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MOLECULAR MARKERS AS A NEW TOOL
TO MONITOR AND FORECAST
POPULATION DENSITIES OF BALSAM
FIR SAWFLY AND RELATED FOREST
INSECT

INTERIM REPORT TO THE WESTERN NEWFOUNDLAND
MODEL FOREST
MARCH 2006

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PROBLEMATIC AND PROPOSED SOLUTIONS

Balsam fir sawfly (BFS) is an important forest insect pest in the province of Newfoundland and Labrador. BFS infestations occur cyclically and usually collapse after 3 or 4 years. However, the current infestation has continually grown since 1991. Moreover, much of the infested area encompasses valuable balsam fir stands that have been pre-commercially thinned to promote growth, a work and investment whose beneficial effects are threatened by the sawfly damages.

Like other wasp species, the reproductive mode of the BFS is arrhenotoky, in which male offspring arise from unfertilized, haploid eggs and female offspring from fertilized, diploid eggs. Females may control the sex of their offspring at oviposition (primary sex ratio) by regulating the sperm access to the egg and thus, control the sex structure of populations. The observed proportion of females in stands varies from 8 to 83% (unpublished data). Because the expected variability in sex ratios, sex pheromone traps or the current egg sampling, both assuming 50:50 sex ratios, will have a low predictive value for assessing BFS population trends. An improved method considering this characteristic would be to determine the sex of overwintering BFS eggs collected during the fall. Since there is a direct relationship between the number of females and the number of eggs counted the following year during the fall, the identification and development of specific molecular markers to estimate BFS sex at the egg stage would permit managers to predict the trend of BFS population densities. This would allow potential forecasting for 2 years compared with the current egg counts now employed, which only forecasts for the next season. Moreover, the use of this new tool may also result in the reduction of the sampling effort required for monitoring this pest. This method should be easy to implement under operational conditions, since foresters already sample overwintering BFS eggs.

The deliverables for 2005-2006 were: (1) to update the literature review on the known potential sequences of the genome of insect and Hymenoptera that are of some interest for our tool development; (2) to screen some coding genes markers; (3) to optimize PCR programs for DNA amplification of potential markers; (4) to develop the protocol to isolate and clean each individual DNA bands from the gel and to purify the DNA in order to prepare the samples for the sequencing step, and (5) to sequence interesting DNA fragments.

MATERIAL AND METHODS

(1) DNA ANALYSIS

The BFS adults used during the screening process have been collected in 1999 and 2000 in balsam fir stands located within the defined WNMFO2 boundaries and kept at -80°C . The nuclear DNA of BFS adults of both sexes was individually extracted using a modified version of the classical extraction method (Sambrook and Russell, 2001) and stored at -20°C .

A review of the DNA sequences of insect genome published on the site of the National Center for Biotechnology Information (NCBI) has been done to update information on DNA regions of

interest in insects.

The list of screened markers appears in Table 13. For each pair of markers the following steps were done: (1) PCR reactions using initially the PCR programs (duration: 2-5.5 h) provided into the corresponding reference (the positive control being the insect species for which the marker pairs were developed when available); (2) a verification of PCR amplification on agarose gel (85 Volts, electrophoresis duration: 1-2 h); and (3) a verification of the degree of polymorphism (identification of possible allelic types) using the single-strand conformational polymorphism (SSCP) technique (2W, electrophoresis duration: 4-17 h, temperature = 22-25°C).

Since the tested markers have not been developed for BFS, they may not bind well to the DNA of BFS or may bind to several locations. This will result in very faint bands or in several bands on the agarose gel while only one is expected. In order to force the binding process and improve the amplification, the annealing temperature program for DNA amplification of potential DNA fragments (showing variation between BFS females and males), as well as the number of PCR cycles, were progressively decreased and/or increased until a minimum number of bands appeared and /or bands became darker (temperature and cycle optimization).

Individual bands of 4 BFS adults of each sex, as well as 2 honeybee adults of each sex, on the agarose gels obtained using the marker pairs, CSDFa-Q16Ra (42°C and 37 cycles) were cut and treated in order to recuperate the DNA. DNA fragments were extracted from the agarose gel using the Quiaquick Gel Extraction Kit. Twenty microlitres of individual band DNA were purified and sent for sequencing at CFL, Quebec.

RESULTS AND DISCUSSION

LITERATURE REVIEW

The NCBI was created in 1988 with the mandate of developing systems for storing and analyzing knowledge about DNA sequences. In 2005, NCBI databases contained about 100 billion bases. Figure 1a shows the exponential increase over time of the number of insect sequences stored in the NCBI databases. The curve of *Drosophila melanogaster* sequences follows closely the growth of the Insecta curve at first since a genomic project was created to sequence the whole genome of this insect model (Fig. 1a). After 2000, the insect studies started to diversify, although the group of Hymenoptera is clearly under-represented (Fig. 1a). The honeybee is the most studied Hymenoptera and the Diprionidae (family in which BFS is included) in the sub-order Symphyta is still poorly studied (Fig. 1b).

The most commonly sequenced regions of insect genome are in decreasing order: the microsatellites, mitochondrial DNA (mtDNA), the elongation factor-1 α gene, the cytochrome b, and the ribosomal DNA (rDNA) (Table 1).

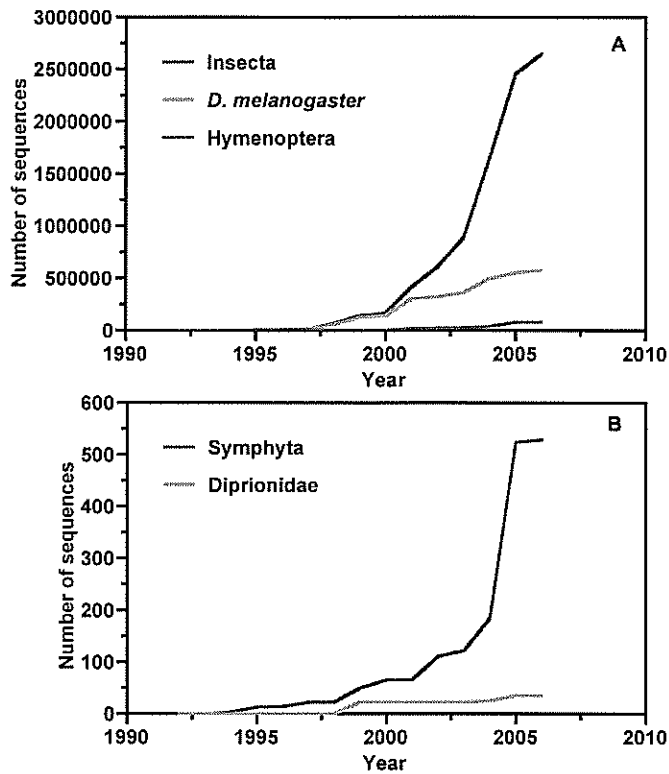


Figure 1: Cumulative numbers of DNA sequences stored in NCBI databases.

Table 1: Known sequences of insect genome in February of each year.

DNA type	# OF HITS 2003	# OF HITS 2004	# OF HITS 2005	# OF HITS 2006
Microsatellites	2333	4951	6679	10633
Mitochondrial DNA	4038	5392	6745	9932
Elongation factor-1 α gene	3929	4700	5820	8145
Cytochrome b	1510	2206	3191	5625
Ribosomal DNA	1942	2685	3304	4280

Microsatellites are short repeating sequences (e.g., (AT)_n). They may be composed of perfect (or pure) repeats or imperfect (or interrupted) repeats, in which the repetitive unit is interrupted by no repeat sequences. Microsatellites vary substantially in numbers and length between individuals, and they are generally highly variable, with an average heterozygosity reaching 90% in some species (Pellissier and Williams, 1998). Hymenoptera represents about 20% of the sequenced microsatellites. Only two species in the suborder Symphyta were explored for microsatellites (Table 2).

Table 2: Known sequences of microsatellites in the suborder of Symphyta of the Hymenoptera Order.

FAMILY	SPECIES	NCBI ACCESSION	REFERENCE
Cephidæ	<i>Cephus cinctus</i> (wheat stem sawfly)	AY145886; AY145890	Hartel et al (2003)
Diprionidae	<i>Diprion pini</i> (pine sawfly)	X92815; X92837	Rouleux-Bonnin et al. (1996)

The elongation factor-1 α gene (EF) becomes widely used in systematic, being informative across a broad range of divergences in insects (Caterino et al., 2000), and thus it is more studied than it was

when we started this project (Table 1). Although the complete sequence of EF was determined only in one species of Hymenoptera, the honey bee, *Apis mellifera* (Danforth and Ji, 1998) when we started this project, Hymenoptera species represents now 13% of the hits. We obtained 243 hits in our search with the suborder Symphyta of the Hymenoptera order. All of them came from one paper on the systematic of Tenthredinidae, which included external controls from families Argidae (*Sterictiphora* sp.), Cimbicidae (*Abia candens*) and Diprionidae (*Diprion similis*) and explored the EF sequences for various genus of the subfamily Nematinae [*Amauronematus* (3 species), *Anoplonyx*, *Ardis*, *Athalia*, *Bacconematus*, *Brachycoluma* (2 species), *Caliroa*, *Cladius*, *Craesus*, *Craterocercus*, *Caulocampus* (2 species), *Dineura*, *Eitelius*, *Endelomyia*, *Endophytus*, *Epicenematus*, *Eriocampa*, *Euura* (3 species), *Fagineura*, *Fallocampus*, *Hemicbroa* (2 species), *Heterarthrus*, *Hoplocampa* (2 species), *Larinematus*, *Melastola* (2 species), *Mesoneura*, *Micronematus*, *Nematinus*, *Nematus* (4 species), *Neopareophora*, *Pachynematus*, *Paranematus*, *Phyllocopa* (2 species), *Phymatocera*, *Pikonema* (2 species), *Platycampus*, *Polynematus*, *Pontania* (5 species), *Pontoprastia*, *Priophorus*, *Pristicampus*, *Pristiphora* (7 species), *Pristola*, *Pseudodineura* (2 species), *Scolioneura*, *Sharliphora*, *Stauronematus*, *Strongylogaster*, *Susana*, *Tenthredo*, and *Trichiocampus*] (Nyman *et al.*, 2006).

The cytochrome b is a coding gene which received a great attention these last few years (Table 1). Only 3% of the hits for the cytochrome b referred to Hymenoptera, and the sequence is complete in only 7 species (Table 3).

Table 3: Known complete sequences of cytochrome b DNA in the Order of Hymenoptera.

SUB-ORDER	FAMILY	SPECIES	GENBANK ACCESSION	REFERENCE
Apocrita	Apidae	<i>Apis mellifera ligustica</i>	NC_001566	Crozier and Crozier (1993)
Apocrita	Apidae	<i>Melipona bicolor</i>	NC_004529	Silvestre and Arias (2004)
Apocrita	Formicidae	<i>Formica candida</i>	AY786144-57	Goropashnaya (2003)
Apocrita	Formicidae	<i>Formica lugubris</i>	AY573856-96	Goropashnaya <i>et al.</i> (2004a); Goropashnaya <i>et al.</i> (2004b)
Apocrita	Formicidae	<i>Formica pratensis</i>	AY584196-233 AY517517 AY604524-5	Goropashnaya <i>et al.</i> (2004a); Goropashnaya <i>et al.</i> (2004b); Seifert and Goropashnaya (2004)
Apocrita	Formicidae	<i>Polyrhuchis turneri</i>	AY437886	Beckenbach <i>et al.</i> (2005)
Apocrita	Formicidae	<i>Tetraponera rufonigra</i>	U02458	Jermiin and Crozier (1994)

The nuclear ribosomal DNA gene cluster generally consists of the 18S, 5.8S, 2S and 28S subunits, separated by two internal transcribed spacer regions (ITS1 and ITS2), one external transcribed spacer region (ETS) and one intergenic spacer (IGS) separating clusters (Fig. 2). This cluster is repeated several time (multiple copies). Among the non-coding region of the rDNA, the ITS1 and ITS2 regions were the most studied in insect (Table 4) and Hymenoptera (Table 5). The complete sequences of the ITS1 (Table 6) and ITS2 (Table 7) were respectively identified in 14 and 22 species of Hymenoptera of suborder Apocrita. The IGS and ETS are poorly studied (Table 4 and 5). The IGS has been sequenced in only 9 insect species from 3 different orders (Table 8) and the ETS is known only in 4 species of 3 different orders of insect (Table 9).

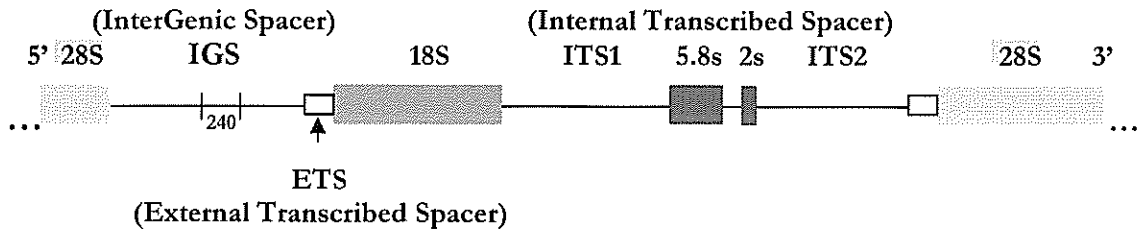


Figure 2: Schematic representation of a nuclear ribosomal DNA cluster (Modified from McKee et al. 1992, and Polanco et al. 1998).

Table 4: Known sequences of ribosomal DNA in insects in February of each year.

GENOME SECTION	SEQUENCE 2003	SEQUENCE 2004	SEQUENCE 2005	SEQUENCE 2006
IGS	3	9	10	12
ETS	4	4	4	4
18S	611	706	864	1045
ITS1	772	1040	1215	1405
5.8S	1142	1576	2054	2868
2S	99	135	137	204
ITS2	927	1364	1821	2507
28S	735	1038	1446	2143

Table 5: Known sequences of ribosomal DNA in Hymenoptera in February of each year.

GENOME SECTION	SEQUENCE 2003	SEQUENCE 2004	SEQUENCE 2005	SEQUENCE 2006
IGS	0	0	0	0
ETS	0	0	0	0
18S	32	32	59	62
ITS1	41	57	92	95
5.8S	54	70	115	130
2S	0	0	0	0
ITS2	39	55	87	102
28S	44	44	66	81

Table 6: Known sequences of ITS-1 gene on ribosomal DNA in the Order of Hymenoptera.

SUB-ORDER	FAMILY	SPECIES	GENBANK ACCESSION	REFERENCE
Apocrita	Aphelinidae	<i>Eretmocerus sp.</i>	AF273639	De Barro et al. (2000)
Apocrita	Aphelinidae	<i>Eretmocerus hayati</i>	AF273637-38	De Barro et al. (2000)
Apocrita	Aphelinidae	<i>Eretmocerus mundus</i>	AF273631-35	De Barro et al. (2000)
Apocrita	Aphelinidae	<i>Eretmocerus queenslandensis</i>	AF273640-48	De Barro et al. (2000)
Apocrita	Aphelinidae	<i>Eretmocerus warrae</i>	AF273621-30	De Barro et al. (2000)
Apocrita	Apidae	<i>Apis cerana</i>	AF176019-21	Sittipraneed et al. (1999)
Apocrita	Braconidae	<i>Peristenus pallipes</i>	AY608602	Ganepy et al. (2005)
Apocrita	Ichneumonidae	<i>Mesochorus sp.</i>	AY588968	Ashfaq et al. (2005)
Apocrita	Mymaridae	<i>Gonatocerus ushmadi</i>	AY542705-18	Vickerman et al. (2004)
Apocrita	Mymaridae	<i>Gonatocerus fasciatus</i>	AY542723-25	Vickerman et al. (2004)
Apocrita	Mymaridae	<i>Gonatocerus morrilli</i>	AY542719-20	Vickerman et al. (2004)
Apocrita	Mymaridae	<i>Gonatocerus norifasciatus</i>	AY542720-22	Vickerman et al. (2004)
Apocrita	Mymaridae	<i>Gonatocerus triguttatus</i>	AY542726-27	Vickerman et al. (2004)
Apocrita	Trichogrammatidae	<i>Trichogramma minutum</i>	AY374440-45 & U36235-36	Jeng et al. (2003)

Table 7: Known sequences of ITS-2 gene on ribosomal DNA in the Order of Hymenoptera.

SUB-ORDER	FAMILY	SPECIES	GENBANK ACCESSION	REFERENCE
Apocrita	Aphelinidae	<i>Eretmocerus mundus</i>	AF273653-50	De Barro <i>et al.</i> (2000)
Apocrita	Aphelinidae	<i>Eretmocerus queenslandensis</i>	AF273654-57	De Barro <i>et al.</i> (2000)
Apocrita	Aphelinidae	<i>Eretmocerus uarue</i>	AF273658-62	De Barro <i>et al.</i> (2000)
Apocrita	Apidac	<i>Apis cerana</i>	AF176019-21	Sittipraneed <i>et al.</i> (1999)
Apocrita	Braconidae	<i>Lipolexis oregruae</i>	AY498553	Persad <i>et al.</i> (2004)
Apocrita	Braconidae	<i>Peristenus pallipes</i>	AY519659; AY608602	Garipey <i>et al.</i> (2005) ; Zhu <i>et al.</i> (2004)
Apocrita	Braconidae	<i>Peristenus pseudopallipes</i>	AY519660	Zhu <i>et al.</i> (2004)
Apocrita	Encyrtidae	<i>Agentiaspis fuscolilis</i>	AF291456-58	Alvarez and Hoy (2000)
Apocrita	Encyrtidae	<i>Agentiaspis citricola</i>	AF291439-55	Alvarez and Hoy (2000)
Apocrita	Eurytomidae	<i>Eurytoma setigera</i>	AB200278	Yara (2006)
Apocrita	Figitidae	<i>Leptopilina heterotoma</i>	DQ218154	Schlenke <i>et al.</i> (2005)
Apocrita	Ichneumonidae	<i>Mesochorus sp.</i>	AY588967-68	Ashfaq <i>et al.</i> (2005)
Apocrita	Myrmariidae	<i>Gonatocerus frigitatus</i>	AY542746	Vickerman <i>et al.</i> (2004)
Apocrita	Myrmariidae	<i>Gonatocerus fasciatus</i>	AY542744-45	Vickerman <i>et al.</i> (2004)
Apocrita	Myrmariidae	<i>Gonatocerus norifasciatus</i>	AY542743	Vickerman <i>et al.</i> (2004)
Apocrita	Myrmariidae	<i>Gonatocerus morrilli</i>	AY542741-42	Vickerman <i>et al.</i> (2004)
Apocrita	Myrmariidae	<i>Gonatocerus ashmeadi</i>	AY542728-40	Vickerman <i>et al.</i> (2004)
Apocrita	Torymidae	<i>Torymus beneficus</i>	AB200270-72	Yara (2006)
Apocrita	Torymidae	<i>Megastigmus nipponicus</i>	AB200279	Yara (2006)
Apocrita	Torymidae	<i>Torymus sinensis</i>	AB200273-75	Yara (2006)
Apocrita	Trichogrammatidae	<i>Trichogramma dendrolimi</i>	AF517576	Li (2002)
Apocrita	Trichogrammatidae	<i>Trichogramma minutum</i>	AY374440-45 U36235-36	Jeng <i>et al.</i> (2003) ; Wang <i>et al.</i> (2003a)

Table 8: Known complete sequences of the intergenic spacer (IGS) of ribosomal DNA in insect.

ORDER	FAMILY	SPECIES	GENBANK ACCESSION	REFERENCE
Dictyoptera	Blattellidae	<i>Blattella germanica</i> (German Cockroach)	AF005243	Mukha <i>et al.</i> (2000)
Diptera	Culicidae	<i>Aedes aegypti</i> (yellow fever mosquito)	AF004986	Wu and Fallon (1998)
Diptera	Drosophilidae	<i>Drosophila funebris</i>	L17048	Mian <i>et al.</i> (1993)
Diptera	Drosophilidae	<i>Drosophila hydei</i>		Tautz <i>et al.</i> (1987)
Diptera	Drosophilidae	<i>Drosophila melanogaster</i> (fruit fly)	AF191295	Tautz <i>et al.</i> (1987); Briscoe and Tomkiel (2000)
Diptera	Drosophilidae	<i>Drosophila oreana</i>		Tautz <i>et al.</i> (1987)
Diptera	Drosophilidae	<i>Drosophila virilis</i>		Tautz <i>et al.</i> (1987)
Diptera	Simuliidae	<i>Simulium sanctipauli</i> (black fly)	AF403821- AF403825	Morales-Hojas <i>et al.</i> (2002)
Lepidoptera	Saturniidae	<i>Attacus ricini</i> (silkworm)	AF463459	Wang <i>et al.</i> (2003b)

Table 9: Known complete sequences of external transcribed sequence (ETS) of ribosomal DNA in insect.

ORDER	FAMILY	SPECIES	GENBANK ACCESSION	REFERENCE
Diptera	Drosophilidae	<i>Drosophila melanogaster</i> (fruit fly)	AF191293-5; X02211; X03501	Briscoe and Tomkiel (2000) ; Di Nocera <i>et al.</i> (1986) ; Simeone <i>et al.</i> (1985)
Diptera	Glossinidae	<i>Glossina morsitans</i> (tsetse fly)	X05007	Cross and Dover (1987)
Hemiptera	Delphacidae	<i>Laodelphax striatellus</i>	AY346092	Liao <i>et al.</i> (2003)
Lepidoptera	Bombycidae	<i>Bombyx mori</i> (domestic silkworm)	X05086	Fujiwara and Ishikawa (1987)

Table 10: Known sequences of 18S gene on ribosomal DNA in the Order of Hymenoptera.

SUB-ORDER	FAMILY	COMMON SPECIES NAME	NCBI ACCESSION	REFERENCE
Apocrita	Aphelinidae	<i>Aphelinus gossypii</i>	AY216700	Weathersbee <i>et al.</i> (2004)
Apocrita	Braconidae	<i>Lipolexis scutellaris</i>	AY216699	Weathersbee <i>et al.</i> (2004)
Apocrita	Braconidae	<i>Lysiphlebus testaceipes</i>	AY216698	Weathersbee <i>et al.</i> (2004)

Table 11: Known sequences of 28S gene on ribosomal DNA in the Order of Hymenoptera.

SUB-ORDER	FAMILY	SPECIES	NCBI ACCESSION	REFERENCE
Apocrita	Chrysididae	<i>Chrysis</i> sp.	AF142520	Carpenter and Wheeler (1999)
Apocrita	Gasteruptiidae	<i>Gasteruption</i> sp.	AF142521	Carpenter and Wheeler (1999)
Apocrita	Mutillidae	<i>Dasymutilla</i> sp.	AF142518	Carpenter and Wheeler (1999)
Apocrita	Pompilidae	<i>Hemipepsis</i> sp.	AF142519	Carpenter and Wheeler (1999)
Apocrita	Rhopalosomatidae	<i>Rhopalosoma</i> sp.	AF142524	Carpenter and Wheeler (1999)
Apocrita	Scoliidae	<i>Campsomeris</i> sp.	AF142517	Carpenter and Wheeler (1999)
Apocrita	Scoliidae	<i>Scolia</i> sp.	AF142523	Carpenter and Wheeler (1999)
Apocrita	Tiphidae	<i>Myzinum</i> sp.	AF142525	Carpenter and Wheeler (1999)
Apocrita	Trigonalidae	<i>Labidognathos</i> sp.	AF142522	Carpenter and Wheeler (1999)
Apocrita	Vespidae	<i>Belonogaster juncea colonialis</i>	AF142529	Carpenter and Wheeler (1999)
Apocrita	Vespidae	<i>Euemes tripunctatus</i>	AF142526	Carpenter and Wheeler (1999)
Apocrita	Vespidae	<i>Polistes tenebricosus</i>	AF142530	Carpenter and Wheeler (1999)
Apocrita	Vespidae	<i>Polybia affinis</i>	AF142528	Carpenter and Wheeler (1999)
Apocrita	Vespidae	<i>Vespa maculifrons</i>	AF142527	Carpenter and Wheeler (1999)

No additional sequence of the 18S (Table 10) and 28S (Table 11) genes was submitted to NCBI last year. Sequences of the 5.8S gene on ribosomal DNA are known for species in 6 Hymenopteran families (Table 12), all in the suborder Apocrita.

Table 12: Known sequences of 5.8S gene on ribosomal DNA in the Order of Hymenoptera.

SUB-ORDER	FAMILY	SPECIES	GENBANK ACCESSION	REFERENCE
Apocrita	Apidae	<i>Apis cerana</i>	AF176019-21	Sittipraneed <i>et al.</i> (1999)
Apocrita	Braconidae	<i>Peristenus digonentis</i>	AF473537	Erlandson <i>et al.</i> (2004)
Apocrita	Braconidae	<i>Peristenus pallipes</i>	AF473538; AY608602	Erlandson <i>et al.</i> (2004); Garipey <i>et al.</i> (2005)
Apocrita	Braconidae	<i>Peristenus stygius</i>	AF473539	Erlandson <i>et al.</i> (2004)
Apocrita	Cynipidae	<i>Andricus curator</i>	AF395161	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Andricus kollari</i>	AF395160	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Barbotinia oranienis</i>	AF395163	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Diplolepis rosae</i>	AF395158	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Panteliella bicolor</i>	AF395164	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Periclistus brandti</i>	AF395165	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Plagiotrochus quercusilicis</i>	AF395162	Rokas <i>et al.</i> (2002)
Apocrita	Cynipidae	<i>Synergus gallaeopomiformis</i>	AF395159	Rokas <i>et al.</i> (2002)
Apocrita	Ichneumonidae	<i>Mesochorus</i> sp.	AY588968	Ashfaq <i>et al.</i> (2005)
Apocrita	Torymidae	<i>Torymus geranii</i>	AB200280	Yara (2006)
Apocrita	Trichogrammatidae	<i>Trichogramma brasiliensis</i>	AY167416	Archna <i>et al.</i> (2005a)
Apocrita	Trichogrammatidae	<i>Trichogramma chilonis</i>	AY167418	Archna <i>et al.</i> (2005a)
Apocrita	Trichogrammatidae	<i>Trichogramma exiguum</i>	AY167419	Archna <i>et al.</i> (2005a)
Apocrita	Trichogrammatidae	<i>Trichogramma japonicum</i>	DQ177151	Archna <i>et al.</i> (2005b)
Apocrita	Trichogrammatidae	<i>Trichogramma minutum</i>	AY359470; AY374438-40; U36235-36	Jeng <i>et al.</i> (2003); Wang <i>et al.</i> (2003a)
Apocrita	Trichogrammatidae	<i>Trichogramma ostrinae</i>	AY167417	Archna <i>et al.</i> (2005a)

MARKER SCREENING AND OPTIMIZATION

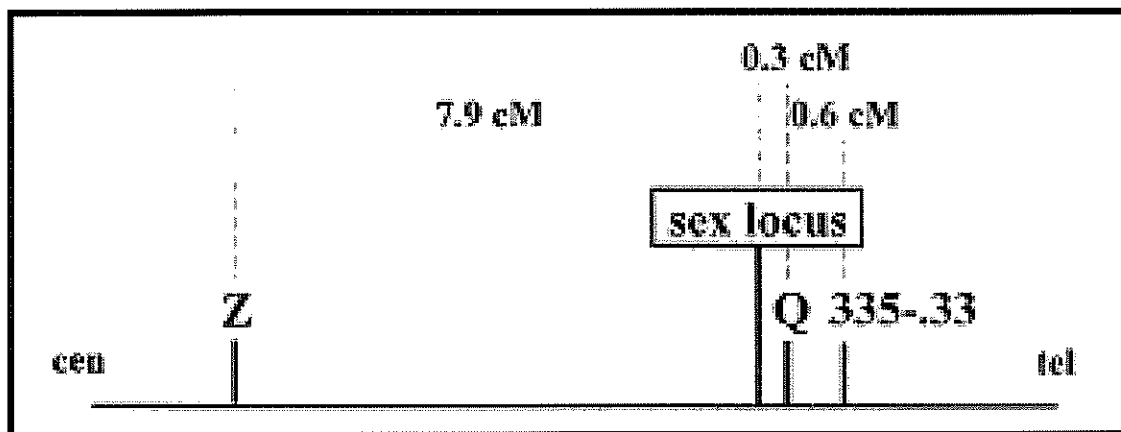


Figure 3: Schematic representation of genes in the sex locus neighborhood of the honeybee (Beye *et al.* 1999).

Beye *et al.* (1999) proposed markers flanking genes Z and Q. He realized that the complementary sex determination locus (CSD), the gene responsible for the sex determination in Hymenoptera, was located between the Z and Q (Fig. 3). Hymenopteran females will always be heterozygote at the CSD, while males will appear like homozygote since they are hemizygote. The identification and the development of specific markers targeting the CSD of BFS might provide the molecular tool required to sex BFS eggs. In 2003, Beye *et al.* published specific markers for the CSD of honeybee and found a high polymorphism at this locus, with 19 different sex-determining alleles. We thus concentrated our effort this year to amplify the CSD region of the BFS, trying different combinations

with the CSD markers developed for the honeybee (Table 13). The potential of the marker pairs for the development of our tool, the conditions tested, the optimal conditions (when determined) and the marker status are indicated in Table 13.

Table 13: List of marker combinations targeting the sex locus tested, their potential and status.

MARKERS	REFERENCE	CONDITIONS TESTED	OPTIMAL CONDITIONS	POTENTIAL (SSCP)	STATUS
CSDFi-CSDR1i	Beye <i>et al.</i> (2003)	48, 50°C; 29, 35 cycles	None	None	Rejected
CSDFi-CSDR2i	Beye <i>et al.</i> (2003)	48, 50°C; 29, 35 cycles	None	None	Rejected
CSDFa-CSDR1a	Modified from Beye <i>et al.</i> (2003)	35, 48, 50°C; 29, 35 cycles	None	None	Rejected
CSDFa-CSDR2a	Modified from Beye <i>et al.</i> (2003)	35, 48, 50°C; 29, 35 cycles	None	None	Rejected
CSDFi-Q16Fa	F: Beye <i>et al.</i> 2003; R: Modified from Beye <i>et al.</i> (1999)	From 38 to 43°C; 35 cycles	On-hold	None	Rejected
CSDFi-Q16Ra	F: Beye <i>et al.</i> 2003; R: Modified from Beye <i>et al.</i> (1999)	From 35 to 43 C; 35 cycles	On-hold	None	Rejected
CSDFa-Q16Fa	F: Modified from Beye <i>et al.</i> 2003; R: Modified from Beye <i>et al.</i> (1999)	From 33 to 43°C; From 32 to 41 cycles	T _a = 40°C; 37 cycles	Poor	On-hold
CSDFa-Q16Ra	F: Modified from Beye <i>et al.</i> 2003; R: Modified from Beye <i>et al.</i> (1999)	From 37 to 44°C; From 35 to 46 cycles	T _a = 42°C; 37 cycles	Good	Sent for sequencing

We obtained no bands for BFS or Newfoundland honeybees when we used the initial marker pairs (CSDFi-CSDR1i; CSDFi-CSDR2i) proposed by Beye *et al.* (2003), suggesting that the CSD of American strains of honeybees would differ significantly from their European kin. We thus shortened the initial markers in order to slightly decrease their specificity. The modified marker pairs did not give better results (CSDFa-CSDR1a; CSDFa-CSDR2a). We assumed that the absence of band was caused by a binding failure of the reverse marker. Beye *et al.* (2003) also ran into a similar problem due to the high variability at the end of the CSD locus of honeybee.

We tried to amplify DNA fragment with the initial and modified CSD forward and either the forward or the reverse of the Q16a gene that were screened earlier. The trials with the initial CSD forward confirmed the poor binding of the long markers. We obtained several bands even for the honeybee (Fig. 4A, 4B), suggesting that the reverse markers was acting as a random amplification of polymorphic DNA (RAPD), amplifying arbitrary sequence fragments of genomic DNA.

While the CSDFa-Q16Fa also produced several bands for both BFS and honeybee (Fig. 5A), we obtained only one band for the honeybee when we used CSDFa-Q16Ra (Fig. 5B). This marker pairs produced 4 bands for BFS males and females, indicating that they bind at several locations on the DNA. However, one of the bands coincided with the honeybee band, suggesting that we might have been successful in amplifying the CSD and Q16 genes in BFS. This hypothesis will be confirmed when we will know the sequence of the DNA fragments.

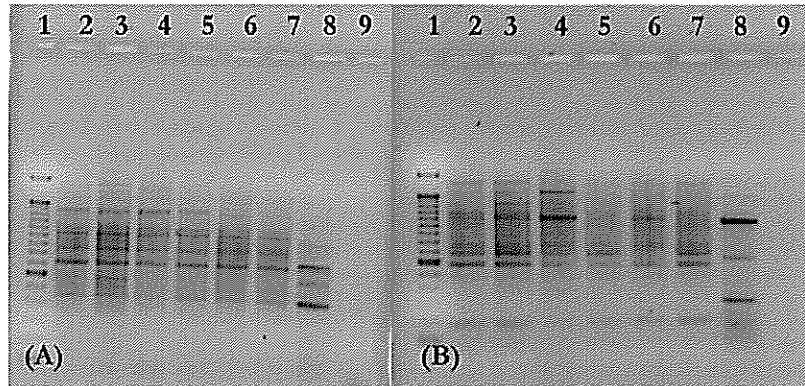


Figure 4: DNA fragments produced with (A) CSDFi-Q16Fa (38°C and 35 cycles) and (B) CSDFi-Q16Ra (36°C and 35 cycles) markers on agarose gels. Lane 1 is the ladder. Lanes 2 to 4 represented females. Lanes from 5 to 6 represented males. Lane 8 is the positive control, which is honeybee DNA. Lane 9 is the negative control containing no DNA, only the reagents.

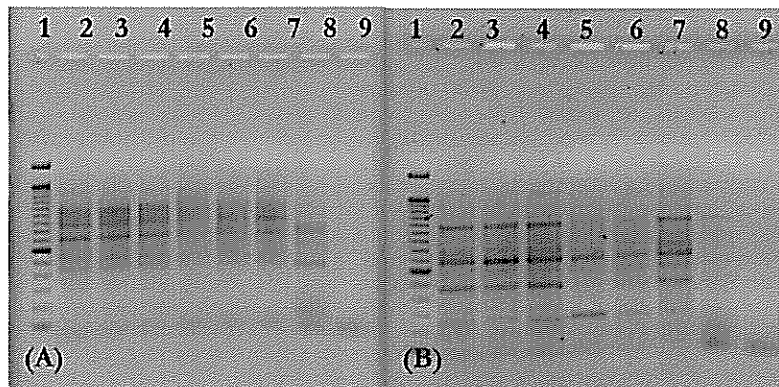


Figure 5: DNA fragments produced with (A) CSDFa-Q16Fa (40°C and 37 cycles) and (B) CSDFa-Q16Ra (42°C and 37 cycles) markers on agarose gels. Lane 1 is the ladder. Lanes 2 to 4 represented females. Lanes from 5 to 6 represented males. Lane 8 is the positive control, which is honeybee DNA. Lane 9 is the negative control containing no DNA, only the reagents.

The technique to extract and purify individual DNA fragments from agarose gels has been developed. Purified samples of PCR products obtained using the marker pairs, CSDFa-Q16Ra, were prepared and sent for sequencing at CFL, Quebec.

Despite the fact that several of our key pieces of equipment (distilled water system, ice machine, heat sterilizer, computer ...) broke and/or malfunctioned during the year, the project has made significant progress towards the development of our molecular tool to determine the sex of BFS.

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