a combination of two laboratory strains (Mont Boron and Valbonne) that were merged in 2004 to prevent extinction. Strains were kept in plexiglass population cages and females were allowed to parasitize once a week on *Ephestia kuehniella* (Lepidoptera) larvae. Parasitised larvae were reared at 25°C for four weeks until the adult wasp emerged. *E. kuehniella* was reared in the lab from eggs obtained from Koppert B.V., The Netherlands.

Crosses and mapping population

Crosses between males and females from different strains were established to maximize the number of informative markers per cross as indicated in Figure 4.1. To increase the chance of interaction, 10 females from one population were put together in a plexiglass cage with 20 males of a different population. When a

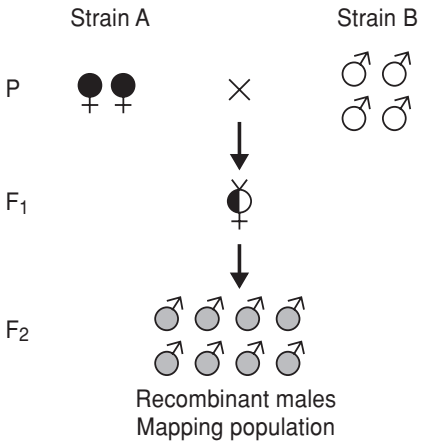

Figure 4.1. Breeding scheme used to produce the male mapping populations. The mothers (black) and fathers (white) originated from different strains with the purpose of increasing the number of informative markers. F_1 virgin females (black/white) produced the F_2 recombinant males for the mapping population (grey).

couple was observed mating, it was secluded from the cage and kept together another 24 hours, after which, the male was frozen at -80°C for genetic analysis. The female was provided with host larvae for three periods of 24 hours to obtain as many offspring as possible. The female was then also frozen for further analysis. Before emergence, F_1 generation pupae were individually separated for collecting virgin females. F_1 virgin females were individually hosted by the same regime as their mothers to produce the haploid F_2 males for mapping and stored at -80°C. Our mapping population consisted of 93 males that originated from two crosses (Antibes x Mont Boron-Valbonne; Valence x Mont Boron-Valbonne). Genotyping of parental and F_1 females was done first and when the F_1 females showed heterozygosity for several markers, we proceeded to score their F_2 for these markers.

DNA extraction and marker analysis

DNA was extracted from the abdomen of the wasps using a standard proteinase K/salt-chloroform protocol. Presence and quality of DNA was checked on a 1% agarose gel. We used a set of 18 microsatellites developed by Butcher *et al.* and published in GenBank that are listed in Table 4.2A plus 36 polymorphic microsatellite markers (Table 4.2B) previously described in Chapter III of this thesis. In addition, we used six pair of selective AFLP primers which produced a set of 78 markers. In addition, the locus *VLP*-p40 (Hellers *et al.*, 1996) was amplified.

PCR reactions for microsatellites were performed in 1X PCR buffer (Promega) with 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Roche), 0.2 μM each primer, 0.4 units of Taq

Table 4.2A. Microsatellite loci for the parasitic wasp *Venturia canescens* (submitted to GenBank by Butcher *et al.* in 2006). Locus name designates the clone number in GenBank; Size range: locus allele range (bp); Repeat motif: repeat comprised between the primers; Tm: annealing temperature (°C); Primer sequences: forward and reverse 5′-3′ primer sequence.

Locus	Accession No.	Size (bp)	Repeat Motif	Tm (°C)	Primer sequence (5′ – 3′)
Vcan001	DQ649235.1	170	(GT)35	58	F: GCAGCTGTATTAGAGTGGTTTGAGG
				58	R: GATTCCGACCGATGTAGTTAGCCTAA
Vcan002	DQ649236.1	112-116	(TG)33	56	F: GAGTTTTCATTCGCTCTCTTTCGC
				55	R: TCCCGAGGTTCGTAAATACAGTG
Vcan003	DQ649237.1	147-151	(GT)63	66	F: TCGCTGGGCGGGTTTAGAGCAACTTTGGC
				60	R: GGCTATTTAGGAGTTCACATTAGGACAGG
Vcan004*†	DQ649238.1	127-153	(GT)30	69	F: AAGCCCCTCTGGCATCCTCCCACGGAGC
				64	R: GGCTCCGGCTGTCACGTAACGTTTGGAT
Vcan005	DQ649239.1	150	(GT)46	54	F: CAGTAAGTTTTCTCGTCTTGTTGATATT
				54	R: CTTAATACAGAATTATTCCGAGCTTAAC
Vcan007	DQ649241.1	137	(CA)17	67	F: CTCACGTGCAGGGCGGGCAACGGTG
				62	R: CCCGAGTATTCACGTCCCCGGTTTG
Vcan009*†	DQ649243.1	087-089	(GT)24	56	F: TAACCATACGCGCATCCACGTTTAT
				49	R: GTACGTAAATATATAATGAGTACAC
Vcan014	DQ649248.1	113	(AC)15	66	F: GGGCAGGACTGCCAGCTCGCCCT
				58	R: CCGTTGTGGAAACGCAAAATTTCATCC
Vcan015	DQ649249.1	117-119	(TG)15	59	F: CCCTCTATAGGTTCAACCTTATACTGTAC
				59	R: GCACCGTCGATAAGATCAGACTACTG
Vcan017	DQ649251.1	142	(GT)30	59	F: GGGAGGAGAACAATTGGACCCTGA
				62	R: GATCCAGACTCCACGGCGCTGG
Vcan023*†	DQ649257.1	136-146	(CAG)10	59	F: CGTTTAACGATAGTGCTCGCTACCC
				62	R: CGACGCCGCAGTCTCAACGTTTTCC
Vcan028	DQ649262.1	116	(CTT)9	59	F: ATCGACCATTGGACTTTCTATCTTCTCAG
				57	R: TAAACCATGCCAATAAGCGAAGACGAAT
Vcan029a	DQ649263.1	122	(AGA)8	60	F: CTTTACACTGGATGCNCTCCGGTC
				60	R: TCGTCTTGGCGTTAAGTCGAATTTCG
Vcan029b†	DQ649263.1	136-146	(AGA)8	59	F: ACACAACGGGCTTTACACTGGATGC
				56	R: AGCATCGAGGACATCGAAATAAGC
Vcan032*	DQ649266.1	120-124	(GT)30	59	F: CCAGGAATGAAATGACGCAATTAATTAGCG
				61	R: GGAGGTCCTAACGGATGAGACCATCT
Vcan033	DQ649267.1	162	(CCA)17	53	F: GGCCAGGAAAATGTTATACTCTG
				60	R: GTTTGCGSTTAACCGGTGGCGG
Vcan042	DQ649276.1	142	(AC)40	57	F: CSATCGCTCTBAGATAACACACAC
				54	R: CGATTTCAGAAGCAAAAATTCTCATTC
Vcan044*	DQ649278.1	108-114	(TC)10	53	F: CTCTTTGCCCATCCCTTCC
				53	R: CTTCTTCTTGAAAAYAATGAGGG

* indicates markers that are mapped; † indicate markers that were used for csd-locus mapping.

polymerase (Promega) and approximately 5 ng of template DNA. The PCR profile was 1 cycle of 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 30 sec at the specific annealing temperature for each primer pair, 1 min at 72°C and a final cycle of 7 min at 72°C.

Table 4.2B. Polymorphic microsatellite loci for the parasitic wasp *Venturia canescens* developed in Chapter III of this thesis. Locus numbers follow on those of Butcher *et al*. Size range: locus allele range (bp); Repeat motif: repeat comprised between the primers; Tm: annealing temperature (°C); Primer sequences: forward and reverse 5′-3′ primer sequence.

Locus	Accession No.	Size (bp)	Repeat Motif	Tm (°C)	Primer sequence (5′ – 3′)
Vcan060*	GU053668	244-253	(GA)9	58	F: GGAGTTACTGAAGGCAAACAAGG
					R: AATGACTGAACGGGACTCGAT
Vcan061	GU053669	186-200	(CT)13	55	F: TACGGTACTGAGAGATGTGTGGA
					R: TCGGTTAGAAGTGAGACCTGAAC
Vcan062*	GU053670	250-266	(CT)8	55	F: TGACTCCCTATGCACTTCTTCTC
					R: GAAGGTGTCGAGATATGGTGAAC
Vcan063*	GU053671	178-186	(AG)39	55	F: CGTATACTCACGCACACACAAAG
					R: CTTGACGATATGGGTTGATGG
Vcan064*	GU053672	277-289	Complex‡	55	F: GTTGCTAACTTCGAGGACAGACT
					R: AGTAGTAACGTTCGATGGCAGAG
Vcan065*†	GU053673	209-233	(TGC)15	55	F: TCATTGTCACTGGTCGGTGT
					R: GATCATAGGCAACAGCAGCA
Vcan066*†	GU053674	238-252	(CTTT)11	55	F: GATACCAGACTCGAGATCTATTCAA
					R: CGACCGACAATCAAGGTTTT
Vcan067*	GU053675	138-157	(CAA)26	55	F: ATGGTTCAGCAGCAACATCA
					R: GTTCCCTTGTAAAGCGGATG
Vcan068	GU053676	141-177	(TTCG)21	58	F: TCCCGACTTTCTCACTCCTC
					R: AGGAAGGAAGGAACGAAGGA
Vcan069*	GU053677	200-226	(AAC)45	55	F: GAATGAGGATCAGCAAAATCG
					R: GATGGCAGAGCAACTCGTTT
Vcan070*	GU053678	221-230	(GCT)10	55	F: TGCTCGCCCTTTTCTTTATT
					R: CATCTGCCACGACTCTCAAG
Vcan071*†	GU053679	222-252	(CAA)11	55	F: CTCCTACGCACTCCCTTCAC
					R: TTGTACGTTGGCACTTGAGC
Vcan072	GU053680	197-203	(GAC)14	55	F: TGAATTTGTCGTTGCTGCTC
					R: CGAGGAAGTTCAGGCTCAAG
Vcan073*†	GU053681	206-260	(TGT)15	60	F: GGTCCAACGGTACTTCCTGA
					R: ACTTCCGTCAGCCCTACCTT
Vcan078*	GU053686	168-171	(ATT)5	61	F: AGGTGATGTTAGGCGGTTTG
					R: TTTTCGCGGGTTTTGTTTAC
Vcan079†	GU053687	146-160	(GA)15	61	F: AGGAACGCAAAATGAAATGG
					R: TCGTTCGAACTTTTCCCCTA
Vcan083*	GU053691	241-250	(TGA)11	60	F: ATAGCTCATCGCTCCTCTGC
					R: CGCCCATCTTGTGCTTATGT
Vcan085	GU053693	146-200	(TTC)8	53	F: AGGTTCAATGGCTTTGCTGT
					R: GCTTTCCGAGCTTTTCCTCT
Vcan088*	GU053696	132-144	(CA)44	60	F: AGTAACCGGTCAGCCTTTGG
					R: CACGTTCCAATTTCCACACA
Vcan091*	GU053699	154-186	(GA)32	60	F: GTAGGCACGTACCGAGGAAA
					R: TCCACGCTCGTGTGTGTACT
Vcan092*	GU053700	208-220	(TTCC)6	60	F: CGTTCGTTCTTTCGTTCGTT
					R: CGGCATTGTCCTTCTTGTTT
Vcan094	GU053702	154-158	(CT)26	60	F: ACGATCGCTCAATCGAAGTT
					R: CTCCCATAAACTCGGAGCAA
Vcan095†	GU053703	080-096	(CA)24	50	F: GTAATCATTTTCGCTCCGTGA
					R: TCGTTTCTCTTTTCGTTCGAG

Table 4.2B. Continued

Locus	Accession No.	Size (bp)	Repeat Motif	Tm (°C)	Primer sequence (5′ – 3′)
Vcan096*†	GU053704	172-178	(AG)39	55	F: CTCACGCACACACAAAGTCC R: TGCTTGACGATATGGGTTGA
Vcan097*†	GU053705	140-152	(GA)15	55	F: AATGGAGACAACGAGGCAAC R: ATCAGAGTCGACCCAGCAAC
Vcan099*	GU053707	126-138	(AG)12	55	F: TGGCCATAACAGGGAGAAAG R: GTCACTGGGGAAGAGTGGTG
Vcan102*	GU053710	161-177	(TC)14	60	F: TTCCAATTCACGAATCAACG R: CCTCTGAGTCACCGAAAAGC
Vcan103	GU053711	154	(TC)10	48	F: CTCAAGCTATGCATCCAACG R: TCTCGGAGTCAATCCCACTC
Vcan104*	GU053712	164	(GA)14	55	F: CAAAAGGGAGGGAAAGGAAG R: CCCACGTTTTCGGTGTACTT
Vcan105	GU053713	156	(TC)15	48	F: TGGGCAATTACCCCACTAAA R: GCACGTGCAATTCTGATGAC
Vcan106*†	GU053714	186-224	(TC)24	60	F: CCTCATCTCGAGGGAGGATT R: ATCGCGAGTTGCGTAGTTTC
Vcan109*	GU053717	188-194	(AG)10	60	F: TTAATTGAACGGGGAAAACG R: GCAGTCGGTGTAGCGTGTTA
Vcan110	GU053718	173-175	(AC)25	55	F: CCATTCATTCGGATCTCACC R: CCGACGTTTGTATCTTCGTTC
Vcan112*†	GU053720	143-178	(TC)15	55	F: GCAGAGATTTTTGCCACAGG R: TGGCTGGATGAAGGGATATT
Vcan114†	GU053722	237-251	(AG)10	55	F: AAAAATGAACGACAGAAGGA R: GTTGCGCTCTTTGTGAATA
Vcan115	GU053723	096-124	(CT)11	55	F: TTTTTCACTCTTCGTTCCTC R: TGCTACCCTCTTGATCTCC

‡ (CAA) CAG (CAA)8 CAG (CAA) CAG (CAA)4 CAG CAT (CAA)4 CCA (CAA)2 (CAG)4 (CAA) CAG (CAA)2 CAC (CAG)2 CAA CAG (CAA)3 CCA.
* indicates markers that are mapped; † indicate markers that were used for csd-locus mapping.

For AFLP analysis (Vos *et al.*, 1995) 125 ng genomic DNA was double-digested with *EcoRI* and *MseI*. Adaptors (Table 4.3) were ligated to the resulting DNA fragments, generating template DNA for pre-amplification. Pre-amplification reactions were carried out in 11.25 µl AFLP core mix (Applied Biosystems) and 19 µM of the *EcoRI* primer and *MseI* primer (Table 4.3). The PCR profile was as follows: first 1 cycle of 120 s at 72°C, 19 cycles of 5 s at 94°C, 30 s at 56°C and 120 s at 72°C and a final step of 4°C. The primers used in the selective-amplification contained three additional nucleotides at the 3′ end (Table 4.3); the *EcoRI* primers were labeled with a fluorescent dye (FAM, HEX or NED). A 1:10 or a 1:100 dilution of the pre-amplification products served as template for the selective-amplification step. Reactions

were carried out in 1X PCR buffer (Promega), 0.2 mM dNTPs (Roche), 1 mM $MgCl_2$, 10 or 1 µM *EcoRI* + NNN labeled primer, 20 or 17 µM *MseI* + CNN primer and 0.4 units of Taq polymerase (Promega). The PCR profile was eight cycles of 5 s at 94°C, 30 s at 65°C and 120 s at 72°C (in each cycle the annealing temperature was lowered by 1°C), twenty-four cycles of 5 s at 94°C, 30 s at 56°C and 120 s at 72°C, elongation at 72°C for 10 min and a final step at 4°C.

All microsatellite and AFLP reactions were carried out in a GeneAmp 9700 PCR machine (Applied Biosystems) and PCR products were visualized with an ABI 377 automated DNA sequencer (Applied Biosystems). Fragment sizes were determined using GeneScan computer software provided by the manufacturer and GenoGrapher version1.6.0 (J.J. Benham, Montana State University, 1999) for the AFLPs.

Linkage analysis and map construction

For the detection of linkage and map building we used software MultiPoint (http://www.multiqtl.com), which infers marker order and map distances using a multipoint likelihood approach (Mester *et al.*, 2003a, 2003 b, 2004). All markers were tested for significant deviation from Mendelian segregation by χ^2 analysis ($p < 0.01$). Markers that deviated significantly from Mendelian expectations were

Table 4.3. Adaptors and primers used for the production of AFLP markers.

Adaptors	Sequence
EcoRI	*EcoRI*-ad1 5'-CTCGTAGACTGCGTACC-3'
	EcoRI-ad2 3'-CATCTGACGCATGGTTAA-5'
MseI	*MseI*-ad1 5'-GACGATGAGTCCTGAG-3'
	MseI-ad2 3'-TACTCAGGACTCAT-5'
Preamplification primers	Sequence (5′–3′)
EcoRI	GACTGCGTACCAATTCCN*
MseI	GATGAGTCCTGAGTAAC
Selective primer combinations	Sequence (5′–3′)
BC	B: Eco-AAG: GACTGCGTACCAATTC**AAG**
	C: Mse-CAG: GATGAGTCCTGAGTAA**CAG**
CB	C: Eco-ACA: GACTGCGTACCAATTC**ACA**
	B: Mse-CAC: GATGAGTCCTGAGTAA**CAC**
ED	E: Eco-ACG: GACTGCGTACCAATTC**ACG**
	D: Mse-CAT: GATGAGTCCTGAGTAA**CAT**
FA	F: Eco-ACT: GACTGCGTACCAATTC**ACT**
	A: Mse-CAA: GATGAGTCCTGAGTAA**CAA**
AB	A: Eco-AAC: GACTGCGTACCAATTC**AAC**
	B: Mse-CAC: GATGAGTCCTGAGTAA**CAC**
CA	C: Eco-ACA: GACTGCGTACCAATTC**ACA**
	A: Mse-CAA: GATGAGTCCTGAGTAA**CAA**

* N = A or T

included in linkage groups if their presence did not alter the order established without them. The assignment of markers to linkage groups was done by clustering markers having LOD scores ≥ 3. The Kosambi mapping function that incorporates the possibility of crossover interference was used to convert recombination frequencies into map distances (Kosambi, 1944).

To estimate the genetic size of *V. canescens* we followed the approach by Fishman *et al.* (2001) where G_{e1} is calculated by adding two time *s* (average marker spacing). This method accounts for chromosome ends outside the terminal marker. We also estimated the genetic size of *V. canescens* by the method used in Chakravarti *et al.* (1991) G_{e2} by multiplying the length of each linkage group by [(m+1)/(m-1)], where *m* is the number of markers of each linkage group. The final genetic size (G*e*) was the average of G_{e1} and G_{e2}.

Csd locus mapping

As for the mapping crosses, males and females from two different populations were put together in a cage and allowed to mate. Mating couples were separated form the rest and females allowed to lay eggs for three periods of 24 hours. F_1 pupae were isolated before emergence and when adult offspring emerged brother-sister crosses were established. Brother-sister crosses (BS or F_1) (Figure 4.2) will either share one sex allele and produce a sex ratio in the F_2 higher than 0.5, indicative of diploid males among the offspring (matched cross) or have no sex allele in common and produce a F_2 sex ratio of ~0.5, indicative of no diploid male production (unmatched cross). F_2 males from the two matched crosses with most offspring (BS1 and BS2) were screened with flow cytometry to determine their ploidy level, as indicated for the physical genome size estimation further in this chapter. A segregation analysis using 15 polymorphic microsatellites (see Tables 4.2A and 4.2B) was carried out for diploid females and diploid males. A microsatellite locus linked to the *csd* locus will be more often heterozygous in diploid females than in

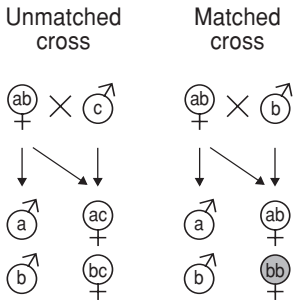

Figure 4.2. Schematic sex allele distribution of a brother-sister cross. Brother-sister crosses will either have no sex allele in common and produce haploid males and diploid females (unmatched cross) or share one sex allele and produce haploid males, diploid females and diploid males (matched cross). Diploid males are indicated in grey.

diploid males. For each marker we compare the expected and observed number of homozygotes under Mendelian segregation (no linkage) among diploid males using Fisher exact test.

***VLP* mapping**

The *VLP*-p40 gene has two alleles that differ in a 54 bp insert: *VLP*+ and *VLP*- which can be easily scored in an agarose gel. For mapping the *VLP* gene we used the haploid male mapping population. Parents and F1 females were genotyped and when they showed polymorphism for this gene F2 males were further screened. For amplification of the *VLP*-p40 gene we used specific primers (VLPF 5'-CTCAATATGTGGGGTGGTGG-3' and VLPR 5'-TCGCAGTGGCTTGTCA-GAGT-3') (Hellers *et al.*, 1996). PCR reactions for *VLP* were performed in 0.4X PCR buffer magnesium free (Promega) with 1 mM $MgCl_2$, 0.08 mM dNTPs (Roche), 0.2 pmol/ml of each primer, 0.4 units of Taq polymerase (Promega) and approximately 5 ng of template DNA. The PCR profile was 1 cycle of 2 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final cycle of 10 min at 72°C. PCR products were checked in a 1.5 % agarose gel.

Physical genome size estimation

The diploid physical genome size of *V. canescens* was determined by standard flow cytometry. Adult female wasp heads were homogenized in Galbraith buffer (21mM $MgCl_2$, 30 mM tri-Na citrate dihydrate, 20mM MOPS, 0.1% Triton X-100, 1mg/l RNase, pH 7.2), filtered (50µm), stained overnight in propidium iodide (Sigma, St. Louis, Missouri, USA) and loaded on a LFRII flow cytometer (BD BioSciences, Franklin Lakes, New Jersey, USA). As size standard we used whole body cells of *Drosophila* and the hymenopteran *Nasonia vitripennis* whose genome sizes are known to be 157 and 312 Mb respectively (Wilfert *et al.*, 2007a).

Results

A total of 54 microsatellites were tested on the parental and F_1 generations to screen for polymorphism. Three microsatellites failed to amplify, 15 were not polymorphic and 36 were polymorphic in at least one of the two mapping families. These were further used for the F_2 male mapping population. In addition, 78 AFLP fragments could be reliably scored from six primer pair combinations (Table 4.3).

We mapped 29 microsatellites and 19 AFLPs fragments in addition to the nuclear loci *VLP* and *csd* onto11 linkage groups (Figure 4.3). The map covers 1040 cM with linkage groups ranging in length from 27.5 to 336 cM, with an average of four markers per linkage group (ranging from 2 to 13 markers) and an average marker spacing of 21.6 cM (with a range from 2 to 39 cM). Linkage group I was the largest group with 13 markers, whereas linkage group II had six marker plus the

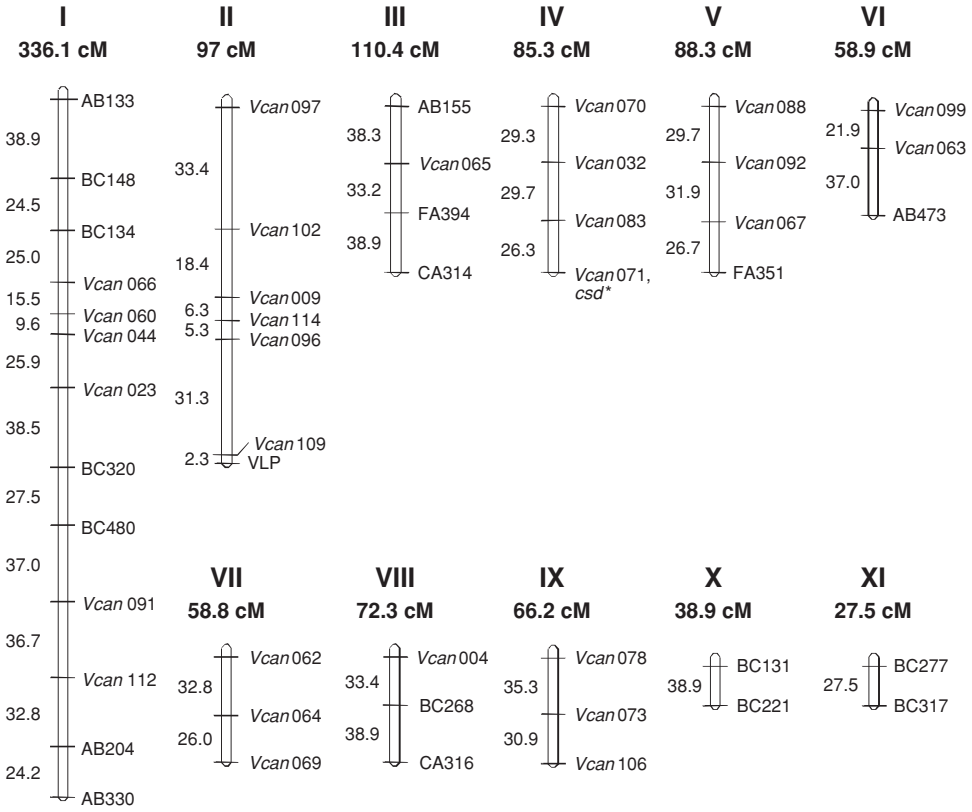

Figure 4.3. Linkage map of *Venturia canescens* based on microsatellites and AFLP markers. Markers are indicated on the right; map distances (in Kosambi cM) on the left of each linkage group. AFLP markers are indicated by two letters that indicate pair of selective primers followed by the size of the amplified fragment in base pairs. The *VLP* locus is located at the tip of linkage group II and the *csd* locus at the tip of chromosome IV.

VLP locus, linkage group IV had five markers plus the *csd* locus, linkage groups III and V had four markers, linkage groups VI, VII and VIII had three markers and linkage groups X and XI had two markers.

The estimated map length was 1654 cM, which is the average of the two methods (see Materials and Methods), respectively 1602 and 1706 cM. The map covers about 60% of the genome, calculated as the observed length of 1040 cM divided by the estimated length of 1654 cM which indicates that the map is not saturated and more markers are needed to get a better resolution. The average recombination density in *V. canescens* is 6 cM/Mb (estimated map length divided by average genome size) which is comparable to other Hymenopterans which do not have exceptionally high recombination rates (Wilfert *et al.*, 2007a), but it is higher than the 1.4-1.5 cM/Mb recently reported for *N. vitripennis* (Niehuis *et al.*, in press)..

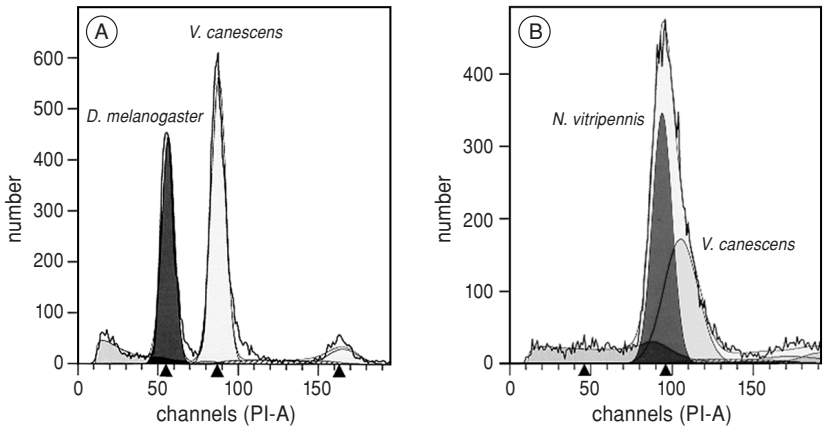

Figure 4.4. Flow cytometry graphs comparing genome sizes of (A) *Drosophila* and *V. canescens* (B) *N. vitripennis* and *V. canescens*. Cell number is indicated on the left. PI-A indicates propidium iodide area as a measure for DNA content.

In the study of localization of the complementary sex determining locus, families BS1 and BS2 produced 37 and 42 individuals in the F_2 (sex ratio of 0.73 and 0.83 respectively). Sixty-two males were checked with flow cytometry from which 7 males in BS1 were diploid and 13 in BS2. Genotyping data of F_2 females and diploid males with 15 polymorphic microsatellites is shown in Appendix I. Microsatellite *Vcan*029b showed significant linkage with the *csd* locus in family BS1 ($p = 0.03$) and *Vcan*071 in family BS2 ($p = 0.003$). Marker *Vcan*029b shows a strange segregation pattern: only one combination of the two possible appears in the F_2. This can be indicative of a segregation distortion or a genotyping mistake (e.g. P female is homozygous 149/149 and not heterozygous 149/161, see Appendix I). Furthermore, when the offspring of both families is pooled, only *Vcan*071 shows significance, indicating linkage with *csd*. The recombination frequency between *Vcan*071 and *csd* is 6% (only one heterozygous diploid individual out of 17). A 6 cM interval separates *Vcan*071 from *csd*, but we can not determine the relative position of *csd* in reference to *Vcan*071.

V. canescens head cells had 1.56 times more DNA than *Drosophila* cells (Figure 4.4A) and 1.12 times less DNA than *N. vitripennis* cells (Figure 4.4B). The physical size of the genome of *V. canescens* was calculated to be between 245 Mb (157 Mb x 1.56 = 244.9 Mb) and 279 Mb (312 Mb / 1.12 = 278.6 Mb).

Discussion

This is the first linkage map constructed for the parasitic wasp *Venturia canescens*. The map comprises 48 markers (29 microsatellites and 19 AFLP fragments) and

two nuclear loci. Fifty-four percent of the microsatellites (N = 54) mapped, whereas only a 24 % of the AFLPs markers (N = 78) could be reliably mapped. This indicates that although microsatellites are costly to produce, they are more robust and reliable markers for mapping purposes than AFLPs. This map spans 1040 cM which is much smaller than the linkage map of honey bee (4115 cM) but larger than the map of *Nasonia vitripennis* (765 cM), and similar to other hymenopteran species, e.g. *Trichogramma brassica* (1330 cM; Wilfert *et al.*, 2007a). The physical genome size of *V. canescens* was estimated to be between 245 - 279 Mb which is slightly smaller than the genome of *N. vitripennis* (312 Mb), but as large as the genomes of Trichogramma (246 Mb) and *A. mellifera* (262 Mb). The recombination rate in *V. canescens* is estimated to be 6 cM/Mb, which is not as high as in *A. mellifera*, which appears to have an exceptional high recombination rate (see Gadau *et al.*, 2001).

We have mapped the *csd* locus in *V. canescens*. Our analysis indicates that *csd* is linked to marker *Vcan*071 on linkage group IV. The sex locus has been mapped in other Hymenoptera such as *Bombus terrestris* (Gadau *et al.*, 2001), *Bracon hebetor* (Antolin *et al.*, 1996) and *Bracon* sp. near *hebetor* (Holloway *et al.*, 2000). In the honey bee *csd* has not only been mapped (Hunt & Page, 1995) and physically located on chromosome VIII (Beye & Moritz, 1996), but it has also been isolated and characterized. Its molecular mechanism is currently being studied (Beye *et al.*, 2003; Beye, 2004).

The CSD trait depends on heterozygosity for femaleness. Under automictic central fusion thelytoky loci distant from the centromere will become homozygous with time (Speicher *et al.*, 1965; Crozier, 1971; Beukeboom & Pijnacker, 2000). CSD is therefore only compatible with thelytoky if the *csd* locus is located in a region were heterozygosity is maintained e.g. close to the centromere or in a region with low recombination. The results presented in this study locate *csd* at the tip of chromosome IV which is inconsistent with the cytogenetic predictions. It is possible that there is an inversion in this region of the chromosome with low recombination. A more likely explanation is that the map presented here is not saturated and both *Vcan*071 and *csd* are actually closer to the centromere than shown in Figure 4.3. Further fine scale mapping of chromosome IV is needed to resolve this issue. In a genetic diversity study carried out in Chapter V of this thesis, several arrhenotokous and thelytokous females, from different field populations were genotyped with 15 microsatellites; one of these markers was *Vcan*071. Eight out of 10 arrhenotokous and 2 out of 3 thelytokous females were homozygous for *Vcan*071 (*Ho* = 0.8 and 0.7 respectively). Despite the low number of individuals tested, this indicates that the level of homozygosity for *Vcan*071 (but not *csd*) is quite high in natural populations. The high number of homozygous females for *Vcan*071 confirms that this marker and the *csd* locus are not tightly linked and that there is no linkage disequilibrium between the *Vcan*071 and *csd* alleles.

This map contains the nuclear locus *VLP* which is located at the tip of linkage group II. We expect arrhenotokous populations to be more heterozygous for *VLP*

than thelytokous populations. As genome homozygosation occurs in thelytokous wasps, a distal *VLP* locus will become homozygous quite rapidly whereas homozygosation is prevented in arrhenotokous populations by fertilization and recombination. We have tested these predictions with the data collected by Malmberg *et al.* (2000). They collected arrhenotokous and thelytokous *V. canescens* females from 25 different locations in the Côte d'Azur (France) in 1997 and 1998. After determining the reproductive mode of the newly emerged wasps, they scored 102 arrhenotokous and 19 thelytokous females for *VLP*. No heterozygous thelytokous females were found, whereas in arrhenotokous populations heterozygotes were common (35 individuals) and the distribution of the genotypes was dependent on the reproductive mode (χ^2 = 18.97, *df* = 2, *P* = 7.6E-05). These observations are in concordance with the position of *VLP* at the tip of linkage group II.

This map provides a starting point for more detailed genetic studies in *V. canescens*, such as for future QTL mapping of behavioural traits. Life history trait differences between arrhenotokous and thelytokous populations have been well studied in the *V. canescens* system. For example, arrhenotokous and thelytokous females have different foraging behaviour strategies (Desouhant *et al.*, 2005), they allocate energy in different ways (Pelosse *et al.*, 2007) and different oviposition strategies have been observed between the two reproductive modes (Amat *et al.*, 2003). Crosses between arrhenotokous males and thelytokous females would allow for studying the genetic basis of these traits, as has been done for thelytokous reproduction in *A. mellifera* (Lattorff *et al.*, 2005, 2007). Although genetic exchange between arrhenotokous males and thelytokous females has been reported, we were not able to repeat these results of Schneider *et al.* (2003). In our crosses, thelytokous females mated with arrhenotokous males and males transferred sperm, but females did not lay eggs that contained the paternal genotype (Mateo Leach, unpublished data). The most likely explanation is that gene exchange between arrhenotokous males and thelytokous females is very rare, as Schneider *et al.* (2003) used one specific laboratory strain.

As we cannot use thelytokous females for mapping purposes, further studies should focus on arrhenotokous populations with different life history traits. Alternative methods such as generating arrhenotokous selection lines or artificial insemination of thelytokous females with arrhenotokous sperm will help to map traits of interest in *V. canescens*.

Acknowledgements

We thank Rense Veenstra for valuable help with the AFLP amplification and Carlos Bernstein for providing some of the wasp strains. We also thank Barbara Feldmeyer for useful discussion during data analysis.

Appendix IA. Genotyping data for the BS1 family with 15 polymorphic microsatellites.

BS 1	*Vcan*114	*Vcan*004	*Vcan*066	*Vcan*071	*Vcan*073	*Vcan*112	*Vcan*096	*Vcan*009	*Vcan*095	*Vcan*065	*Vcan*029b	*Vcan*097	*Vcan*106	*Vcan*023	*Vcan*079
F_1 female	241247	144146	239252	231234	228250	147151	172176	7687	8096	209218	149161	140142	208214	144144	155155
F_1 male	241	171	239	234	250	144	176	87	80	215	149	142	214	144	158
female 01	241247	146171	239252	234234	228250	144151	0	7687	8096	0	149149	142142	214214	144144	155158
female 12	241241	144171	239252	234234	228250	144151	176176	8787	8080	0	149149	140142	214214	144144	155158
female 17	241241	144171	239239	231234	250250	144151	176176	7687	8080	215218	149149	142142	214214	144144	155158
female 25	241247	144171	239252	234234	228250	144151	172176	0	8080	0	149149	140142	214214	144144	155158
female 26	241247	144171	239252	234234	250250	144151	172176	7687	8096	0	149149	142142	214214	144144	155158
female 32	241247	146171	239252	234234	228250	144151	172176	8787	8096	0	149149	142142	214214	144144	155158
female 33	241241	0	239252	231234	228250	0	0	0	0	209218	0	0	0	0	0
female 34	241241	144171	239252	234234	0	144151	176176	7687	0	209215	149149	142142	0	144144	155158
female 35	241241	144171	239239	231234	228250	144151	176176	8787	8096	215218	149149	140142	208214	144144	155158
female 36	241241	146171	239239	234234	228250	0	0	0	0	0	149149	142142	214214	144144	155158
2n male 02	241247	146171	0	231234	228250	0	172176	7687	8080	0	149149	140142	0	144144	155158
2n male 03	0	146171	239239	0	0	144151	172176	0	8080	0	149149	140142	214214	144144	155158
2n male 06	241241	146171	0	231234	250250	144151	0	0	8096	0	149149	0	0	144144	155158
2n male 07	241241	146171	239239	231234	0	144151	176176	8787	8080	0	149149	142142	214214	144144	155158
2n male 09	241247	144171	239239	0	228250	144151	0	7687	8096	209215	149149	140142	208214	144144	155158
2n male 11	241241	146171	239252	234234	250250	144151	176176	8787	8096	0	149149	0	0	0	0
2n male 15	241247	146171	239252	0	250250	144151	172176	0	8080	209215	149149	142142	214214	144144	155158

Appendix IB. Statistical comparison of observed and expected homozygous diploid males in family BS1 using Fisher exact test.

BS1 (17)	*Vcan*114 female x male		*Vcan*004 female x male		*Vcan*066 female x male		*Vcan*071 female x male		*Vcan*073 female x male	
P	241247	241	144146	171	239252	239	231234	234	228250	250
F1	241247	241241	144171	146171	239252	239239	231234	234234	228250	250250
Obs 2n female	4	6	6	3	7	3	3	7	7	2
Obs 2n male	3	3	1	6	2	3	3	1	2	4
Fisher *p*	0.23		0.05		0.24		0.16		0.11	

BS1 (17)	*Vcan*112 female x male		*Vcan*096 female x male		*Vcan*009 female x male		*Vcan*095 female x male		*Vcan*065 female x male	
P	147151	144	172176	176	7687	87	8096	80	209218	215
F1	147144	144151	172176	176176	7687	8787	8096	8080	209215	215218
Obs 2n female	0	8	3	4	4	3	4	3	1	2
Obs 2n male	0	6	3	2	2	2	3	4	2	0
Fisher *p*	1.00		0.38		0.30		0.20		1.00	

BS1 (17)	*Vcan*029b female x male		*Vcan*97 female x male		*Vcan*106 female x male		*Vcan*023 female x male		*Vcan*079 female x male	
P	149161	149	140142	142	208214	214	144144	144	155155	158
F1	149161	149149	140142	142142	208214	214214	0	144144	155158	0
Obs 2n female	0	9	3	6	1	7	0	9	9	0
Obs 2n male	0	7	3	2	1	3	0	6	6	0
Fisher *p*	0.03	*	0.25		0.24		1.00		1.00	

* Significant result.

Appendix IIA. Genotyping data for the BS2 family with 15 polymorphic microsatellites.

BS 2	*Vcan*114	*Vcan*004	*Vcan*066	*Vcan*071	*Vcan*073	*Vcan*112	*Vcan*096	*Vcan*009	*Vcan*095	*Vcan*065	*Vcan*029b	*Vcan*097	*Vcan*106	*Vcan*023	*Vcan*079
F_1 female	241247	144146	239252	231234	228234	151151	172180	7676	8296	209218	149149	140142	214214	128144	155155
F_1 male	241	171	239	234	250	144	176	87	82	215	149	142	214	144	158
female 01	241241	146171	239252	234234	228250	144151	176180	7687	8080	0	149149	140142	214214	144144	155158
female 14	241241	146171	239239	231234	228250	144151	0	7687	8080	215218	149149	142142	214214	144144	155158
female 28	241247	144171	239252	0	228250	144151	172176	7687	8096	0	149149	142142	214214	144144	155158
female 29	241241	146171	239252	231234	228250	144151	176180	7687	8096	0	149149	142142	214214	144144	155158
female 30	241241	146171	239252	231234	228250	144151	176180	7687	8080	0	149149	142142	0	144144	155158
2n male 02	241247	144171	239239	234234	228228	144151	172176	7687	8096	209215	149149	140142	214214	144144	155158
2n male 06	241247	146171	239252	234234	234250	144151	176176	7687	8080	0	149149	0	214214	144144	155158
2n male 08	241247	144171	239239	234234	228250	144151	0	7687	8080	0	149149	140142	214214	144144	155158
2n male 12	241247	146171	239239	234234	250250	144151	172176	0	8080	0	149149	0	214214	144144	155158
2n male 13	241247	144171	239252	234234	234250	144151	0	0	8096	209215	149149	140142	214214	144144	155158
2n male 15	241241	146171	239252	234234	228250	144151	176180	0	8096	0	149149	142142	214214	128144	155158
2n male 17	241241	146171	0	234234	228250	144151	0	0	8080	0	149149	0	0	144144	155158
2n male 21a	241247	144171	239252	234234	228250	144151	176180	7687	8096	0	149149	140142	214214	128144	155158
2n male 21b	241241	146171	239239	234234	234250	144151	176180	7687	8096	215218	149149	142142	214214	144144	155158
2n male 23	241241	146171	0	234234	228250	0	0	0	0	0	149149	0	0	0	158158
2n male 25	241241	146171	239239	234234	228250	144151	176180	7687	8080	0	149149	140142	214214	128144	155158
2n male 26	241241	144171	239252	234234	228250	144151	176180	7687	8096	209215	149149	140142	214214	128144	155158
2n male 28	241241	146171	239239	234234	228250	144151	176180	7687	8096	0	149149	142142	214214	128144	155158

Appendix IIB. Statistical comparison of observed and expected homozygous diploid males in family BS2 using Fisher exact test.

BS2 (18)	*Vcan*114 female x male		*Vcan*004 female x male		*Vcan*066 female x male		*Vcan*071 female x male		*Vcan*073 female x male	
P	241247	241	144146	171	239252	239	231234	234	228234	250
F1	241247	241241	144171	146171	239252	239239	231234	234234	228250	234250
Obs 2n female	1	4	1	4	4	1	3	1	5	0
Obs 2n male	6	7	5	8	5	6	0	13	8	3
Fisher *p*	0.15		1.00		0.17		0.003	*	1.00	

BS2 (18)	*Vcan*112 female x male		*Vcan*096 female x male		*Vcan*009 female x male		*Vcan*095 female x male		*Vcan*065 female x male	
P	151151	144	172180	176	7676	87	8096	80	209218	215
F1	144151	-	172176	176180	7687	-	8096	8080	209215	215218
Obs 2n female	5	0	1	3	5	0	2	3	0	1
Obs 2n male	12	0	2	6	8	0	7	5	3	1
Fisher *p*	1.00		1.00		1.00		0.15		1.00	

BS2 (18)	*Vcan*029b female x male		*Vcan*97 female x male		*Vcan*106 female x male		*Vcan*023 female x male		*Vcan*079 female x male	
P	149149	149	140142	142	214214	214	128144	144	155155	158
F1	0	149149	140142	142142	-	214214	128144	144144	155158	0
Obs 2n female	0	5	1	4	0	4	0	5	5	0
Obs 2n male	0	13	6	3	0	11	5	7	12	0
Fisher *p*	1.00		0.16		1.00		0.15		1.00	

* Significant result.

Appendix III. Statistical comparison of observed and expected diploid males in families BS1 and BS2 using Fisher exact test.

BS1 + BS2 (35 indv)	*Vcan*114 Het	*Vcan*114 Hom	*Vcan*004 Het	*Vcan*004 Hom	*Vcan*066 Het	*Vcan*066 Hom	*Vcan*071 Het	*Vcan*071 Hom	*Vcan*073 Het	*Vcan*073 Hom
Obs 2n female	5	10	14	0	11	4	6	8	12	2
Obs 2n male	9	10	20	0	7	9	3	14	13	4
Fisher *p*	0.25		1.00		0.25		0.04	*	not	applic.

BS1 + BS2 (35 indv)	*Vcan*112 Het	*Vcan*112 Hom	*Vcan*096 Het	*Vcan*096 Hom	*Vcan*009 Het	*Vcan*009 Hom	*Vcan*095 Het	*Vcan*095 Hom	*Vcan*065 Het	*Vcan*065 Hom
Obs 2n female	13	0	7	4	9	3	6	6	4	0
Obs 2n male	18	0	11	2	10	2	10	9	6	0
Fisher *p*	1.00		not	applic.	not	applic.	0.25		0.23	

BS1 + BS2 (35 indv)	*Vcan*029b Het	*Vcan*029b Hom	*Vcan*97 Het	*Vcan*97 Hom	*Vcan*106 Het	*Vcan*106 Hom	*Vcan*023 Het	*Vcan*023 Hom	*Vcan*079 Het	*Vcan*079 Hom
Obs 2n female	0	14	4	10	1	11	0	14	14	0
Obs 2n male	0	20	9	5	1	14	5	13	18	0
Fisher *p*	not	applic.	0.20		not	applic.	not	applic.	1.00	

* Significant result.
Not applic. means that this marker was informative in only one family which prevented pooling the data of the two families.