Genome Mapping and Genomics in Animals

Chittaranjan Kole
Series Editor

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Editors

Genome Mapping and Genomics in Arthropods

Springer

Genome Mapping and Genomics in Animals
Volume 1

Series Editor: Chittaranjan Kole

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(Editors)

Genome Mapping and Genomics in Arthropods

With 25 Illustrations, 3 in Color

Springer

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ISBN 978-3-540-73832-9 e-ISBN 978-3-540-73833-6
DOI 10.1007/978-3-540-73833-6

Library of Congress Control Number: 2007934674

Cover design: WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

Preface to the Series

The deciphering of the sequence of a gene for the first time, the gene for bacteriophage MS2 coat protein to be specific, by Walter Fiers and his coworkers in 1972 marked the beginning of a new era in genetics, popularly known as the genomics era. This was followed by the complete nucleotide sequence of the same bacteriophage in 1976 by the same group; DNA sequencing of another bacteriophage (Φ X174) in 1977 by Fred Sanger, Walter Gilbert, and Allan Maxam, working independently; and first use of any DNA marker in gene mapping in 1980 for the human system by David Botstein. These landmark discoveries were immediately embraced by the life science community and were followed by an array of elegant experiments leading to the development of several novel concepts, tools, and strategies for elucidation of genes and genomes of living organisms of academic and economic interests to mankind.

The last two decades of the twentieth century witnessed the invention of the polymerase chain reaction; several types of molecular markers; techniques of cloning large DNA segments in artificial chromosomes; approaches to isolate and characterize genes; and tools for high-throughput sequencing, to name just a few. Another noteworthy development had been the formulation of different computer software to analyze the huge amount of data generated by genome mapping experiments, and above all deployment of information technology to store, search, and utilize enormous amounts of data particularly of cloned genes, transcripts, ESTs, proteins, and metabolites. This sweet and swift marriage of biology and information technology gave birth to bioinformatics and the new "omics" disciplines such as genomics, transcriptomics, proteomics, and metabolomics.

The tide of genome mapping and genomics flooded all phyla of the animal kingdom and all taxa of the plant kingdom and most obviously the prokaryotes. In the animal systems, we already had the gene sequence for the CFTR protein in humans in 1989; genome sequence of the model organism *Caenorhabditis elegans* in 1998; genetic maps of many higher animals with map positions of genes and gene-clusters during the nineties. We also happily witnessed the beginning of genome sequencing projects of three domestic animals (cow, dog, and horse) and poultry in 1993. All these achievements and endeavors culminated in the whole-genome sequence of the fruit-fly *Drosophila*, the garden pea of the animal system, in 2000 declaring a successful and pleasant ending of the genome science efforts of the twentieth century. The new millennium in 2001 started with the publication of the draft sequence of the human genome on February 15th by The International Human Genome Mapping Consortium and on February 16th by The Celera Genomics Sequencing Team.

A flurry of new concepts and tools in the first few years of the first decade of the twenty-first century has enriched the subject of genomics and the field has broadened to include the young and fast-growing disciplines of structural genomics, functional genomics, comparative genomics, evolutionary genomics, and neutraceutical genomics, to name just a few. We now have more, faster, cheaper, and cleverer mapping and sequencing strategies, association mapping and the

454 for example; several tools, such as microarrays and cDNA-AFLP to isolate hundreds of known and unknown genes within a short period, elegantly assisted by transcript-profiling and metabolic-profiling; identifying new genes from the knowledge-base of homologous genomes; and precise depiction of the road map of evolution of human and other members of the animal kingdom and their phylogenetic relationships with members of other species or genera. Within less than a decade of the deciphering of the first complete genome sequence for an animal species in 1998, we have complete sequences of some seventeen species of the animal kingdom including nematodes (2), arthropods (4), domestic animals and poultry (2), marsupial (1), wild animals (2), aquatic animals (4), human (1), and non-human primate (1). Many more genome mapping projects are progressing rapidly and their results are expected to be published soon.

The list of achievements in the fields of genome mapping and genomics in human and other members of the animal kingdom is enormous. It is also true that in today's world, in the global village of the new millennium, we have access to almost all information regarding the initiation, progress, and completion of all endeavors of animal genome sciences and can enrich our knowledge of the concepts, strategies, tools, and outcomes of the efforts being made in animal genome mapping and genomics. However, all this information is dispersed over the pages of periodicals, reviews on particular types of animals or their specific groups in hard copy versions, and also in electronic sources at innumerable links of web pages for research articles, reports, and databases. But we believe that there should be a single compilation, in both hard copy and electronic versions, embodying the information on the work already done and to be done in the fields of genome mapping and genomics of all members of the animal kingdom that are of diverse interests to mankind: academic, health, company, or environment.

We, therefore, planned for this series on Genome Mapping and Genomics in Animals with six book volumes dedicated to Food and Fiber Animals; Wildlife and Companion Animals; Fishes and Aquatic Animals; Arthropods; Laboratory Animals; and Human and Non-human Primates. We have included chapters on the species for which substantial results have been obtained so far. Genomes of many of these species have been sequenced or are awaiting completion of sequencing soon. Overview on the contents of these volumes will be presented in the prefaces of the individual volumes.

It is an amazingly interesting and perplexing truth that only four nucleotides producing only twenty amino acids in their triplet combination could create anywhere between five to thirty million species of living organisms on the earth. An estimated number of about a half million vertebrate animal species have been described so far! Genomes of the few animal species from this enormous list that we know today are also too diverse to elucidate. It is therefore daring to edit a series on depiction of the diverse genomes we are presenting in over sixty chapters in the six volumes. Seven globally celebrated scientists with knowledge and expertise on different groups of animal systems, and human and non-human primates provided me with the inspiration and encouragement to undertake the job of the series editor. Noelle (Noelle E. Cockett), Paul (Paul Denny), Wayne (Wayne B. Hunter), Tom (Thomas D. Kocher), Ravi (Ravindranath Duggirala), Tony (Anthony G. Comuzzie), and Sarah (Sarah Williams-Blangero) were always available for consultations and clarifications on any aspect while editing the manuscripts of this series. While working on this series, I have been a student first, a scientist

second, and an editor third and last, with the mission to present a comprehensive compilation of animal genome mapping and genomics to the students, scientists, and industries currently involved and to be involved in the study and practice of animal genome sciences. I express my thanks and gratitude as a humble science worker to these seven volume editors for giving me an opportunity to have an enriching and pleasant view of the wide canvas of animal genome mapping and genomics. I also extend my thanks and gratitude to all the scientists who have generously collaborated with their elegant and lucid reviews on the rationale, concepts, methodologies, achievements, and future prospects of the particular systems they are working on, and for the subtle touches of their own experiences and philosophies.

As expected, the editing jobs of this series comprised communication with the volume editors, authors, and publishers; maintenance of the files in hard and soft copies; regular internet searches for verification of facts and databases; and above all maintenance of an environment to practice and enjoy science. My wife Phullara, our son Sourav, and our daughter Devleena were always with me on my travels as a small science worker on a long road of "miles to go before I sleep," not only for the successful completion of this series but also in all my efforts for teaching, research, and extension wherever I worked and stayed in my life.

We have already completed a seven-volume series on Genome Mapping and Molecular Breeding in Plants with Springer that has been very popular among students, scientists, and industries. We are also working on a series on Genome Mapping and Genomics in Microbes with Springer. It was, is, and will be enriching and entertaining to work with the experienced and wonderful people involved in the production of this series, including Sabine (Dr. S. Schwarz), Jutta (Dr. J. Lindenborn), and Cornelia (Mrs. C. Gründer), among many from the Springer family. I record my thanks and gratitude to them, here (and also submit in the databanks for future retrieval) for all their timely co-operation and advice when publishing these volumes.

I trust and believe that we must have missed deliberations on many significant animal species and left many mistakes on the pages of these volumes. All these lapses are surely mine, and all the credits must go to the volume editors, the authors, and the publisher. In the future these errors will be rectified on receipt of suggestions from the readers, and also there will be further improvement of the contents and general set-up of the volumes of this series.

Clemson — Chittaranjan Kole
September 5, 2007

Preface to the Volume

The field of genomics continues to provide new and inspiring answers to the most intriguing questions about life on earth and beyond. This influence will most likely continue even as humans turn toward the colonization of space. Genomics and bioinformatics have accelerated the rate of discovery in all fields of science, but even more so in those fields related to entomology. Arthropods, such as insects, greatly impact our world, yet few really understand how insects affect our lives. Most have seen the pollinators, bees and butterflies, and can see their roles in the production of fruits, food, and fiber crops. Few have seen the benefits we all receive when the parasitoid lays its eggs inside of a pest insect. The numerous, invisible, and never-ceasing interactions of insects and arthropods entwined with their host plants, pathogens, and as food for other animals within the chain of life are as common and as necessary as the air we breathe.

Insects and arthropods contribute hundreds of billions of dollars to our well-being. However, they also cost us hundreds of millions of dollars each year in lost yields, structural damage, and in efforts to manage them and/or in reducing the diseases they spread. Estimates suggest that for every *single dollar* spent in biological control of insect pests the general public receive a *$5 return* or more in benefits to their quality of life. When you factor in the other benefits that agricultural research has produced, which include more nutritious foods, reduced use of insecticides, environmentally safer herbicides and pesticides, development of new products in fiber, food, feed, and products that impact human health, one can see that this has been and will continue to be one of the best investments to maintain the growth and welfare of our world. The combination of biology, genomics, and computers in bioinformatics has changed the face of entomology forever. The wonder of these advances have yet to be fully realized, but are only a small jump from "thought" to "understanding" as we continue to explore all the possibilities. *Genomics* has opened a new gateway to examine existing and emerging questions concerning arthropods. While insects as a group make up more species walking on the earth than all other animal groups combined, very few species have had any genomic data produced. We are still only at the beginning in our quest to understand insects and other arthropods.

The completion of the *Drosophila* genome initially demonstrated the broad applications of using an insect as a model system to conduct functional genomics studies, thereby elucidating gene functions which were also found to be present in many non-dipteran species. Now several insect genomes have been completely sequenced such as of fruit flies and mosquitoes, the honey bee, the flour beetle, the silkworm, the pea aphid, and a parasitic wasp, with others scheduled to follow: a medfly, the hemipteran *Rhodnius prolixus*, a bedbug, a body louse, and two-spotted spider mites, as well as other agricultural pests such as whiteflies. These studies on the evolution and phylogeny of insects will continue to advance our understanding of their similarities and differences, which was not possible using traditional methods.

The insects covered in this volume are only the early stages of some of the projects that are being pursued in genomics of insects, ticks, and other arthropods. In the preparation of this volume we wish to express our gratitude to all of the authors for their contributions and expert cooperation. The experience allowed us to grow professionally and it was a pleasure to work with Dr. Jutta Lindenborn, Life Science Editorial Springer. We also wish to acknowledge and thank many publishers and authors for their generosity and goodwill to permit authors to gain permission of previously published works. We wish to acknowledge and thank Dr. Xiomara Sinisterra for her patience and assistance in many levels in the completion of this project.

Clemson and Fort Pierce — Wayne B. Hunter
September 5, 2007 — Chittaranjan Kole

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Abbreviations

AChE	Acetylcholinesterase
AFLP	Amplified fragment length polymorphism
AIL	Advanced intercross line
ARS	Agricultural Research Service
Avr	*Avirulence* (gene)
BAC	Bacterial artificial chromosome
bp	Base pair
CATS	Comparative anchor-tagged sequences
CBP	Carotenoid-binding protein
CDC	Center for Disease Control and Prevention
cDNA	Complementary DNA
CIM	Composite interval mapping
cM	Centimorgan
csd	Complementary sex determination
DEN-2	Dengue-2 virus
DHF	Dengue hemorrhagic fever
E chromo.	Supernumerary chromosomes eliminated from the soma
EST	Expressed sequence tag
FISH	Fluorescent in situ hybridization
FPC	Fingerprint contig
Gbp	Giga base pairs
H	Hessian fly resistance (gene)
HBC	High backcross population
HBGP	Honeybee Genome Project
IGP	*Ixodes* Genome Project
ISSR	Inter-simple sequence repeat
kb	Kilobase
LBC	Low backcross population
LD	Lyme disease
LG	Linkage group
MAS	Marker-assisted selection
Mb	Megabase
MBC	Map-based cloning
Mbp	Mega base pairs
MIM	Multiple interval mapping
mRNA	Messenger-RNA
Mya	Million years ago
NCBI	National Center for Biotechnology Information
NPV	Nucleopolyhedrosis virus
OP	Organophosphate
PCR	Polymerase chain reaction
pg	Picogram
QTL	Quantitative trait loci

RAPD	Random(ly) amplified polymorphic DNA
rDNA	Ribosomal-DNA
RFLP	Restriction fragment length polymorphism
RNAi	RNA interference
S chromo.	Autosomes and X chromosomes found in both the germline and the soma
SNP	Single nucleotide polymorphism
SSGP	Secreted salivary gland protein
SSR	Simple sequence repeat
STAR	Sequence-tagged RAPD marker
STS	Sequenced tagged sites
TC	Tentative consensus
TIGR	The Institute for Genomic Research
TOT	Transovarial transmission
USDA	United States Department of Agriculture

1 Honeybee

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1.1 Introduction

All honeybees of the genus *Apis* are obligate social organisms that exist in colonies usually containing a single reproductive female, the queen, and many thousands of facultatively sterile females, the workers. Hundreds to a few thousand males are produced during the spring and summer months in temperate climates, but they die or are driven from the hive in the autumn. During the spring and summer, the queens of the species most commonly used commercially, *Apis mellifera*, may lay up to 2,000 eggs or more per day. If the eggs are fertilized, they develop into diploid females, while unfertilized eggs develop into haploid males. If the egg-laying rate of the queen begins to falter, the workers will feed some of the diploid larvae a special diet that causes them to develop into queens, whereas the remainder of the diploid eggs develop into smaller, less reproductively capable females whose egg laying is suppressed by queen pheromones. Workers typically live about five weeks during the summer, but have a longer life expectancy during the winter. The queen is capable of living for up to five years, but is typically killed by a replacement queen within two years. During late spring and early summer when colony numbers are at their highest in temperate climates, the workers rear replacement queens and the old queen leaves with a portion of the colony members in a swarm. In this way, colony reproduction is achieved. A similar type of colony dynamic occurs in tropical regions, but the seasonal variation is much reduced.

In temperate climates, honeybees store resources (honey and pollen) to support the colony over the winter months when resources may not be available. In both temperate and tropical regions, storage of resources also supports colony expansion and reproductive swarming. The strong resource-collecting instinct has resulted in several species of *Apis* being kept as honey producers and pollinators. At least two species are kept commercially, the Western or European Honeybee, *Apis mellifera*, and the Asian honeybee, *Apis cerana*. Another species, *Apis dorsata*, which is common in southern Asia, produces open combs in trees and on cliff faces. Though it is not managed commercially, it is often raided in wild populations. No fertile hybrids have been produced between species.

This chapter will deal with the most common and commercially important of these species, *Apis mellifera*. As a species it is native to the Old World: Europe, Africa, Eurasia, and Asia. Within *Apis mellifera* more than two dozen subspecies are recognized that correspond to the great variety of climatic zones to which the species is endemic (Sheppard and Meixner 2003). Colonies from several European subspecies were first brought to the New World in the early seventeenth century and to Australia in the early nineteenth century. Beekeeping has a very ancient and varied history, with evidence that honeybees have been kept for thousands of years. There are depictions of beekeeping from ancient Egypt and China. Remarkably many bee husbandry practices have not changed in millennia. In fact, hollow log hives as depicted in wall murals from several thousand years ago are still used in northern Africa and the Mediterranean.

Despite the extended relationship between humans and bees, the history of beekeeping is not one of domestication. Genetic improvement of honeybees is complicated by the fact that they suffer from severe inbreeding depression and cannot therefore be inbred to any great degree. Bees have even developed a vari-

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ety of behavioral characteristics that reduce the possibility of inbreeding. These behavioral traits further complicate the breeding of bees. For example, honeybees are adapted to mate during flight presumably to prevent mating with closely related males in the hive. As a result, it is not possible to get bees to mate in laboratory cages. Mating takes place in aerial "drone congregation areas" where males (drones) from the surrounding region congregate, awaiting the arrival of virgin queens. Queens will mate with about 12 to 17 drones over a period of one to several days and will not mate again for the remainder of their lives. The remote nature of mating and the large number of partners make it extremely difficult to control mating in the field. Queen breeders attempt partial control of mating by providing many drone source colonies from selected stocks but drones from the surrounding 5–10 kilometers can access the drone congregation area. Inbreeding is also prevented by specific genetic and biochemical mechanisms. Homozygosity at the sex-determining locus (*csd*) results in a phenotype known as "shot brood," in which half of the diploid larvae are killed (Beye et al. 2003). Heterozygosity at the sex-determining locus is required for female development. While males are usually haploid and therefore hemizygous at the sex-determining locus, homozygous diploids also begin to develop as males, but they apparently produce a pheromone (dubbed the "eat me" pheromone) that results in them being consumed by the worker bees within 48 hours after eclosion from the egg (Woyke 1998). As a result, the pairing of identical *csd* alleles caused by mating between a queen and closely related drones greatly reduces the overall vigor of honeybee colonies and the maximal size of a colony that can be produced. In addition, shot brood is a drain on the finite egg-laying capacity of the queen and stimulates the workers to destroy the queen whom they perceive to be failing.

The technique of artificial insemination has been developed to overcome the limitations that aerial mating to multiple drones imposes on bee breeding. It has been shown, however, that the limited volume of semen supplied to artificially inseminated queens (when using single-drone inseminations) tends to produce colonies that are weak and therefore prone to queen replacement or loss by attrition without careful management. Inbreeding, a tool typically used in genetic improvement programs, greatly exacerbates the problems associated with maintaining colonies produced by artificially inseminated queens, due to the compounding effect of shot brood and inbreeding depression on the lack of colony vigor. As a result, it is very difficult to select for certain traits by classical breeding methods. Three final limitations to breeding of bees all relate to the reproductive unit of honeybees being the colony. Many traits, particularly behavioral traits, are only apparent at the colony level and therefore must be selected at the colony level, rather than at an individual level. Thus, rapid phenotypic screening is often impossible. Furthermore, the need to maintain genotypes as colonies, limits the number of genotypes that can be screened and maintained. Finally, long-term cryogenic storage of germplasm, while theoretically possible, is not without its own inherent problems (Stort and Goncalves 1986) and as yet is not a practical option. Thus, "reference" strains must be maintained in the field as sperm has only been maintained in short-term storage for a period of up to 6 weeks (Collins 2000).

1.1.1 Taxonomic Description

The honeybee *Apis mellifera* is in the Order Hymenoptera, Suborder Apocrita, Superfamily Apoidea, Family Apidae, Subfamily Apinae, Tribe Apinini. All *Apis* species have complex societies. There are also other genera of "stingless bees" in the Apidae that are highly social. Honeybee (*Apis*) nests consist of vertical combs formed from secreted wax. Some honeybee (*Apis*) species construct their combs in the open but the two species commonly kept for honey production and pollination are cavity nesters. Honeybees are found worldwide from the tropics to subarctic temperate zones and on all continents except Antarctica. There are various subspecies of *Apis mellifera*, all of which interbreed readily, suggesting that observed differences reflect selection for adaptation to local environmental conditions. While subspecies are generally distinguished by morphometrics, color, and region of origin, behavior and size are also important characteristics. For example, the "Africanized " honeybees ranging from South to North America are derived from *A. mellifera scutellata* from sub-Saharan Africa. Even though these honeybees hybridize readily with European subspecies, they retain the small size and highly defensive stinging behavior of *A. m. scutel-*

lata. The adaptation of these bees to tropical environments has allowed them to proliferate across tropical and subtropical regions of the Americas where they are considered a major problem because of their stinging behavior.

1.1.2 Economic Importance

Honeybees are of greatest economic importance as pollinators of fruit, nut, vegetable, and pasture crops as well as many uncultivated flowering plants. Payments made to beekeepers for pollination services in the USA alone have been estimated to be approximately US$150–220 million annually (USDA 2003) with a total value of the crops that are pollinated at US$14.5 billion (Morse and Calderone 2000). Honeybees are also commercial producers of honey; according to FAO statistics (http://faostat.fao.org) the total value of global honey production was 1,345,672 metric tons in 2004 and 82,000 metric tons in the USA. Other products harvested from honeybees include beeswax, pollen, royal jelly, and propolis, often taken as nutritional supplements, used for medicinal purposes or in cosmetics and other products.

1.1.3 Breeding Objectives

One of the distinguishing characteristics of honeybees is that the most important traits for commercial beekeeping are behavioral and not physiological as with most domestic animals. Although behavioral traits are heritable, heritability tends to be much less than what is typically expected of a physiological characteristic. Assays for behavioral traits tend to be difficult, as they often depend on actions at the colony level, which result from interactions between individuals, communication, and collective "decision making."

The most positive heritable traits desirable for commercial breeding are described below.

Disease Resistance

Resistance against parasites, such as the *Varroa jacobsoni* (Kulincevic et al. 1992; deGuzman et al. 1996) and *Acarapis woodi* mites (deGuzman et al. 1998), and pathogens such as *Paenibacillus larvae* (Spivak and Reuter 2001), the causative agent of American Foulbrood (AFB), is of high importance to the beekeeping industry. Many studies suggest that a major component of colony level resistance to both pathogens and mites is the tendency to remove diseased brood from the hive, thereby significantly reducing the abundance of pathogens and pests. This activity is referred to as hygienic behavior and consists of removal of the wax caps from the brood cells and removal of infected brood (Rothenbuhler 1964; Spivak and Reuter 2001; Lapidge et al. 2002). The bees also line the interior surfaces of the hive with antibiotic-rich propolis which is collected from resins and gums secreted by plants. Significant differences in propolis collection have been observed between subspecies and propolis is a minor commercial product, but selection is most often employed to reduce propolis collection because this sticky substance makes it more difficult for the beekeeper to remove combs for inspection. Insects also contain humoral and cellular immune systems (Iwanaga and Lee 2005), but genetic variability for these traits has neither been described nor exploited in a breeding program.

Pollen Foraging

The value of honeybee-pollinated crops makes crop pollination the single most important activity of bees in terms of human economic significance. When a worker collects pollen from a flower as a protein source for the hive, this activity results in more efficient pollination than the activities of a nectar-collecting worker (Cane and Schiffhauer 2001). Bidirectional selection for the amount of pollen stored in hives showed that the trait is genetically controlled and that selection for more pollen stored in the hive increases the proportion of foragers specializing in pollen collection (Hellmich et al. 1985; Hunt et al. 1995; Page et al. 2000) Genetic markers linked to this trait are available and have been used in a breeding program (Hunt et al. 1995; Page et al. 2000). Breeding for pollen hoarding has not been carried out commercially due to a lack of commercial incentive as pollination services are contracted on a per hive basis without regard for specific genotypes. It is not known whether excessive pollen hoarding may negatively impact nectar collection and may reduce the ability of the bees to respond to resource availability or colony needs.

Table 1 First generation maps constructed in honeybee

Population used	Marker type(s)	Marker number	Coverage (cM)	Marker interval (cM)	References
Arbitrary cross H_1 drones	RAPD STS	365	3,110	9.1	Hunt and Page (1995)
European × Africanized H_1 drones	RAPD STS	Approx. 350	N/A	N/A	Hunt et al. (1998)
European × Africanized H_1 drones	RAPD	N/A	N/A	N/A	Hunt et al. (1999)
Hygenic × non-hygenic H_1 drones	RAPD STS Allozyme	358	3,406.2	9.5	Lapidge et al. (2002)
High pollen hoarding × low pollen hoarding					Rueppell et al. (2004a, b)
HBC (backcross to high line) workers	AFLP STS	387	3,897		
LBC (backcross to low line) workers		396	3,702		
Selected "fast" and "slow" reversal learning lines H_1 drone progeny	RAPD	153 154	2,100 2,650	Approx. 8	Chandra et al. (2001)
Defensive × gentle HBC (backcross to defensive) workers LBC (backcross to gentle line) workers	AFLP	233 326	N/A	N/A	Arechavaleta-Velasco and Hunt (2004)

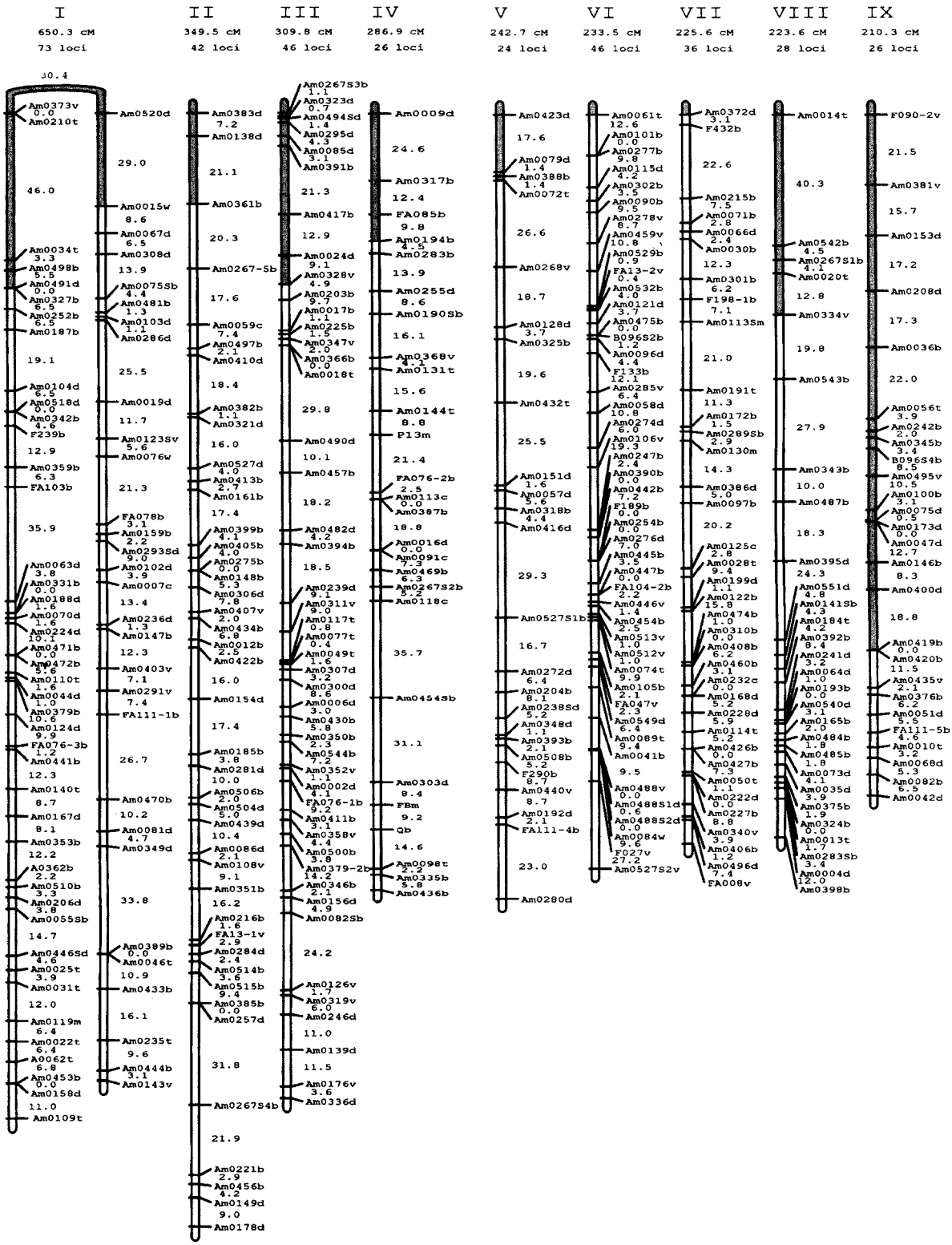
Nectar Collecting

Increased honey collection by individuals seems to correlate genetically with reduced pollen hoarding, but this has not been demonstrated at the level of the colony. Workers of low pollen hoarding strains of bees return to the hive with nectar far more frequently than workers of high pollen hoarding strains (Calderone and Page 1988; Page and Fondrk 1995). This could contribute to greater honey production, but less efficient crop pollination. Strains containing either of these traits could be used for different cropping systems and purposes. Another interesting characteristic of high pollen strains is that they have a faster rate of behavioral development and initiate foraging at a younger age than low pollen strains (Rueppell et al. 2004a). In practical application, queen breeders select for honey production based on colony yields and marker-assisted selection has not been attempted.

Defensive Behavior

One of the most undesirable traits in any commercial apiary is defensive behavior (stinging activity), as an increase in this behavior leads to hives being less manageable by beekeepers. Queen producers have long

Fig. 1 Microsatellite genetic map for the honeybee. The map is composed of 541 markers and 24 linkage groups (I–XXIV), 19 major groups and 5 minute ones. The number of loci and the genetic length are indicated above every group. See Solignac et al. (2004) for nomenclature of markers. The Kosambi distance is indicated between two adjacent markers. The *shaded section* in the top part of most of the groups indicates the position of the centromeric region. Reproduced with permission from Solignac et al. (2004)


I
650.3 cM
73 loci
II
349.5 cM
42 loci
III
309.8 cM
46 loci
IV
286.9 cM
26 loci
V
242.7 cM
24 loci
VI
233.5 cM
46 loci
VII
225.6 cM
36 loci
VIII
223.6 cM
28 loci
IX
210.3 cM
26 loci

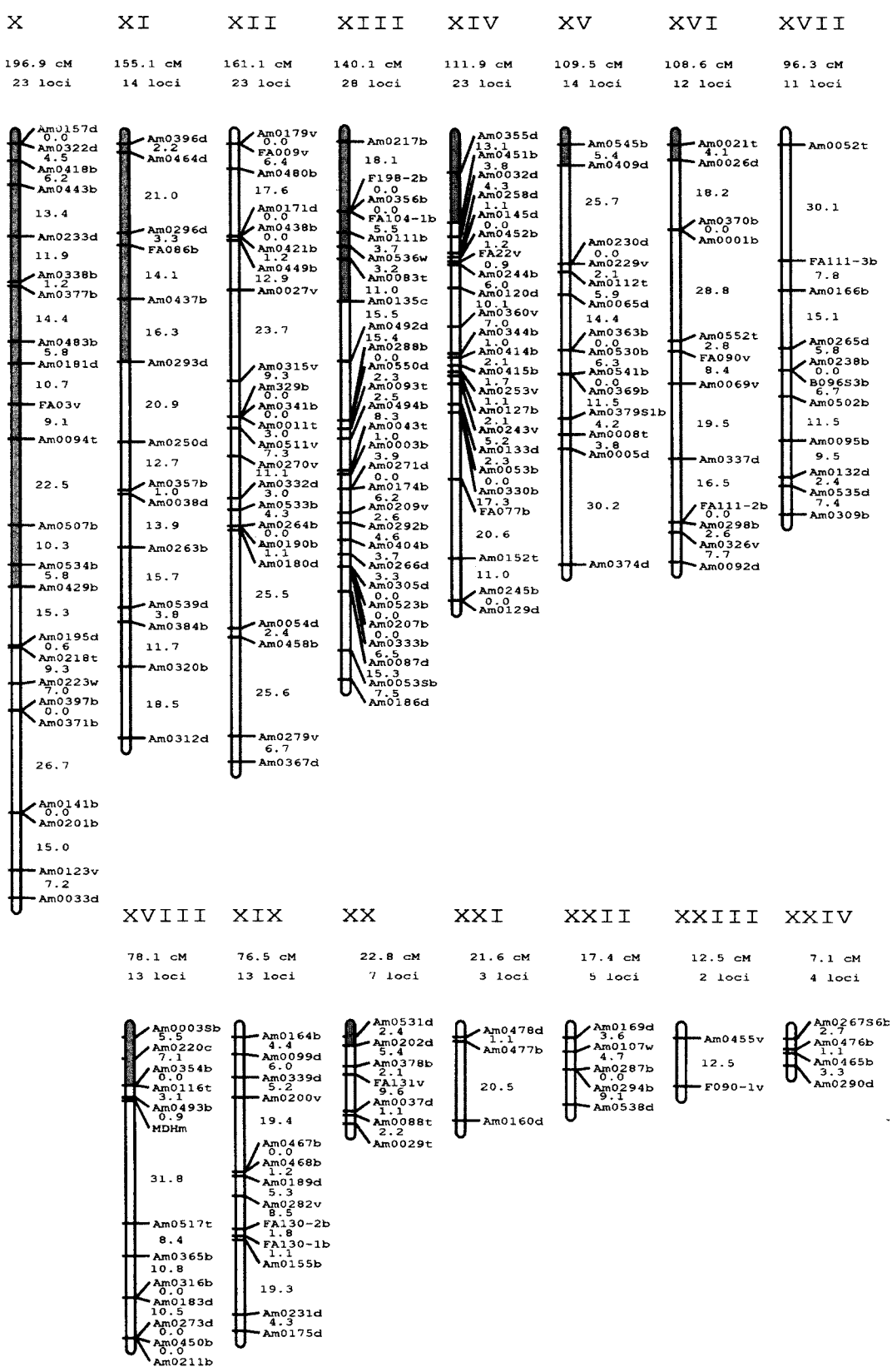


Fig. 1 (continued)

advertised the gentleness of colonies produced from their Queens. As with selection for honey production, the "breeding" efforts of the commercial queen producers have largely consisted of culling colonies from the queen-rearing and drone-producing apiaries that exhibit excessive defensive behavior. Heightened concern about defensive behavior in the Americas has resulted from the spread of Africanized bees that exhibit such strong defensive behavior that they constitute a public health hazard. DNA markers are available to assist with selection against the defensive behavior of Africanized bees but have not yet been put into commercial use (Hunt et al. 1998). Recently, it was shown that the excessive stinging response of Africanized bees shows a paternal effect because hybrids of African paternity were much more defensive than hybrids with European paternity (Guzmán-Novoa et al. 2005). Paternal effects on stinging behavior increase the need for providing gentle drone stocks near mating yards.

Other Traits

A range of additional traits varies between subspecies and could be incorporated into a breeding program. As well as the traits listed above, subspecies also vary in tendency to swarm, ability to survive in extreme climates, and the numbers of queens which they will produce. Each of these traits is potentially of commercial interest.

1.1.4 Classical Breeding Achievements

Some classical breeding achievements have been summarized by Kulincevic (1986). Generally, there are two types of breeding programs, the first of which includes small pioneering projects usually undertaken by research scientists to investigate the genetic basis of specific traits of interest in carefully defined populations that are genetically controlled by artificial insemination (Hunt et al. 1995; Rueppell et al. 2004a). Programs designed to identify DNA markers linked to traits are discussed later in this chapter. As scientific exercises, these projects have been highly successful. However, maintaining genotypes in a free-ranging feral (rather than domesticated) species like the honeybee takes a considerable amount of effort. For this reason very few projects using molecular markers have translated into commercial successes. Perhaps one example, however, is the use of mitochondrial DNA assays combined with selection for larger size of wings, which reduced defensiveness in an Africanized area (Guzmán-Novoa and Page 1999). The second type of breeding program usually consists of field selection for useful traits in commercial breeding programs involving large number of colonies in isolated apiaries from which all but the most promising colonies have been culled. Commercial queen production ultimately relies on open mating of queens to achieve optimal insemination and maximal genotypic diversity in resulting colonies through multiple mating. Some level of control over mating is achieved by inducing elite colonies to produce a larger than normal number of drones in an attempt to saturate drone congregation areas where the queens will mate with improved genotypes. Drone production is readily achieved by putting "drone comb" in the brood chamber of the colonies. Drone comb has a larger cell size than combs in which workers are reared. The slightly larger cells cause the queen to lay unfertilized eggs that develop into haploid drones. Traditional commercial breeding programs are generally poorly or only anecdotally documented (Kulincevic 1986). Such programs have included development of the "Buckfast bee " by "Brother Adam" (born Karl Kehrle) who for many years was in charge of beekeeping at Buckfast Abbey in England. The Buckfast bee is a hybrid bee that includes among its desirable attributes resistance to acarine mites that had decimated populations in the British Isles in the early twentieth century. Breeder queens are usually produced by artificial insemination, and methods have been developed to minimize the occurrence of shot brood in closed population breeding programs (Laidlaw and Page 1986). Daughter queens from the breeders are open mated for sale to beekeepers. Another commercial breeding objective is to produce bees that exhibit hybrid vigor as with crosses between somewhat inbred stocks, as has been done to create the "Starline" and "Midnight" hybrids (Witherell 1976). Many of these commercially available stocks have certain desirable traits, such as resistance to foulbrood diseases and higher honey production, but such traits must be maintained by repeated culling of substandard queens. Breeders often lose these traits in succeeding generations due to

outbreeding (Page and Laidlaw 1992). Less sophisticated programs that are almost universally adopted by queen producers involve identifying the best honey producing colonies from throughout their operations. These colonies are then moved to a queen production apiary. Before such colonies are accepted for queen production, however, they are usually evaluated for reduced defensive behavior. This usually is achieved by the experience of the queen breeder in the tendency of the bees to sting during colony manipulations. Increasingly, however, breeders are also concerned with traits such as hygienic behavior and mite resistance.

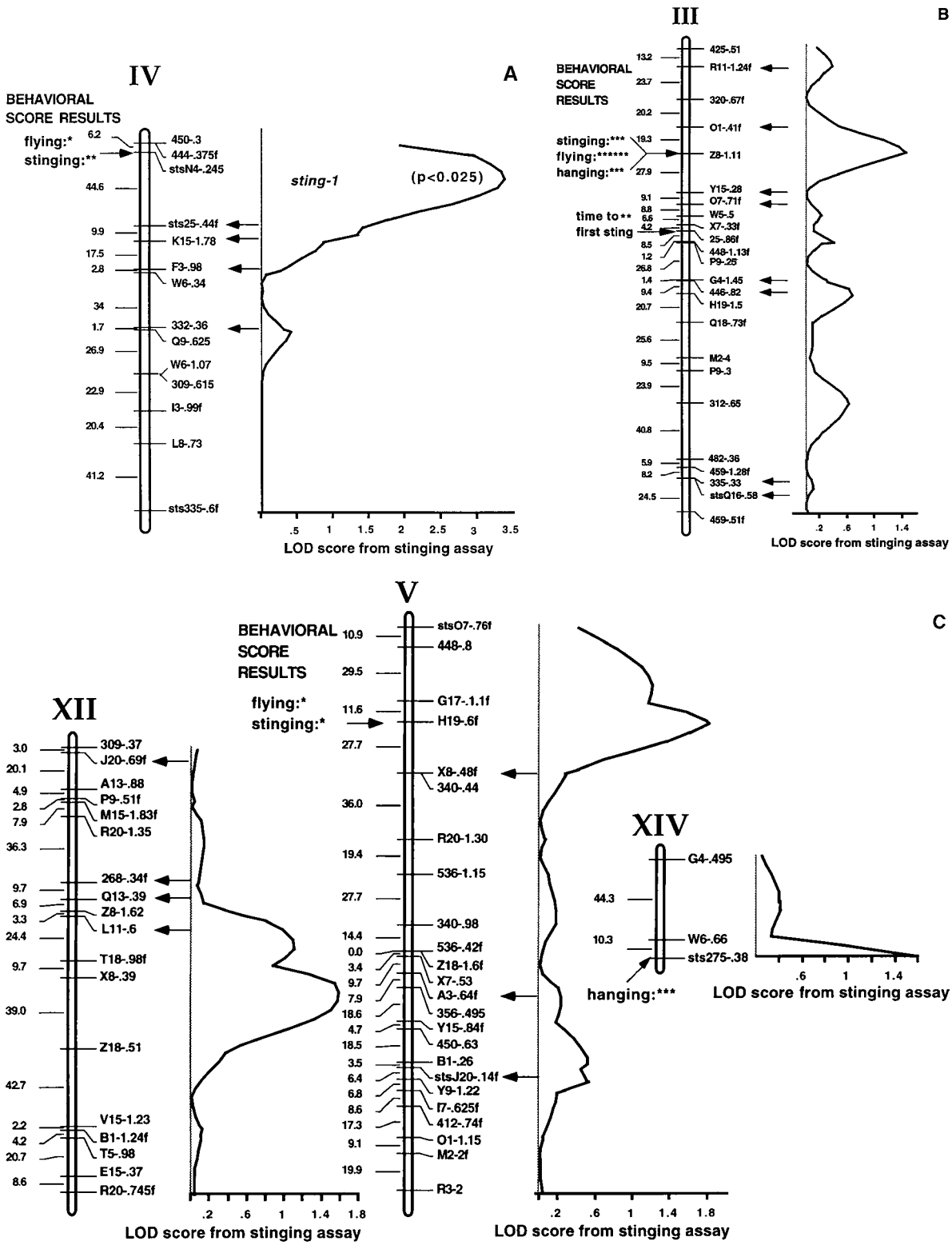
1.1.5 Limitations of Classical Endeavors and Utility of Molecular Mapping

Sophisticated bee breeding is extremely difficult, primarily because honeybees suffer from inbreeding depression. The severity of inbreeding depression, coupled with the inability to maintain lines under controlled conditions makes it nearly impossible to maintain stable, improved, highly inbred lines. Artificial insemination can be used in the development of improved lines, but it is time consuming and requires a degree of skill and specialized equipment which makes it relatively costly. Artificial insemination is also inadequate for the commercial production of highly productive, mated queens. Ultimately, commercial apiaries rely on queens that are naturally mated on the wing. Honey production, provision of pollination services, and even queen production are labor-intensive, low profit margin activities. The economic reality, together with the breeding difficulties as just outlined, limits the application of classical genetics to bee improvement. Molecular tools have been effective in following introgression of Africanized DNA into commercial stocks (Suazo et al. 2001). In addition, mitochondrial DNA combined with selection for larger wings, which are characteristic of European subspecies has been used to breed less defensive bees without instrumental insemination in areas experiencing Africanization (Guzmán-Novoa and Page 1999) but this has required considerable effort and has been driven by a very significant perceived benefit (Guzmán-Novoa and Page 1994). This example does demonstrate that application of modern genetic tools can be used effectively to enhance what would otherwise be limited to a casual selection program. The example also demonstrates success at breeding for behavioral characteristics, which can be extremely plastic selection targets. The breeding effort was also successful despite the tremendous capacity of Africanized colonies to produce swarms which complicate genotype maintenance given the requirement for open mating during queen production. It is anticipated that the recently completed characterization of the honeybee genome will provide a wealth of potential genetic breeding targets that may ultimately be effective in limiting problems currently being encountered with pests and pathogens. Only the most sophisticated queen producers will be able to integrate molecular breeding programs into their queen production efforts. The difficulty of distributing improved breeding stocks, a trivial endeavor in plant or animal breeding, will favor close linkages between research laboratories and progressive queen producing establishments.

1.2 Construction of Genetic Maps

Honeybees have an unusually high recombination frequency (approximately 40 kb/cM) (Hunt and Page 1995), which explains why early efforts relying on

Fig. 2 Possible quantitative trait loci influencing honeybee colony stinging response. (**A**) Sting-1 on linkage group IV. The significance level determined by permutation tests appears near the peak logarithm of the odds (LOD) score. (**B**) A possible QTL on linkage group III. (**C**) Other possible QTLs affecting colony stinging behavior. Behavioral scores (tendency to fly up, hang from comb, and to sting) were made during colony inspections. Kruskal-Wallis tests at marker loci were performed to evaluate effects of QTLs on these behavioral scores ($P < 0.05^{*}$; $P < 0.01^{**}$; $P < 0.005^{***}$; $P < 0.0001^{******}$). Linkage group numbers are from Hunt and Page 1995. RAPD markers are designated by the primer names followed by approximate size of the marker fragment. Markers indicated by *arrows* were common to both linkage maps. Sequences for the sequence-tagged sites are designated by the "sts" prefix. Results shown are the output of multiple QTL model interval mapping (MQM). Reproduced with permission from Hunt et al. (1998)


A
IV
BEHAVIORAL
SCORE RESULTS
flying:*
stinging:**
6.2
450-.3
444-.375f
stsN4-.245
44.6
9.9
sts25-.44f
K15-1.78
17.5
2.8
F3-.98
W6-.34
34
1.7
332-.36
Q9-.625
26.9
W6-1.07
22.9
309-.615
I3-.99f
20.4
L8-.73
41.2
sts335-.6f
sting-1
(p<0.025)
.5 1 1.5 2 2.5 3 3.5
LOD score from stinging assay
B
III
BEHAVIORAL
SCORE
RESULTS
stinging:***
flying:******
hanging:***
time to**
first sting
13.2
425-.51
23.7
R11-1.24f
320-.67f
20.2
O1-.41f
19.3
Z8-1.11
27.9
9.1
Y15-.28
8.8
O7-.71f
6.6
W5-.5
4.2
X7-.33f
8.5
25-.86f
1.2
448-1.13f
26.8
P9-.25
1.4
G4-1.45
9.4
446-.82
20.7
H19-1.5
Q18-.73f
25.6
9.5
M2-4
P9-.3
23.9
312-.65
40.8
5.9
482-.36
8.2
459-1.28f
335-.33
24.5
stsQ16-.58
459-.51f
.2 .6 1 1.4
LOD score from stinging assay
C
V
BEHAVIORAL
SCORE
RESULTS
flying:*
stinging:*
10.9
stsO7-.76f
448-.8
29.5
11.6
G17-.1.1f
H19-.6f
27.7
X8-.48f
340-.44
36.0
R20-1.30
19.4
536-1.15
27.7
340-.98
14.4
0.0
536-.42f
3.4
Z18-1.6f
9.7
X7-.53
7.9
A3-.64f
18.6
356-.495
4.7
Y15-.84f
18.5
450-.63
3.5
B1-.26
6.4
stsJ20-.14f
6.8
Y9-1.22
8.6
I7-.625f
17.3
412-.74f
9.1
O1-1.15
M2-2f
19.9
R3-2
.2 .6 1 1.4 1.8
LOD score from stinging assay
XII
3.0
309-.37
20.1
J20-.69f
4.9
A13-.88
2.8
P9-.51f
7.9
M15-1.83f
R20-1.35
36.3
9.7
268-.34f
6.9
Q13-.39
3.3
Z8-1.62
24.4
L11-.6
9.7
T18-.98f
X8-.39
39.0
Z18-.51
42.7
2.2
V15-1.23
4.2
B1-1.24f
20.7
T5-.98
E15-.37
8.6
R20-.745f
.2 .6 1 1.4 1.8
LOD score from stinging assay
XIV
44.3
G4-.495
10.3
W6-.66
sts275-.38
hanging:***
.6 1 1.4
LOD score from stinging assay

phenotypic markers for linkage analysis produced few examples of linked loci (Tucker 1986). The first proper genetic linkage map was produced by Hunt and Page (1995) using 365 random amplified polymorphic DNA (RAPD) markers. They used a population of haploid male progeny of a single female F_1 queen bee, which they called the H_1 progeny. More recently, other maps have been produced, mostly using haploid drone progeny of an F_1 queen (H_1), while others have used a backcross to generate maps and analyze quantitative trait loci (QTLs) for various behavioral traits. These maps are summarized in Table 1. Most recently, second-generation high-resolution maps that use markers of known sequence have been constructed by Solignac et al. (2004) and Hunt (http://www.ncbi.nlm.nih.gov/mapview/static/beesearch.html#HUNT). The map produced by Solignac et al. (2004) (Fig. 1) is a high-density, saturated map consisting of 541 loci, 474 of which were microsatellite markers and additional sequence-tagged site (STS) markers with three sex-linked markers (Q and FB loci and one RAPD band). It consists of 24 linkage groups with average interval between markers of 7.5 cM and a total length of at least 4,381 cM. The population used for this linkage analysis was different from most of the other maps previously produced, in that they used genotypes from the worker progeny of two back-crossed queens, which were multiple mated. They also used Carthagene software for linkage detection. The map has recently been greatly expanded to include about 2,000 microsatellite markers and has been integrated into the Honeybee Genome Project (HBGP) and used as the scaffold for building the physical map of the genome. The Hunt map has also been integrated with the Honeybee Genome Project and was constructed using 1,063 markers, consisting of 628 amplified fragment length polymorphism (AFLP), 45 sequence-tagged sites (STS), 342 RAPD, and 48 microsatellite markers. The AFLP map was integrated with the genomic sequence with 300 marker sequences obtained by cloning and sequencing marker fragments, as well as the microsatellites shared by the AFLP and microsatellite maps. The map consists of 25 linkage groups with an average interval between markers of approximately 4.7 cM and a total length of 4,996 cM.

1.3 Gene Mapping

There have been a few genes mapped in the various breeding studies to date, a couple that are actually polymorphic markers (one allozyme locus and one morphological locus), several of candidate genes for foraging behavior, and the sex determination locus. These genes are summarized in Table 2. The Honeybee Genome Project (The Honeybee Genome Sequencing Consortium 2006) includes the majority of mapped and annotated genes. For more information on the genome project and genetic resources visit Beebase at http://racerx00.tamu.edu/bee_resources.html.

1.4 Detection of Quantitative Trait Loci

Most QTL studies have looked at colony level traits usually achieved by genotyping haploid drone progeny of an F_1 queen, i.e., the H_1 progeny (Hunt and Page 1995) to make a genetic linkage map. These H_1 progeny are then used as sperm donors for artificial insemination of virgin queen "supersisters" (sharing 75% or more genetic background) from one of the parental lines resulting in backcross hybrid colonies, and the colony can be tested for the trait being studied. Alternatively, worker progeny may be tested for certain individual traits, such as learning and memory (Chandra et al. 2001) or guarding behavior (Arechavaleta-Velasco and Hunt 2004). In this case, the genetic background is reduced by testing and genotyping the worker offspring of a single-drone cross. Often, the various populations are individually bred for a particular study and crossing schemes can be quite complex and tailored to suit the individual research program. A summary of detected QTLs is listed in Table 3 and a typical QTL analysis is shown in Fig. 2.

1.5 Map-based Cloning

Recent map-based cloning efforts include cloning of the complementary sex determination locus (*csd*) by

Table 2 Summary of genes mapped in honeybee

Target trait	Population used	Marker type(s) used	Gene mapped	Gene symbol	Linkage group(s)[a]	Nearest/flanking marker(s)	References
Sex determination	European × African European × European H_1 drone progeny	RAPD STS	X-locus, complementary sex determination locus	*csd*	(3)	stsQ16-.58h (1.6 cM) sts335-.33 (0.6 cM) Q marker (0.3 cM) Z marker (7.9 cM)	Hunt and Page (1995); Beye et al. (1994, 1999, 2003)
Allozyme polymorphism	Arbitrary cross H_1 drones	Allozyme polymorphism RAPD STS	Malate dehydrogenase	*Mdh*	(18)	450-.64 (6.9 cM)	Hunt and Page (1995)
Body color	Arbitrary cross H_1 drones	Morphological RAPD STS	Black body color locus	*blk*	(6)	Q16-.34f (30.3 cM)	Hunt and Page (1995)
Age of first foraging, pollen foraging (*pln2*)	"High" and "low" lines for pollen hoarding HBC, LBC	STS	Tyramine receptor	*tyr*	(10)	sts-tyr	Rueppell et al. (2004a, b)
Age of first foraging, pollen foraging	"High" and "low" lines for pollen hoarding HBC, LBC		*period* ortholog	*Amper*	N/A	sts-per-ecap	Rueppell et al. (2004a, b)
Age of first foraging, pollen foraging	"High" and "low" lines for pollen hoarding HBC, LBC		*foraging* gene, cGMP-dependent protein kinase (PKG)	*Amfor*	N/A	sts-pkg-ecap	Rueppell et al. (2004a, b)

[a] Linkage group numbers in parentheses indicate independent linkage group/chromosome assignment, i.e., may not be the same group in each case

Table 3 Summary of quantitative trait loci (QTL) detected in honeybee

Target trait	Population used	Marker type(s)	Strategy	Software used	QTLs detected	Gene action/ allele effects	Linkage group(s)[a]	Nearest marker(s)	References
Stinging and guarding-defensive behavior	"High" defensive backcross "Low" defensive backcross H_1 drone progeny	RAPD AFLP STS	Composite interval mapping ANOVA BTL	Mapmaker MapQTL QTL Cartographer	*sting1* *sting2* *sting3* 7 other QTL detected for guarding	Stinging/guarding Guarding Guarding	(4) (3) (5)	stsN4-.245 Z8-1.11 H19-.6f A43-.320 A18.155 A23.310 A11.310 A28-.190 A16.175 A16.330b A31.240	Hunt et al. (1998, 2007); Guzmán-Novoa et al. (2002); Arechavaleta-Velasco et al. (2003); Arechavaleta-Velasco and Hunt (2004)
Alarm pheromone levels	European × African backcross H_1 drone progeny	RAPD STS	Multiple-QTL model (MQM)	MapQTL	n-Decyl acetate (1) n-Octanol (3) Isopentyl acetate (4) Hexyl acetate (1)	African allele decreases Decreases Increases Decreases Decreases Increases Decreases Increases Decreases	(11) (11) (13) (3) (11) (9) (13) (1) (9)	460-.67 C2-1.05 C14-2.1f O1-.41f C2-1.05 L18-1.41 425-.3f Q9-.38f G4-1.25	Hunt et al. (1999)
Wing length/ body size	European × African backcross H_1 drone progeny	RAPD STS	MQM, interval mapping	QTL Cartographer	7 QTLs detected	Worker wing length, drone wing length	(6) (12) (2) (2) (11) (10) (1)	R11-.43 T5-.98 Q9-.34 536-.735 460-.67 H7-.5 sts275-.67	Hunt et al. (1998)
Associative learning	H_1 drone progeny	RAPD	Composite interval mapping	MapQTL	*lrn1* *lrn2* *lrn3*	Latent inhibition Reversal learning	(10) N/A N/A	R3-.86 X12-.87 A10-.70	Chandra et al. (2001)

[a] Linkage group numbers in parentheses indicate independent linkage group/chromosome assignment, i.e., may not be the same group in each case

Table 3 (continued)

Target trait	Population used	Marker type(s)	Strategy	Software used	QTLs detected	Gene action/ allele effects	Linkage group(s)[a]	Nearest marker(s)	References
Foraging, pollen hoarding, sucrose responsiveness	"High" line "Low" line HBC, LBC	AFLP STS RAPD	Interval mapping ANOVA	Mapmaker3.0b QTL Cartographer MapQTL4.0	*pln1* *pln2* *pln3* *pln4*	Complex interaction of: nectar concentration, nectar-load size, pollen-load size, pollen proportion, sucrose response (PER)	(2) (10) (6) N/A	stsD8-.33f sts-tyr Y15, sts-q4-ecap sts-pkg-ecap, (Amfor)	Hunt et al. (1995, 2007); Page et al. (2000); Rueppell et al. (2004a, b, 2006)
Hygienic behavior	H_1 drone progeny	RAPD STS Allozyme	Interval mapping	Mapmaker3.0	7 QTLs detected	Uncapping, removal of brood	(2) (4) (5) (6) (13) (15) (22)	336_0.71 397_0.9 242_0.24 172_0.5f 395_0.7 G17_1.0 123_1.0	Lapidge et al. (2002)
Age of first foraging	"High" and "low" lines for pollen hoarding HBC, LBC	AFLP STS	Interval mapping ANOVA	Mapmaker3.0b MapQTL4.0 QTL Cartographer	*aff1* *aff2* *aff3* *aff4*	Behavioral maturation	N/A	E1M4270 E6M5259 E2M9126 E6M8348	Rueppell et al. (2004a, b)
Sex determination (X-locus)	European × African European × European H_1 drone progeny	RAPD STS Micro-satellite	Restriction mapping, linkage mapping	Mapmaker	*csd*	Sex determination	(3)	*csd* (gene cloned)	Beye et al. (1994, 1999, 2003)

Beye et al. (2003). Another attempt to clone a QTL was by Lobo et al. (2003) in which an 81-kb bacterial artificial chromosome (BAC) clone was identified by hybridization of a sequenced marker associated with the *sting-2* locus for aggressive behavior to a BAC library (Tomkins et al. 2002). In the sequenced BAC clone there were 13 putative genes predicted by *ab initio* techniques and two transcripts present in the honeybee EST database. All but one of the putative genes were confirmed to be expressed in the honeybee brain by reverse transcription analysis of mRNA from honeybee heads, and they lacked similarity to any known invertebrate or vertebrate proteins or transcripts.

With the advent of the Honeybee Genome Project (HBGP), it is expected that map-based cloning efforts will become easier and shall increase in frequency (The Honeybee Genome Sequencing Consortium 2006). Already, the genome sequence has been used to identify candidate genes for QTLs that influence division of labor in foraging (pollen versus nectar), and also for defensive behavior. Pollen foragers tend to have more highly developed ovaries than nectar foragers and candidate genes for foraging behavior suggest that pollen foraging is influenced by insulin-like signaling (Amdam et al. 2006; Hunt et al. 2007). The candidate genes identified for defensive behavior QTLs suggest that particular genes involved in sensory tuning and neural development are involved in this behavior (Hunt et al. 2007). These efforts were facilitated by the high recombination rate of the honey bee. Because of high meiotic recombination rates, the confidence intervals for QTL location contained a manageable number of candidate genes (Beye et al. 2006).

1.6 Future Scope of Works

The first phase of the Honeybee Genome Project was completed in 2006. The genome sequences show that the bee is highly polymorphic and many single-nucleotide polymorphisms (SNPs) are present even in coding sequences. The high recombination rate has resulted in a situation in which only about 50 candidate genes are present within 97% confidence intervals for each behavioral QTL region (Hunt et al. 2007). The genome sequence is expected to facilitate many more genetic studies on honeybees ranging from behavioral and developmental, to answering basic questions about the evolution of social behavior. RNA interference (RNAi) and linkage disequilibrium studies could be used to test candidate genes for mapped QTL. The honeybee is unique to other commercially farmed organisms including other insects; their sociality enables them to be excellent scientific research models but their need for genetic variability in order for colonies to survive makes them difficult to breed for commercial purposes. However strains and subspecies have enough variability to overcome any regional challenge.

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2 Bumblebee

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2.1 Introduction

Bumblebees are important natural pollinators of flowers in cool and temperate regions, including many commercial crops (Velthuis and van Doorn 2006). Indeed, they are second only to honeybees as commercial pollinator insects. They are of particular importance for tomatoes and other Solanaceae crops requiring "buzz pollination" as well as for greenhouse crops in general (Dag and Kammer 2001; Morandin et al. 2001). The commercial production of bumblebees has thus developed into a thriving branch of agribusiness, generating an estimated yearly economic value of $1.25 billion in pollination services in the US alone (Ghazoul 2005). Currently, five species of bumblebees are used commercially, *Bombus* and *B. occidentalis* in Northern America, *B. ignitus* and *B. lucorum* in East Asia, and *B. terrestris* in Europe, South America, Asia, and New Zealand. The use of non-native bumblebee species is a major ecological concern (Velthuis and van Doorn 2006). Introduced pollinators could change the local flora, e.g., by increasing the pollination rate of exotic weeds, and threaten native pollinators by increasing competition (Goulson 2003). These concerns are reinforced by results showing that even the introduction of non-native subspecies, such as *B. t. sassaricus* from Southern Europe to Western Europe, could lead to the displacement of native bees (Ings et al. 2005).

In basic research, bumblebees have been intensively studied as model organisms in such fields as behavior, physiology, or foraging strategies (Goulson 2003). *B. terrestris* has been developed as a model organism in ecology and evolution, particularly for the study of host-parasite interactions, ecological immunology, social behavior, and developmental biology (Baer and Schmid-Hempel 1999; Moret and Schmid-Hempel 2000; Lopez-Vaamonde et al. 2004; Pereboom et al. 2005). Due to increased efforts, this species can now also serve as a genetic and genomics model for basic and applied research on bumblebees.

2.1.1 Taxonomic Description

Worldwide, there are some 250 species of bumblebees (Michener 2000), which form the monophyletic genus *Bombus* within the family Apidae (Hymenoptera) (Kawakita et al. 2004). Most bumblebees are primitively eusocial; they live in monogynous colonies with reproductive division of labor and have an annual life cycle. An exception to this rule are some species of the New World subgenus *Fervidobombus*, which show some characteristics of highly eusocial hymenoptera, such as perennial colonies and sophisticated colony defense systems (Cameron and Williams 2003). The subgenus *Psithyrus* contains the cuckoo bumblebees, which are social parasites of primitively eusocial bumblebees. The family Apidae harbors many related species differing in their social systems, ranging from solitary bees to the primitively eusocial bumblebees and the highly eusocial honeybees and stingless bees. Bumblebees can thus be very useful for comparative studies on the evolution of sociality.

2.1.2 The Model Organism *Bombus terrestris*

B. terrestris is one of the most common European bumblebee species in the wild and has been effec-

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W. Hunter, C. Kole (Eds.)

tively domesticated (Velthuis and van Doorn 2006). It can be studied both under natural conditions in the field and under a controlled environment in the laboratory. *B. terrestris* is a ground-nesting primitively eusocial species. After hibernation, an individual single-mated queen (Estoup et al. 1995; Schmid-Hempel and Schmid-Hempel 2000) will found a colony and start producing female workers. Toward the end of the season, she will produce sexuals, including up to several hundred haploid males, if conditions are favorable. Due to the production of large numbers of haploid offspring, *B. terrestris* is particularly amenable to genetic linkage mapping and QTL (quantitative trait loci) studies.

2.1.3 The *B. terrestris* Genome

The haploid karyotype of *B. terrestris* comprises 18 chromosomes, two more than those found in the honeybee *A. mellifera* (Gadau et al. 2001). The physical genome size has been estimated at 625 Mb via flow cytometry using propidium iodide staining and heparinized red blood cells from female chicken as a reference independent from the honeybee (Wilfert et al. 2006). The physical genome size of *B. terrestris* is, therefore, estimated to be 2.7 times larger than that of *A. mellifera*. So far, no mutant markers have been identified in this species.

2.2 Genetic Linkage Maps

2.2.1 Construction of Linkage Maps

In social hymenoptera, the haplodiploid genetic make-up and the potentially large number of progenies of single queens can facilitate genetic linkage mapping. Because the haploid males arise from unfertilized eggs, a mother's meiotic recombination frequency can be directly assessed in her haploid sons. If a queen produces a sufficient number of sons, i.e., more than one hundred, her male progeny can be used as a mapping population. Genotyping this population allows the construction of a genetic linkage map based on the mother's recombination frequency. This approach has been applied successfully in the bumblebee *B. terrestris* (Gadau et al. 2001; Wilfert et al. 2006) and, recently, in the ant *Acromyrmex echinatior* (Sirviö et al. 2006). *B. terrestris* is particularly well-suited for genetic mapping because its colonies are monogynous and can be maintained under controlled conditions in the laboratory. Under optimal conditions and ad libitum food (Gerloff et al. 2003), a queen can produce up to a thousand haploid sons, providing sufficient meiotic information for the construction of high-resolution linkage maps (Hunt and Page 1995). An additional advantage of using haploid mapping populations is that dominant genetic markers can be used without loss of information. This is of particular importance in non-model organisms with little available genetic resources, where the use of dominant informative markers allows the jump-starting of genomic studies.

In large *B. terrestris* colonies, workers will often reproduce by laying male eggs (Alaux et al. 2005). It is important to remove worker-produced males, since they would introduce random variation into the linkage information, which could result in inflated estimates of recombination frequencies. Removing worker-produced males can easily be achieved in this system by using codominant markers, such as microsatellites. In monandrous haplodiploid species, all females receive the single paternal allele. Males have a 50% chance of inheriting the paternal allele. Thus, at any codominant locus at which paternal and maternal alleles differ, on an average 50% of worker-produced males can be distinguished. Wilfert et al. (2006) demonstrated that worker-produced males can be effectively removed from the mapping populations by genotyping several variable codominant markers.

Colonies can be started either by queens collected from the field after hibernation or by queens bred seminaturally in the laboratory. Using mapping populations originating from field-collected queens is particularly rewarding in QTL studies, since this approach potentially reveals genetic variation underlying phenotypic variation of fitness-relevant traits as shaped by natural selection in the wild (Slate 2005). In classical linkage mapping, the phase of alleles, i.e., whether an allele was provided by the F_0-mother or the F_0-father, needs to be known. This information cannot be obtained for field-caught bumblebee queens. Gadau et al. (2001) have developed a phase-unknown

Table 1 Summary of published linkage maps in *B. terrestris*

Population	Marker types	Number of markers	Map size (cM)	Estimated total size (cM)	Coverage (%)	Linkage groups	Marker spacing (cM)	Reference
Sex-1	Microsatellites, RAPDs	76	953	–	34.5	21	12.1	Gadau et al. (2001)
BBM-1	Microsatellites, AFLPs	246	2,222	2,734	80.1	21	10.3	Wilfert et al. (2006)
BBM-2	Microsatellites, AFLPs	124	1,223	2,786	44.3	25	12.5	Wilfert et al. (2006)
BBM-3	Microsatellites, AFLPs, RAPDs	118	1,124	2,761	40.7	30	12.8	Wilfert et al. (2006)

mapping procedure for this system, which has been experimentally validated (Wilfert et al. 2006). Here, linkage mapping is conducted with a doubled data set in which each genetic marker is represented twice in the complimentary phases, which produces two identical, but phase-inverted linkage maps (see Wilfert et al. 2006, for a detailed description). Although this procedure cannot provide information on the alleles' true phase (maternal or paternal), it fixes the linkage phases of the markers within individual linkage groups, which is sufficient information for the construction of linkage maps and for QTL studies.

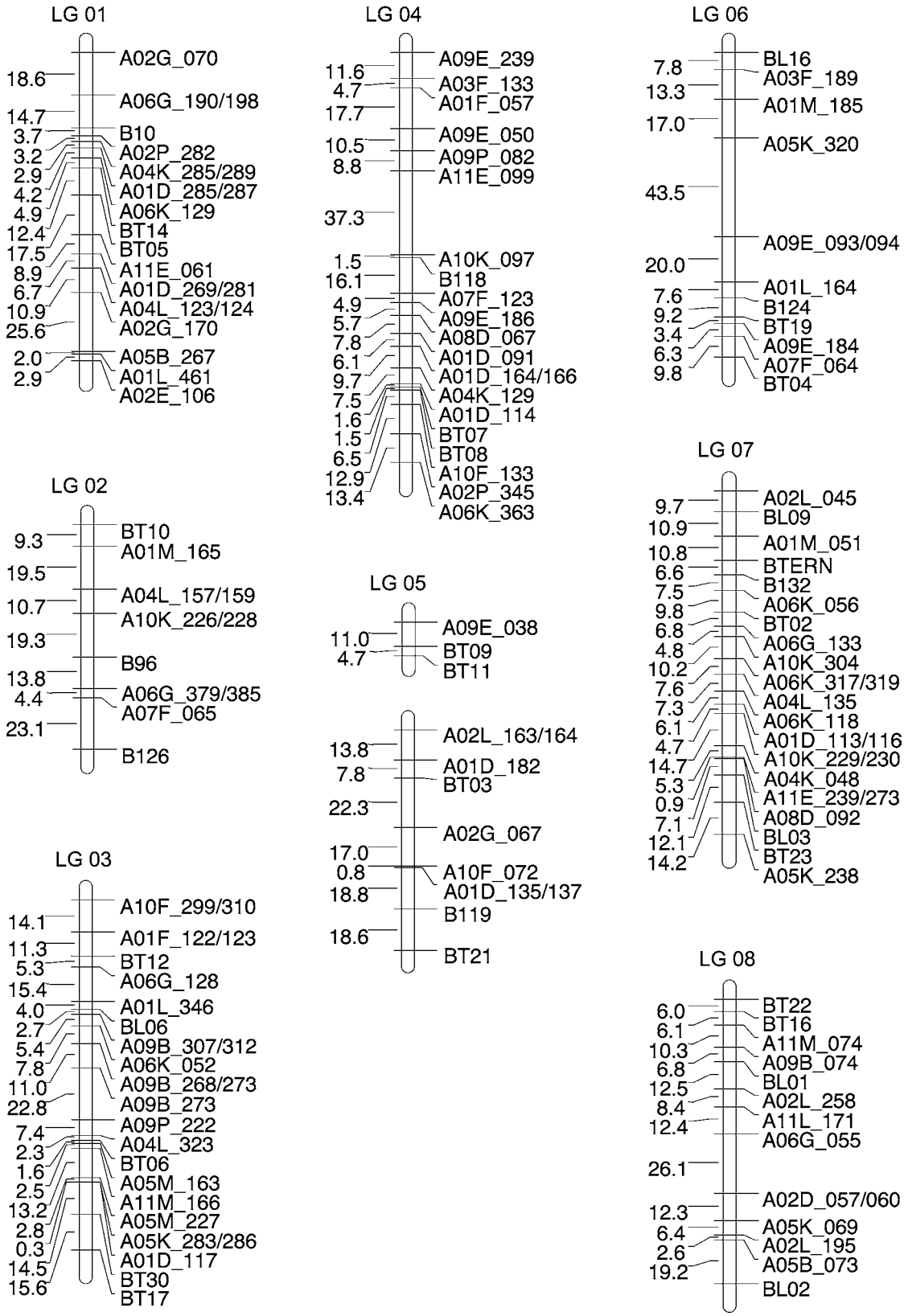
2.2.2 Published Genetic Maps

Four independent genetic linkage maps of *B. terrestris* have been published (see Table 1; Gadau et al. 2001; Wilfert et al. 2006). Using a methods-of-moments approach (Hulbert et al. 1988; Chakravarti et al. 1991), the total recombination genome length of this species has been consistently estimated to be in the range of 2,700–2,800 cM, with a mean of 2,760 cM (Wilfert et al. 2006), resulting in a ratio of 226 kb/cM. The phase-known map BBM-1 (Fig. 1; Wilfert et al. 2006) is nearly saturated, with an average marker spacing of 10.3 cM, covering 80.1% of the estimated total recombination genome. With 21 linkage groups, this map also closely matches the haploid karyotype of *B. terrestris* (18 chromosomes). Wilfert et al. (2006) used recurring groups of microsatellites and potentially homologous amplified fragment length polymorphism (AFLP) markers to extract a core of 14 homologous linkage groups from the three independent linkage maps constructed in their study. The core map can serve as a reference between different mapping populations and experiments. For example, this tool has been used to compare the genetic architecture of host susceptibility and other fitness-relevant traits across dif ferent natural populations (Wilfert et al. 2007a).

2.3 Mapping of Genes and QTLs

Because genetic linkage maps can be generated with ease from mapping populations originating from wild-caught queens, the phenotypic variation of quantitative traits in natural populations can be studied in *B. terrestris*. While QTL studies are widely used in model organisms, they remain exceptional in natural, unmanipulated populations (Slate 2005). The approach adopted here allows uncovering the genetic variation of quantitative traits as relevant to natural

Fig. 1 Phase-known linkage map BBM-1 in *B. terrestris*. Linkage groups LG01 to LG14 have been homologized between three independent maps and can therefore be used as a reference tool for comparative studies. The non-homologized linkage groups are referred to as BB1_15 to BB1_20. Modified after Wilfert et al (2006)


LG 01
A02G_070
18.6
A06G_190/198
14.7
B10
3.7
A02P_282
3.2
A04K_285/289
2.9
A01D_285/287
4.2
A06K_129
4.9
BT14
12.4
BT05
17.5
A11E_061
8.9
A01D_269/281
6.7
A04L_123/124
10.9
A02G_170
25.6
A05B_267
2.0
A01L_461
2.9
A02E_106
LG 02
BT10
9.3
A01M_165
19.5
A04L_157/159
10.7
A10K_226/228
19.3
B96
13.8
A06G_379/385
4.4
A07F_065
23.1
B126
LG 03
A10F_299/310
14.1
A01F_122/123
11.3
BT12
5.3
A06G_128
15.4
A01L_346
4.0
BL06
2.7
A09B_307/312
5.4
A06K_052
7.8
A09B_268/273
11.0
A09B_273
22.8
A09P_222
7.4
A04L_323
2.3
BT06
1.6
A05M_163
2.5
A11M_166
13.2
A05M_227
2.8
A05K_283/286
0.3
A01D_117
14.5
BT30
15.6
BT17
LG 04
A09E_239
11.6
A03F_133
4.7
A01F_057
17.7
A09E_050
10.5
A09P_082
8.8
A11E_099
37.3
A10K_097
1.5
B118
16.1
A07F_123
4.9
A09E_186
5.7
A08D_067
7.8
A01D_091
6.1
A01D_164/166
9.7
A04K_129
7.5
A01D_114
1.6
BT07
1.5
BT08
6.5
A10F_133
12.9
A02P_345
13.4
A06K_363
LG 05
A09E_038
11.0
BT09
4.7
BT11
A02L_163/164
13.8
A01D_182
7.8
BT03
22.3
A02G_067
17.0
A10F_072
0.8
A01D_135/137
18.8
B119
18.6
BT21
LG 06
BL16
7.8
A03F_189
13.3
A01M_185
17.0
A05K_320
43.5
A09E_093/094
20.0
A01L_164
7.6
B124
9.2
BT19
3.4
A09E_184
6.3
A07F_064
9.8
BT04
LG 07
A02L_045
9.7
BL09
10.9
A01M_051
10.8
BTERN
6.6
B132
7.5
A06K_056
9.8
BT02
6.8
A06G_133
4.8
A10K_304
10.2
A06K_317/319
7.6
A04L_135
7.3
A06K_118
6.1
A01D_113/116
4.7
A10K_229/230
14.7
A04K_048
5.3
A11E_239/273
0.9
A08D_092
7.1
BL03
12.1
BT23
14.2
A05K_238
LG 08
BT22
6.0
BT16
6.1
A11M_074
10.3
A09B_074
6.8
BL01
12.5
A02L_258
8.4
A11L_171
12.4
A06G_055
26.1
A02D_057/060
12.3
A05K_069
6.4
A02L_195
2.6
A05B_073
19.2
BL02

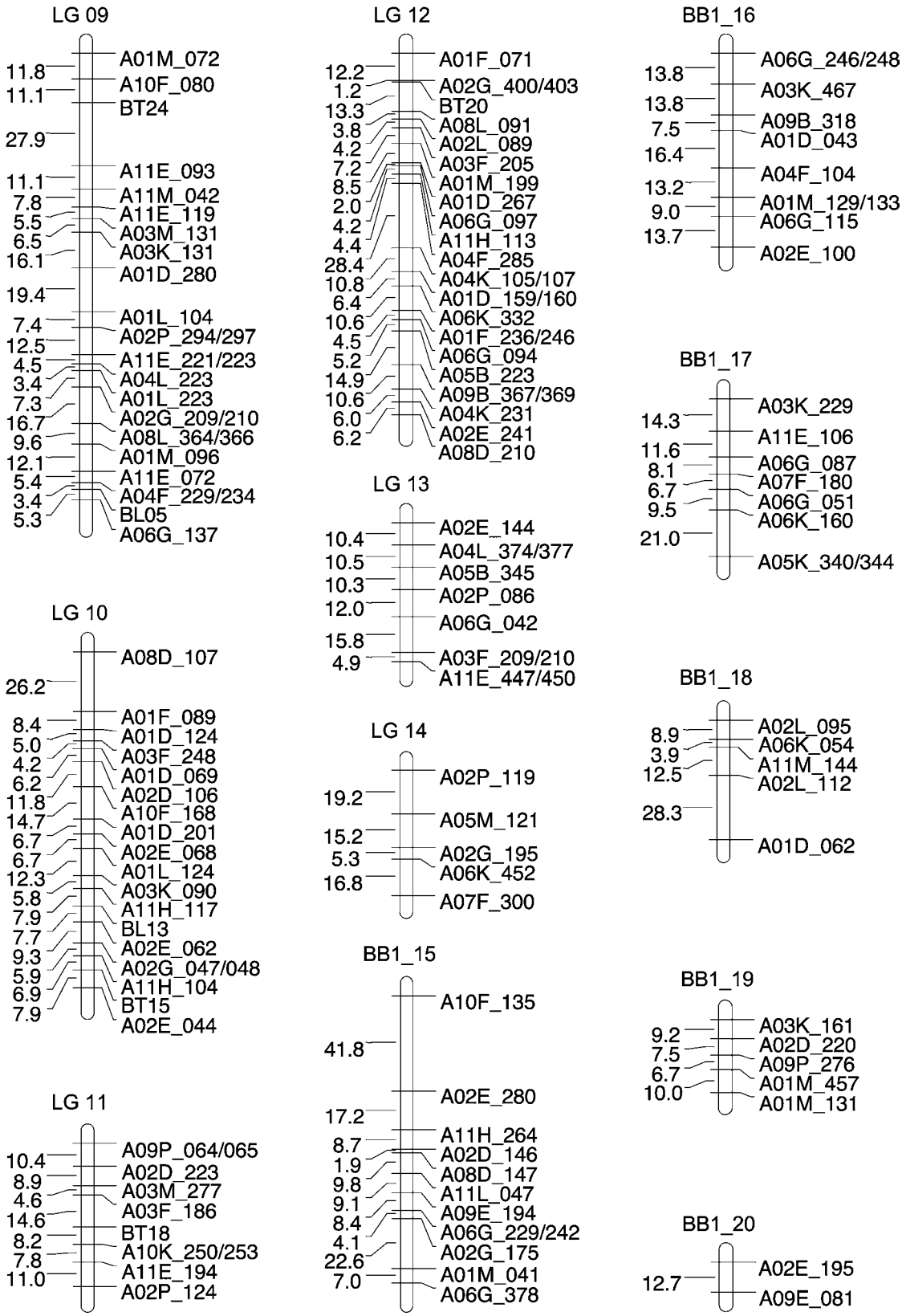


Fig. 1 (continued)

Table 2 Summary of QTL studies in *B. terrestris* (MapQTL was used for all QTL analysis)

Trait	Population	Number of QTL	Number of pair-wise epistatic interactions	Reference
Host susceptibility	BBM-1	2	3	Wilfert et al. (2007a)
	BBM-2	2	3	
	BBM-3	2	3	
Body size	BBM-1	6	5	Wilfert et al. (2007a)
	BBM-2	4	1	
Immune defense traits:				
Encapsulation	BBM-1	3	3	Wilfert et al. (2007a)
	BBM-3	2	3	
PO-activation	BBM-1	1	4	Wilfert et al. (2007b)
Zone of inhibition				Wilfert et al. (2007b)
– *Arthrobacter globiformis*	BBM-1	2	1	Wilfert et al. (2007b)
– *Paenibacillus alvei*	BBM-1	2	1	Wilfert et al. (2007b)
Male sexual traits:				
Accessory gland size	BBM-1	7	3	Wilfert et al. (2007b)
Sperm number	BBM-1	1	2	Wilfert et al. (2007b)
Sperm length	BBM-1	0	4	Wilfert et al. (2007b)

selection and studying the distribution of QTL effects within a population. Another important aspect is that epistatic interactions can be easily identified using a haploid mapping population. Firstly, there are no obscuring effects of dominance. Secondly, when analyzing pair-wise interactions, only four allele combinations have to be compared, providing high power for the detection of epistatic interactions even with limited sample sizes. The results of QTL studies are given in Table 2.

2.3.1 Host Susceptibility

The genetic basis of the susceptibility of *B. terrestris* to its trypanosome gut parasite *Crithidia bombi* has been studied in three natural mapping populations. This host-parasite model system shows strong genotype-by-genotype interactions (e.g., Schmid-Hempel et al. 1999). Therefore, this is an interesting system for studying the variation of genetic architecture across populations. The authors found a generally similar genetic basis of this trait in different populations – few minor additive QTLs and an important contribution of epistatic interactions – but little sign of recurring loci (Wilfert et al. 2007a). These QTLs are the starting point for developing informative markers that may allow alleles to be tracked in natural populations. The QTLs promise to provide stringent tests for predictions that have so far not been amenable to testing (Little 2002), such as the occurrence of negative frequency-dependent selection inherent in the Red Queen hypothesis (e.g., Hamilton et al. 1990).

2.3.2 Immune Defense

The encapsulation response is a general insect immune defense mechanism, which has been implied in the defense against gut parasites (Kaslow and Welburn 1996). Evidence for a genetic association of *Plasmodium* refractoriness and encapsulation has been found in *Anopheles gambiae* mosquitoes (Gorman et al. 1997; Zheng et al. 1997, 2003). Wilfert et al. 2007a) and has shown that there is a significant overlap in the genetic architecture of susceptibility to *C. bombi* and the strength of the encapsulation reaction in the two studied mapping populations. No such association was found with the simultaneously measured trait of body size.

Trade-offs between costly phenotypic traits have been cited as a mechanism for the maintenance of genetic variation in the underlying genes (e.g., Siva-Jothy et al. 2005). Wilfert et al. (2007b) have analyzed the genetic architecture of immune and sexual investment traits in male bumblebees. The genetic basis of the traits was generally characterized by minor QTLs and several epistatic interactions. There was no evidence of trade-offs between investment in immune defense and reproduction. On the contrary, the authors found a positive correlation between the hemolymph's antibacterial activity and the number of stored sperm, a potential sexual investment trait. They also found a negative correlation, possibly indicating a trade-off, between two immune traits. Both phenotypic correlations were backed by a significant overlap in their genetic architecture.

2.3.3 Sex Locus

In many hymenopteran species sex is allocated via a complementary sex determination system (CSD; Cook 1993). In this model individuals who are haploid (= hemizygous) or homozygous at the CSD develop the male phenotype, whereas heterozygotes develop as females (Whiting 1943). Empirical evidence from breeding experiments indicates a single locus CSD in *B. terrestris* (Duchateau et al. 1994). The finding was supported by bulked segregant analysis, which found only one genetic locus responsible for sex allocation (Gadau et al. 2001). Single locus CSD has been proven in the honeybee *A. mellifera*. The sex locus region has been fine mapped (Hasselmann et al. 2001) and the *csd* gene has been identified (Beye et al. 2003). These findings will allow further comparative studies of sex determination in honeybees and bumblebees.

2.4 Available Techniques and Resources

Although so far comparatively few genomic tools are available, *B. terrestris* is developing into a model organism for ecological genetics and genomics. To advance genetic studies, a large number of microsatellite loci are being developed (R.F.A. Moritz 2006, personal communication). These markers will be incorporated into the core linkage map (Wilfert et al. 2006). A bacterial artificial chromosome (BAC) library with sixfold coverage has been constructed (Wilfert et al., unpublished data). The library is currently being screened for candidate genes and is being used for further development of informative markers linked to the QTLs. Gene expression has been the focus of research into the regulation of caste determination; for these studies, cDNA libraries and subtractive libraries enriched for larval development genes have been produced (Pereboom et al. 2005).

Genetic and genomic research on bumblebees profits greatly from developments in the honeybee *A. mellifera*. For example, sequence homology is great enough to allow cross-hybridization of bumblebee cDNA to honeybee macroarrays (L. Wilfert, personal observation, 2003). The honeybee genome has been sequenced and annotated (The Honeybee Genome Sequencing Consortium 2006). This resource has been successfully used for the isolation of candidate genes in *B. terrestris* (R. Schmid-Hempel, personal communication 2006). While the *A. mellifera* genome represents a useful resource, basic and applied research in *B. terrestris* as well as comparative studies across the Apoidea would profit greatly if its genome would be sequenced.

2.5 Future Scope of Works

B. terrestris is an ecological and evolutionary model organism and, since it lends itself with ease to genetic and genomic studies, it is poised to become even more important for basic research in the future. Two exemplary areas of research are the innate immune system and the co-evolution of host-parasite interactions. Both these fields are at the center of basic research; host-parasite co-evolution, for example, is considered as a potential cause for the maintenance of sexual reproduction and recombination (e.g., Hamilton et al. 1990). Innate immunity is the first line of defense of any organism against parasites and pathogens, yet its studies in vertebrates are obscured by the adaptive immune system. The insect immune system, in particular in the bumblebee *B. terrestris*, can thus serve as a model for innate immune systems in general.

As bumblebees become ever more important in agriculture, understanding their host-parasite interactions becomes economically relevant. In this respect, genomic techniques such as QTL mapping may be of practical interest for marker-assisted selection, including the selection for disease resistance in livestock (Andersson 2001). The approach relies on breeders genotyping their stocks for markers associated with relevant QTLs, e.g., resistance to pathogens or product quality, in order to monitor and increase the breeding success (see Collard et al. 2005). So far, little effort has been invested into increasing the agricultural value of bumblebees, e.g., their pollinating efficiency. With the ongoing domestication of bumblebees and their increased mass-rearing, infectious diseases are an emerging agricultural problem. For example, the commercial rearing of *B. occidentalis* in western North America has been set back due to heavy infestations by the microsporidian parasite *Nosema bombi* (Velthuis and van Doorn 2006). In such cases, selective breeding for parasite resistance may represent a practicable resort. Classical selection in bumblebees is at the moment prohibitively difficult and time-intensive. Particularly, the single locus complementary sex determination system limits selection since inbreeding will result in a high proportion of colonies producing sterile diploid males (Duchateau et al. 1994; Zayed 2004). These experimental difficulties could be by-passed by the use of marker-assisted breeding programs. Genetic and genomics studies may thus allow us to profit even more from this efficient and versatile pollinator.

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3 The Jewel Wasp – *Nasonia*

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3.1 Introduction

Nasonia belongs to a large and diverse group of insects, the parasitoid Hymenoptera. Adult parasitoid wasps are free-living and lay their eggs in or on various life stages of arthropods (e.g., insects, ticks, mites). Unlike parasites, their larvae develop from the nutrients of a single host organism, either externally or internally, and thereby kill the host (Grimaldi and Engel 2005). Most arthropods are attacked by multiple parasitoid wasp species, which are typically specialized on different life stages of the host (e.g., egg, larva, pupa). As a consequence, parasitoids are arguably the most important biological regulators of arthropod populations in nature.

Parasitoid wasps include more species beneficial to humans than any other insect group. Since parasitoid Hymenoptera are known to attack significant vectors of human diseases, such as house flies, roaches, and ticks, and pests of agricultural importance (Quicke 1997), they received great attention from areas such as applied entomology and integrated pest management. They are important regulators of agricultural pests and as such have a major impact on human food resources. Their impact on humans by reducing the destruction of agricultural products is indeed staggering. In the US alone, biological control programs using parasitoid wasps are estimated to save approximately $20 billion dollars annually by reducing crop losses caused by new invasive species (K. Hackett, USDA personal communication). While this number does not include the vast savings incurred from the biological control of other native pests, biological control programs save billions of dollars a year and reduce environmental risks during the management of agricultural pests. Direct benefits to human health derived from the use of parasitoid Hymenoptera, which regulate native and invasive pest species, are the reduction in the number of applications of pesticides, thus improving water and crop qualities that are directly linked to improved human health.

In recent years, *Nasonia* has become an important model system for evolutionary, ecological, and developmental genetics. Examples include studies of sex ratio (Werren 1980; Reece et al. 2004; Shuker and West 2004; Shuker et al. 2005), sex determination (Trent et al. 2006; Beukeboom 1995; Beukeboom et al. 2007), speciation (Breeuwer and Werren 1995; Gadau et al. 1999), genetic conflict (Werren et al. 1987; Nur et al. 1988; Beukeboom and Werren 1992), development (Pultz et al. 2000; Pultz and Leaf 2003; Lynch et al. 2006), behavior (van den Assem 1986; Beukeboom and van den Assem 2001), morphological evolution (Weston et al. 1999; Gadau et al. 2002), and symbiosis (Breeuwer and Werren 1990; Bordenstein et al. 2001). Due to haplodiploid sex determination (males are haploid and females are diploid), *Nasonia* spp. are excellent organisms for investigating the genetics of complex traits. This is because the interactions between genes influencing a male trait can be investigated without the added complexity of dominance interactions. Finally, these insects are excellent laboratory organisms, with short generation time (approximately two weeks at 25 °C) and ease of rearing. *Nasonia* has been studied genetically for over 50 years

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W. Hunter, C. Kole (Eds.)

and several visible marker-based linkage maps have been developed (summarized in Saul 1993). Many *Nasonia vitripennis* lines with visible mutation are kept in culture in the laboratories of J.H. Werren (University of Rochester) and L.W. Beukeboom (University of Groningen). In more recent years, mapping efforts using different molecular markers, for example amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR = microsatellites), sequence tagged site (STS), and random amplified polymorphic DNA (RAPD), have been undertaken (Gadau et al 1999, 2002; Rütten et al. 2004; Velthuis et al. 2005). Recently, genome sequencing projects have been completed for *N. vitripennis* and its two sibling species *N. girault*i and *N. longicornis* (Werren et al. 2004; http://www.hgsc.bcm.tmc.edu/projects/nasonia/). Here we describe the current state of knowledge of genetic mapping in *Nasonia* and its potential for further research.

3.1.1 Systematic Position

Nasonia belongs to the Chalcidoidea, one of the two most species-rich superfamilies of parasitoid Hymenoptera (the other being the Ichneumonoidea) (Grimaldi and Engel 2005). The genus *Nasonia* is part of the Pteromalidae, a huge family of exclusively parasitic wasps with about 340 described North American species (Triplehorn and Johnson 2005). Chalcidoidea is the most important insect group in applied biological control: over 800 species have been used in biocontrol programs in one way or another (http://www.nhm.ac.uk/research-curation/projects/chalcidoids/introduction.html).

3.1.2 The Genus *Nasonia*

The genus *Nasonia* consists of three closely related species: *N. vitripennis, N. girault*i, and *N. longicornis* (Darling and Werren 1990). *N. girault*i and *N. longicornis* are sister taxa, which split about 0.2 million years ago. Sister to this species cluster is *N. vitripennis*, which shared a last common ancestor with the other two species about one million years ago (Campbell et al. 1993). *N. girault*i occurs in eastern North America and *N. longicornis* in western North America. *N. longicornis* has recently also been recorded in the eastern US, however. *N. girault*i and *N. longicornis* are parasitoids of *Protocalliphora* fly pupae, which are found in bird nests. *N. vitripennis* has a worldwide distribution and parasitizes various fly pupae that are found on carcasses and in bird nests. Where its range overlaps with that of the other two species, the taxa occasionally occur in microsympatry, i.e., in the same bird nest (Darling and Werren 1990; B. Grillenberger unpublished observation).

Under natural conditions the three *Nasonia* species are reproductively isolated from each other due to their infection with different *Wolbachia* bacteria strains which cause cytoplasmic incompatibility (Breeuwer and Werren 1990; Bordenstein et al. 2001). However, once the wasps are cured of their *Wolbachia* endosymbionts by means of antibiotics, they can produce viable and fertile hybrid offspring (Breeuwer and Werren 1990, 1995).

The life cycle of *Nasonia* is similar to that of other parasitoids (Fig. 1). All three *Nasonia* species sting and lay their eggs upon the pupae of flies, specifically blowflies, fleshflies, and houseflies. *Nasonia* and its close relatives (i.e., *Muscidifurax, Spalangia*, and *Trichomalopsis*) are all used as biological control agents of *Musca* (Patterson and Rutz 1986; Legner 1995). They are routinely sold by commercial insectaries to control for houseflies and other filth flies in dairy farms, feedlots, and poultry rearing operations (Axtel and Rutz 1986). One company in North America sells *Nasonia* for teaching purposes in genetic classes due to its visible mutations and haplodiploid sex determination system (Ward's Natural Science, Rochester, New York).

All three *Nasonia* species are easy to work with in the laboratory. They have a comparatively short generation time (two weeks at 25 °C) and produce a large number of offspring (up to 500 per female). Individuals are easily sexed in their immobile pupal stage, and adults of laboratory lines do not readily fly, which allows handling them without their anesthetization. Pupae and adults can be maintained in the refrigerator for 1–2 months. It is possible to induce diapause in *Nasonia*, which allows the storage of important strains in the refrigerator for up to 1.5 years without further maintenance. Hosts (*Sar-*

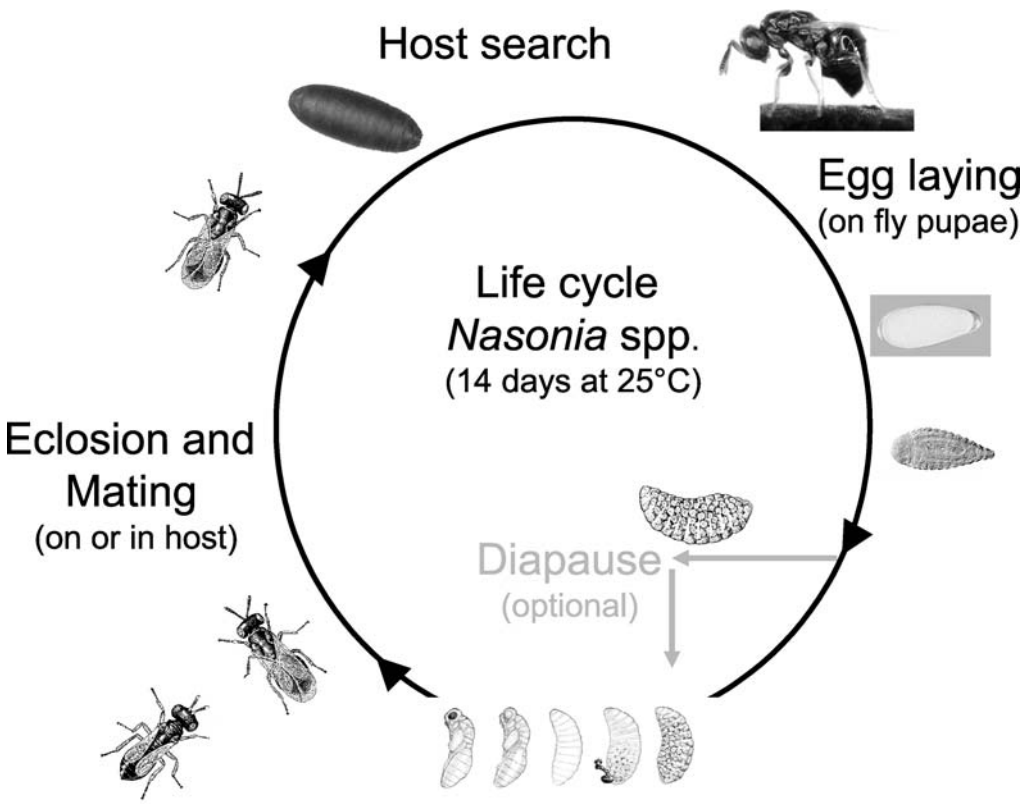

Fig. 1 Life cycle of *N. vitripennis* at a temperature of 25 °C

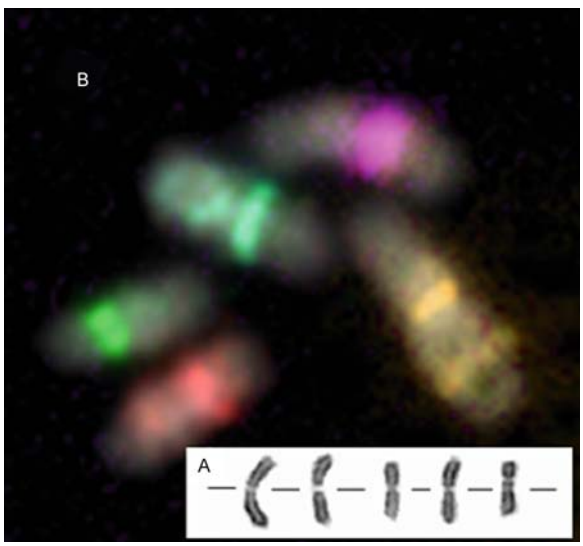

Fig. 2 **A** Karyotype of a male *N. vitripennis* (1n). **B** FISH of *N. vitripennis* chromosomes with chromosome-specific probes (for details see Rütten et al. 2004)

Table 1 Mutant markers, their linkage group association, and recombinational distances based on crosses between two mutant lines (Saul 1993 and unpublished data of J.H. Werren and L. Beukeboom). The mutant lines in *bold* were mapped on the linkage map of Gadau et al. (1999) using interspecific crosses between the *N. vitripennis* mutant lines (e.g., or123) and R16A (*Nasonia girault*i). The *underlined* mutant lines were mapped using crosses between the *N. vitripennis* mutants and the *N. longicornis* line IV7 (Beukeboom unpublished data)

Percent recombination	Symbol	Name	Phenotype
Linkage Group I			
	rep	Red-eye pupae	Slightly red-eyed pupae
2			
	rdh 1	Reddish	Dark red eyes
11			
	rev 421	Reverend	Legs of pupae extend toward ventral midline
3			
	ga 251	Garnet	Red eyes
1*			
	hb 441	Hunchback	Thoracic segments compressed
3			
	R	**R-locus**	Many eye color mutants in region of no recombination, multiple loci. Includes stDR, oyDr, **red 833**, and others
4			
	cur 321	Currant	Red eyes
3			
	cop 362	Copper	Frons copper-yellow
1			
	cop 2	Copper	Frons copper-yellow
1*			
	stp 211	Stumpy	Abdomen shortened
2			
	ga 351	Garnet	Red eyes
2			
	gl	Glass	Eye facets poorly differentiated and number reduced
1			
	pu	Purple	Dorsal thorax purple and frons blue (absence of yellow sheen)
1			
	ga 120	Garnet	Red eyes
4			
	cop 1	Copper	Frons copper-yellow
5			
	wa 362	White appendages	Appendages white (entire body white in young pupae)
1*			
	stp 361	Stumpy	Abdomen shortened
1			
	bk362	Black	Black eyes
17			
	vg	Vestigial	Rudimentary wings

Table 1 (continued)

Percent recombination	Symbol	Name	Phenotype
Linkage Group II			
	bl 108	Blue	Frons blue
15			
	rdh 5	**Reddish**	**Red eyes**
<1			
	crw	Crumpled wings	Wings crumpled in adults, female sterile
<1			Position with respect to cl 131, bl 106 not yet determined
	cl 131	Cleft	Ocellar region reduced w/ dorsal cleft between eyes number of antennal segments reduced
<1			
	bl 106	Blue	Frons blue
<1			
	se 121	Small eyes	Eyes small, fewer facets than normal
<1			
	mh 493	Mahogany	Dark red eyes; rdh 810 is allelic
1			
	bl 109	Blue	Frons blue
25			
	unf 441	Unfolded	Incomplete eclosion from pupal case in dorsal thorax; small mesothoracic wings
Linkage Group III			
	tl 627	Tile	Rust-red eyes; pe 100 allelic
<1			
	bl 5101	Blue	Frons blue
2			
	fx 331	Flexed	Mesothorax duplicated; metathorax reduced; both pairs or wings of equal size, pupa flexed ventrally
9			
	cop 411	Copper	Frons yellow-copper
37			
	bk 424	**Black**	**Black eyes**
Linkage Group IV			
	or 123	Orange	Light orange-red eyes
≪1			
	bk 576	Black	Eyes slightly darker browner than wild type
<1			
	bl 13	Blue	Frons blue
<1			
	ws1	Wing size	Wing size locus, introgressed from *N. girault*i into *N. vitripennis*
~ 20			
	st 5219[a]	Scarlet	Scarlet eyes

[a] Saul mistakenly placed this marker on linkage group III

Table 1 (continued)

Percent recombination	Symbol	Name	Phenotype
Linkage Group V			
	ga 561	Garnet	Red eyes; tom's red is allelic
10			
	pel 311	Pellucid	Gray-white eyes
12	mod 306	Modifier	Changes red and scarlet eyes to yellow and orange
	st 318	**Scarlet**	**Bright red eyes**
13			
	mm 251	Mickey mouse	Eyes protuberant, dorsal head defective
5			
	pm 541	Plum	Frons blue or reddish-blue
6			
	sw 561	Short wings	Small mesothoracic wings; metathoracic wings project out from body

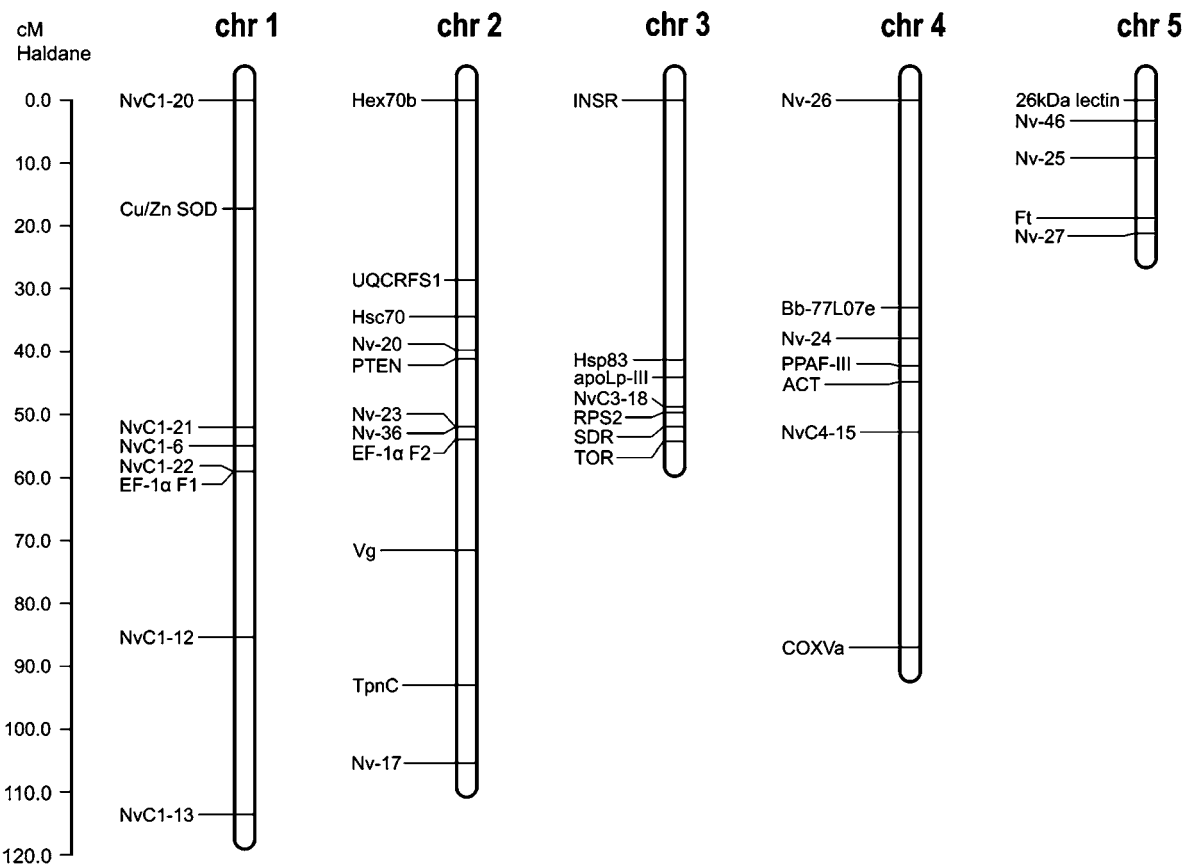

Fig. 3 Linkage map based on 225 embryos of F_2 hybrids between *N. vitripennis* (AsymCX) and *N. giraulti* (RV2X) (Niehuis et al. unpublished data)

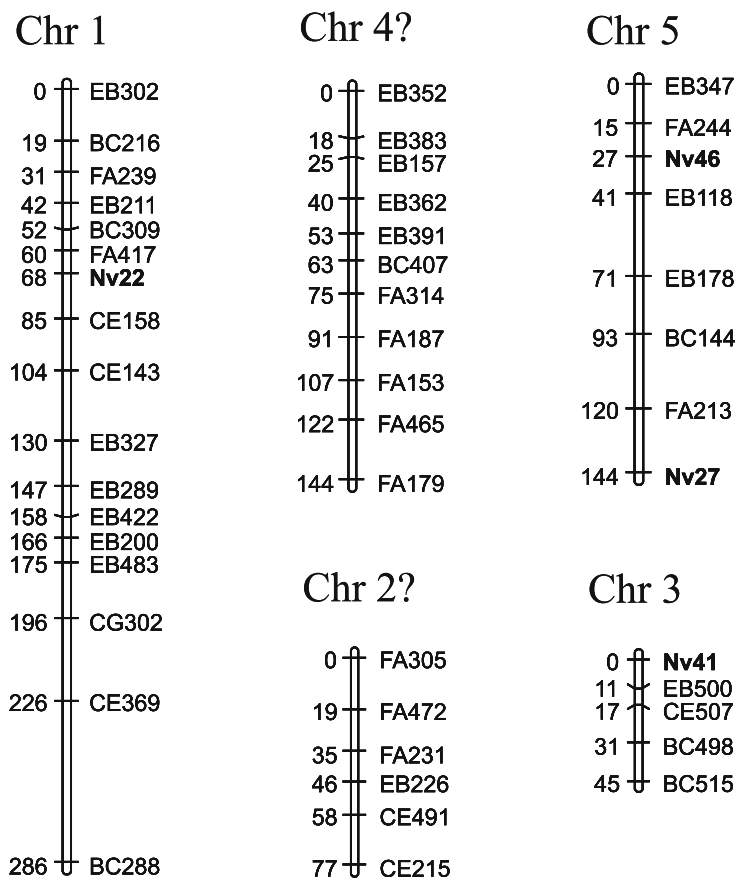

Fig. 4 Linkage based on 97 F_2 hybrid males from a cross between *N. vitripennis* (AsymC) and *N. longicornis* (IV7R2) in an *N. longicornis* cytoplasm (Peire et al. in preparation). Since overlapping markers were missing, it was not possible to assign chromosomes 2 and 4. Therefore, we added a ? after the chromosome association

cophaga spp. in North America; *Calliphora* spp. in Europe) are available commercially and easily cultured.

As in all Hymenoptera, *Nasonia* has a haplodiploid sex determination. In contrast to the well-studied honey bee, however, its specific sex determination system (molecular details yet to be explored) allows extensive inbreeding and hence, the rearing of isogenic lines. The genetics of *Nasonia* has been studied since the 1950s (the earlier work on *Nasonia* is summarized by Whiting 1967), but its potential "for the investigation of the biological problems connected with the parasitic Hymenoptera" was already recognized by Girault and Sanders in 1909 (p. 13 in Whiting 1967). In summary, *Nasonia* uniquely combines properties of a genetic model organism, such as a short generation time, laboratory tractability, and species interfertility, with features of haploid genetics in an otherwise complex eukaryotic organism.

3.1.3 Classic Mapping Efforts

All *Nasonia* species have five metacentric to submetacentric chromosomes (Gokhman and Westendorf 2000; Rütten et al. 2004; Fig. 2). Compared to other Hymenoptera, the chromosomes of *Nasonia* are very large. Based on recombination maps and cytology of hybrids, a high level of synteny is expect between the three species (Figs. 3, 4, 5).

All mapping efforts in *Nasonia* exploited the advantage of haplodiploid genetics, i.e., all mapping populations consisted of haploid males. Natural markers and induced mutant markers in *N. vitripennis* were used in initial mapping projects. These mutants were characterized by a unique eye or body color, morphological deformations, or male lethality (Table 1). Saul (1993) summarized all linkage information of earlier studies and published an *N. vitripennis* linkage

Table 2 Summary of published linkage maps for *Nasonia*

Crosses used	Marker types	Number of markers	Map size	References
Several mutant strains of *N. vitripennis*	Visible markers (mostly eye or body color mutants)	14	n. a.	Saul and Kayhart (1956)
Several mutant strains of *N. vitripennis*	Visible markers	47	264 cM (Kosambi)	Saul (1993)
N. vitripennis × *N. giraulti* F_2 males	RAPD	80	829 cM (Kosambi)	Gadau et al. (1999)
N. longicornis × *N. vitripennis*	RAPD	63	764.3 cM (Kosambi)	Rütten et al. (2004)
N. longicornis × *N. vitripennis*	RAPD, microsatellites, STS markers	48	624.5 cM (Kosambi)	Rütten et al. (2004)
N. longicornis × *N. vitripennis*	AFLP, microsatellites, STS markers	51	249.2 cM (Kosambi)	Pietsch et al. (submitted)
N. vitripennis × *N. giraulti*	microsatellites, STS markers	38	381.5 cM (Haldane)	Niehuis et al. (submitted)
N. longicornis (cross between isofemale lines from Idaho and California)	AFLP	57	228 cM (Kosambi)	Velthuis et al. (2005)
N. vitripennis × *N. longicornis*	AFLP, microsatellites	47	696 cM (Kosambi)	Pietsch (2005)

map based on 47 mutant markers. This map consists of five linkage groups spanning a total of 264 cM. All maps published so far are listed in Table 2.

3.2 Construction of Genetic Maps

Linkage mapping in *Nasonia* is straightforward because of the availability of highly inbred strains and the opportunity to perform interspecific crosses. The only limitation is the small size of the wasps, resulting in a limited amount of DNA per individual. However, two technical developments help to overcome this obstacle. First, the increase of polymerase chain reaction (PCR)-based genotyping techniques, such as AFLP, RAPD, and microsatellites, allow the study of a large number of markers per individual even with small quantities of genomic DNA (e.g., Pietsch et al. 2004; Rütten et al. 2004). Second, the development of molecular techniques that reliably amplify a whole genome can generate a potentially unlimited amount of DNA from a small quantity of genomic DNA (e.g., GenomiPhi, Amersham Biosciences). Niehuis et al. (in preparation) used GenomiPhi to amplify DNA from embryos to construct a linkage map (Fig. 3).

The classic linkage maps based on visual markers used intraspecific crosses of *N. vitripennis* mutant strains (*N. giraulti* and *N. longicornis* were first described in 1990 by Darling and Werren) whereas almost all linkage maps based on molecular markers used interspecific crosses (for an exception, see Velthuis et al. 2005; Table 2). A standard mapping population of *Nasonia* consists of F_2 males that are generated by crossing two highly inbred *Wolbachia*-cured strains and then letting the genetically identical hybrid F_1 females lay unfertilized eggs (Fig. 6). The desired F_2 males eventually hatch from these unfertilized eggs, and each of these males has a unique

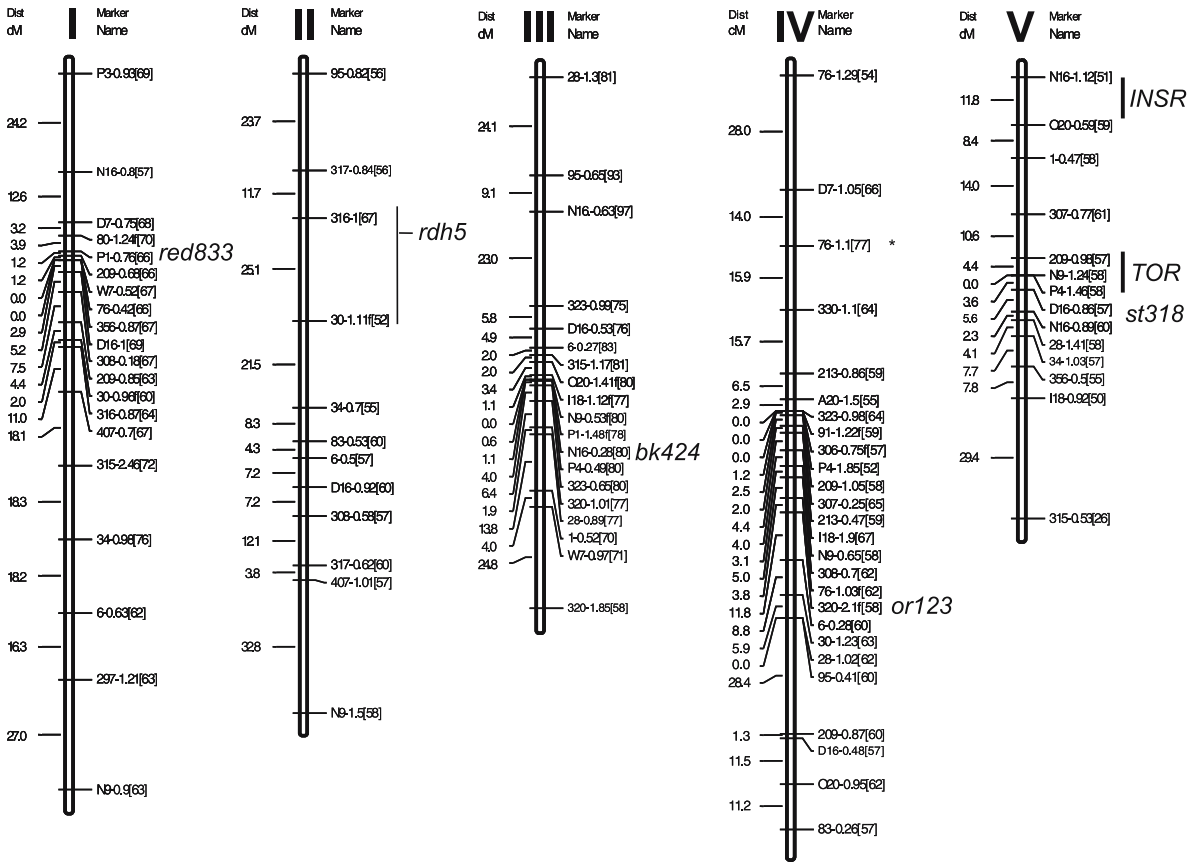

Fig. 5 Linkage map based on 178 F_2 hybrid males from a cross between AsymC (*N. vitripennis*) and R16A (*N. girault*i) in an *N. vitripennis* cytoplasm (modified from Gadau et al. 1999)

combination of both species' genomes because of meiosis during oogenesis.

If females are needed for mapping, to conduct, for example, a quantitative trait locus (QTL) analysis of a female trait, the best way would be to backcross F_1 hybrid females. In that way, each F_2 female has an identical set of chromosomes coming from the father of the backcross lineage, but varies in the chromosomes coming from the mother because of recombination during oogenesis in the F_1 females (Fig. 6). F_1 intercrosses are not possible, because males develop parthenogenetically and thus, F_1 hybrid males do not exist. F_1 equivalent hybrid males can be generated, however, by allowing F_1 females to sting hosts as virgins and subsequently keeping them alive at lower temperatures (4 °C) until their sons (F_2 hybrid males) have hatched. The virgin F_1 mothers can then be mated with their own sons.

Backcrossing a single F_2 male with females of one of the highly inbred parental lineages generates a large number of genetically identical females. These females are clones, because a haploid male produces genetically identical sperm and most parental lines currently used in the laboratory for cross experiments (AsymC = *N. vitripennis*, RV2 = *N. girault*i, IV7 = *N. longicornis*) are isogenic. These females can be used in the analysis of complex phenotypes to reduce the environmental variance of the measured phenotypes by using the mean phenotype of multiple genetically identical females (e.g., Velthuis et al. 2005; Fig. 6).

Starting with an interspecific cross, one can construct either introgression lines (IL) by repeated backcrossing male or female hybrids or generate recombinant inbred lines (RIL) with a varying combination of the two parental genomes by initiating inbreeding after the second or third backcross experiment (Fig. 6).

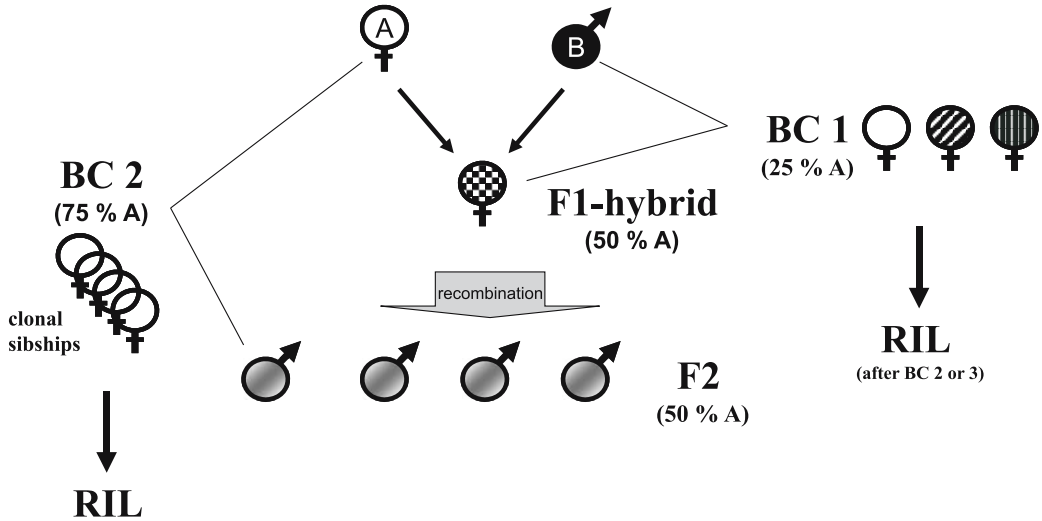

Fig. 6 Possible breeding schemes between the three *Nasonia* species and nuclear genetic composition (e.g., 50% of genome A) of individuals. Recombinant inbred lines (RIL) can be produced by repeated inbreeding (e.g., brother-sister mating) after BC_1 or BC_2. Introgression lines (IL) can be constructed by backcrossing for more than 10 generations to one parental line followed by inbreeding. Clonal sibships can be produced by backcrossing F_2 males to either of the parental lines

3.3 Gene Mapping

Due to haplodiploidy, the complete genome of *Nasonia* can be efficiently screened for mutations in the F_2 generation because recessive mutations in the genome are immediately identifiable (e.g., Saul and Kayhart 1956; Pultz et al. 2000; Pultz and Leaf 2003). To search for new mutations, males are mutagenized and crossed to control females. The F_1 females are then set as virgins, thus producing exclusively haploid F_2 males that can be screened for any phenotype of interest because all mutations are expressed in haploid males. F_1 females that produce interesting mutant phenotypes in the F_2 hybrid males can then be mated to standard lines to maintain recessive lethal or sterile mutations (Saul and Kayhart 1956; Pultz et al. 2000). An alternative to transgenic techniques is the introgression of specific genomic regions or genes from one *Nasonia* species in the genetic background of another. Using this method, genomic regions around QTL of interest that cause species differences can quickly be narrowed down.

A comparison of coding sequences from expressed sequence tag (EST) libraries suggest on average of about one percent sequence difference between *N. vitripennis* and *N. giraulti* (unpublished). If this estimate is representative for the whole genome, we expect approximately one single nucleotide polymorphism (SNP) every 100 bp of coding DNA. This allows tracing SNP markers in virtually every exon of the genome, making mapping of specific genes in F_2 males straightforward. So far, about 20 genes (Fig. 3; Niehuis et al. unpublished data) and the insulin pathway genes (Baudry and Werren, unpublished data) have been mapped using this technique.

3.3.1 Comparing Published Linkage Maps and Marker Associations

The major goals for the integration of the different linkage maps (Tables 2 and 3) were to connect Sauls' (1993) visible markers with the new molecular marker maps and to assign previously published linkage groups to physical chromosomes using the anchored linkage map by Rütten et al. (2004) and markers therein. We tried to homologize chromosomes and linkage groups of all published maps as far as possible (Table 3). For example, it was possible to assign the linkage groups of the RAPD linkage map of Gadau et al. (1999) to physical chromosomes by mapping three visible markers and two insulin pathway genes

Table 3 Marker and linkage group/chromosome association and proposed set of chromosomal standard markers to reference new markers. The table lists chromosome-specific markers that were used for the in situ hybridization (in *bold*, e.g., NvC1-6; Rütten et al. 2004). Microsatellite and STS markers are named Nv plus a number (e.g., Nv22). SNP markers are named after the gene sequence from which they were developed (e.g., PTEN, Niehuis et al. in preparation). For further details, we refer the reader to the original literature cited in the first column. To position visible mutant markers in our molecular maps, Beukeboom (unpublished data) crossed the *N. longicornis* strain IV7R2 with *N. vitripennis* mutant strains and genotyped six mutant and wildtype F_2 males each for eleven markers on all five chromosomes. He found that rdh5 was linked to Nv22 (Fisher's Exact, two-tailed: $P = 0.021$) on chromosome 1, st5219 to Nv30 on chromosome 4 (Fisher's Exact, two-tailed: $P = 0.077$), and the two R-complex loci STDR and red 833 to Nv27 on chromosome 5 (Fisher's Exact, two-tailed: $P < 0.001$)

	Chr. 1	Chr. 2	Chr. 3	Chr. 4	Chr. 5
Rütten et al. (2004)	**NvC1-6**; **NvC1-12**; **NvC1-13**; **NvC1-20**; **NvC1-21**; **NvC1-22**; Nv22	**NvC2-14**; Nv20; Nv23	**NvC3-17**; **NvC3-18**	**NvC4-15**; **NvC4-16**; Nv21; Nv24; Nv26; Nv37; *or123*[a]	**NvC5-19**; Nv25; Nv27; *stDR*[a]; *st5215*[a]
Saul et al. (1993)	LG-II *rdh-5*[a]	LG-III *BK424*[a]	LG-V *st318*[a]	LG-IV *or123*[a]	LG I R-complex; *stDR*[a]; *red 833*[a]
Gadau et al. (1999) (Fig. 5); Baudry and Werren (unpublished data)	LG-II *rdh-5*[a] linked to Nv22	LG-III *BK424*[a]	LG-V TOR[b]; INSR[b]; *st318*[b]	LG-IV *or123*[b]	LG-I *red 833*[b] linked to Nv27[a]
Pietsch (2005); Pietsch et al. (in preparation)	Nv22; Nv32; Nv40; Nv44	Nv-20; Nv23; Nv36	Nv41	Nv24; Nv37	Nv25; Nv27; Nv45; Nv46
Velthuis et al. 2005 (AFLP markers only)	–	–	–	–	*stDR* is part of the R-complex
Niehuis et al. (in preparation)	**NvC1-6**;**-12**;**-13**;**-20**; **-21**;**-22**	Nv-20; Nv23; Nv36; PTEN[b]	**NvC3-18**; TOR[b]; INSR[b]; Nv41	**NvC4-15**; Nv24; Nv26; Bb-77L07e[c]	Nv25; Nv27; Nv45; Nv46
Peire et al. (in preparation)	Nv19; Nv22; Nv40; Nv44	Nv23	Nv41	Nv37	Nv27; Nv46
Standard markers to place and orient new markers	Nv22 Nv40	Nv20 Nv23	NvC3-18 Nv41	Nv24 Nv26	Nv27 Nv46

[a] Visible markers described in Saul et al. (1993), the mutant strains are available from Beukeboom (University of Groningen) and Werren (University of Rochester)

[b] Genes derived from an EST library and mapped in the mapping population of Gadau et al. (1999) (Baudry and Werren unpublished data)

[c] BAC clone completely sequenced as part of the Nasonia Genome sequencing project by Baylor Human Genome Sequencing Center, Department of Molecular and Human Genetics, Baylor College of Medicine (GenBank accession: AC185133)

mapped in the RAPD linkage map to chromosomes [*stDR* is on chromosome 5 and *or123* and *ST5219* are on chromosome 4 (Rütten et al. 2004, Beukeboom unpublished data); *rdh5* mapped on chromosome 1 (Beukeboom unpublished data); TOR and INSR were associated with linkage group 5 and chromosome 3 (Niehuis et al. in preparation; Baudry and Werren, unpublished data)]. With four linkage groups assigned to a specific chromosome, linkage group 3 in Gadau et al. (1999) has to be homologous to chromosome 2.

It is to be noted that chromosome associations of two microsatellite markers in Pietsch et al. (2004) had to be changed and Nv24 and Nv26 are now on chromosome 4 due to renaming of chromosomes by Rütten et al. (2004). Also, there is an error in Table 1 of Rütten et al. (2004): Nv26 is listed under chromosome 2 instead of chromosome 4. However, all markers are correctly placed on the linkage map shown in the article.

3.4 Detection of Quantitative Trait Loci

Nasonia is an excellent system for positional cloning, in particular for genes of complex genetic traits. By studying closely related interfertile species, an almost unlimited number of molecular markers are available for fine-scale mapping (see above). Other important features facilitating QTL analyses in *Nasonia* are the short generation time, a high recombination rate of the genome, and the ease of generating linked visible and lethal markers to assist in recombinant walking. Thus, *Nasonia* is well-positioned for quickly proceeding from mapped QTL to the underlying genes. The potential for studying the underlying genetic architecture of QTL has increased by sequencing the whole *Nasonia* genome.

3.4.1 Wing Size

Males of *N. vitripennis* and *N. giraulti* differ in wing size, probably due to differences in their wing cell size. By studying F_2 hybrids obtained from crosses between five eye-color mutant strains of *N. vitripennis* (corresponding to the five chromosomes of *Nasonia*), Weston et al. (1999) assessed the effects of individual chromosomes on the quantitative trait "wing size." The authors found that at least three chromosomes have a significant effect on wing size and that one or more wing size loci are tightly linked with the eye color mutation *or123* on linkage group IV (chromosome 4). Weston et al. (1999) further introgressed a small fragment of linkage group IV with the mutant marker *or123* from *N. giraulti* into the genetic background of *N. vitripennis*. The single region accounted for 44% of the species difference in normalized wing size between *N. vitripennis* and *N. giraulti*. In a subsequent study, Gadau et al. (2002) found three QTL for a normalized wing size variable and multiple QTL for other wing and head size associated traits (see Table 4).

Three major QTL from *N. giraulti* have now been backcrossed into *N. vitripennis* background and linked visible and lethal mutations have been used to reduce the size of the introgressed regions to (in one case) less than 0.01 cM (J.H. Werren, unpublished results). The available genome sequence of *N. vitripennis* (Human Genome Sequencing Center, Baylor College of Medicine) will allow scanning for genes and regulatory elements in this region. As outlined above, it is possible to trace SNP markers in every exon of potential genes and genotype further introgressed males for recombinants. Only those genes/exons causally linked to the phenotype of interest are expected to strictly cosegrate with the phenotype. As an alternative approach, one could silence the genes in question using RNA interference (RNAi) techniques (Lynch and Desplan 2006). Similar approaches are in progress for behavioral genes (courtship behavior, Table 4) and can be applied to a wide range of traits, utilizing the related species, strains, or selection lines.

3.4.2 Male Courtship Behavior

Male courtship behavior differs quantitatively and qualitatively between all three *Nasonia* species (van den Assem and Werren 1994) and may serve as a prezygotic hybridization barrier in sympatric populations of *Nasonia* species. The courtship behavior can be divided into multiple quantifiable components. Hybrid haploid males derived from interspecific crosses between inbred *N. vitripennis* and *N. longicornis* lines were phenotyped for five quantitative and two qualitative components of the courtship behavior, which differed significantly between the two parental species. In a QTL analysis of these species differences, we found QTL for almost all components, which mapped to all five chromosomes (Pietsch et al. in preparation). No QTL with a major pleiotropic effect on the full courtship display had been found, indicating a complex genetic architecture for the differences in male courtship behavior between *N. vitripennis*

Table 4 Summary of QTL studies in *Nasonia* (MapQTL was used for all QTL analyses)

Trait	Population	Marker type	Number of QTL detected/trait	Epistatic QTL	Reference
Wing and head size	Interspecific cross *N. vitripennis* × *N. giraulti*	RAPD	2 *wing length* 1 *wing width* 3 *normalized wing size* 5 *wing setae density* 4 *head width*	2 *wing length* 2 *normalizes wing size* 3 *wing setae density*	Gadau et al (2001)
Wing and head size	Interspecific cross *N. vitripennis* × *N. longicornis*	STS, microsatellites	1 *wing length* 1 *normalized wing width*	*None detected*	Rütten et al. (2004)
Courtship behavior	Interspecific cross *N. vitripennis* × *N. longicornis*	STS, microsatellites, AFLP	2 *latency* 1 *fix-1st nod* 5 *cycle* 2 *headnod* 3 *total series*	2 *latency* – 4 *cycle* 2 *headnod* 1 *total series*	Pietsch et al. (in preparation)
Mate discrimination	Intraspecific *N. longicornis*	AFLP	3 *mate discrimination*	*None detected*	Velthuis et al. (2005)

and *N. longicornis* rather than a major gene influencing multiple components of the male courtship behavior.

3.4.3 Epistasis

Major QTL and non-additive (epistatic) interactions among QTL are readily detected in haploid males, since they are not obscured by dominance interactions. Additionally, compared to diploid organisms one can use smaller sample sizes to test for significant two-way interactions in the genome because in a haploid genome there are only four possible genotypes for two-way interactions. We found extensive two-way epistatic interactions between QTL for different courtship components, located on different chromosomes. The observed epistatic interactions have different patterns, e.g., in conditional QTL the effect of the primary QTL is sometimes conditioned upon an *N. vitripennis* allele (head nods, total number of series) but in other cases upon the *N. longicornis* allele (latency, cycle time) (Pietsch et al. in preparation.). Thus, epistatic interactions appear to be an important component of the genetic basis of species differences in *Nasonia*. This has also been shown for wing size differences (Gadau et al. 2002).

3.5 Available Techniques and Resources

The development of new genetic/genomic tools for *Nasonia* are rapidly advancing. Among the resources currently available are an 11x-covering bacterial artificial chromosome (BAC) library (Clemson University Genomic Institute; http://www.genome.clemson.edu/projects/nasonia/), chromosome specific libraries (Rütten et al. 2004), two embryonic cDNA libraries (M.A. Pultz and D. Leaf) and a male and female head cDNA library (R. Bertossa, University of Groningen). EST from different tissues and life stages (pupae and adults) of two species (*N. vitripennis* and *N. giraulti*) have been sequenced (J.H. Werren et al. unpublished data) and an additional 12 EST libraries are planned to be available by the end of 2007 (http://daphnia.cgb.indiana.edu/). RNAi has recently been successfully used in *Nasonia*

(Lynch and Desplan 2006) and a transformation system is under development (C. Desplan personal communication). In addition, a large number of *Wolbachia*-free *Nasonia* strains from all three species and recombinant inbred lines (RILs) between these species are available [L. Beukeboom (Nv × Nl) and J.H. Werren (Nv × Ng)]. These RILs can be utilized for mapping interspecies differences in behavior, development, and physiology. Finally, the *N. vitripennis* genome has been sequenced with a 6x coverage and the genome of the two other *Nasonia* species with a 1x coverage; http://www.hgsc.bcm.tmc.edu/projects/nasonia/). The publication of the annotated genome is planned for the end of 2008.

3.6 Future Scope of Works

Nasonia will be an excellent model system to address demanding questions in basic and applied biology due to its haplodiploid genetics and the availability of the genome sequences for its three species. Understanding the genetic basis and architecture of host preference, host finding, sex allocation, pheromone communication, and diapause is essential for using any kind of parasitoids in integrated pest management. With the annotation and publication of the *Nasonia* genome, it is expected that parasitoid genomics will take off and will help us to use parasitoids more effectively for the benefit of human agriculture, health, and nutrition.

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4 Silkworm

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4.1 Introduction

The silkworm, *Bombyx mori* , is thought to have been domesticated from a mulberry-feeding moth, *Bombyx mandarina*, which is naturally distributed among East Asian countries. *B. mori* belongs to the superfamily, Bombycidae, of the Lepidoptera, one of the most abundant insect orders, which includes butterflies and moths. Cytological studies reveal that the number of chromosomes is 56 in somatic cells and 28 in germ cells (Yatsu 1913). In wild populations of *B. mandarina*, two karyotypes (n = 28 or 27) coexist, both of which can be crossed with *B. mori* to make fertile hybrids. The difference in the two karyotypes is that two chromosomes corresponding to linkage group (LG) 14 and an unidentified LG of *B. mori* are joined in a single chromosome in *B. mandarina* (n = 27; Banno et al. 2004). The haploid genome size of *B. mori* is estimated to be 530 Mb (Gage 1974) by Cot analysis, though more recent measurements using flow cytometry suggest it is closer to 474–493 Mb (JS Johnston personal communication), 2.7 times that of *D. melanogaster* and one-sixth to one-eighth the size of the mammalian genome. In *B. mori,* the female has Z and W sex chromosomes like most lepidopterans (Traut and Marec 1996), and the W chromosome determines femaleness (Hasimoto 1933).

It is difficult to find direct archaeological evidence of domestication, since silkworm body parts and instruments for sericulture are fragile and rarely found in ancient ruins. However, it is at least certain that sericulture was already recorded in documents from the first dynasty in China (seventeenth century B.C.). Images of spinning cocoons of wild moths into thread have appeared widely around the world. Sericulture was probably started in this way, and human controls were gradually added in feeding, protection from natural enemies, artificial selection, reproduction, and cultivation of mulberry.

The extent of genetic diversity in conventional races also supports the origin of sericulture in China (Yoshitake 1968). Sericulture spread throughout East Asia until the beginning of the Christian era, and was introduced into the West through the Silk Road in the sixth century. Silk has been fascinating to the upper classes of all ages and countries because of its fine texture and beautiful appearance, although it was very expensive and accounted for less than 1% of total fiber production. However, chemical fibers invented in the twentieth century competed for the demand for silk and reduced its price. Since sericulture is typically a labor-intensive agriculture, farmers in advanced countries have moved to more profitable crops and developing countries in Asia, such as China and India, as well as Brazil, are now the world's major silk producers.

Currently, people intend to modify the silkworm to produce more valuable substances by the use of genetic engineering, using nucleopolyhedrosis virus (NPV) vectors or transgenesis techniques. Genome projects and related work are underway using *B. mori* as a model organism for Lepidoptera, the most serious group of agricultural pests (for review, see Goldsmith et al. 2004).

4.1.1 Classical Mapping Efforts

It was in 1906 that K. Toyama first made clear that traits of larval spots and yellow cocoon were inherited in *B. mori* in a mendelian fashion. In 1913, these

Genome Mapping and Genomics in Animals, Volume 1
Genome Mapping and Genomics in Arthropods
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two genes, larval markings (plain, *p*, 2-3) and yellow hemolymph (*Y*, 2-28.6), were shown to be linked, and the first linkage group (LG) was discovered. Since then, other LGs have been clarified by genetic studies. Multiple alleles were found in several loci such as the *p*, for example normal (p^3), plain (p), dark color (p^M), and black stripe (p^S), and *E* (6-21.1; extra legs, the first published example of a homeotic transformation of larval segment identity) genes. Subsequently, many morphological mutations concerning egg, larva, pupa, cocoon, and adult have been genetically analyzed, and 15 LGs and 56 genes were mapped by 1951. In the following 20 years, an additional seven LGs were found and a total of 131 genes were described. In the 1960s, genetic studies on protein polymorphisms began, and many enzymes such as alkaline phosphatase and blood esterase were analyzed. However, it was not until 1968 that *Alb* (albumin, 20-6.2) was placed onto the linkage map as the first isozyme marker (Gamo 1968). To date, 246 genes, including nine isozyme genes and an unlinked mutation, *E-tr*, have been assigned to 27 LGs (Banno et al. 2005; http://kaiko.kyushu-u.ac.jp/about/home%20of%20materials/map.pdf).

4.1.2 Objectives of Breeding

Needless to say, the main objectives of breeding are to increase silk production, improve the quality of cocoons, and maintain the stability of the yield. Silkworm breeding has strived from ancient times to increase cocoon shell weight and the number of eggs laid, to acquire higher resistance against pathogens, humidity, and temperature, to improve the ratio of the cocoon shell to the total cocoon (shell plus pupa), and to increase the hatchability of eggs.

In Japan, the advantages of F_1 heterosis have been widely recognized, and two hybrid strains, Ogusa and Hakuryu, were generated in the 1790s to increase silk yields. Use of F_1 hybrid races has spread since 1914, because farmers understood the advantages of larger cocoon and healthy growth. Nearly all silkworms reared in Japan were converted to F_1 hybrids by 1931. Because F_1 individuals display traits inherited from both parents and there is frequently a trade-off among important traits, it is necessary for breeders to have considerable experience to find the best combination of parental races in order to express the most important desirable traits. For this purpose, various conventional races have been collected from around the world and selected for breeding of parental races, which have become the basis of bioresources of *B. mori*.

Classical genetic studies have also contributed to practical breeding. For example, translocation of the *Y* gene (yellow cocoon) to the W chromosome, which carries virtually no morphological markers, has been utilized to identify female pupae easily in a white cocoon background. This is not only beneficial to egg producers by facilitating matings, but also meets demands from the textile industry, because male cocoons are superior to those of females in reelability, cocoon fiber content, and loss of cocoon shell weight during the degumming process (whereby the sericin "glue" proteins are removed from the silk fiber).

However, elimination of yellow female cocoons is not an ideal solution to reduce cost and labor, since feeding female larvae is still performed. If balanced recessive lethal genes are introduced to the paternal Z chromosomes, elimination of females will be realized earlier and completely (Strunnikov 1979; Ohnuma 1988). Such a strain, Platinum Boy (http://www.silk.or.jp/sangiken/tamago.htm), has been generated by A. Ohnuma.

4.1.3 Limitations of Classical Endeavors and Utility of Molecular Mapping

Many of the genetic markers on the classical linkage map of *B. mori* are spontaneous morphological mutations, but there are limitations for mutagenesis and finding novel mutations. Further, it is difficult to use many morphological markers in a single experiment, so that genetic distances among multiple markers cannot be calculated directly. Moreover, some mutant phenotypes are unstable, which makes genotyping difficult and leads to inaccuracies in the resultant genetic distances.

Because of the relatively large number of chromosomes, many mutant strains are needed for sufficient matings to map a trait onto the classical linkage map, which takes considerable labor and time. Since systematic maintenance of mutant strains is limited to Japan (http://kaiko.kyushu-u.ac.jp/mutation.

html; http://ss.nises.affrc.go.jp/nisesDB/bombygen/tablemaster-eng.html), South Korea, and China, classical mapping is significantly more difficult for researchers in other countries. Therefore, the advantages of molecular mapping are particularly great in *B. mori*.

4.2 Construction of Genetic Maps

The important features of *B. mori* for linkage mapping are that it is short-lived (<1 month for larval stages), small in body size (<5 g), and easily reared en masse. These are distinct advantages; however, the relatively small body size limits the yield of DNA and proteins isolated from an individual, which make it difficult to employ conventional mapping techniques such as isozyme and restriction fragment length (RFLP) analyses multiple times. In addition, unlike in plants and mammals, sampling DNA and reproducing progeny are usually incompatible.

Polymerase chain reaction (PCR)-based methods like random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) greatly address these limitations. However, these PCR-based markers are highly specific, and it is difficult to integrate results obtained from experiments using different parental strains. Therefore, PCR-based markers universally utilized for many different combinations of strains have been urgently needed. Yasukochi (1999) showed that co-dominant and conserved markers could be established by conformation-sensitive gel electrophoresis of PCR products amplified from known single-copy genes and unique genome sequences, which enabled mapping of hundreds of genes and molecular markers (Yasukochi et al. 2006).

A major feature of lepidopteran oogenesis is that genetic recombination does not occur (Tanaka 1913), which makes it easy to identify linkage groups. Using BF_1 progeny from a mating of an F_1 female and a recurrent parental male, markers on the same linkage group always co-segregate. In F_2 intercross analysis, no individual can be homozygous for both maternal and paternal dominant markers on the same autosome, which can be used to confirm whether linkage groups are really independent (Yasukochi 1998).

Table 1 lists the summary of published linkage maps. Two molecular linkage maps of *B. mori* were first published in 1995. One was based on RAPDs (Promboon et al. 1995), and the other was based on

Table 1 Summary of data in published linkage maps of *Bombyx mori*

Publication	Number of LGs[a]	Number of tested progeny	Number of markers					
			Total	Known genes and cDNAs	RAPDs	AFLPs	Micro-satellites	Others
Promboon et al. (1995)	29 (2)	101 F_2	169		169			
Shi et al. (1995)	15 (8)	52 F_2	51	51				
Yasukochi (1998, 1999); Yasukochi et al. (2006)	28 (26)	166 F_2	1,577	244 (427)[b]	1,010			323
Tan et al. (2001)	30 (1)	47 BF_1	356			356		
Nguu et al. (2005)	28	100 BF_1	189	189				
Prasad et al. (2005)	8 (1)	60 BF_1	29					
Miao et al. (2005)	29 (26)	189 BF_1	547	29			518	
Yamamoto et al. (2006)	28 (26)	190 BF_1	534	(107)[c]				534

[a] Numbers in parentheses indicate numbers of LGs assigned to classical LGs

[b] In addition to 244 directly mapped genes and ESTs, 183 genes and ESTs were confirmed to be localized on BAC contigs assigned to the linkage map

[c] 107 ESTs were estimated to be localized on BACs whose end sequences were mapped by in situ hybridization experiments using BAC high-density filters

Table 2 Mapped genes in *Bombyx mori*

Genes	Accession number	Linkage group[a]	References[b]
18 wheeler	AB070579	23	1
18S rDNA	X05086	11	1
30K lipoprotein (major plasma protein)	X54734-X54736	20	1, 2
30kP protease A precursor	AB026735	16	1
45-kDa immunophilin FKBP45	DQ085777	21	1
50-kDa lectin	D14168	19	1
90-kDa heat shock protein	AB060275	U(28)	1
A disintegrin and metalloproteinase with thrombospondin-like motifs 1	AB194270	9	1
A disintegrin and metalloproteinase with thrombospondin-like motifs	AB194271	20	1
Abdominal-A	X62620	6	1
Abdominal-B	X62619	6	1
Actin 2	X06363	8	1
Actin A3	U49845	17	1
Actin A4	U49644	17	1
Acyl-CoA binding protein	AF246695	10	1
Acyl-CoA binding protein	AF246696	10	1
AD10 (fibrillin-like protein)	AB062683	9	1
Ala-tRNA synthetase	M55993	4	1
Allatostatin preprohormone	AF309090	14	1
Alpha-amylase	U07847	8	1, 2
Alpha-tubulin	AB072305	9	1
Annexin B13b	AB063190	10	1
Antennapedia	D16684	6	1
Apolipophorin III	AY341912	16	1
Apterous	AB024903	1	1
Armadillo/beta-catenin gene	AF538874	10	1
ATP-dependent transmembrane transporter protein (wh3)	AF229609	10	1
Attacin	D76418	6	1, 2
Bacteriophage T7 lysozyme-like protein 1	AB017519	16	1
Bacteriophage T7 lysozyme-like protein 2	AB017520	9	1
Beta-1,3-glucan recognition protein	AB026441	11	1
Beta-tubulin	AB003287	20	1
Beta-tubulin	AB072309	20	1
Beta-tubulin	AB072310	15	1
BHR38	X89247	4	1
Bm101 gene	AY091586	18	1
Bm44	AB158647	23	1
BmGATA beta isoforms	U16320	15	1
BmLSP-T	AB158646	20	1
BmPI-T1	AB158645	8	1
BmRelA	AB096087	12	1
Bmwcp10 (cuticle protein)	AB091694	21	1
Bmwcp1a (cuticle protein)	AB047477	22	1

[a] The definition of LG 27 and 28 has not yet been unified

[b] 1 Yasukochi et al. (2006), 2 Miao et al. (2005)

Table 2 (continued)

Genes	Accession number	Linkage group[a]	References[b]
Bmwcp2 (cuticle protein)	AB047480	22	1
Bmwcp3 (cuticle protein)	AB047481	22	1
Bmwcp7a (cuticle protein)	AB047485	22	1
Bmwcp9 (cuticle protein)	AB047488	22	1
Bombyxin G1	AB010380	11	1
Bomopsin1	AB047924	15	1, 2
Bomopsin2	AB047925	11	1
Bras2	AB206960	7	1
Broad complex	AY166728	8	1
Bursicon beta subunit precursor	BN000690	23	1
Cadherin-1	AB002395	7	1
Cadherin-like membrane protein btr175a	AB041508	6	1
Calcineurin B	AF287251	18	1
Calreticulin	AB090887	22	1
Carotenoid-binding protein	AB062740	2	1
Casein kinase 2 alpha subunit	AB206394	5	1
Casein kinase 2 beta subunit	AB206395	15	1
Casein kinase I alpha subunit	AY769969	26	1
Caspase-1	AF448494	10	1
Cathepsin D	AY297160	23	1
Caudal	D16683	13	1
Cdc2	D85134	16	1
cdc2-related kinase	D85135	5	1
Cecropin A1	D84395	6	1
Cecropin B	D25321	26	1
Cecropin D	AB010825	26	1
Cell cycle checkpoint kinase 2	AB194683	8	1
Chemosensory protein CSP1	AF509239	19	1
Chemosensory protein CSP2	AF509238	19	1
Chitinase precursor	AB052914	7	1
Chorion A/B.L12-11	X15557	2	1
Chorion b-ZIP transcription factor	AY618899	6	1
Chymotrypsin inhibitor CI-8A	AY361483	19	1
Collagen type IV	AY099069	4	1
Conventional protein kinase C	AB184961	8	1
CP8 precursor	AY387408	10	1, 2
Cu/Zn SOD	AY461705	23	1
Cubitus interruptus	AF529422	U(28)	1,2
Cuticle protein	AB017550	22	1
Cyclin B	D84452	5	1
Cyclophilin-like protein	AB206402	9	1
Cysteine proteinase	S77508	23	1
Cytosolic juvenile hormone binding protein	AF098303	10	1
DEAD box protein 1	AB003909	11	1
Defensin-like protein	DQ118523	4	1
Deformed	D83534	6	1
Deoxynucleoside kinase	AF226281	5	1
DH-PBAN	D16230	11	1
Diapause bioclock protein	DQ086424	10	1

Table 2 (continued)

Genes	Accession number	Linkage group[a]	References[b]
Dmc1 homolog	U94994	11	1
DNA cytosine-5 methyltransferase	AB194009	11	1
Dopa decarboxylase	AF372836	4	1
Dopamine transporter	DQ105979	10	1
Ecdysone receptor	D43943	10	1
Ecdysteroid-inducible angiotensin-converting enzyme-related gene product	AB026110	9	1
Ecdysteroid-phosphate phosphatase	AB107356	25	1
Egg specific protein	D12521	19	1, 2
Elongation factor 1 alpha	D13338	5	1, 2
Elongation factor 1 delta	AB046366	5	1
Elongation factor 1 gamma	AB046361	18	1
EN16(annexin)	AB041636	7	1
Enbocin	U30289	26	1
Engrailed	M64335	2	1
Early chorion	X58446-X58448	2	1
Esterase-like protein	AB017522	14	1
Eukaryotic translation initiation factor 2	AJ715870	23	1
Even-skipped	D38486	2	1
Fatty-acyl reductase	AB104896	12	1
Fibroin light chain	M76430	14	1
FK506-binding protein FKBP59 homolog	AB206403	6	1
FNTA(farnesyltransferase/geranylgeranyltransferase) type I alpha subunit	AB201555	23	1
FTZ-F1	D10953	1	1
Furin-like convertase	AB120705	21	1
G protein alpha subunit Gs2	AB187517	20	1
General odorant binding protein 2	X94989	19	1
GGY cuticle protein 1	AB055661	18	1
Gloverin-like protein 1	AB190863	P(27)	1
Gloverin-like protein 2	AB190864	17	1
Gloverin-like protein 3	AB190865	P(27)	1
Gloverin-like protein 4	AB190866	P(27)	1
Glucosidase	AY272037	5	2
Glutathione S-transferase	AJ006502	6	1
Glutathione S-transferase 1	AY192575	7	1
Glutathione S-transferase 1-6	AB176691	6	1
Gly tRNA synthase	L08106	15	1
Glycerophosphoryl diester phosphodiesterase	AB115083	15	1
Glycine-rich protein	AB197877	8	1
Gq-like G protein alpha subunit	AB105070	9	1
Gram(-) bacteria-binding protein	L38591	11	1
Heat shock 70–kDa protein cognate	AB016836	15	1
Heat shock cognate protein BmHSC70-4	AB084922	9	1
Heat shock protein hsp19.9	AB195970	U(28)	1
Heat shock protein hsp20.1	AB195971	U(28)	1
Heat shock protein hsp20.8A	AF315317	U(28)	1
Heat shock protein hsp21.4	AB195972	13	1
Heat shock protein hsp23.7	AB195973	U(28)	1

Table 2 (continued)

Genes	Accession number	Linkage group[a]	References[b]
Hemolin-interacting protein	AY461708	13	1
Hemolymph juvenile hormone binding protein	AF098304	23	1
Heparanase-like protein	AB079860	1	1
High-affinity octopamine transporter protein	DQ097788	20	1
Homeodomain protein PBX	AB192342	15	1
Hormone receptor 39	AB005600	15	1
Humoral lectin	D29738	10	1
Immulectin	AY297159	4	1
Inducible nitric oxide synthase-like protein	AB017521	10	1
Inhibitor of apoptosis protein	AF281073	23	1
Intersex	AY648340	7	1
Invected	M64336	2	1
J domain containing protein	AF176014	12	1
Juvenile hormone diol kinase	AY363308	3	1
Juvenile hormone esterase	AF287267	25	1
Juvenile hormone-regulated protein gene marker	AY547263	8	1
Kazal-type serine proteinase inhibitor 1	AF352583	25	1
Kinesin-like protein	D21206	7	1
Kiser	AY579746	4	1
Kynurenine 3-monooxygenase	AB063490	10	1
Lab	AB120761	6	1
Larval serum protein	D12523	20	1
LCP(larval cuticle protein)17	AB004766	8	1
LCP(larval cuticle protein)18	AB021883	9	1
LCP(larval cuticle protein)22	AB004767	22	1
LCP(larval cuticle protein)30	X74321	22	1
Lebocin 3	AB003035	10	1
Leu-rich repeat G protein-coupled receptor	AF177772	8	1
Lim protein	AY461436	13	1
Lipase-1	AB076385	5	1
Lysosomal-associated transmembrane protein	AF317420	8	1
Lysozyme	L37416	12	1
Mago-nashi-like protein gene	AY863106	15	1
Masquerade-like serine proteinase homolog	AF513368	24	1
MCM7	AB177622	15	1
Membrane-type alkaline phosphatase	D90454	3	1
ADP/ATP translocase	AY227000	24	1, 2
Mn superoxide dismutase	AB190802	3	1
Molybdenum cofactor sulfurase	AB090243	9	1, 2
Moricin 2	AB019538	23	1
Myosuppressin receptor	AB188257	10	1
NAD-dependent deacetylase sirtuin 2 homolog	AB194684	25	1
NADPH cytochrome P450 reductase	AB042615	10	1
N-ethylmaleimide sensitive fusion protein	AY864804	22	1
Nonclathrin coat protein gamma2-COP	AB040670	8	1
NPV promoting protein	AB030701	18	1
NPV resistance marker	AY380834	U(28)	1
Nuclear matrix association region	AF130337	5	1
Nuclear orphan receptor	AF237663	U(28)	1

Table 2 (continued)

Genes	Accession number	Linkage group[a]	References[b]
Nuclear receptor GRF	AF124981	15	1
Octopamine receptor	X95607	23	1
Olfactory receptor-1	AB059431	1	1
Olfactory receptor-4	AB186506	1	1
Olfactory receptor-5	AB186507	14	1
Olfactory receptor-6	AB186508	12	1
Orphan nuclear receptor E75A	AF332550	10	2
p109	AB008449	12	1
p23-like protein	AB206399	6	1
p270	U67867	16	1
Paralytic peptide	AB064522	13	1
Phenol UDP-glucosyltransferase	AF324465	15	1
Pheromone-binding protein	X94987	19	1
PKG(cGMP-dependent protein kinase)-I	AF465600	3	1
PKG(cGMP-dependent protein kinase)-II	AF465602	14	1
Polyubiquitin	AB021974	21	1
POU-M1	L01266	22	1
Profilin	AY690617	4	1
Proliferating cell nuclear antigen	AB002264, AB002265	22	1
Prolyl 4-hydroxylase alpha subunit	AY099068	12	1
Prophenoloxidase activating factor 3	AY061936	25	1
Prophenoloxidase subunit 1	D49370	16	1
Protein disulfide-isomerase-like protein ERp57	AB210112	5	1
Protein kinase C inhibitor	AY860950	2	1
Protein tyrosine phosphatase	AB126695	23	1
Protein-O-fucosyltransferase 1	AJ831491	4	1
Protein-O-fucosyltransferase 2	AJ831834	24	1
Prothoracicotropic hormone	D90082	22	1, 2
Pupal cuticle protein	X68930	16	1
Pyruvate kinase	AB126230	9	1
Rad51	V94993	25	1
Ras3	AB170011	6	1
Receptor-type guanylyl cyclase	AB047558	7	1
Replication protein A middle subunit	AB194777	8	1
Replication protein A small subunit	AB194778	25	1
Ribonuclease L inhibitor homolog	AB164193	7	1
Ribosomal protein L3	AB024901	4	1
Ribosomal protein L4	AY769271	11	1
Ribosomal protein L5	AY769272	15	1
Ribosomal protein L6	AY769273	16	1
Ribosomal protein L7A	AY769275	15	1, 2
Ribosomal protein L9	AY769277	12	1
Ribosomal protein L10A	AY769279	15	1
Ribosomal protein L11	AY769280	3	1
Ribosomal protein L12	AY769281	19	1
Ribosomal protein L13	AY769282	5	1
Ribosomal protein L13A	AY769283	3	1, 2
Ribosomal protein L14	AY769284	11	1
Ribosomal protein L15	AY769285	6	1

Table 2 (continued)

Genes	Accession number	Linkage group[a]	References[b]
Ribosomal protein L18	AY769287	11	1
Ribosomal protein L19	AY769289	5	1
Ribosomal protein L21	AY769290	13	1
Ribosomal protein L22	AY769291	17	1, 2
Ribosomal protein L23A	AY769293	10	1
Ribosomal protein L24	AY769294	9	1
Ribosomal protein L31	AY769301	13	1
Ribosomal protein L32	AB048205	24	1
Ribosomal protein L37	AY769308	22	1
Ribosomal protein P0	AJ457827	15	1
Ribosomal protein P1	AJ490511	15	1
Ribosomal protein P2	AY769269	16	1, 2
Ribosomal protein S5	AY769319	15	1
Ribosomal protein S6	AY769320	21	1
Ribosomal protein S7	AY769321	20	1
Ribosomal protein S8	AY769322	15	1
Ribosomal protein S11	AY706955	P(27)	1
Ribosomal protein S14	AY706956	9	1
Ribosomal protein S15	AY706957	21	1
Ribosomal protein S15A	AY769331	26	2
Ribosomal protein S16	AY769332	14	1
Ribosomal protein S17	AY769333	21	1
Ribosomal protein S20	AY769336	20	1
Ribosomal protein S21A	AY578154	2	1
Ribosomal protein S23	AY706958	15	1
Ribosomal protein S24	AY578155	17	1
Ribosomal protein S25	AY769340	25	1
Ribosomal protein S26	AY769341	3	2
Ribosomal protein S27	AY769342	7	1
Ribosomal protein S28	AY583363	2	1
Ribosomal protein S29	AY769345	25	1
Ribosomal protein S30	AY769346	23	1
Ribosomal protein SA	AB062685	17	1
Ribosomal SOP2	AY763110	11	1
SC element binding protein	M69262	21	1
Sensory neuron membrane protein-1	AJ251958	18	1
Sericin 1	AB007831	11	1
Serine protease	AB073673	7	1
Serine protease-like protein	DQ118520	6	1
Seroin 2	AF352585	5	1
Serotonin receptor	X95604	10	1
Serpin-4A	AY566164	9	1
Serpin-5	AY566165	P(27)	1
Sex comb reduced	D83533	6	1
Sex lethal	AB207267	16	1
SGF-1	D38514	25	1
Silk protein P25	X04226	2	1
Small GTP-binding protein (rabB)	AF013572	25	1
Soluble alkaline phosphatase	AB013386	3	1

Table 2 (continued)

Genes	Accession number	Linkage group[a]	References[b]
Sorbitol dehydrogenase	D66906	21	1, 2
Star	AF455272	26	1
Storage protein 1 (SP1)	X12978	23	1
Storage protein 2 (SP2)	M24370	3	1
Sui1 translation initiation factor	AY426343	24	1, 2
Supercoiling factor	D49948	13	1
Testis-specific tektin	AB056651	P(27)	1
Testis-specific ATPase inhibitor-like protein	AB056704	14	1
Thiol peroxiredoxin	AY438331	15	1
Topoisomerase II	AF013277	11	1
Trachealess	AB007832	5	1
Transcription factor BmEts	AB115082	17	1
Transformer-2	AY626066	8	1
Translation initiation factor 2 alpha subunit	DQ073458	20	1
Trehalase	D86212	17	1
Triosephosphate isomerase	AY734490	1	1
Troponin I	AY873787	13	1
Ultrabithoraxbx	X62618	6	1
Ultraspiracle	U06073	4	1
Urbain	Z47409	18	1
VAP-peptide	AB001053	6	1
Vasa-like gene	D86601	19	1
Vitellin degrading protease	D16233	P(27)	1
Vitelline membrane associated protein P30	AF294885	17	1
Vitellogenin	D30733	U(28)	1
Vitellogenin receptor	AY676607	20	1
Wing disc-specific protein CB10	AB062684	5	1, 2
Wnt	D14169	4	1
Xanthine dehydrogenase 1	D38159	12	1
Xanthine dehydrogenase 2	D43965	12	1
Y-box protein	AB098537	1	1
Zerknült	AB120763	6	1

RFLPs of cDNA probes (Shi et al. 1995). In 1998, Yasukochi constructed a dense linkage map based mainly on RAPD markers consisting of 28 linkage groups (LGs) corresponding to the 28 haploid chromosome number of *B. mori* (Yasukochi 1998). These three maps were developed using the same strains C108 and Daizo (Dazao, in Chinese); the latter strain was also used for whole-genome shotgun sequencing (Mita et al. 2004; Xia et al. 2004). In addition, four maps based on microsatellites, a fluorescent polarization dye terminator, SNP-detection assay, and RFLP markers were made using the same strains (Miao et al. 2005; Yamamoto et al. 2006), as well as using different strains (Nguu et al. 2005; Prasad et al. 2005). As a consequence, there are too many independent linkage maps of *B. mori* and it is likely that many of them will not be widely utilized unless they are integrated. Table 2 lists genes mapped onto the linkage maps (Miao et al. 2005; Yasukochi et al. 2006).

Integration of classical and molecular linkage groups is critical for optimal utilization of the information accumulated in classical mapping efforts. The classical LGs were thought to be composed of 27 groups and an unlinked gene, *E-tr,* equivalent to 27 autosomes and the Z chromosome. However, classical LG 24 and 27 were recently found to be identical,

and the 26 classical LGs have now been assigned to molecular counterparts (Yasukochi et al. 2005).

4.3 Efforts in Forward Genetics

Various genetic traits were previously characterized and mapped onto the classical linkage map as described above. Some of them have been characterized developmentally or physiologically, and candidate genes are already assumed. Table 3 lists cloned genes whose functions are closely related to assumed mechanisms of mutation and whose polymorphisms co-segregate with described mutations. These are described in more detail below.

The *E* locus, which represents a number of dominant homeotic mutations affecting the identity of larval abdominal segments, is closely linked to the *Nc* locus, another homeotic mutation. The *BmUbx* and *Bmabd-A* genes were not detected in homozygous E^N/E^N embryos (Ueno et al. 1992), and the 3′-portion of the *BmAntp* gene is deleted in the *Nc* mutant (Nagata et al. 1996). Linkage analysis using molecular markers derived from a bacterial artificial chromosome (BAC) contig covering the Hox gene cluster (Yasukochi et al. 2004) also suggests that the *E* locus is composed of independent mutations that occurred in the *BmUbx*, *Bmabd-A*, and *BmAbd-B* genes (Y. Yasukochi, and M. Hirokawa, unpublished results).

"Oily" refers to mutations of similar phenotype in which larval skin appears translucent, just like oiled paper, instead of opaque, as in wild type. Twenty-four oily mutations have been placed on the classical linkage map (Banno et al. 2005), and most of them are recessive. This phenotype is caused by lack of uric acid in the hypodermis, but there is a different deficiency for each mutation (Goldsmith 1995). In other words, the white larval skin color of the silkworm seems to be simple at a glance, but is maintained by complex mechanisms required for various essential genes. Putative genes responsible for two mutations, *oq* (12-26.3) and *og* (9-23.6), have been characterized and correspond to enzymes in the uric acid biosynthetic pathway (Table 2; Yasukochi et al. 1998; Komoto et al. 2003).

Recently, a mutation of the *Y* gene, one of the oldest genes known in *B. mori*, was shown to be caused by insertion of a non-LTR retrotransposon into the second exon of the carotenoid-binding protein (CBP) gene in wild type strains (Sakudoh et al. 2005, Sakudoh et al. 2007). This insertion inhibits expression of a functional CBP and prevents the transfer of carotenoids derived from mulberry leaves to the hemolymph. A functional CBP is also expressed in *B. mandarina*, which produces yellow silk. It seems somewhat confusing that "normal" *B. mori* strains have mutations; however, similar events are thought to have occurred frequently during domestication.

Complementation of a mutant phenotype by introducing a wild type gene via transgenesis has not yet been accomplished in *B. mori*. There is one report

Table 3 Genes associated with mutant phenotypes

Loci	LG	Phenotypes	Genes	References
E complex	6	Addition or lack of legs and crescents	*BmUbx, Bmabd-A, BmAbd-B*	Ueno et al. (1992)
Nc	6	Lack of crescents	*BmAntp*	Nagata et al. (1996)
Nd-s	14	Cocoons lacking fibroin	Fibroin light chain	Mori et al. (1995)
og	9	Translucent skin	Molybdenum cofactor sulfurase	Komoto et al. (2003)
oq	12	Translucent skin	Xanthine dehydrogenase	Yasukochi et al. (1998)
w-1	10	White egg	Kynurenine 3-monooxygenase	Quan et al. (2002)
w-3	10	White egg	ABC transporter	Abraham et al. (2000)
Y	2	Yellow hemolymph and cocoon	Carotenoid-binding protein	Sakudoh et al. (2005), Sakudoh et al. (2007)

of the partial rescue of a "naked pupa" mutation by introduction of a cDNA for a light chain of the cocoon filament, which is required for normal secretion of the silk (Inoue et al. 2004). However, further improvement of transgenic or RNAi technology is needed for reliable confirmation of candidate gene identification.

4.4 Detection of Quantitative Trait Loci

Resistance to densovirus, a 5-kb DNA picornavirus, seems to be relatively easily evolved in *B. mori* and four loci, *nsd-1* (21-83), *nsd-2* (17-24.5), *Nid-1* (17-31.1), and *nsd-Z* (LG15), have been reported (Banno et al. 2005). In contrast, there are no reported loci for resistance to nucleopolyhedrosis virus (NPV), presumably because it has a genome of 130 kb and its strategy of infection is too complicated to allow resistance by a single mutation.

B. mori originated in East Asia, and most practical breeding has been carried out there. Races of high yield and good quality bred in East Asia are not necessarily adapted to tropical climates, and it is worthwhile to characterize quantitative trait loci (QTLs) concerning high silk yield, good cocoon quality, and physiological stress resistance for marker-assisted breeding (MAS) of tropical races. This work has just begun in *B. mori*, and yield traits in a few strains have been analyzed by using inter-simple sequence repeat (ISSR) markers (Chatterjee and Mohandas 2003).

Like many lepidopterans, *B. mori* feeds on leaves of a narrow range of plants (*Morus* and some species of *Maclura*, *Broussonetia*, and *Cudrania*). Attempts have been made to isolate mutants feeding on a broader range of plants, and several loci, *Bt* (1-40.7), *Np* (11-32.7), *ph* (LG1), and *Nps/Ph/pph* (LG3), were mapped onto the classical linkage map (Banno et al. 2005), although the relationship among loci located on LG3 is unclear. It is expected that analysis of these "polyphagous" mutants will reveal molecular mechanisms in feeding traits conserved among lepidopterans, which may lead to development of novel means of pest control.

B. mori and the honeybee, *Apis mellifera*, are the most fully domesticated insects, but changes in their behavior during domestication are in marked contrast. Whereas honeybees remain essentially wild, striking differences are observed in behaviors of *B. mori* and *B. mandarina*, such as the domesticated species' loss of ability to fly or escape predation and its abundant immediate laying of eggs, which the wild species deposits in small amounts over several days and wide areas. Since matings between *B. mori* and *B. mandarina* generate fertile F_1 hybrids, several preliminary projects are underway to identify the genetic basis of such differences in behavior using BF_1 progeny.

4.5 Advanced Projects

4.5.1 Sequencing Projects: ESTs and Whole-genome Shotgun

Large-scale sequencing projects of expressed sequence tags (ESTs) (Mita et al. 2003; Cheng et al. 2004) and whole-genome shotgun (Mita et al. 2004; Xia et al. 2004) have been performed. The data can be used for homology search at dedicated databases "Kaikoblast (kaikoblast.dna.affrc.go.jp/)" and "SilkDB" (Wang et al. 2005), as well as at public databases. As a result, our knowledge of the silkworm genome sequence has been dramatically increased.

However, the scale of these projects was insufficient considering the size and complexity of the *Bombyx* genome and assembled sequence contigs are still highly fragmented. Therefore, integration of sequence data from different projects has been essential and in April 2006 a collaborative agreement toward this end was reached between the two sponsoring agencies, the National Institute of Agrobiological Sciences (NIAS, Japan) and Southwest Agricultural University (China) (http://www.nias.affrc.go.jp/pressrelease/20060418/index.html in Japanese). It is expected that more reliable sequences will be published in the near future.

cDNA microarrays are now widely utilized to find specific expression of genes. The first microarray of *B. mori* was prepared by spotting PCR products amplified from cDNA clones (Ote et al. 2004). A second-generation microarray has already been designed from clustered EST sequences under Affymetrix tech-

nology, and experimentally distributed from NIAS (H. Noda personal communication).

4.5.2 Physical Mapping Efforts

BAC libraries play critical roles in map-based cloning (MBC) and genome sequencing. BAC libraries have been constructed from strains p50 and C108, and a PCR-based screening system has been developed (Wu et al. 1999). Using this library and screening system, 874 BAC contigs have been constructed, which cover nearly all of the genes published in public databases and approximately 23% of the whole genome (Yasukochi et al. 2006). One well-characterized example is a contig spanning the Hox gene cluster on LG6 (Yasukochi et al. 2004).

The utility of BAC clones for determining large and complex genome sequences was shown in the sequencing of the fibroin heavy chain gene (Zhou et al. 2000) and the Broad-Complex gene (Ijiro et al. 2004). Another application of BAC clones is to use probes for chromosomal fluorescence in situ hybridization (FISH) analysis. Large-insert clones are excellent probes for FISH; however, there are numerous transposable/repeated elements in the *B. mori* genome which generate non-specific signals. Utilization of previously mapped BAC clones as probes with excess genomic DNA competitor enabled highly specific detection of their chromosomal locations, allowing visualization of a complete karyotype for a lepidopteran with the assignment of LGs to chromosomes for the first time (Yoshido et al. 2005). BAC-FISH analysis is particularly effective for detection of chromosomal aberrations (Sahara et al. 2003).

4.5.3 Integrated Genetic Map

Linkage maps unrelated to genome information such as genome sequences, ESTs, linkage maps, BAC contigs and chromosomal locations are of little use for modern genetics, since maps themselves are not a goal but tools to find clues to identify molecular mechanisms underlying phenotypes. A second-generation linkage map has been constructed for *B. mori*, which contains more than 567 polymorphic markers useful for QTL analysis and marker-assisted breeding (Yasukochi et al. 2006) and has a close relationship to the cytogenetic analysis carried out by BAC-FISH (Yoshido et al. 2005).

4.5.4 Toward Comparative Genomics of Lepidoptera

The question of whether genome structure is conserved among lepidopteran insects has intrigued geneticists, especially considering the holocentric nature of lepidopteran chromosomes. If synteny exists, genetic studies on other lepidopteran species would be greatly facilitated by utilization of genome information from *B. mori*.

Recently, a linkage map was constructed in a mimetic butterfly, *Heliconius melpomene* (Jiggins et al. 2005). All 13 conserved genes in four LGs of *H. melpomene* available for a test of synteny were found to be colocalized in the same LGs in *B. mori*, and the gene order in LG10 and 11 of *H. melpomene* was conserved in LG5 and 15 of *B. mori,* respectively (Yasukochi et al. 2006). More detailed analysis was carried out for *H. melpomene*, and revealed extensive synteny between *H. melpomene* and *B. mori* (Pringle et al. 2007).

Syntenic analysis requires mapping of conserved genes. However, there is usually low intraspecific polymorphism in coding regions of such genes, making it difficult to perform linkage analysis by conventional genetic mapping approaches. BAC-FISH is a high-throughput tool for determining the order of conserved genes, especially for less well-characterized species. Since a number of BAC libraries have been constructed from other lepidopteran species (e.g., d'Alençon et al. 2004), detailed comparison with *B. mori* is now feasible by BAC-FISH. If synteny maps are established for major groups of Lepidoptera, lepidopteran research will be fundamentally changed by making use of *Bombyx* genome information.

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5 Pea Aphid

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5.1 Introduction

Aphids have long been of interest because of their complex life cycles, environmentally induced morphologies, and importance to agriculture. However, only recently has the resulting wealth of ecological and population genetic data begun to be supplemented by genomic and genetic mapping approaches. In 2004, the International Aphid Genomics Consortium, a collaboration of aphid researchers, chose to establish the pea aphid, *Acyrthosiphon pisum*, as the aphid of choice for the development of genomic resources. Here we introduce the pea aphid and discuss a number of biological questions for which the species is well suited. We then review previous mapping and quantitative trait loci (QTL) studies, ending with a discussion of the genomic tools that are currently available, including the recently initiated genome-sequencing project.

The pea aphid is one of approximately 4,400 species of aphids in the order Hemiptera. Pea aphids belong to the family Aphididae, subfamily Aphidinae, and are named after their host plants, members of the pea family Fabaceae (Leguminosae). The historical range of the pea aphid was palaearctic, but pea aphids are now distributed worldwide, introduced to North America within the last 125 years, presumably by transportation on their host plants (Blackman and Eastop 2000). Like other aphids, they are soft-bodied animals that have a pair of long antennae that stretch much of the length of their body, and a pair of cornicles on the dorsal fifth abdominal segment that excrete alarm pheromones in response to predators. They probe the plant surface with their proboscis and use a piercing stylet to tap phloem and passively ingest sap. Because this diet is carbohydrate-rich and deficient in a number of essential amino acids, aphids rely on obligate bacterial endosymbionts to meet their nutritional requirements. Adult pea aphids reach up to 4.5 mm in length, are pink or green, and can be winged or unwinged.

The pea aphid has a complex life cycle that seasonally alternates between sexual and asexual reproduction (Fig. 1). In the spring, a female pea aphid emerges from an egg that has overwintered. This female is asexual and produces or "founds" a population of genetically identical females, which continue to reproduce asexually during the summer months. Asexual reproduction is accomplished via a modified meiosis in which the reduction division is effectively skipped and no recombination occurs (Blackman 1987; Hales et al. 2002). Daughter embryos complete embryogenesis within the mother's ovarioles and mothers give live birth to first instar nymphs, up to 12 per day. After progressing through four nymphal instars (approximately 10 days), the resulting adult females are capable of producing offspring. The short asexual generation time allows aphid clones to undergo many generations during the summer months and, along with their high fecundity, to quickly colonize host plants.

In the fall, asexual females respond to the cues of shortened day length and colder temperatures by asexually producing a generation of sexual females and males (MacKay et al. 1983; Lees 1990). Males, which are XO, are produced genetically by the random loss of one X chromosome due to a failure to attach to spindle fibers on the metaphase plate during the single maturation division (Orlando 1974; Blackman 1987; Wilson et al. 1997). Mating between sexual females, which are oviparous, and males produces an egg containing a female (XX) embryo, presumably because only sperm that carry an X chromosome are viable. The egg is specially adapted to withstand winter conditions and hatches

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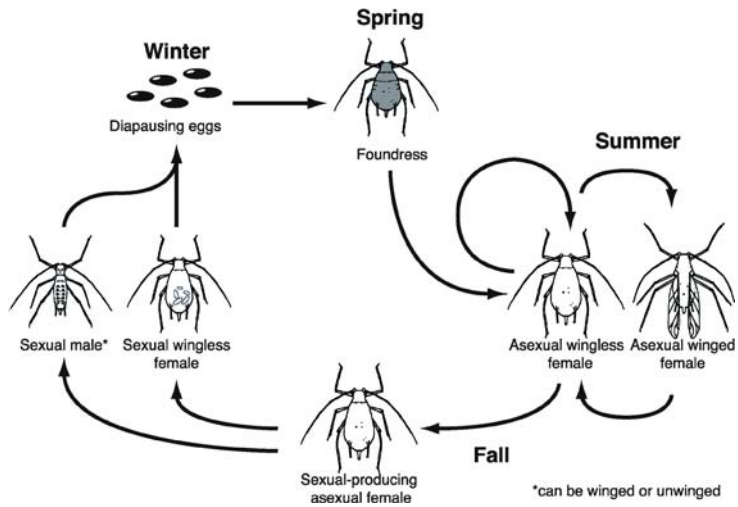

Fig. 1 Life cycle of the pea aphid (copyright: A. Shingleton)

approximately 100 days later, in the spring, allowing a newly emerged asexual female to found a new clone.

Among other phenomena, the pea aphid is a model system for examining polyphenisms, which are environmentally induced, discrete, alternative morphologies. Displayed by a wide variety of insects, polyphenisms are typically adaptive, allowing insects to cope with environments that change in predictable ways. In the case of the pea aphid, the principal polyphenisms are the so-called wing and reproductive polyphenisms.

In the summer months, asexual females typically develop without wings. However, under stressful conditions, such as when the plant becomes overcrowded, they produce offspring that develop wings and can fly to a new host plant. Details of the wing polyphenism and the genomics and genetic approaches that have been taken to better understand it are discussed below. The reproductive polyphenism, already described as part of the life cycle, refers to the alternative production of sexual and asexual females as determined primarily by day length. Although sexual and asexual females possess subtle differences in external morphology, the principal difference between the two is the presence of either large, yolk-filled, haploid oocytes or much smaller, developing diploid embryos within the ovarioles. The developmental decision for the females to become either sexual or asexual is made during embryogenesis based on the day length-induced state of the mother. In the case of either polyphenism, pea aphids offer the advantage that the alternative morphologies can be found among members of the same clone, which possess identical genotypes, greatly simplifying analysis of the phenomena.

5.1.1 Agricultural Importance

Aphids are best known as agricultural pests. As a group, they are estimated to be responsible for the annual loss of hundreds of millions of dollars worth of crops in the USA alone (Oerke 1994; Morrison and Peairs 1998). Their high fecundity and short generation time result in large populations that can destroy crops. Feeding alone can result in either plant death or cosmetic damage that can make a crop undesirable. The primary damage caused by aphids, however, is due to their ability to vector devastating plant viruses (Nault 1997; Blackman and Eastop 2000; Nault et al. 2004). Aphids are efficient virus vectors due in part because the winged morphs disperse widely. Winged morphs can traverse large areas via active flight or via passive migratory flights in upper air currents, traveling as far as 1,000 km in a single flight (Robert 1987). They are, therefore, capable of traveling from one agricultural field to another, spreading viral diseases as they disperse.

The pea aphid specifically is classified as a mild agricultural pest on alfalfa and clover, vectoring more than 30 viral diseases (Blackman and Eastop 2000). As

a member of the Macrosiphini, pea aphids are closely related to most major pest aphid species, including the peach-potato aphid (*Myzus persicae*) and the Russian wheat aphid (*Diuraphis noxia*). Hence, investigations into potential means of controlling pea aphid populations are likely to be applicable to these and other aphid pest species.

5.1.2 Breeding Objectives

Pea aphids are relatively easy to rear in the laboratory. They can be housed in small Petri dishes containing a single leaf of *Medicago arborea* inserted into agar containing fertilizer, or in closed buckets containing whole alfalfa plants. The asexual phase of the life cycle is highly amenable to laboratory culture: lines of interest can be kept as clones indefinitely, without recombination, in incubators replicating summer-like conditions. If a sexual generation is required, for genetic crosses, for example, individuals can be placed in an incubator replicating fall-like conditions (Via 1992). Resulting eggs are subsequently placed in incubators mimicking winter-like conditions.

Here we briefly describe three aspects of pea aphid biology, the wing polyphenism, host plant specialization, and bacterial symbioses, that have been examined from a genetic mapping, quantitative trait loci (QTL), and genomics perspective, respectively. Specific studies representing the latter approaches will be described when we revisit each of these topics later in the chapter. Although we have chosen to focus on these three areas, they are by no means the only aspects of pea aphid biology that are likely to be amenable to future genomics or genetic mapping approaches (for excellent reviews about aspects of pea aphid biology, see Heie 1980; Minks and Harrewijn 1980; Moran 1992; Blackman and Eastop 1994, 2000; Dixon 1998).

Winged and Unwinged Morphs

As described above, asexual females typically develop without wings. However, under stressful conditions such as a decline in host plant quality or an overcrowded plant, they produce offspring that develop wings and can fly to a new host plant (Sutherland 1969). Other stressors that have been documented to induce winged offspring include exposure to predators (Dixon and Agarwala 1999; Podjasek et al. 2005) and parasitoids (Sloggett and Weisser 2002). In the pea aphid at least, such cues, rather than directly influencing developing nymphs, are instead first perceived by the mother, who then somehow transmits a permissive signal to develop with wings to her embryos before they are born (Sutherland 1969).

The sexual males produced in the fall are also found as winged and unwinged forms. Though in contrast to the environmentally cued wing dimorphism in females, wing production in males is determined by an unidentified X-linked genetic polymorphism at the *aphicarus* (*api*) locus (Smith and MacKay 1989; Caillaud et al. 2002; Braendle et al. 2005a). The male wing dimorphism is, therefore, referred to as a polymorphism. Interestingly, genetic variation for the female polyphenism is linked to the *api* locus (Braendle et al. 2005b).

Although referring to the female polyphenism as a "wing dimorphism" is convenient shorthand, many aspects of the phenotype in fact differ between winged and unwinged individuals (Kring 1977). For example, winged morphs have ocelli on the vertex of their head and greatly expanded thoraces with flight musculature, whereas unwinged morphs do not. Also, the cuticle of the winged morph is more heavily sclerotized than that of the unwinged morph and the dimensions of the legs and siphunculi differ. Moreover, winged individuals are active and fly to new host plants, whereas unwinged morphs are sedentary. Winged females also have reduced fecundity relative to unwinged morphs (MacKay and Wellington 1975; MacKay et al. 1983).

The existence of two distinct morphologies is generally thought to be a trade-off between resources dedicated to reproduction in the unwinged morph versus resources dedicated to dispersal in the winged morph (reviewed in Zera and Denno 1997). The pea aphid provides a unique advantage for studies of wing dimorphism, which are common in insects, by exhibiting both environmental (the female polyphenism) and genetic (the male polymorphism) mechanisms of determination. Investigations into the molecular basis of the genetically determined polymorphism may thus reinforce our understanding of the molecular basis of the extreme phenotypic plasticity shown by the polyphenism.

Host Plant Specialization

The pea aphid is also well suited for examining the genetics of adaptation and speciation. Although some clones of pea aphids are generalist feeders, others prefer to feed on particular host plants such as pea, alfalfa, or clover (Via 1991; Sandstrom 1994). This specialization may have preceded their introduction to North America (Birkle and Douglas 1999; Simon et al. 2003; Frantz et al. 2006). Especially well studied are the host races that specialize on either alfalfa (*Medicago sativa*) or red clover (*Trifolium pretense*) in North America (Via 1991; Caillaud and Via 2000). When a pea aphid encounters a host plant, it decides whether it is an acceptable food source by probing it several times with its stylet (Caillaud and Via 2000). Upon acceptance, the aphid will settle on a plant for feeding. Because aphids tend to breed where they spend their time feeding, this creates a situation where gene flow between the two host plant specialists is low (Via 1994, 1999). These pea aphid host plant specialists may, therefore, represent incipient species produced by sympatric speciation (Hawthorne and Via 2001).

Bacterial Symbioses

Pea aphids have been exceptionally well studied with regard to their bacterial endosymbionts. The species of bacteria that aphids require to provide them with essential amino acids, *Buchnera aphidicola*, resides within specialized cells of the aphid called bacteriocytes (Buchner 1965; Braendle et al. 2003) and is transmitted vertically, from mother to daughter. This obligate relationship between aphids and *Buchnera* is an ancient one, dating to 150 to 250 million years ago (Munson et al. 1991). As with other obligate endosymbionts, the genome of *Buchnera* is highly reduced (approximately 650 kb; Gil et al. 2002), but retains genes required for the synthesis of essential amino acids (Baumann et al. 1999; Shigenobu et al. 2000).

In addition to *Buchnera*, the pea aphid harbors at least five less well characterized facultative secondary symbionts of the *Rickettsia*, *Spiroplasma*, *Regiella*, *Serratia*, and *Hamiltonella* genera (Chen et al. 1996; Fukatsu et al. 2001; Moran et al. 2005). These symbionts affect traits such as resistance to elevated temperature (Montllor et al. 2002), parasitoid resistance (Oliver et al. 2003, 2005), host plant specialization (Tsuchida et al. 2004), and induction of winged forms (Leonardo and Mondor 2006).

5.1.3 Limitations of Genetic Linkage Mapping

As with many other systems, one of the greatest limitations of genetic mapping in the pea aphid is its long sexual generation time. Although stock populations can be induced by fall-like conditions to produce sexual individuals on a rolling basis, the eggs require about 100 days of winter-like conditions (alternating between 13 hours at 4 °C and 11 hours at 0 °C) to complete development (Via 1992). Unfortunately, rearing eggs at higher temperatures generally does not successfully speed up development: at 10 °C hatching success is significantly lower, and at 16 °C embryos show severe malformations, with no embryos hatching (Shingleton et al. 2003). It is possible, however, to induce eggs to hatch sooner by shifting them to 16 °C following a critical period, though such eggs typically show decreased hatching rates: eggs transferred from 0–4 °C to 16 °C at day 49 instead of day 98 showed a 20% reduction in hatching rate (Shingleton et al. 2003).

5.2 Construction of Genetic Maps

The densest pea aphid genetic map to date was developed by Hawthorne and Via (2001) in order to study host plant specialization. They developed a linkage map of 173 dominant amplified fragment length polymorphism (AFLP) markers. These markers group into four linkage groups (Fig. 2), agreeing with a previous report of four chromosomes in the pea aphid (Sun and Robinson 1966). Braendle et al. (2005a) developed an additional seven AFLP markers, all on the X chromosome (Fig. 3).

A number of studies have identified microsatellites that are variable in the pea aphid (Caillaud et al. 2002, 2004; Kurokawa et al. 2004). However, only a subset of these microsatellites have thus far been localized to any particular linkage group (Caillaud et al. 2002). Sabater-Munoz et al. (2006) identified 921 microsatellite repeats based on the expressed sequence tag (EST) collection (discussed below). It is likely that a portion of these will vary and hence be useful markers. Future map construction will undoubtedly be aided by the forthcoming genome sequence.

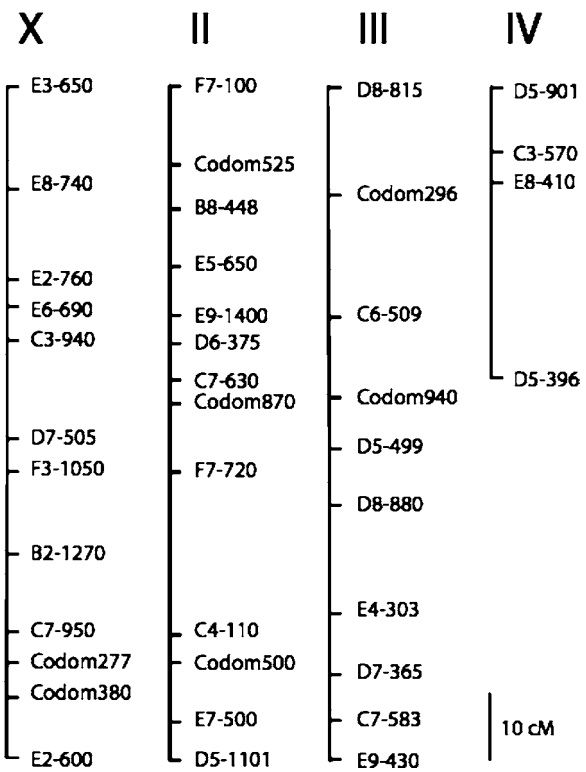

Fig. 2 Map of the four linkage groups (X, II, III, IV) of the pea aphid clover specialist, illustrating the location of co-dominant markers (those with a prefix of "Codom") and AFLP markers (all others). An additional set of AFLP markers and their corresponding positions for the alfalfa pea aphid specialist can be found in Hawthorne and Via (2001). Figure modified from Hawthorne and Via (2001)

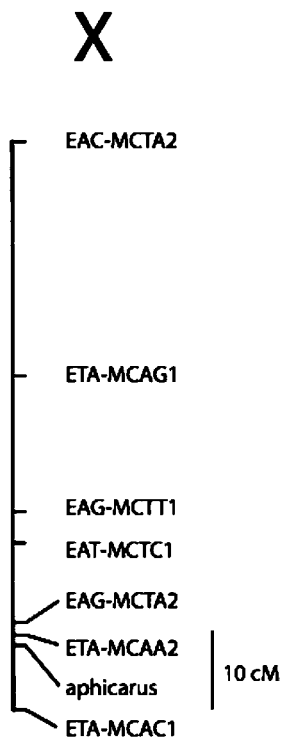

Fig. 3 Genetic map of the pea aphid X chromosome near *aphicarus*, illustrating the location of AFLP markers. Figure modified from Braendle et al. (2005a)

5.3 Gene Mapping by Linkage Analysis

The previously discussed male wing dimorphism is the only trait in the pea aphid that has been mapped by classical linkage analysis. Clones collected from nature produce either all winged males, all unwinged males, or winged and unwinged males in an equal ratio. Based on this observation, and the fact that males have only one X chromosome, Smith and MacKay (1989) hypothesized that the winged state of males is determined by a locus on the X chromosome. This hypothesis was supported by later work by Caillaud et al. (2002), who showed that the trait is determined by a single locus and segregates accordingly in the F_2 generation of a mapping panel produced from an initial cross between a clone that only produced winged males, and a clone that only produced unwinged males. They further confirmed that the locus was on the X chromosome by showing that the trait co-segregated with three X-linked microsatellite markers. Braendle et al. (2005a) then detected AFLP markers flanking this locus (Fig. 3), and named the locus *aphicarus* (*api*) after Icarus, the tragic figure of Greek mythology whose wax-cemented feather wings melted when he flew too closely to the sun.

5.4 Detection of Quantitative Trait Loci

The sole quantitative trait loci (QTL) study conducted in pea aphids aimed to identify the loci that underlie host plant specialization and mate choice. Hawthorne and Via (2001) reciprocally crossed an alfalfa specialist to a red clover specialist in order to test the hypothesis that specialization for a particular host plant and mate choice are genetically correlated, which

would suggest a mechanism to facilitate reproductive isolation. They measured fecundity on each of the two host plants (two traits) as a proxy for host plant specialization, and acceptance of each host plant (two traits) as a proxy for mate choice given that pea aphids mate where they feed.

Quantitative trait loci were identified on all four linkage groups that together explained 10–57% of the genetic variance in the four traits. On two of the linkage groups, they found four complexes of QTLs in close enough proximity to suggest that either the same locus might affect more than one of the traits or that there was tight linkage between the QTLs. These QTLs were all in the direction of promoting fecundity and acceptance on the host plant for which each aphid host race was specialized, while decreasing fecundity on the opposite host plant. They concluded that the genetic correlations between these traits could have facilitated divergence between the host plant specialists. Further studies using higher-resolution mapping will be necessary to confirm this result.

5.5 Advanced Work

5.5.1 Physical Mapping Efforts

A 6× coverage bacterial artificial chromosome (BAC) library has been constructed, consisting of 27,648 clones with an average insert size of 130 kb (Chris Amemiya, personal communication). As this library has only recently been completed (June 2006), it has not yet been used in any mapping attempts.

5.5.2 Sequencing Projects: ESTs and Whole-genome Shotgun

To date, over 67,000 expressed sequence tags (ESTs) from the pea aphid are publicly available in dbEST. These ESTs complement smaller numbers of ESTs from other aphid species, including *Myzus persicae* (approximately 14,000), *Aphis gossypii* (approximately 8,400), *Toxoptera citricida* (approximately 4,300), and *Rhopalosiphum padi* (approximately 500). In comparisons of the pea aphid to other Macrosiphini (including *Myzus*) and to Aphidini (including *Aphis*, *Toxoptera*, and *Rhopalosiphum*), nucleotide divergences for sequenced open reading frames of orthologous genes range from 5% to 10% and up to 15%, respectively (Moran et al. 1999; Von Dohlen and Teulon 2003).

A recent study analyzed 40,904 of the pea aphid ESTs derived from cDNA libraries made from antennae, bacteriocytes, digestive tracts, heads, parthenogenetic embryos, and multistage whole-bodies (Sabater-Munoz et al. 2006). These ESTs formed 12,082 contigs and singletons with an overall GC content of 33%. Of the unique transcripts 59% showed no homology with known proteins, although 25% of these transcripts are less than 300 bp and many did not have open reading frames (over 70% for transcripts <1,000 bp and over 30% for transcripts >1,000 bp). Of the unique transcripts that showed homology to known proteins, only 34% were present in *Drosophila melanogaster*. Further, 741 *D. melanogaster* genes showed similarity to more than one pea aphid contig, raising the possibility that gene duplications have occurred in the pea aphid.

Sabater-Munoz et al. (2006) also found tissue-specific gene expression in the bacteriocyte- and parthenogenetic embryo-derived libraries relative to the other six libraries. The bacteriocytes, which house the endosymbiotic bacteria, exhibited gene expression associated with amino acid metabolism and defense reactions, while 75% of the unique transcripts from the parthenogenetic embryo library had no known homology with *Drosophila*.

A pea aphid cDNA microarray has been constructed, consisting of quadruplicate spots of approximately 1,750 unique ESTs from pea aphids, and 117 unique genes from the bacterial endosymbiont *Buchnera aphidicola*. The pea aphid/*Buchnera* microarray has been used to study the heat shock response of the two organisms in parallel (Wilson et al. 2006) and to study the transcriptional basis of the winged and unwinged morphs of both females and males (Brisson et al. 2007).

Whole-genome shotgun sequencing of the 525-Mb pea aphid genome (Spencer Johnson personal communication) at 6× coverage was initiated in June 2006 at the Baylor College of Medicine, Human Genome Sequencing Institute, with funds provided by the National Human Genome Research Institute (http://www.hgsc.bcm.tmc.edu/

projects/aphid/, aphidbase.com). The genome will be assembled and computer-annotated in 2007. The strain used in the sequencing effort is the New York LSR1 line, which has been inbred for one generation and has had its secondary endosymbionts removed via ampicillin treatment. The strain, referred to as LSR1.G1.AC (for LSR1, one generation of inbreeding, antibiotic-cured), was also heat treated at 30 °C for four days prior to DNA extraction in order to decrease the amount of DNA contributed by its primary obligate endosymbiont, *Buchnera aphidicola*.

5.6 Future Foci

The recent and continuing acquisition of ESTs, a newly constructed BAC library, and a soon to be completed genome sequence will surely be a boon for those interested in the molecular and genetic processes underlying the phenomena exhibited by aphids. For example, the study of aphid insecticide resistance, which has thus far focused primarily on *Myzus persicae*, should benefit by easing the identification and cloning of insecticide targets and detoxifying enzymes that have been implicated in resistance more generally in insects (Ishaaya 2001) and more specifically in *Myzus* (Field et al. 1988; Field and Foster 2002). Our comparative genomics picture of arthropods is also likely to be enriched by the addition of one of the first genomes from a hemimetabolous insect (in addition to the hemipteran *Rhodnius prolixus* which is currently scheduled to be sequenced). Finally, the fact that the genomes of both *Medicago trunculata* (http://www.medicago.org/genome/), one of the pea aphid's primary host plants, and *Buchnera* (http://buchnera.gsc.riken.go.jp/), its primary endosymbiont, are also available should grant researchers the ability to explore the interactions of these organisms at a depth not previously possible.

Efforts must now focus on accurately annotating the genome and developing post-genomics technologies, including the development of whole-genome microarrays. Also needed are technologies that will allow us to test gene function, such as RNA interference (RNAi) and transgenesis. To date, there is one report of successful RNAi, in which a gene that is abundantly expressed in the adult salivary gland is knocked down by small interfering RNAs (siRNAs) injected directly into the hemolymph (Mutti et al. 2006). Apparently siRNAs are able to move into salivary gland cells, whereupon they exert their effects. It remains to be seen if this will be possible for other organs and tissues of the adult or the developing embryos found in asexual females. Transgenesis allows one to both misexpress a gene as well as test the regulative ability of its putative enhancers. To date, there are no reports of successful transgenesis in aphids, though successes in several other insect groups with at least two vectors (Wimmer 2003; Pavlopoulos et al. 2004) suggest that it is almost certainly possible. It is our hope that increasing interest in the pea aphid will facilitate such developments in the near future. Interested parties are encouraged to sign on to the aphid genomics list server (http://www.eco.princeton.edu/mailman/listinfo/aphidgenomics) to keep abreast of future developments.

Acknowledgement. JAB and GKD acknowledge support from the NIH for NRSA postdoctoral fellowships. We also thank David Stern, Teresa Leonardo, and Dayalan Srinivasan for helpful comments on the manuscript.

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6 Mosquito

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6.1 Introduction

6.1.1 History

Over 3,500 mosquito species have been described; this list continues to expand, particularly as contemporary techniques have facilitated identification of cryptic species within the taxa previously recognized as a single species (Munstermann and Conn 1997). This chapter concentrates on two species, *Aedes aegypti* and *Anopheles gambiae* (Order: Diptera, Family: Culicidae). These two species are the best characterized members of the mosquito subfamilies Culicinae and Anophelinae, respectively, including complete genome sequencing efforts. They had and continue to have profound impacts on global human health. Both these species originated in sub-Saharan Africa and, with human assistance, have become established in other tropical regions of the world. As an extreme example of an invasive species, *A. aegypti* is presently found in nearly all subtropical and tropical areas. Global eradication efforts for both these two species failed, largely due to rapid emergence of insecticide resistance. *A. aegypti* is recognized to have three subspecies that exhibit morphological and behavioral differences that include the type form *A. aegypti aegypti*, a sylvan form *A. aegypti formosus*, and a pale form *A. aegypti queenslandensis* (Christophers 1960). *A. gambiae* consists of a cryptic complex of at least seven species (Coluzzi et al. 2002). The complex is denoted as *A. gambiae sensu lato* whereas *A. gambiae sensu stricto* (*s.s.*) refers to the major African malaria vector species. In addition, *A. gambiae s.s.* exists in several chromosomal forms in Africa and is likely undergoing speciation (Coluzzi et al. 2002). Hereafter in this chapter, *A. gambiae* will be used to refer to *A. gambiae s.s.*

The superfamily Culicoidea that includes mosquitoes, likely existed in the Upper Triassic period, approximately (∼) 215 million years ago (Mya) (Hennig 1981). The fossil record indicates that the family Culicidae was well evolved by 58–35 Mya (Eocene period) with most fossils coming from the Oligocene period (38–26 Mya). The *Drosophila* and mosquito lineages diverged ∼250 Mya, and the *Anopheles* lineage diverged from the *Aedes* lineage ∼150 Mya (Krzywinski et al. 2006).

While *A. aegypti* and *A. gambiae* share many characteristics, they also show considerable species-specific divergence in many others (Christophers 1960; Gillies and De Meillon 1968). As with most mosquitoes, *A. aegypti* and *A. gambiae* females actively seek blood meals which subsequently stimulate oviposition. Both are highly anthropophilic (prefer human blood meals) and endophagic (readily enter human habitations for blood feeding), and as such retain very close associations with humans. *A. aegypti* typically oviposit in small containers, often man-made including within houses, with the eggs deposited at the water-air interface. Eggs are resistant to desiccation and remain viable for several weeks in dry conditions; these can readily be stored in the laboratory and induced to hatch by simply placing them in deoxygenated water. *A. gambiae* typically oviposit in shallow pools, essentially any natural or artificial (man- or animal-made) surface depressions that hold water for sufficient time for larvae to complete development. Eggs are laid at the water surface and are intolerant of desiccation and, therefore, the life cycle in the laboratory must be maintained continuously. As with nearly all mosquitoes, both species carry a haploid complement of three chromosomes (Rai and Black 1999). Their respective genome architectures, however, reflect the divergent evolution of the Culicinae and Anophelinae. *A. aegypti* (and other Culicinae) does not have dimorphic sex chro-

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W. Hunter, C. Kole (Eds.)

mosomes, with sex apparently determined by a single autosomal locus. *A. gambiae* shows the more typical X/Y sex chromosome dimorphism. Reported genome sizes in mosquitoes vary considerably from 0.23 to 1.9 pg, with the Anophelinae typically much smaller size than the Culicinae (Severson and Black 2005). Genome size in *A. gambiae* is ~273 million base pairs (Mbp) while that of *A. aegypti* is ~5 times larger with ~1,376 Mbp. This relates not to the relative gene complement, but to the abundance of repetitive DNA present in the *A. aegypti* genome (Black and Rai 1988). The *A. aegypti* genome shows "short period interspersion" wherein ~1–2 kilobase (kb) unique sequence regions alternate with 200–600 base pair (bp) and 1–4 kb repetitive regions across the genome. Conversely, *A. gambiae* shows "long period interspersion" wherein long repeat sequences of ~5.6 kb or more alternate with unique sequence regions of ~13 kb or more.

6.1.2 Importance

Mosquito-borne diseases have emerged or re-emerged as significant human health problems due to a number of factors including the lack of progress in vaccine development (or in some cases, delivery), emergence of drug resistance in pathogens and insecticide resistance in mosquitoes, and the decline in socioeconomic conditions and political will in many disease endemic countries that limits disease monitoring and mosquito control efforts (Gubler 1998).

A. aegypti is the primary global vector for the yellow fever and dengue fever viruses. Although an effective vaccine exists for yellow fever, there are ~200,000 yellow fever cases per year resulting in ~30,000 deaths (WHO 2001). About 2.5 billion people are at risk for dengue, with ~50 million cases per year and ~500,000 cases of dengue hemorrhagic fever (DHF) for which fatality rates exceed 20% without intensive supportive hospital therapy (WHO 2002). No effective vaccines or treatments for dengue exist.

A. gambiae is the primary vector of the malaria parasite, *Plasmodium falciparum*, in sub-Saharan Africa. This is the most lethal form of malaria, with ~500 million acute cases per year that result in 1.5–2.7 million deaths (Phillips 2001). About 90% of the mortality occurs in Africa, mainly among children under the age of five. No effective vaccines exist and resistance to most antimalarial drugs has emerged and spread globally at an alarming rate.

6.1.3 Objectives of Genetic Studies

Genetic studies in mosquitoes have largely been focused on increasing the understanding of mosquito/pathogen interactions, with the view that this knowledge may lead to novel control strategies for mosquito-borne diseases. It has long been recognized that mosquito species and individuals/populations within species vary in their intrinsic ability to support development of a given pathogen and subsequently to transmit a pathogen to the vertebrate host (Curtis and Graves 1983; Hardy et al. 1983). Selection experiments clearly showed that high levels of resistance or susceptibility to a pathogen could usually be achieved within only a few generations. Furthermore, selected strains maintained stable phenotypes, and classical crosses between the resistant and susceptible forms generally approximated Mendelian expectations for genetic control of the phenotypes. For *A. aegypti*, this includes genetic competence to vector yellow fever virus (Wallis et al. 1985; Miller and Mitchell 1991), dengue virus (Gubler et al. 1979; Bennett et al. 2005a), filarioid nematodes responsible for human lymphatic filariasis (Macdonald 1962), and to the avian malaria parasite *Plasmodium gallinaceum* (Kilama and Craig 1969; Thathy et al. 1994). With *A. gambiae*, strains refractory to *Plasmodium* were selected that displayed two phenotypes: (1) melanotic encapsulation of developing oocysts on the midgut epithelium (Collins et al. 1986) and (2) intracellular ookinete killing (Vernick et al. 1995).

6.1.4 Classical Mapping Efforts

The value of applying genetic tools to mosquitoes has been readily acknowledged and new genetic marker technologies have enthusiastically been investigated and applied to them. Early genetic studies were stimulated by the emergence of insecticide resistance and the need to better understand the associated mech-

anisms and population dynamics. The inheritance of DDT resistance as a single dominant trait was among the first genetic studies for both *A. aegypti* and *A. gambiae* (Coker 1958; Haridi 1972). These efforts led to organized searches for useful morphological mutations and the identification of linkage associations, and eventually some rudimentary genetic maps (Craig and Hickey 1967; Kitzmiller and Mason 1967). Demonstration of enzymes as polymorphic genetic markers (isozymes) re-invigorated this effort in the late 1960s and led to the first broad-scale linkage maps for mosquitoes. The *Est-6* locus in *A. aegypti* was the first isozyme marker developed for genetic studies in mosquitoes (Trebatoski and Craig 1969). Shortly thereafter, nine isozyme markers had been investigated for a number of mosquito species including both *A. aegypti* and *A. gambiae* (Bullini and Coluzzi 1974), although few linkage studies had been performed. The best populated linkage map for a mosquito was constructed for *A. aegypti*, with > 70 loci that included morphological mutant, insecticide resistance, and isozyme markers (Munstermann and Craig 1979; Munstermann 1990). Of note, availability of these markers facilitated the identification of individual loci associated with vector competence for the filarial worm (Macdonald 1962) and *P. gallinaceum* (Kilama and Craig 1969) parasites. For *A. gambiae*, > 20 morphological mutant, insecticide resistance, and isozyme loci were identified during this era, although only a limited number of linkage associations were determined among the individual markers (Narang and Seawright 1982; Hunt 1987).

Application of classical genetic maps and mapping tools for in depth studies of mosquitoes remained limited for a number of reasons. First, while some mosquito species like *A. aegypti* readily adapt to laboratory culture, others like *A. gambiae* can be difficult. Further, once colonized *A. gambiae* requires continuous culture, meaning colony maintenance is highly labor-intensive. Second, mosquito stocks carrying morphological mutations can be difficult to maintain as these can have negative effects on fitness. In addition, each mutation is generally maintained as a separate colony, adding to the number of stocks that must be maintained. This led to the untimely loss of mutant stocks for which considerable effort had been expended in their genetic characterization. Third, and likely the most significant limitation, individual genetic studies could only be performed with crosses involving a relatively small number of marker loci, usually only two or three. That is, few multi-mutant marker genetic stocks were successfully generated and isozyme loci often showed limited polymorphism within individual crosses.

6.2 Construction of Genetic Maps

6.2.1 Genetic Maps for *Aedes aegypti*

The demonstration that DNA-based molecular markers could readily be adapted for constructing multilocus linkage maps (Botstein et al. 1980) ushered in a new era in mosquito genetics. The first complete mosquito map was constructed for *A. aegypti* using restriction fragment length polymorphism (RFLP) markers based largely on cDNA clones representing both known genes and random expressed sequence tags (ESTs) (Severson et al. 1993). This map consisted of 50 DNA molecular markers that identified 53 loci covering 134 cM across the three linkage groups. This was a composite map generated from results for five independent F_1 intercross populations with ~100 individuals per population. In addition, four morphological marker loci were included, which facilitated partial integration of the molecular map with the classical linkage map (Munstermann and Craig 1979). While effective for genetic studies, utility of RFLP markers is limited for small bodied organisms like mosquitoes, as DNA quantity becomes a limiting issue such that a maximum of 15–25 marker loci can be evaluated in an individual.

Development of the polymerase chain reaction (PCR) (Saiki et al. 1988) and PCR-based genetic markers created new opportunities for mosquito genetics as DNA quantity was no longer a problem. A second map for *A. aegypti* using PCR-generated random amplified polymorphic DNA (RAPD) markers was constructed based on two BC_1 populations that consisted of 96 RAPD loci covering 168 cM, and again included some morphological marker loci (Antolin et al. 1996). A third map was constructed using single-strand conformation polymorphism (SSCP) analysis of PCR-amplified cDNA sequences with a single F_1 intercross population that consisted of 57 loci cov-

ering 134 cM (Fulton et al. 2001). A composite map that included RFLP, SSCP, and single nucleotide polymorphism (SNP) markers consisted of 146 loci and covered 205 cM (Fig. 1). An additional map using 148 amplified fragment length polymorphism (AFLP) and six SSCP loci was constructed with two F_1 intercross populations and covered 180.9 cM (Zhong et al. 2006).

Of note, although microsatellite sequences or simple sequence repeats (SSRs) have been shown to be very abundant and useful as PCR-based markers in

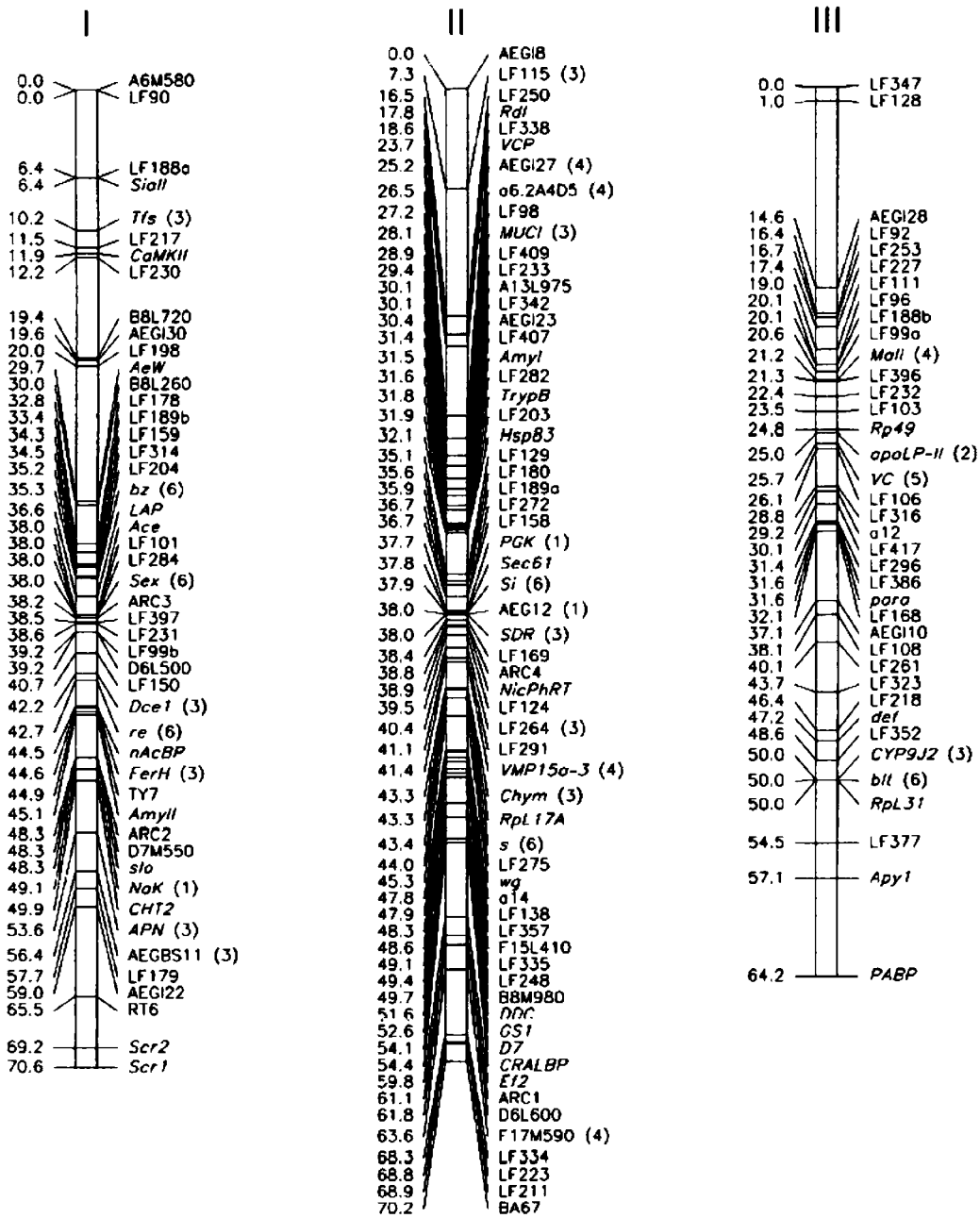

Fig. 1 Composite RFLP, SNP, and SSCP genetic linkage map for *Aedes aegypti*. Map distances in Kosambi centiMorgans. Markers were mapped as RFLP loci except those indicated by "()", (1) SNP only, (2) SSCP only, (3) SNP and RFLP, (4) SSCP and RFLP, (5) SSCP and SNP, (6) morphological. Reproduced with permission from Severson et al. (2002) and the Royal Entomological Society

many organisms (Tautz 1989), efforts to construct a genetic map for *A. aegypti* using SSR markers were unsuccessful due to the paucity of useful microsatellites in its genome (Fagerberg et al. 2001; Chambers et al. 2007). This is due to an apparent reduction in microsatellite frequency across the genome and the frequent association of those that do exist with repetitive elements that negate their utility (Chambers et al. 2007). Still, about 20 useful SSR loci have been identified, with eight placed to chromosome positions (Huber et al. 1999, 2001; Chambers et al. 2007).

6.2.2 Genetic Maps for *Anopheles gambiae*

In contrast to *A. aegypti*, success in developing RFLP marker loci for *A. gambiae* was minimal (Romans et al. 1991, 1999; Gorman et al. 1997), largely due to the technical difficulties involved with this marker system with the smaller *A. gambiae*. A few RAPD markers have been identified for *A. gambiae*, with 15 RAPDs placed as single loci on the genetic map, and 35 developed as sequenced tagged sites (STS) and placed on the physical map (Favia et al. 1994; Dimopoulos et al. 1996). Major difficulties evident with RAPD markers in mosquitoes and other organisms are their dominant inheritance and general lack of reproducibility (Black 1993). Microsatellite sequences are, however, quite abundant in the *A. gambiae* genome and a well-populated map consisting of 131 SSR loci was constructed using several BC_1 families (Zheng et al. 1993, 1996). An integrated map that included SSR, RAPD, morphological, and insecticide resistance loci covering 215 cM was developed (Zheng et al. 1996).

6.2.3 Genetic Maps for Other Mosquitoes

The *Aedes albopictus* genetic linkage map was based on an F_1 intercross family with a total of 68 RAPD loci covering 225 cM across all three linkage groups (Mutebi et al. 1997). This mosquito is recognized as a vector for dengue viruses and is a competent vector for a number of other arboviruses. The *Ochlerotatus (Aedes) triseriatus* map was based an F_1 family with 56 RAPDs and 10 cDNAs analyzed as SSCP markers covering 210 cM (Graham et al. 2003). This mosquito is the primary vector for the La Crosse encephalitis virus.

6.2.4 Comparative Genetic Mapping Among Mosquitoes

Because the *A. aegypti* RFLP map was based largely on cDNA sequences as the marker loci (Severson et al. 1993, 2002), these frequently shared sufficient sequence identities to hybridize with Southern blots containing genomic digests of other mosquito species (Severson et al. 1994a). This facilitated the construction of genetic linkage maps for several mosquito species using the *A. aegypti* cDNAs as probes to identify genome locations of comparative loci.

The first comparative RFLP linkage map was developed for *A. albopictus* and consisted of 18 RFLP loci that covered 98.4 cM across the three linkage groups using F_1 intercross families (Severson et al. 1995a). Linkage groups and marker linear orders remained identical to *A. aegypti*. However, the same markers covered 129.1 cM in *A. aegypti*, with the difference due largely to reduced recombination on chromosome 1 in *A. albopictus*.

The *Armigeres subalbatus* comparative map consisted of 26 RFLP loci covering 181.5 cM using F_1 intercross families (Ferdig et al. 1998). For 25 of these loci, linkage groups were conserved, with chromosomes 1 and 3 reflecting colinearity. For chromosome 2 in *A. subalbatus*, markers in the middle region of the linkage group (likely containing the centromere region) were inverted in linear order compared to *A. aegypti*. The sex locus is located on chromosome 1 in *A. aegypti*, but is on chromosome 3 in *A. subalbatus*.

The *Culex pipiens* comparative map was developed using 21 RFLP markers that identified 22 loci covering 165.8 cM using F_1 intercross families (Mori et al. 1999). The *C. pipiens* complex includes the primary global vectors for *Wuchereria bancrofti* derived human lymphatic filariasis. Chromosome 1 markers were consistent with *A. aegypti* linear orders, but recombination rates in *C. pipiens* were extensively reduced. *C. pipiens* chromosomes 2 and 3 showed near whole chromosome arm translocations with some rearrangement evident within arms compared to *A. aegypti*.

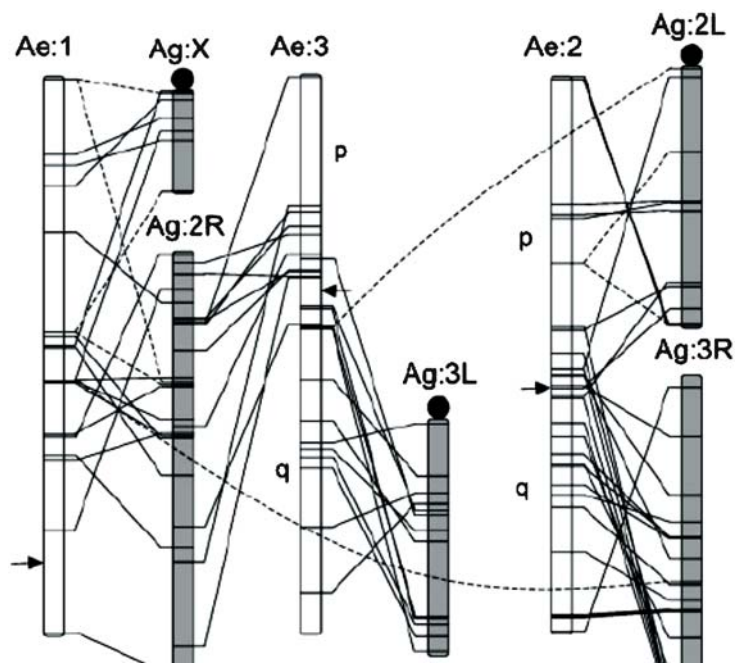

Fig. 2 Comparative genome positions of orthologous genes identified between *Aedes aegypti* (*Ae*) and *Anopheles gambiae* (*Ag*). Linkage map positions for *A. aegypti* after Severson et al. (2002). *Lines* link each ortholog to the corresponding cytological position for the A. *gambiae* ortholog. *Dashed lines* identify putative duplicate loci or transpositions. Putative centromere positions in *A. aegypti* extrapolated from Brown et al. (2001) are indicated by *arrows*, with *p* and *q* referring to individual arms on chromosomes 2 and 3. Reproduced with permission from Severson et al. (2004)

The *Culex tritaeniorhynchus* comparative map was developed using 14 RFLP makers covering 155.1 cM using BC_1 families (Mori et al. 2001). This mosquito is the primary vector for the Japanese encephalitis virus. The *C. tritaeniorhynchus* map was completely consistent with the *C. pipiens* map, with the exception that the sex locus is located on chromosome 3 in *C. tritaeniorhynchus* versus chromosome 1 in *C. pipiens*. Evaluation of recombination among F_1 intercross families confirmed that no recombination occurs in *C. tritaeniorhynchus* females.

The *O. triseriatus* comparative map was developed using 21 RFLP markers that identified 22 loci covering 270.7 cM using F_1 intercross families (Anderson et al. 2001). Linkage group associations of chromosome 1 markers were conserved with *A. aegypti*, but rearrangement in linear order was evident. Chromosomes 2 and 3 reflected near whole arm rearrangements with *A. aegypti* and within-arm inversions. Two RFLP loci located near the ends of chromosomes 1 and 3 in *A. aegypti*, although segregating, could not be placed to linkage group in *O. triseriatus* suggesting recombination frequencies beyond resolution with the existing marker set.

Comparisons of *A. aegypti* RFLP loci based on 75 cDNAs to ortholog positions in the *A. gambiae* genome also showed near whole arm conservation between the two species (Fig. 2). Chromosome 1 in *A. aegypti* contained orthologs to the X chromosome and the 2R arm in *A. gambiae*, suggesting a break/fusion event in karyotype evolution in *A. aegypti*. Chromosome 2 in *A. aegypti* contained orthologs to the 2L and 3R arms in *A. gambiae*, while chromosome 3 in *A. aegypti* contained orthologs to 2R and 3L in *A. gambiae*. Extensive paracentric inversions were evident indicating linear orders are likely maintained only at the microsyntenic level.

A panel of *A. aegypti* RFLP markers were adapted for use as comparative anchor-tagged sequences (CATS), represented as primer pairs with sufficient interspecific sequence conservation to allow PCR amplification of the target fragment from a broad array of Diptera species (Chambers et al. 2003). Species tested included the mosquitoes *A. gambiae*, *Aedes togoi*, *A. subalbatus*, *C. pipiens*, and *O. triseriatus*, as well as two non-mosquito dipterans, *Drosophila melanogaster* and *Rhagoletis pomonella*. The 23 CATS primer pairs were each demonstrated to amplify with at least one non-*Aedes aegypti* species. Polymorphisms were identified by SSCP analysis or SNP analysis following fragment sequencing. Fifteen CATS markers, including several newly developed ones, were mapped in an interspecific

segregating population derived from a cross between *O. triseriatus* and *Ochlerotatus hendersoni* (Anderson et al. 2005).

Comparative mapping studies done to date, completely support the suggestion by Matthews and Munstermann (1994) based on common isozyme loci that karyotype evolution in mosquitoes has largely involved gross structural changes, including Robertsonian translocations and paracentric inversions.

6.3 Detection of Quantitative Trait Loci

Quantitative trait loci (QTL) studies in mosquitoes have largely focused on efforts to identify genomic regions associated with the ability of the mosquito to serve as a competent vector for transmitting pathogens to humans. Classical genetic studies had indicated that vector competence was a quantitative trait, and development of DNA-based saturated linkage maps provided the mechanism for beginning to resolve these complex phenotypes into their discrete genetic components (Severson et al. 2001). The number and genomic position of QTLs detected for a given trait is known to vary among segregating populations, with each likely representing valid QTLs (Beavis 1994). This is expected as QTL detection power is impacted by population size, magnitude of trait heritability, and marker density, and therefore, no single mapping effort is likely to define the complete genetic basis for a quantitative trait. A number of QTL analysis programs have been developed and thresholds for declaring QTLs are determined by permutation test (Churchill and Doerge 1994). Both experiment-wise and comparison-wise 95% thresholds are usually calculated. The stringency of the experiment-wise threshold, while reducing the likelihood of declaring false-positive QTLs (type I error), also increases the potential for rejecting true-positive QTLs (type II error) (Churchill and Doerge 1994). Several QTL studies in mosquitoes have reported QTL results relative to the comparison-wise thresholds or have reported as tentative QTLs, those genetic markers showing significant associations with a phenotype using standard statistical tests. Table 1 summarizes results from QTL studies in mosquitoes.

6.3.1 Quantitative Trait Loci Detected in *Aedes aegypti*

Filarial Worms

Classical mapping efforts determined that susceptibility to the filarioid nematode parasite of humans, *Brugia malayi*, and the related *Brugia pahangi* and *Wuchereria bancrofti* was primarily influenced by a single, sex-linked (chromosome 1) gene designated f^m (Macdonald 1962; Macdonald and Ramachandran 1965). Two QTLs were identified across each of three populations; a major QTL was located to the genomic region likely containing the f^m locus, while the second QTL had a minor effect (Severson et al. 1994b). Individual QTL effect varied across populations dependent on genetic background. Another study (Beerntsen et al. 1995) identified a QTL influencing the number of microfilariae ingested by the mosquito while taking an infected blood meal and the number that penetrate the midgut. This QTL was located in the same genomic region containing the minor effect QTL determining susceptibility (Severson et al. 1994b).

Plasmodium gallinaceum

Classical mapping efforts identified a major locus (*pls*) that determined susceptibility to the avian malaria parasite, *Plasmodium gallinaceum* (Kilama and Craig 1969). Two QTLs were identified in each of two populations; a major QTL was located to the genomic region likely containing the *pls* locus, while the second QTL had a minor effect (Severson et al. 1995b). Zhong et al. (2006; Fig. 3), using a densely saturated linkage map, identified six QTLs determining *P. gallinaceum* susceptibility in each of two populations; four QTLs explained the majority of the phenotypic variance in each population. Lack of common markers across studies prevents comparisons of QTL locations.

Dengue Virus

Several studies have investigated vector competence for the dengue-2 (DEN-2) virus in *A. aegypti*. Two genetic barriers to virus infection have been identified in this mosquito: a midgut infection barrier (ability for the virus to infect and replicate in the midgut epithelium) and a midgut escape barrier (ability for the virus to escape from the midgut epithelial cells and disseminate throughout the body) (Bosio

Table 1 Quantitative trait loci in mosquitoes

Species	Trait	Phenotype assay	Pedigree[a]	Number of progeny[b]	Marker types	Number of QTL	Detection method	Reference
Aedes aegypti	Filarial worm susceptibility	L3 stage larvae	IC	187/102/276	RFLP (13/13/14)	2	Mapmaker/QTL[c]	Severson et al. (1994b)
	Filarial worm midgut penetration	Microfilariae	IC	167	RFLP (6)	1	Mapmaker/QTL	Beerntsen et al. (1995)
	Plasmodium gallinaceum susceptibility	Oocysts	IC	154/122	RFLP (16/16)	2	Mapmaker/QTL	Severson et al. (1995b)
	Plasmodium gallinaceum susceptibility	Oocysts	IC	146/122	AFLP (148) SSCP (6)	6	Map Manager QTX[d]	Zhong et al. (2006)
	Dengue virus susceptibility	Den-2 midgut infection	IC	207	RAPD-SSCP (57) cDNA-SSCP (14) SSR (5) STAR (6)	4	BINARYQTL[e] QTL Cartographer[f]	Bosio et al. (2000)
	Dengue virus susceptibility	Den-2 midgut escape	IC	36	nd	1	BINARYQTL QTL Cartographer	Bosio et al. (2000)
	Dengue virus susceptibility	Den-2 midgut infection	AIL-F_5	147	SSCP (44)	2	BINARYQTL QTL Cartographer 2.0[g]	Gomez-Machorro et al. (2004)
	Dengue virus susceptibility	Den-2 midgut escape	IC AIL-5	62/33 244/241	SSCP (44/34)	3	QTL Cartographer 2.0	Bennett et al. (2005b)
	Dengue virus susceptibility	Den-2 midgut infection	IC AIL-5	76/40 436/296	SSCP (44/34)	2	QTL Cartographer 2.0	Bennett et al. (2005b)
Anopheles gambiae	*Plasmodium cynomolgi* B susceptibility	Encapsulated oocysts	BC_1	150	SSR (50)	3	Mapmaker/QTL	Zheng et al. (1997)
	CM-Sephadex bead encapsulation	Encapsulated beads	BC_1	90	SSR (15) RFLP (5)	1	Mapmaker/QTL	Gorman et al. (1997)

[a] *IC* F_1 intercross, *AIL* advanced inbred line; [b] Number of progeny per family, *nd* data not given; [c] Lander et al. (1987); [d] Manly et al. (2001); [e] Xu et al. (1998); [f] Basten et al. (1997); [g] Basten et al. (2002); [h] Seaton et al. (2002); [i] Xu and Xu (2003)

Table 1 (continued)

Species	Trait	Phenotype assay	Pedigree[a]	Number of progeny[b]	Marker types	Number of QTL	Detection method	Reference
	Plasmodium cynomolgi Ceylon susceptibility	Encapsulated oocysts	IC	167	SSR (34)	3	Linear Regression	Zheng et al. (2003)
	Plasmodium falciparum susceptibilty	Oocysts	IC	83/82	SSR (24)	1	Kolmogorov-Smirnov test	Niaré et al. (2002)
	Plasmodium falciparum susceptibility	Oocysts	F_1/IC	38/41/58/69/47	SSR (20)	2	QTL Express[h] QTL Cartographer	Menge et al. (2006)
	Plasmodium falciparum susceptibility	Oocysts Encapsulation	F_1/IC	83/82/152/45/ 62/40/21	SSR (25)	7	Wilcox-Mann-Whitney test	Riehle et al. (2006)
	Insecticide resistance	DDT resistance	BC_1/IC	129/192	SSR (27)	2	Mapmaker/QTL	Ranson et al. (2000)
	Insecticide resistance	Permethrin resistance	IC/F_4	122/96/36/80	SSR (29/6)	3	Mapmaker/QTL	Ranson et al. (2004)
Anopheles gambiae × *Anopheles arabiensis*	Hybrid sterility	Sperm development	BC_1/BC_2	nd	SSR (21)	16	QTL Cartographer	Slotman et al. (2004)
	Hybrid sterility	Ovary development	BC_1	nd	SSR (23)	10	QTL Cartographer	Slotman et al. (2005)
Ochlerotatus triseriatus	La Crosse virus transovarial transmission	Ovary infection	IC	28	RAPD-SSCP (56) cDNA-SSCP (10)	3	BINARYQTL	Graham et al. (2003)
	Sex ratio	Sex	IC	146	RAPD-SSCP (72) cDNA-SSCP (7)	6	BINARYQTL	Graham et al. (2004)
Ochlerotatus hendersoni × *Ochlerotatus triseriatus*	La Crosse virus susceptibility	Oral transmission	IC	164	SNP (16) RFLP (1) SSCP (8)	4	QTL-by-SAS[i] Fisher's exact test	Anderson et al. (2005)

Table 1 (continued)

Species	Trait	Phenotype assay	Pedigree[a]	Number of progeny[b]	Marker types	Number of QTL	Detection method	Reference
	Body size	Wing length	IC	164	SNP (16) RFLP (1) SSCP (8)	3	QTL Cartographer Linear Regression	Anderson et al. (2005)
	Larval saddle hairs	Hair number	IC	121	SNP (24)	1	QTL-by-SAS Fisher's exact test	Anderson et al. (2006)
	Larval siphon hair branches	Branch number	IC	123	SNP (24)	1	QTL-by-SAS Fisher's exact test	Anderson et al. (2006)
	Larval acus attachment	Acus attachment	IC	130	SNP (24)	3	QTL-by-SAS Fisher's exact test	Anderson et al. (2006)
	Larval anal papillae shape	Anal papillae shape	IC	125	SNP (24)	3	QTL-by-SAS Fisher's exact test	Anderson et al. (2006)
	Larval anal papillae index	Dorsal gill/saddle length	IC	144	SNP (24)	2	QTL Cartographer Linear Regression	Anderson et al. (2006)
	Larval dorsal/ventral papillae ratio	Dorsal gill/ventral gill	IC	144	SNP (24)	1	QTL Cartographer Linear Regression	Anderson et al. (2006)

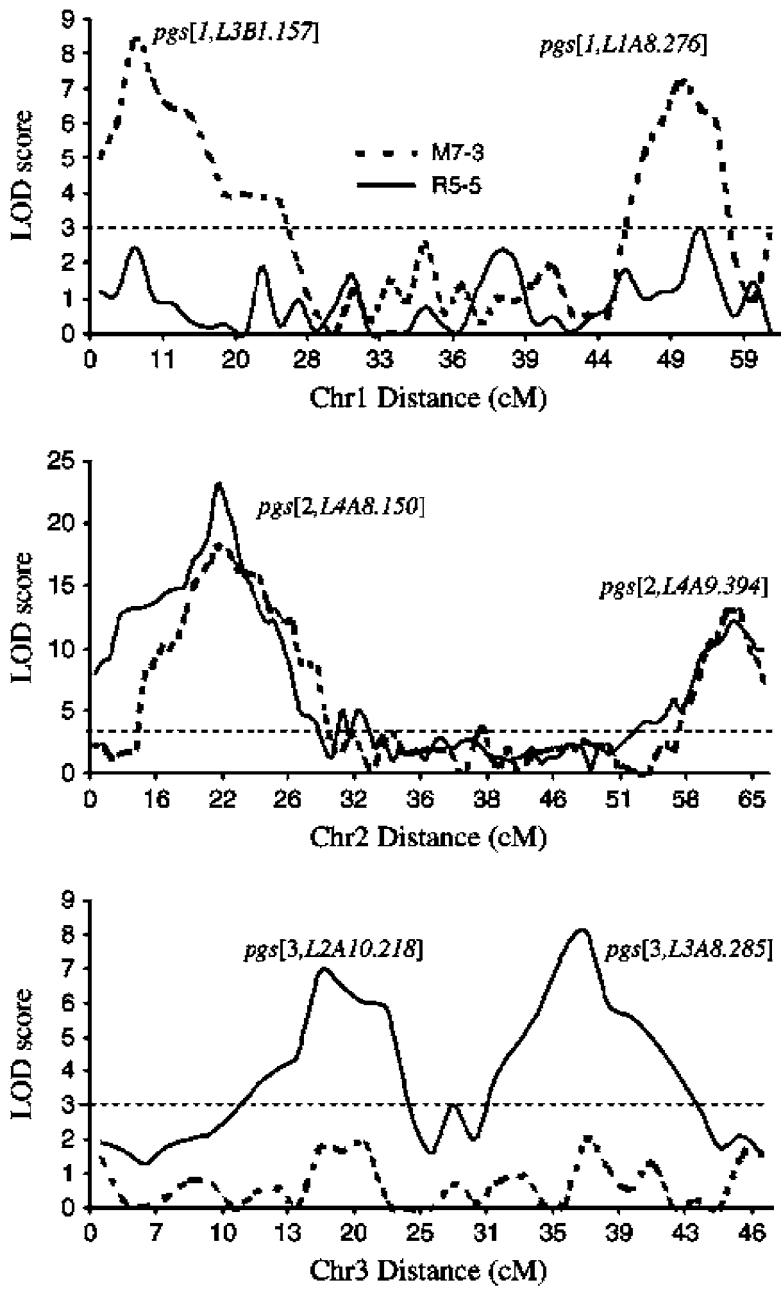

Fig. 3 Composite interval mapping of *Plasmodium gallinaceum* susceptibility in *Aedes aegypti*. F_2 segregating populations R5-5 and M7-3 represent reciprocal crosses between a susceptible and refractory *A. aegypti* strain. Significance thresholds are indicated by *dashed horizontal lines*, logarithm of the odds (LOD) = 3.0 (genomewide $P < 0.001$) as determined by 1,000 permutations. Reproduced with permission from Zhong et al. (2006)

et al. 1998). Bosio et al. (2000) identified four QTLs controlling midgut infection using standard interval mapping (IM) with comparison-wise thresholds; only one of these QTLs was identified using composite interval mapping (CIM) with comparison-wise thresholds. One QTL controlling midgut escape was identified using IM. Two QTLs were identified using an advanced intercross line strategy (AIL) to effectively increase recombination frequency using IM and multiple interval mapping (MIM) with experiment-wise thresholds (Gomez-Machorro et al. 2004); neither QTL mapped to the same genomic regions as those previously identified (Bosio et al. 2000). Another study that included AIL populations (Bennett et al. 2005b) identified two QTLs controlling midgut infection and three QTLs controlling midgut escape us-

ing MIM and CIM; general genomic locations for one QTL for midgut infection and two QTLs for midgut escape were similar to those previously reported (Bosio et al. 2000; Gomez-Machorro et al. 2004), suggesting reproducibility of some QTLs across populations.

6.3.2 Quantitative Trait Loci Detected in *Anopheles gambiae*

A number of QTL studies in *A. gambiae* have focused on *Plasmodium* susceptibility. A strain that encapsulates developing ookinetes and oocytes of *Plasmodium cynomolgi* B was selected (Collins et al. 1986) and used for initial QTL studies. Zheng et al. (1997) identified three QTLs that controlled the encapsulation response. A related study (Gorman et al. 1997) evaluated melanotic encapsulation of CM-Sephadex beads as a proxy for the parasite and identified one QTL that corresponded with the major QTL identified by Zheng et al. (1997). Two QTL locations were re-confirmed and a novel QTL identified with *P. cynomolgi* Ceylon when tested with *A. gambiae* of different genetic background (Zheng et al. 2003). Interestingly, melanotic encapsulation is infrequently observed among *A. gambiae* populations in response to infection by the most devastating malaria parasite, *Plasmodium falciparum*, indicating phenotypic variability across *Plasmodium* parasites in recognition by the mosquito immune system (Collins et al. 1986).

Several studies have performed QTL analyses with natural *A. gambiae* populations. Resistant mosquitoes typically mount an immune response that results in intracellular lysis of the developing parasite (Vernick et al. 1995). Evaluation of segregating populations from field-collected females blood-fed on human gametocyte carriers identified a single QTL using the non-parametric Kolmogorov-Smirnov test (Niaré et al. 2002). In a similar study, two QTLs were identified and one of these colocalized to the same genomic region previously identified for both the lysis phenotype and the encapsulation phenotype (Menge et al. 2006). Riehle et al. (2006) identified up to seven potential QTLs in natural populations, although four of these fall within the same genomic region and could be independent loci or common effects of a single locus; several QTLs colocalized to previously reported QTL regions for both the lysis phenotype and the encapsulation phenotype.

Two studies examined QTL for insecticide resistance in *A. gambiae*. Two QTLs were identified accounting for > 50% of the phenotypic variance in DDT resistance due to increased metabolism by glutathione S-transferase activity (Ranson et al. 2000). Three QTLs were identified for permethrin resistance; one of these colocalized with a sodium channel gene known to be associated with resistance, while another was located near a cluster of cytochrome P450 genes that are often associated with metabolic resistance (Ranson et al. 2004).

The genetic basis for male hybrid inviability and sterility was examined among reciprocal backcross populations between *A. gambiae* and the sibling species *Anopheles arabiensis* (Slotman et al. 2004). A total of 16 QTLs were identified using CIM and MIM with eight QTLs associated with each of the reciprocal backcrosses; none of the QTL positions were common across these backcrosses, indicating that *A. gambiae* sterility regions are different than those for *A. arabiensis*. In a related study (Slotman et al. 2005), female sterility was investigated using the same crossing strategy and QTL analysis; a total of 10 QTLs affecting female hybrid sterility were identified.

6.3.3 Quantitative Trait Loci Detected in Other Mosquito Species

Several QTL studies have involved *Ochlerotatus triseriatus*, the primary vector for the La Crosse encephalitis virus. Females are capable of transovarially transmitting (TOT) the virus to their progeny, and TOT is the primary mechanism for virus persistence in northern temperature areas. Three QTLs with additive inheritance were identified that account for ~53% of the phenotypic variance (Graham et al. 2003).

Laboratory colonies of *O. triseriatus* often exhibit a female-biased sex ratio, whereas male biases are usually observed with most mosquitoes. In addition to the recognized autosomal sex locus on chromosome 1, Graham et al. (2004) identified six QTLs that determine sex ratio in *O. triseriatus*. They suggest that

these represent multiple female-biased sex distorter loci.

Vector competence to transmit La Crosse virus to suckling mice was examined among segregating progeny of an interspecific cross between the vector species, *O. triseriatus*, and a non-vector sibling species, *Ochlerotatus hendersoni* (Anderson et al. 2005). Four tentative QTLs determining La Crosse vector competence were identified. In addition, three QTLs for body size were identified, with mosquitoes smaller than the mean being more likely to transmit virus than those larger. The body size QTLs were located in the same genome regions as the three QTLs for vector competence, suggesting tight linkage or pleiotropy.

Another interspecific cross between *O. triseriatus* and *O. hendersoni* examined the inheritance of six species-specific larval characteristics (Anderson et al. 2006). From one to three QTL(s) was/were identified for each trait, and several of these were located in the same genomic regions, again suggesting tight linkage or pleiotropy.

6.4 Map-based Cloning

Success in QTL mapping and advances in mosquito genomics have facilitated efforts to identify the underlying genes. Examination of a 528-kb genomic region encompassing the *Pen1* QTL for melanotic encapsulation in *A. gambiae* identified 48 genes, several of which were proposed as possible candidate genes (Thomasová et al. 2002). Riehle et al. (2006) examined a genomic region in *A. gambiae* associated with both the melanotic encapsulation and the lysis phenotypes for candidate genes and identified 72 candidates. Of these, three met all their filter criteria and RNAi confirmed that a novel leucine-rich repeat gene (*APL1*) mediates significant protection from *Plasmodium* infection and is a viable candidate. With *A. aegypti*, early trypsin is the only proteolytic enzyme present in the midgut immediately following a blood meal and, therefore, a candidate for genes influencing susceptibility to dengue virus; however, association mapping efforts with the early trypsin gene did not uncover consistent SNP associations among mosquito populations from Mexico suggesting limited influence (Gorrochotegui-Escalante et al. 2005).

6.5 Advanced Works

6.5.1 Physical Mapping Efforts

Aedes aegypti

Physical mapping in *A. aegypti* has been based on fluorescence in situ hybridization (FISH) of cosmid or bacterial artificial chromosome (BAC) genomic DNA probes to metaphase chromosomes (Brown et al. 1995). Polytene chromosome preparations in *A. aegypti* and other culicines are largely unsuitable for physical mapping, likely due to the large genome repetitive content that interferes with proper chromosome spreading. Partial integration of the physical and genetic maps was accomplished by FISH mapping of a number of RFLP-mapped cDNAs or cosmids that carried the cDNA sequences (Brown et al. 2001).

Anopheles gambiae

An excellent physical map based on polytene chromosomes has been prepared for *A. gambiae* (della Torre et al. 1996; Dimopoulos et al. 1996), and was integrated with the genetic linkage map (Zheng et al. 1996). A variety of probes have been placed to the physical map including sequences from microdissected divisions of the polytene chromosomes (Zheng et al. 1991), as well as a number of microsatellites, cDNAs, RAPD-associated sequences, and cosmid and BAC genomic clones.

Other Anophelines

Polytene physical maps have been created for *Anopheles funestus* (Fig. 4; Sharakhov et al. 2004) and for *Anopheles stephensi* (Sharakhov et al. 2006). An integrated genetic and physical map was prepared for *A. funestus* using microsatellite, RFLP, and SNP markers (Wondji et al. 2005). Polytene physical mapping of orthologous genes identified large-scale chromosome arm conservation between *A. gambiae* and *A. funestus*, yet within-arm rearrangements were quite frequent, especially given only a ~5-Mya divergence between them (Fig. 5).

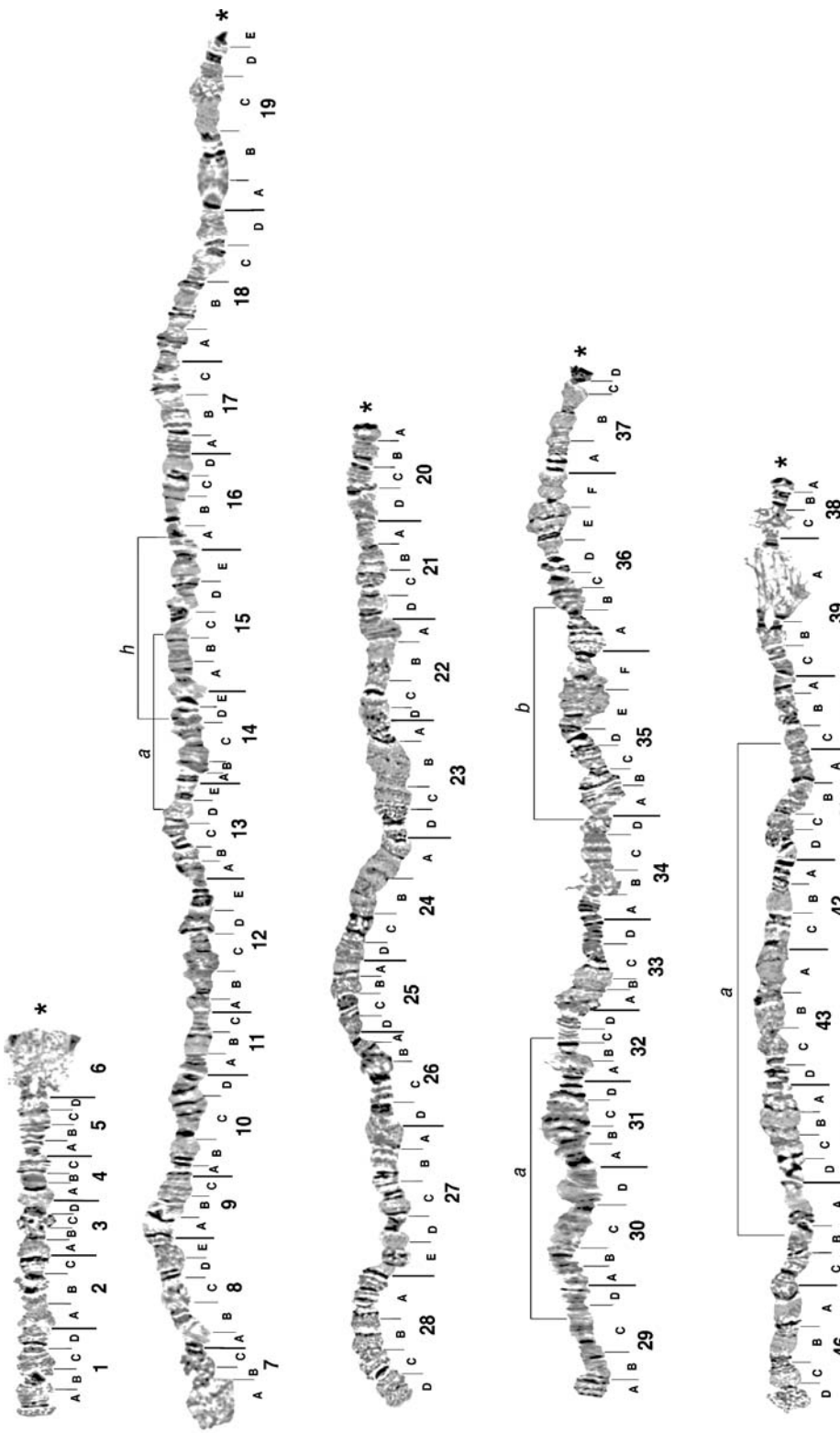

Fig. 4 Photomap of *Anopheles funestus* polytene chromosomes. *Brackets* represent inversions found in specimens from coastal Kenya. *Stars* indicate centromeric regions. Reproduced with permission from Sharakhov et al. (2001)

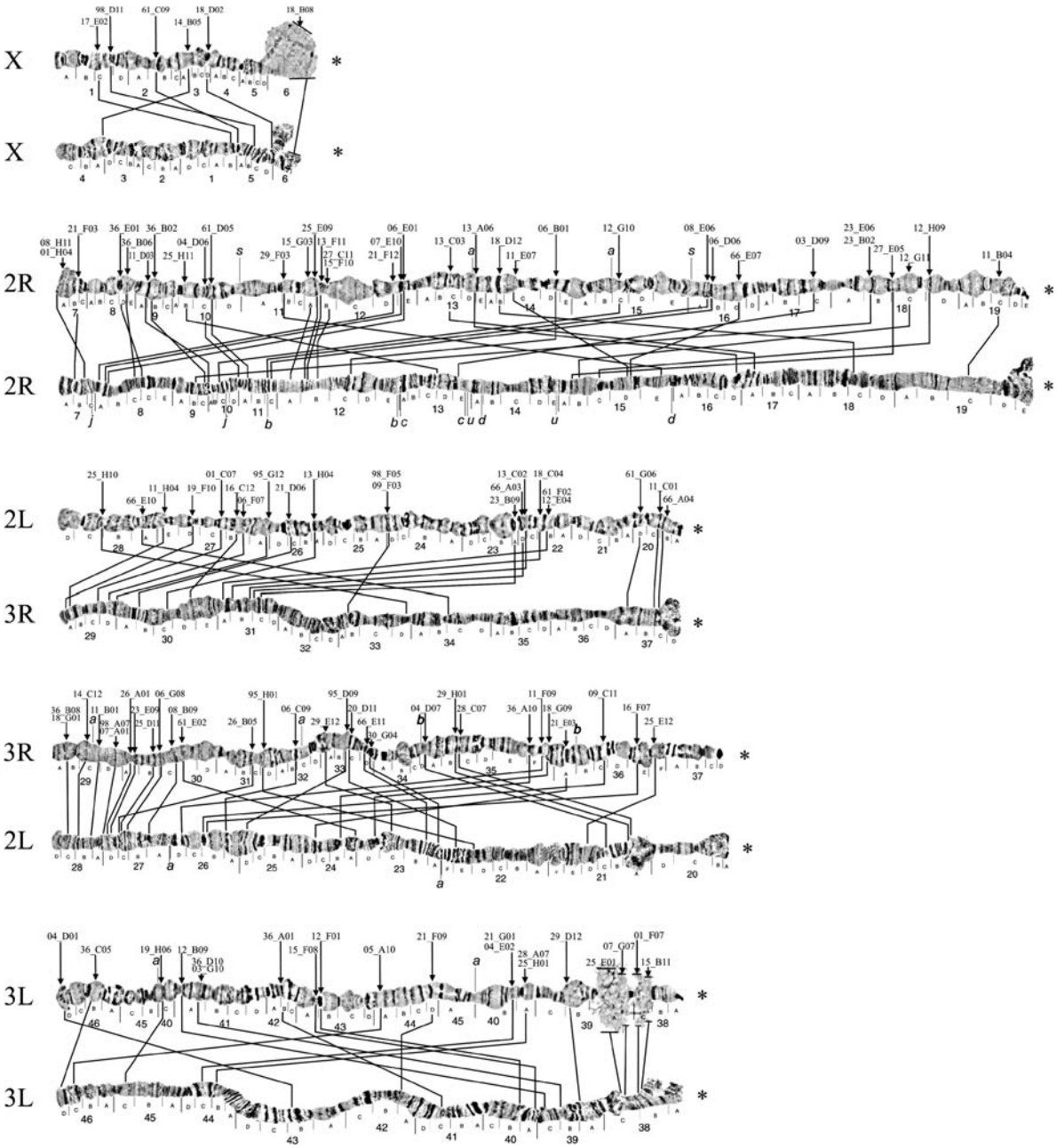

Fig. 5 Physical location of *Anopheles funestus* cDNA clones and location of the putative *Anopheles gambiae* orthologs, given with respect to the polytene chromosome photomaps of both species. Homologous chromosome arms are compared, with *A. funestus* shown above those of *A. gambiae*. Centromeres are indicated by *asterisks*. Approximate breakpoints of common polymorphic inversions are identified by *lowercase italicized letters*. Reproduced with permission from Sharakhov et al. (2002)

6.5.2 Sequencing Projects: Whole-genome Shotgun

Whole-genome sequences using the whole-genome shotgun sequencing strategy have been obtained for *A. aegypti* and *A. gambiae* and are being obtained for *Culex quinquefasciatus* (http://www.vectorbase.org/Sections/Other/addtl_org_includes/cpipiens_genome_justification.pdf) and the M and S forms of *A. gambiae* (http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/AnophelesSeq.pdf); undoubtedly, more species will be added to this list within the near term. Availability of whole-genome sequences will accelerate efforts to understand all aspects of mosquito biology and, particularly, will provide the opportunity to compare and contrast the molecular underpinnings unique to each species. Development of novel disease control strategies derived from this information is eagerly anticipated.

Aedes aegypti

The whole-genome sequence was obtained for the *A. aegypti* Liverpool strain that had been subjected to intense inbreeding to minimize polymorphism (Nene et al. 2007). The sequence was assembled into a relatively large number of continuous DNA sequences (contigs) and linked scaffolds, which span ~1.38 gigabase pairs (Gbp) (Table 2). The general fragmentation of the assembly into moderate-sized scaffolds was likely due to the highly repetitive nature of the genome as ~70% of the genome was determined to be repetitive. A total of 15,419 genes were identified using two automated annotation pipelines. Transcriptional validation was obtained for ~80% of these and, of note, for ~76% of genes annotated as hypotheticals. Genetic and physical mapping information allowed general assignment to chromosome arms of ~31% of the genome. Development of high-resolution physical mapping techniques for *A. aegypti* remains a priority.

Anopheles gambiae

The whole-genome sequence was obtained for the *A. gambiae* PEST strain that had been selected for the standard karyotype (Holt et al. 2002). The sequence was also assembled into a large number of contigs and scaffolds that span ~273 Mbp (Table 2), although ~93% of the assembly was contained in only 303 scaffolds. Contig and scaffold sizes were on average larger than those for *A. aegypti*, with the largest *A. gambiae* scaffolds containing 23.1 Mbp and that of *A. aegypti* 5.83 Mbp. A total of 13,111 genes were identified using two automated annotation pipelines. Physical mapping information obtained from in situ mapped and end-sequenced BAC clones allowed assignment of ~84% of the genome to polytene chromosome position.

6.5.3 Microarrays

Efforts in mosquito genomics have driven efforts to understand the dynamics of the mosquito tran-

Table 2 Genome features in *Aedes aegypti* and *Anopheles gambiae*

	Aedes aegypti[a]	*Anopheles gambiae*[b]
Genome size (Mbp)	1,376	272.9
Sequence coverage	7.6X	10.2X
Number of contigs	36,206	18,962
Number of scaffolds	7,758	8,987
Number of protein coding genes	15,419	13,111
Mean protein coding length (bp)	1,397	1,154
Mean exons per gene	4.0	3.9
Mean intron size (bp)	4,685	808
Longest intron (bp)	329,294	87,786

[a]Nene et al. (2007)
[b]Holt et al. (2002)

Table 3 Microarray studies in mosquitoes

Species	Platform	Elements	Comparison	Target source	Reference
Aedes aegypti	Spotted cDNAs	1,778	Effects of blood feeding	Female midgut	Sanders et al. (2003)
	Spotted cDNAs	1,302	Response to *Plasmodium gallinaceum*	Female midgut	Chen et al. (2004)
	Spotted cDNAs	2,170	Sindbis virus infection	Female midgut	Sanders et al. (2005)
Anopheles gambiae	Spotted cDNAs	3,840	Bacteria and *Plasmodium berghei* infection	Cell culture/adult females	Dimopoulos et al. (2002)
	Spotted cDNAs	3,840	*Plasmodium berghei* melanization	Adult females	Kumar et al. (2003)
	Affymetrix	14,000	Salivary gland activity	Adult males and females	Arcà et al. (2005)
	70-mer oligos	287	Odor reception	Adult male/female palps/antennae/head/carcass	Biessman et al. (2005)
	Spotted cDNAs	3,168	Effects of blood feeding	Adult females	Dana et al. (2005)
	70-mer oligos	230	Insecticide resistance	Adult females	David et al. (2005)
	Affymetrix	14,000	Effects of blood feeding	Adult females	Marinotti et al. (2005)
	Spotted cDNAs	3,840	Effects of REL2	Cell culture	Meister et al. (2005)
	Spotted cDNAs	20,000	Response to O'nyong-nyong virus	Adult females	Sim et al. (2005)
	Spotted cDNAs	20,000	Insecticide resistance	Adult females	Vontas et al. (2005)
	Affymetrix	142,065[a]	Genomic DNA SNPs	Adult females	Turner et al. (2005)
	60-mer oligos	14,180	Bacteria/*Plasmodium falciparum/berghei* infection	Adult female midgut/carcass	Dong et al. (2006)
	70-mer oligos	230	Insecticide resistance	Adult female/male, pupae, larvae	Strode et al. (2006)
Anopheles stephensi	Spotted cDNAs	4,987	*Plasmodium berghei* infection	Female midguts	Xu et al. (2005)

[a]This study examined the 25-mer oligos as individual elements for comparisons

scriptome, particularly under conditions that impact vector competence. The *A. aegypti* and *A. gambiae* genome projects have generated large collections of ESTs as well as the complete annotated gene sets, with ~265,000 ESTs for *A. aegypti* and ~217,000 for *A. gambiae*. These have been applied toward microarray studies involving a wide range of platforms, element densities, and target transcripts (Table 3).

The *A. aegypti* genome project only recently generated resources for in-depth microarray studies (Nene et al. 2007), and at this point three microarray experiments have been published. These involved spotted cDNA arrays containing relatively modest (~2,000) numbers of elements. Each examined midgut transcription, but evaluated responses to general blood feeding, *Plasmodium gallinaceum* infection, and Sindbis virus infection, respectively. Several large-scale cDNA and long-oligo microarray studies are anticipated with the recent release of the genome sequence.

The longer-term impact of genome sequence availability is evident with the level of microarray activity in *A. gambiae*. Thirteen microarray studies have been published, using the spotted cDNA ($n = 6$), long oligo ($n = 4$), and Affymetrix ($n = 3$) platforms. These have addressed a variety of phenotypes including response to pathogens (bacteria, O'nyong-nyoung virus, and *Plasmodium*), general blood feeding, odor reception, and insecticide resistance. Three studies have utilized targeted microarrays with small numbers (~200–300) of elements specifically designed to evaluate gene families likely to be involved in odor reception (Biessman et al. 2005) and insecticide resistance (David et al. 2005; Strode et al. 2006). A novel use of microarray technology was employed by Turner et al. (2005). They used the *A. gambiae* Affymetrix array to evaluate genomic DNA from the M versus S forms, and were able to identify genome regions carrying SNPs that they defined as genomic "speciation islands"; these reflect differentiated regions likely involved in ecological and behavioral isolation of the forms. For this analysis, they examined hybridization intensities across the 25-mer probes and were able to identify perfect versus SNP-containing signals, and subsequently align these along the genome.

Microarray technology has been applied to one other mosquito species, *Anopheles stephensi* (Xu et al. 2005). Using spotted cDNA arrays, midgut responses to *Plasmodium berghei* infections were examined across a temporal continuum spanning development of the parasite.

6.5.4 Integration of Genome Information

VectorBase (http://www.vectorbase.org), a web-based repository for information on invertebrate vectors, has been established as a central point for accessing genome information on individual vectors and easily making comparisons between them (Lawson et al. 2007). VectorBase has established a central role in annotation efforts for vectors, including protein feature predictions, gene ontology codes, and enzyme classification numbers. At present, the database contains complete information for *A. aegypti* and *A. gambiae*, with several additional species already planned for inclusion. All forms of sequence data are available for download.

6.6 Future Scope of Works

The common goal of genome studies in mosquitoes is to develop novel, sustainable methods for preventing them from transmitting diseases to humans. It is clear that genome information has accelerated efforts to tease out individual components driving mosquito morphology, physiology, and behavior. Development of novel genetic control strategies has gained considerable momentum and, particularly, efforts to import genetically modified mosquitoes resistant to pathogen infection into natural populations are active areas of research (Jacobs-Lorena 2003; Sanchez-Vargas 2004; Christophides 2005; James 2005). The next decade will likely establish mosquito genomics at the forefront of arthropod-borne disease prevention.

Acknowledgement. This effort was supported in part by NIH-NIAID grants UO1 AI50936 and RO1 AI059342. This chapter is dedicated to the memory of Dr. George B. Craig Jr for his pioneering efforts in making mosquito genomics a reality.

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7 Hessian Fly

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7.1 Introduction

The Hessian fly (*Mayetiola destructor*) was the first invasive insect to cause economic havoc in the USA (Pauly 2002). It is believed to have originated in Southwest Asia, along with its primary host plant, wheat (*Triticum* spp.) (Harris et al. 2003). It clearly had a long association with small grains in Europe (Fitch 1846; Barnes 1956) and, at about the time of the American Revolutionary War, it was transported to the Americas, probably aboard sailing vessels carrying wheat straw. Its common name reflects the disdain American farmers had for both the insect and the mercenary Hessian soldiers that fought the American Revolutionary Army (Hunter 2001). Today, the insect remains one of the most important pests of wheat in North America, North Africa, Western Europe, New Zealand, and Southwest Asia (Hatchett et al. 1987; Naber et al. 2000, 2003; Harris et al. 2003). Its pest status has been the primary motivation for genetic studies. Nevertheless, there are additional features that make it a subject worthy of investigation. Among these are its specialized relationship with its host plant, its evolutionary position, and an unusual chromosome cycle.

The Hessian fly is a gall-forming insect (Harris et al. 2006) with several attributes that make it suitable for genetic analysis: a short life cycle (30 days), a small genome (158 Mb) (Johnston et al. 2004), and polytene chromosomes. The reproductive biology and behavior of the insect also make it an attractive model (Harris and Rose 1989, 1990; Bergh et al. 1990; Kanno and Harris 2000; Morris et al. 2000; Harris et al. 2001). Compared to most plant-feeding insects, Hessian flies can be reared in a small space. Unlike gall-forming aphid species, the Hessian fly is always sexually reproducing. Females mate only once and deposit 100–400 eggs on the adaxial surfaces of wheat leaves in a short time (approximately 3 hours). Eggs hatch in only 3–4 days at 20 °C. Newly hatched larvae move to the base of the nearest node where their feeding causes abnormal stem and leaf growth, stunting, and the eventual death of seedlings (Anderson and Harris 2006). Larvae normally feed for only 10–12 days, and up to 50 larvae can survive on a single wheat seedling. Non-feeding (third instar) larvae can be easily maintained in diapause at 4 °C for more than a year. This makes it possible to conveniently maintain collections of various Hessian fly populations and genotypes without continual breeding. It also makes it possible to screen thousands of wheat plants for resistance to specific genotypes of the pest. Screening has discovered over 30 Hessian fly resistance genes (*H* genes named *H*1, *H*2, *H*3, etc.) (Martin-Sanchez et al. 2003; Sardesai et al. 2005). It has also permitted the discovery of Hessian fly phenotypes (commonly referred to as biotypes) that differ with respect to their ability to survive on wheat plants carrying different *H* genes. The desire to understand the mechanisms that underlie these phenotypes remains the major impetus for studying the genetics of this pest. This chapter provides a brief history of these investigations, gives an overview of the current state of Hessian fly genomics, and draws attention to areas for future Hessian fly research.

7.1.1 Taxonomic Description

The Hessian fly is a member of the family Cecidomyiidae, one of the largest families within the Order of

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true flies (Diptera) (Mamaev 1975; Gagne 1994). It was the first gall midge identified in North America (Gagne 1989). Its scientific name was changed from *Phytophaga* destructor to *Mayetiola destructor* in the mid-1960s. Cecidomyiids are grouped with other primitive flies (e.g., mosquitoes, midges, gnats, and black flies) in the paraphyletic suborder Nematocera (Yeates and Wiegmann 1999). They belong to the infraorder Bibionomorpha with the Mycetophylidae and the fungus gnats (Sciaridae) (Friedrich and Tautz 1997). The family Cecidomyiidae has been divided into the subfamilies Lestremiinae, Porricondylinae, and Cecidomyiinae. The Lestremiinae and the Porricondylinae are considered to be the more primitive and their members generally feed on fungus or decaying organic matter. The Cecidomyiinae, to which the Hessian fly belongs, comprise the youngest and largest subfamily. Its 3,850 described species represent about 80% of all known cecidomyiids (Harris et al. 2003). A few of these are beneficial predators of aphids and other plant-feeding insects, but most are plant feeders. A significant number of these are important pests; the sorghum midge (*Contarinia sorghicola*), the Asian rice gall midge (*Orseolia oryzae*), the African rice gall midge (*Orseolia oryzivora*), the wheat midge (*Sitodiplosis mosellana*), the sunflower midge (*Contarinia schulzi*), and the Swede midge (*Contarinia nasturtii*) are just a few of the more important examples. The greatest proportion of species in the subfamily Cecidomyiinae is divided into two monophyletic supertribes, the Lasiopteridi and the Cecidomyiidi (Harris et al. 2003). The Hessian fly belongs to the Lasiopteridi. The genus *Mayetiola* includes 26 additional species in Europe and one additional species in North America (Gagne 1989). All *Mayetiola* live on grasses. Other economically important species attack barley (*M. hordei*), rye (*M. secalis*), brome (*M. bromicola*), and oat (*M. avena*) (Harris et al. 2003).

7.1.2 Economic Importance

The economic importance of the Hessian fly is closely associated with that of wheat, which ranks first among all crops in total production and acreage and provides more nourishment for people than any other food source (Briggle and Curtis 1987). Hessian fly resistant wheat cultivars are the most popular and effective means of Hessian fly control (Hatchett et al. 1987; Buntin et al. 1990, 1992; Ratcliffe and Hatchett 1997). Thus, the deployment of Hessian fly resistance into elite lines and cultivars remains a priority in many wheat breeding programs. The acreage planted to resistant wheat (now over 40% across the USA) is expected to continually increase (Patterson et al. 1990; Ratcliffe and Hatchett 1997). Unfortunately, Hessian fly resistance genes typically lose their effectiveness in 7–10 years (Buntin and Chapin 1990; Ratcliffe et al. 1994, 1996). This is caused by the selection of "virulent" Hessian fly genotypes (genotypes that are capable of living on an otherwise resistant wheat) by the wide utilization of single *H* genes (Cox and Hatchett 1986; Gould 1986; Hatchett et al. 1987). Improving the durability of Hessian fly *H* genes is a goal of wheat breeding programs throughout the world. One approach is to pyramid *H* genes in wheat cultivars and elite lines. These efforts are ongoing.

7.1.3 Classical Genetics and Cytology

Accounts of resistance to the Hessian fly date back to 1785 (Painter 1951). It was Reginald Painter (Painter 1930), however, who discovered that field populations of the insect are composed of a mixture of distinct genotypes that differ in their ability to survive and stunt various wheat cultivars. Painter's observations were followed by the development of screens for Hessian fly resistance in wheat (Cartwright and LaHue 1944), and later, by the selection of four Hessian fly strains that differed in their ability to survive on wheats derived from the resistant spring wheat "W38" and the resistant durum wheat P.I. 94587 (Gallun et al. 1961). Several genetic experiments then identified resistance genes *H3* in "W38," *H5* in "Ribeiro," and *H6* in P.I. 94587 (Caldwell et al. 1946; Shands and Cartwright 1953; Allan et al. 1959). These accomplishments set the stage for the classical genetic experiments that firmly associated this insect with the gene-for-gene hypothesis (Flor 1956). Hessian fly virulence to each of these resistance genes was conditioned by simply inherited recessive alleles (Gallun and Hatchett 1969; Hatchett and Gallun 1970; Gallun 1977, 1978). Importantly, the alleles conditioning virulence to these

genes were shown to be non-allelic (Gallun 1978). Thus, consistent with the gene-for-gene hypothesis, it appeared that for each resistance gene in wheat there was a corresponding *Avirulence* (*Avr*) gene in the Hessian fly. Similar experiments later showed that the gene-for-gene hypothesis holds for resistance genes *H*9 and *H*13 (Formusoh et al. 1996; Zantoko and Shukle 1997) and demonstrated that *Avr* genes *vH3* and *vH5* are autosomal whereas *vH6*, *vH9*, and *vH13* are X-linked.

Before it was possible to perform gene mapping experiments in the Hessian fly, it was necessary to develop an understanding of its genome organization and its unusual chromosome cycle (Fig. 1). Chromosome elimination was observed during both embryogenesis and spermatogenesis (Metcalfe 1935; Bantock 1970; Stuart and Hatchett 1988). These studies found that the Hessian fly has a variable number (30–40) of E chromosomes (chromosomes limited to the germline). The Hessian fly's S chromosomes (chromosomes present in both the germline and the soma) are composed of two autosomes (A1 and A2) and two X chromosomes (X1 and X2). Female somatic cells are diploid for both the autosomes and the X chromosomes (A1A2X1X2/A1A2X1X2) whereas male somatic cells are diploid for the autosomes, but haploid for the X chromosomes (A1A2X1X2/A1A2OO). The segregation of a recessive, X-linked, *white-eye*, mutation, and X-linked *Avr* genes showed that the X chromosomes that are eliminated from the male soma are always paternally derived (Shukle and Stuart 1993; Formusoh et al. 1996; Zantoko and Shukle 1997). Thus, both chromosome imprinting and post-zygotic chromosome elimination were clearly associated with sex determination in the Hessian fly. In most females, maternal genotype clearly influences the retention or elimination of the paternally derived X chromosomes from the somatic cells of their offspring. Those females produce either all-female or all-male offspring (Stuart and Hatchett 1991).

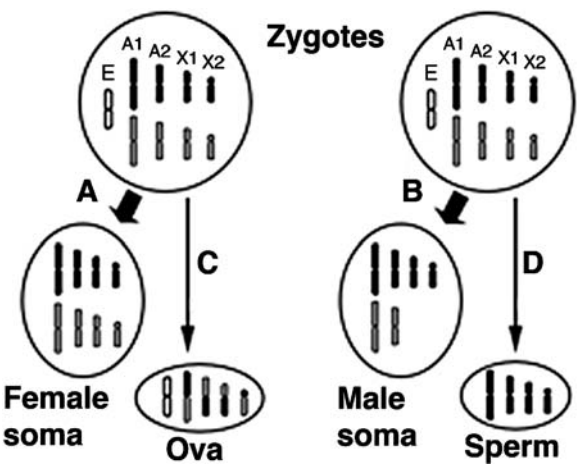

Fig. 1 The chromosome cycle of the Hessian fly. Each zygote contains 30–40 germline-limited E chromosomes (shown as a single chromosome in *outline*), two autosomes (A1 and A2) and two X chromosomes (X1 and X2). During embryogenesis (*A*, *B*) the E chromosomes are eliminated from the presumptive somatic nuclei, but are retained in the germline. When the maternally derived autosomes and X chromosomes (*black* chromosomes) and the paternally derived autosomes and X chromosomes (*grey* chromosomes) are retained in the soma (*A*) the embryo develops as a female. However, if the paternally derived X chromosomes are eliminated from the soma (*B*) the embryo develops as a male. The autosomes and X chromosomes recombine and the E chromosomes divide mitotically during oogenesis (*C*). Each ovum normally contains a haploid set of autosomes and X chromosomes and a full complement of E chromosomes. The E chromosomes and the paternally derived autosomes and X chromosomes fail to segregate into the spermatozoa during spermatogenesis (*D*). Every sperm cell contains only a haploid complement of autosomes and X chromosomes. Males therefore transmit only their maternally derived alleles to their offspring

Chromosome imprinting was also observed during spermatogenesis. During meiosis I of spermatogenesis a monopolar spindle forms that carries only the maternally derived S chromosomes to the spermatozoa. The remaining chromosomes form a "residual" nucleus that gradually disintegrates. Thus, males transmit only their maternally derived alleles to their offspring. Chromosome imprinting, chromosome elimination, and post-zygotic sex determination have since become additional rationale for the study of Hessian fly genetics and genomics. Work in progress is focused on mapping and characterizing the maternal effect locus that controls post-zygotic paternal X chromosome loss.

7.1.4 Limitations of Classical Endeavors and Utility of Molecular Mapping

The greatest barrier to classical genetic analysis in the Hessian fly is the limited ability to perform mu-

tagenesis. This difficulty results from the life history of the insect and its atypical chromosome cycle. The insect is an obligate plant parasite. As a consequence, a suitable artificial diet has not been developed. Spermatogenesis and oogenesis are completed while the insect is a pupa. Adult males will drink fluids, but they live only 2–3 days. Combined, these life history traits make it difficult to have Hessian flies consume a chemical mutagen before mating. Gross chromosome rearrangements induced with γ-irradiation and selected on the basis of semi-sterility have been induced (Stuart et al. 1997). However, these experiments clearly illustrated the second liability; i.e., because males transmit only their maternally derived chromosomes to their offspring, novel mutations are subject to loss each generation they pass through a male.

Other factors also limit the utility of the Hessian fly as a genetic model. The short life of the adult prevents backcrosses between offspring and parents. Full-sib mating among the offspring of unisexual families is also obviously impossible. In addition, although females may deposit up to 400 eggs, one rarely obtains more than 100 offspring from a single female. The small size of the insect limits the quantity of DNA isolated from individuals to about 1 µg. This limits the number and types of molecular markers for which each individual can be scored. Polymerase chain reaction (PCR)-based markers work well, but conventional restriction fragment length polymorphisms (RFLPs) are obviously problematic. The sex determination system and a tendency for inbreeding depression also make full-sib matings difficult. As a consequence, the generation of inbred reference strains and recombinant inbred lines has been complicated. Nevertheless, unlike most gall midge species, the Hessian fly can be reared efficiently and economically in a small space; making the Hessian fly–wheat relationship one of the more thoroughly studied insect–plant interactions (Harris et al. 2003).

7.2 Construction of Genetic Maps

The first genetic maps of the Hessian fly were constructed with seven allozyme loci (Black et al. 1996). This work demonstrated the feasibility of map construction and confirmed the atypical pattern of chromosome inheritance that had previously been proposed based on cytological data (described above). A second linkage map of the three X-linked *Avr* genes *vH6*, *vH9*, and *vH13* was also constructed (Schulte et al. 1999). However, additional large-scale genetic mapping efforts were postponed in favor of experiments designed to identify DNA polymorphisms linked to specific *Avr* genes using bulked segregant analysis (described below) and physical mapping. The ability to physically position cloned DNA with relatively high precision on the larval salivary gland polytene chromosomes of the Hessian fly was performed as a relatively inexpensive alternative to first discovering and then genetically mapping polymorphic DNA sequences (Shukle and Stuart 1995). This strategy was used to position genomic clones on the polytene chromosomes by fluorescence in situ hybridization (FISH), sequence fragments derived from those clones, and develop sequenced tagged site (STS) markers from the sequence data. The construction of Hessian fly bacterial artificial chromosome (BAC) libraries has greatly improved this approach (Fig. 2). Clones in three BAC libraries have been used to position arbitrary genomic DNA fragments as well as ESTs (expressed sequence tags) and STS markers. These efforts are expected to culminate in the development of an FPC-based BAC-contiged map (Soderlund et al. 2000), currently under construction, that is physically anchored to the polytene chromosomes of the Hessian fly and for which the BAC-end sequences of each clone are available for the development of a variety of DNA-based markers.

In addition to these efforts, an amplified fragment length polymorphism (AFLP)-based genetic map of the Hessian fly genome was constructed (Behura et al. 2004). A small mapping female population was developed ($n = 55$) and each female was DNA fingerprinted using 16 selective primer combinations. A total of 183 polymorphic AFLP bands were observed, and 101 of these were used in combination with six STS markers to construct a genetic map consisting of 69 genetic loci on four linkage groups. The complete map covered 443.4 cM and the loci on the map were separated by an average of 6.0 ± 4.8 cM. To physically anchor the genetic map to the polytene chromosomes of the Hessian fly, 35 AFLP bands were converted

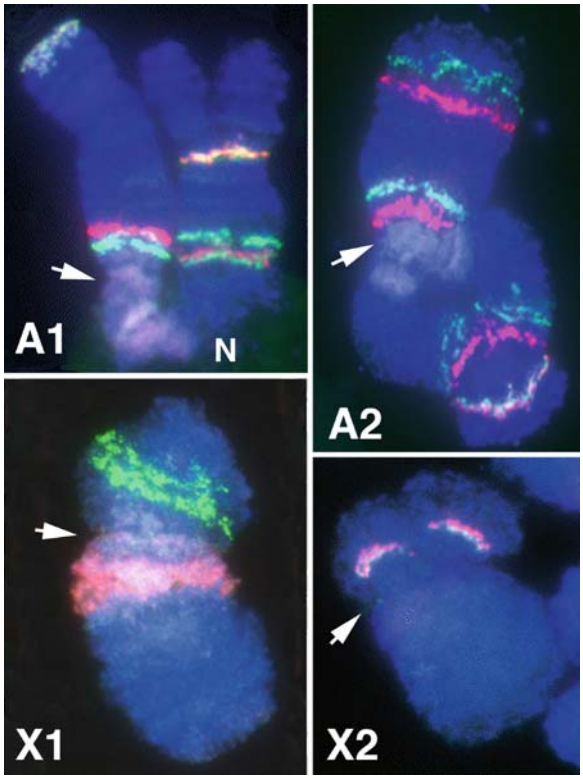

Fig. 2 FISH mapping of BAC clones to the larval salivary gland polytene chromosome of the Hessian fly. Hybridization of 21 BAC clones labeled with either biotin or digoxigenin on autosome 1 (*A1*), autosome 2 (*A2*), chromosome X1 (*X1*), and chromosome X2 (*X2*). The positions of the biotin-labeled clones are seen as *green* fluorescence. The positions of the digoxigenin-labeled clones are seen as *red* fluorescence. *Yellow* fluorescence is visible where biotin- and digoxigenin-labeled probes overlap. *Arrows* indicate the positions of the centromeres. The position of the nucleolus (*N*) on chromosome A1 is also indicated

into STS markers and used as probes in BAC library screens. BAC clones containing 20 of these markers were successfully used in FISH experiments. Those experiments established the correspondence between the genetic linkage groups and the polytene chromosomes. They also established the orientation of the linkage groups on the chromosomes. The proximal region of chromosome A2 was associated with genetic recombination suppression. The long arm of chromosome A1 and the long arm of chromosome X1 were associated with a relative abundance of markers and thus may also be associated with recombination suppression.

7.3 Gene Mapping

Our first approach to positioning *Avr* genes in the Hessian fly genome utilized RAPD-PCR and AFLP-PCR in combination with bulked segregant analysis (Stuart et al. 1998; Schulte et al. 1999; Rider et al. 2002; Behura et al. 2004). Compared to building a genetic map of the entire genome, this approach was relatively efficient and permitted a mapping effort that was focused exclusively on the identification of markers near the *Avr* genes of interest. These efforts identified polymorphic DNA markers linked to *Avr* genes *vH3*, *vH5*, *vH6*, *vH9*, and *vH13*. The polymorphic DNA markers were subsequently cloned, sequenced, and converted into co-dominant STS markers. These STS markers were subsequently used as probes to identify larger genomic fragments within lambda and BAC libraries that contained linked sequence. The larger fragments were then used as probes in FISH experiments to position those fragments, and the associated markers and *Avr* genes, on the polytene chromosomes of the Hessian fly. The most successful effort placed *vH13* and five linked STS markers near the telomere of the short arm of chromosome X2 (Rider et al. 2002). To date the traits of interest in the Hessian fly have been qualitative in nature. Therefore, quantitative trait loci (QTL) analyses still have to be conducted.

7.4 Map-based Cloning

An attempt to clone *vH13* was made by chromosome walking (Lobo et al. 2006). Due to the presence of repetitive DNA and DNA that may be resistant to BAC cloning in this region of the genome, *vH13* has not yet been cloned. However, the genomic DNA near *vH13* was analyzed in considerable detail. This analysis determined that recombination frequencies near the telomere on the short arm of chromosome X2 (approximately 100 kb/cM) are favorable to a map-based cloning effort. Several genes with orthologs in the genomes of the mosquito, *Anopheles gambiae*, and *Drosophila melanogaster* were also discovered. Interestingly, there was synteny among several of these genes between the three species.

7.5 Future Scope of Works

The development of genetic and genomic maps of the Hessian fly arose from the necessity to improve our understanding of this important insect pest. We hope these developments will attract other scientists to investigate the biology, genetics, and evolution of this insect. Below, we briefly describe some of the more obvious rationale for its continued study and address the part that genomics will play in the scope and direction of that research.

7.5.1 Insect–Plant Interactions

The Hessian fly–wheat interaction is one of the more thoroughly studied and often cited examples of host–parasite co-evolution (Harris et al. 2003). It shares many features of pathogenesis and host resistance exhibited by microbes, nematodes, plant pathogenic fungi, and other insects (Subramanyam et al. 2005). It is clearly not the only insect to have such a relationship with its host (Bentur et al. 2003). However, unlike the majority of the others that do, its biology affords an opportunity to explore this relationship genetically. Upon the completion of the FPC-based genomic map, the Hessian fly–wheat relationship will provide an insect–host system that is genetically tractable on both sides of the insect–plant interaction.

One goal is to use the physical genomic map to clone the three X2-linked *Avr* genes, *vH6*, *vH9*, and *vH13*. The position of *vH13* has been narrowed to a region less than 500 kb (Rider et al. 2002; Behura et al. 2004; Lobo et al. 2006). Experiments are in progress to identify the contig in the map that encompasses this gene. Portions of the clones in that contig will be sequenced to generate molecular genetic markers for high-resolution genetic mapping of *vH13* within the contig. With the high recombination rate observed near *vH13*, it is expected that its position will be refined to a single BAC clone. A similar approach will be directed at identifying the genes *vH6* and *vH9*. Still lacking is a functional assay for testing candidate *Avr* genes. Efforts to develop RNAi for this purpose are ongoing in several laboratories.

7.5.2 Understanding the Process of Insect Gall Formation

The Hessian fly has recently been shown to modify plant cells at feeding sites, creating a neoplasm, or gall, that benefits the parasite by the creation of a nutrient sink (Harris et al. 2006). Hessian fly genomics, therefore, offers a means of answering a question first posited by Malpighi in the seventeenth century: How does the control over the fate of plant cell development pass from the plant to the gall-forming parasite? We suspect that the Hessian fly larva injects a salivary product into epidermal cells that triggers complex changes in plant cell development. In fact, recent analyses of first instar larval salivary gland EST libraries indicate that the insect secretes over 200 families of small proteins as it begins feeding on the plant (Chen et al. 2004, 2006; Liu et al. 2004). No obvious homologs of these proteins exist in GenBank. Nevertheless, the timing of their expression suggests some of them may be important to the process of gall formation. Moreover, some may also be elicitors of Hessian fly resistance (*Avr* gene products). Genomic investigations are ongoing to determine the organization of these genes, their regulatory sequences, and their distribution in the genome. Genetic analysis is being pursued to test their association with mapped *Avr* loci. Microarray technology will likely play a valuable role in determining the regulation of the expression of the genes encoding these secreted salivary gland proteins (SSGP) in various Hessian fly genotypes as they feed on resistant and susceptible plants.

7.5.3 Evolutionary Biology

In combination with the genome sequences of *Drosophila*, *Anopheles*, *Aedes*, *Apis*, and other insect genomes presently being sequenced, Hessian fly genomics will extend evolutionary and comparative knowledge of the Insecta. Genetic and physical map positions of heterologous genes will be determined in order to observe trends in insect genomic evolution. Within the insect order Diptera, comparative studies between Hessian fly and *Drosophila* spp., *Anopheles* spp., and *Aedes* spp. are ongoing. Within

the Cecidomyiidae, comparative studies between the Hessian fly, the rice gall midge (*Orseolia oryzae*), and the wheat midge (*Sitodiplosis mosellana*) are in progress. The results of these investigations will permit an evaluation of the potential of the Hessian fly as a comparative model of other important gall midge species. As a member of the Nematocera, comparative analyses may permit an understanding of how primitive flies were able to take two drastically different paths of parasitic evolution: one that led to a lifestyle of feeding on vertebrates, and another that led to a lifestyle feeding on plants.

7.5.4 Population Biology

Managing Hessian fly damage to wheat has long been a matter of population biology. In this context, it is important to point out that it is relatively easy to manipulate Hessian fly populations with resistant wheat cultivars on a scale that mimics conditions relevant to important and current agricultural issues. For example, early experiments demonstrated the relevance of the Hessian fly to the management of transgenic crops (Cox and Hatchett 1986; Gould 1986, 1998), and the application of autocidal insect control (Foster and Gallun 1972). We also envision investigations that address the role wild hosts have on the evolution of pest biotypes. Hessian fly genomics will facilitate these types of investigations by permitting the discovery of the DNA polymorphisms that are necessary for this work and the genetic interactions between genes in various environments.

7.5.5 Chromosome Biology

The function, evolution, and behavior of the germline-limited E chromosomes have long been a subject of speculation (Painter 1966). Hessian fly genomics provides an opportunity to determine the sequence and syntenic relationship that exist between the S and E chromosomes of the Hessian fly, and identify sequences that are unique to each type of chromosome.

7.5.6 Genomic Imprinting

Originally discovered in the sex determination pathway of another nematoceran fly (Metz 1938; Crouse 1960), genomic imprinting regulates gene expression based on whether the gene in question was inherited from the mother or the father. Precisely how it functions, or why it evolved, is still unknown. However, it is clearly involved in aging and reproduction (Reik and Walter 2001; Clayton-Smith 2003) and it is known that its malfunction results in human diseases such as Rett syndrome (Horike et al. 2004), Angelman's syndrome (Jiang et al. 1999), Beckwith-Weidemann syndrome (Maher et al. 2003; Weksberg et al. 2003), transient neonatal diabetes (Temple and Shield 2002), and cancer (Feinberg et al. 2002; Clayton-Smith 2003). Genomic imprinting may be an ancient and highly conserved mechanism (Constância et al. 1998). Therefore, the Hessian fly presents an opportunity to improve our understanding of this mechanism because imprinting is so clearly evident during its chromosome cycle. Future investigations will utilize genomics to develop markers that will make it possible to easily follow genomic imprinting during both meiosis (spermatogenesis) and mitosis (embryogenesis) thereby permitting the identification of the controlling sequences and genes involved in these processes.

7.5.7 Assembly of a Full Shotgun Genome Sequencing Effort

By providing a framework onto which shotgun sequenced contigs can be positioned, an FPC-based physical map of the genome has tremendous utility (Soderlund et al. 2000). Therefore, completion of a physical map of the Hessian fly may advance the possibility of a fully sequenced Hessian fly genome. In addition to making it easier to discover the *Avr* genes, determine the structures of the *SSGP-encoding* genes, and advance population biology and comparative genomics, an assembled draft sequence would permit the development of a genomic-DNA-based microarray chip that could be used to complement gene profiling experiments in wheat, and the discovery of genes that might be exploited as targets for the management of Hessian fly.

Acknowledgement. We thank Rajat Aggarwal and Thiago Benatti for FISH presented in Fig. 2.

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8 Tick

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8.1 Introduction

Ticks (subphylum Chelicerata: class Arachnida: subclass Acari: superorder Parasitiformes: order Ixodidae) are obligate blood-feeding ectoparasites of global medical and veterinary importance. Ticks live on all continents of the world (Steen et al. 2006). There are approximately 899 species of ticks; the majority are ectoparasites of wildlife and approximately 10% of these are recognized as disease vectors or for their ability to cause direct damage through blood feeding (Jongejan and Uilenberg 2004). Ticks transmit a greater variety of viruses, bacteria, and protozoa than any other blood-feeding arthropod (Dennis and Piesman 2005) and are second only to mosquitoes in terms of their medical and veterinary impact (Sonenshine 1991). Other forms of injury attributed to ticks include anemia, dermatosis, and toxicosis. Worldwide there is growing concern because tick-borne infectious diseases are emerging and resurging (Walker 1998, 2005; Telford and Goethert 2004). Many aspects of tick biology have been investigated at the organismal level. However, efforts to understand the genetic basis of host seeking and selection, attachment and feeding, tick-host-pathogen interactions, development and reproduction, and acaricide resistance have been hindered by a lack of tick nucleotide sequence. This situation is rapidly changing with the recent initiation of large-scale sequencing efforts for several tick species. There has been some effort to develop genetic and physical maps to support and exploit tick genomic data but further advances are urgently required. This chapter provides an overview of the current state of tick genomics and highlights areas for future research.

8.1.1 Phylogeny and Evolution of the Ixodida

Ticks and mites are members of the subclass Acari within the subphylum Chelicerata. The chelicerate lineage is thought to be ancient, having diverged from Trilobites during the Cambrian explosion (Brusca and Brusca 1990). It is estimated that is has been approximately 490–550 million years since arthropods in the subphylum Mandibulata, containing the order Hexapoda (Insects), shared a common ancestor with species in the subphylum Chelicerata (Klompen et al. 1996). Not surprisingly then, ticks differ from other blood-feeding arthropods in many aspects of their biology.

Numerous papers have reviewed the phylogeny, evolution, and historical zoogeography of ticks and mites. Unfortunately, phylogenetic studies of the Acari have been confounded by the lack of fossil evidence, specimens, and molecular data. The current understanding of Ixodida phylogeny is represented in Fig. 1. The suborder Parasitiformes includes the order Ixodida which comprises three families, namely the Argasidae (soft ticks), the Ixodidae (hard ticks), and the Nuttalliellidae (comprising a single species which has not been collected for many years). It is generally accepted that the Ixodidae are divided into two lineages, the Prostriata which consists of the single genus *Ixodes* (subfamily: Ixodinae) containing approximately 249 species, and the Metastriata (all other genera) which contains approximately 464 species. The Prostriata are thought to be a paraphyletic lineage, with one distinct clade comprising Australasian species, and the other clade of non-Australasian species (Klompen et al. 1996). The Metastriata contains four subfamilies, namely Amblyomminae, Bothriocrotoninae, Haemaphysalinae,

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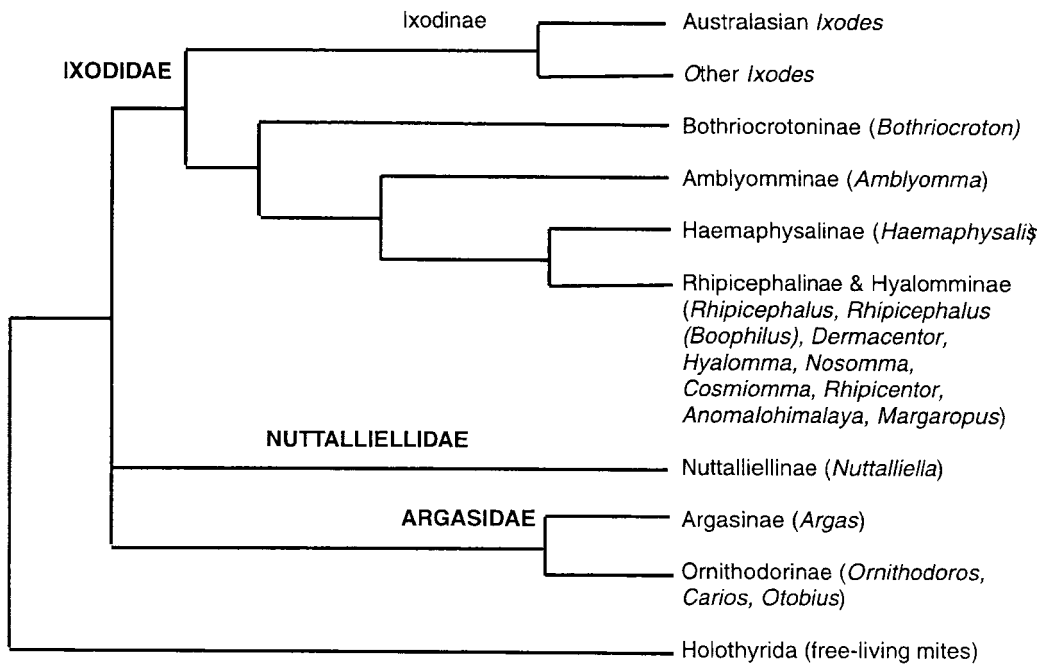

Fig. 1 Current hypothesis of the phylogeny of the subfamilies of ticks. [Reprinted from Toxicon, vol 47, NA Steen, SC Barker, PF Alewood, Proteins in the saliva of the Ixodida (ticks): pharmacological features and biological significance, pp 1–20, Copyright (2006), with permission from Elsevier]

and Rhipicephalinae. The evidence for *Amblyomma* indicates a paraphyletic lineage where *Amblyomma* from Africa form a single lineage and several subgenera of *Amblyomma* exist in South America and Australia. Bothriocrotoninae is a recently added subfamily comprised of the single genus *Bothriocroton*, a basal lineage of endemic Australian ticks previously classified in the genus *Aponomma*. The remaining *Aponomma* species are now considered *Amblyomma* (Klompen et al. 2002). The position of the Haemaphysalinae has yet to be resolved, due in part to incomplete specimens for this genus, as well as morphological similarity to certain *Amblyomma* spp. Hyalomminae (Barker and Murrell 2004) is now considered an invalid subfamily as molecular data suggest that it arises within the Rhipicephalinae (Barker 1998).

Hard ticks are thought to have evolved from bird-feeding soft ticks similar to *Argas* (Black and Piesman 1994; Ribeiro et al. 2006). Klompen et al. (2000) applied a total-evidence based approach utilizing both molecular and morphological characters to propose that the close relationship between holothyrid mites (Acari: Parasitiformes) and the Ixodida suggests that ticks may have evolved from scavengers and not predators. The hypothesis of Steen et al. (2006) suggests that ticks evolved from a saprophytic, carrion-feeding lifestyle, to an obligate hematophagous lifestyle through behavioral, anatomical, and salivary gland adaptations. There has been little molecular analysis of the Argasidae to date although the morphology and systematics of this family have been studied by Klompen (1992) and Klompen and Oliver (1993). Two main hypotheses have been proposed to explain the origin of the hard ticks and their subsequent dispersal around the globe, both of which suggest an origin in that part of Gondwana that eventually became Australia. The first proposes that ticks evolved in Australia on early amphibians in the Devonian ca. 390 Mya (Dobson and Barker 1999) and the other suggests that the first hard tick lived much later (120 Mya) after Australia became relatively isolated (Klompen et al. 1996).

Despite the importance of many *Ixodes* species as parasites and vectors, little is known about the phylogeny of the Ixodinae and there is some debate as to whether the genus *Ixodes* is mono- or para-

phyletic (Barker and Murrell 2002; Xu et al. 2003). Murrell et al. (2001) utilized a total-evidence approach to propose their hypothesis of the origin of Rhipicephalinae. They speculate that the *Dermacentor* lineage evolved in Afrotropical forest and subsequently dispersed on mammals to Eurasia during the Eocene (50 Mya). These ticks subsequently dispersed from Eurasia to the Neartic through the Bering land bridge, and from Europe via Greenland during the Oligocene (35 Mya). The dispersal of the *Dermacentor-Anocentor* ticks from the Neartic to the Neotropics through the Isthmus of Panama took place much later, approximately 2.5 Mya. The *Nosoma-Hyalomma* lineage evolved in the Orient and dispersed during the Miocene (19 Mya). The lineage of *Boophilus* evolved in Africa and dispersed to Eurasia during the Miocene period (14 Mya). Lastly, the *Rhipicentor* species are thought to have evolved in Africa where they have remained confined (Murrell et al. 2001).

8.1.2 Medical, Veterinary, and Economic Importance of Ticks

Some of the most significant tick-borne diseases of humans and animals and their tick vectors are shown in Table 1. The causative agents of these diseases include bacteria (both extracellular and intracellular), viruses, and piroplasm protozoans. The success of ticks as vectors of disease-causing agents can be attributed to a number of factors including wide host range, feeding on multiple hosts, as well as the mechanism and length of time required to blood feed. The long life span (1–2 years) of most hard ticks also enhances vector capability because it provides sufficient time for ticks to become a reservoir host. Both trans-stadial and transovariole mechanisms of pathogen transmission are documented in the Ixodida. Thus, in certain species, both immature stages and adult ticks are competent vectors.

The *Ixodes ricinus* species complex comprises a group of ticks that are distributed in almost all geographic regions of the world and includes a number of species of significance to human health because they vector tick-borne encephalitis virus, rickettsiae, piroplasma, and the *Borrelia* spirochete (Delaye et al. 1997). This complex includes the *Ixodes scapularis* (black-legged or deer tick) and *I. pacificus* (western black-legged tick) vectors of Lyme disease (LD) in the USA, southern Canada, and northern Mexico and the *I. ricinus* and *I. persulcatus* vectors of *Borrelia* in the Palearctic and Oriental regions (Xu et al. 2003). LD is the most common vector borne disease in the USA. Despite federal, state, and local efforts to prevent and control LD, a total of 23,763 cases were reported in 2002 (CDC 2002) representing an almost threefold increase since 1991. The average direct and indirect medical expenses associated with LD patient care are estimated at $2,970 and $5,202 respectively, which translates to a nationwide estimated annual economic impact of approximately US $203 million (in 2002 dollars) (Zhang et al. 2006).

Rhipicephalus (Boophilus) microplus (hereafter *Boophilus*), the tropical or southern cattle tick, has colonized most of the world's tropical and subtropical countries (McCosker 1979; Murrell et al. 2001) and is the most economically important *Boophilus* species. *R. microplus* is a vector of the protozoan (*Babesia bovis* and *B. bigemina*) and bacterial (*Anaplasma marginale*) organisms which cause bovine babesiosis and anaplasmosis ("tick fever"), respectively. The tick-disease complex of *Boophilus* spp.–*Babesia* spp.–*Anaplasma marginale* is probably the most important affecting worldwide livestock production (deCastro 1977), leading to severe economic losses in milk and beef production and restriction in traffic of animals, costing more than US $2.5 billion annually. Chemical treatments (acaricides) are relied on for tick control, however tick resistance to synthetic pyrethroid, organophosphate, and amitraz acaricides is widespread (Foil et al. 2004). Control of cattle ticks is required to minimize production losses and industries incur more than US $200 million in annual losses due to the impact of ticks and tick-borne diseases and costs of treatment to ensure compliance with regulatory protocols for intrastate, interstate, and international livestock movement (Playford and Services 2005).

Other species of ticks that are of medical or veterinary importance include *Rhipicephalus appendiculatus* (brown ear tick) which vectors *Theileria parva*, the causative agent of East Coast fever. In eastern and southern Africa, this disease severely limits cattle production. The tropical bont tick, *Amblyomma variega-*

Table 1 Diseases transmitted by ixodid ticks, showing causative agents and primary tick vector(s)

Disease	Causative agent	Primary tick vectors
Tularemia	*Franciscella tularemia*	*Amblyomma americanum, Dermacentor variabilis, D. nuttalli, Ixodes ricinus*
Human granulocytic ehrlichiosis	*Anaplasma chaffeensis*	*A. americanum*
Heartwater	*Ehrlichia ruminantium*	*A. variegatum, A. hebraeum*
Bovine anaplasmosis	*Anaplasma marginale*	*Boophilus* spp.
Bovine babesiosis	*Babesia bigemina, B. bovis*	*Boophilus* spp.
Colorado tick fever	Reoviridae	*D. andersonii*
Rocky mountain spotted fever	*Rickettsia rickettsii*	*D. variabilis, D. andersonii*
Northern Asian tick typhus (Siberian tick typhus)	*Rickettsia sibirica*	*D. marginatus, D. silvarium, D. nuttalli*
Tick paralysis	Salivary toxin	*D. andersoni, D. variabilis, I. holocylus*
Crimean Congo hemorrhagic fever	Bunyaviridae	*Hyalomma marginatum, H. anatolicum, H. rufipes, H. marginatus*
Kyasanur forest disease	Flaviviridae	*H. spinigera*
Lyme disease	*Borrelia burgdorferi* sensu stricto	*I. ricinus* complex
American babesiosis	*Babesia microti*	*I. scapularis*
Human tick-borne ehrlichiosis	*Ehrlichia spp.*	*I. ricinus*
Tick-borne encephalitis	Flaviviridae	*I. ricinus, I. persulcatus, D. marginatus*
Epizootic bovine abortion	*B. coriaceae*	*Ornithodorus coriaceus*
Relapsing fever	*Borrelia recurrentis*	*Ornithodoros* spp.
Canine ehrlichiosis	*E. canis*	*Rhipicephalus sanguineus*
East Coast fever	*Theileria parva*	*R. appendiculatus*
Boutonneuse fever	*Rickettsia conorii*	*R. sanguineus, R. appendiculatus, Haemaphysalis leachi*

tum and the bont tick, *A. hebraeum* are also of medical and veterinary importance because they are the primary vectors of *Ehrlichia ruminantium* which causes 'Heartwater'. Heartwater is one of the more important cattle diseases in sub-Saharan Africa and Madagascar, and has recently appeared on a few islands in the Caribbean. The lone star tick, *Amblyomma americanum* is also of increasing importance due to changes in its geographical distribution, discovery of new pathogens for which it is a vector, and increased frequency of transmission of those zoonotic infectious agents to humans (Childs and Paddock 2003). *Amblyomma americanum* is the vector of *E. chaffeensis* which causes human ehrlichiosis. Multiple species of *Dermacentor* have also been implicated as major disease vectors in the USA and elsewhere. *Dermacentor andersonii* and *D. variabilis*, the Rocky mountain wood tick and the American brown dog tick, vector Rocky Mountain spotted fever, a disease caused by *Rickettsia rickettsii*.

8.1.3 Overview of Tick Biology

All ticks share the same basic developmental pattern; the egg hatches into a six-legged larva, which molts to an eight-legged nymph. Depending on the species, there may be one or multiple nymphal molts before the final molt to an eight-legged adult. Ticks, with rare exception, are obligate blood feeders at all life stages but are considered to be nonpermanent parasites in that they must find a new host each time they feed. Tick life cycles are defined by the number of hosts upon which a species will feed (Fig. 2). Argasid ticks feed on multiple hosts over a lifetime, even within a life stage and their most common hosts are generally small nesting vertebrates, such as birds and bats. In contrast, ixodid ticks will molt to the next life stage after each feeding on a host. In the Ixodidae, a mated female will deposit a single, large egg batch, and die shortly there-

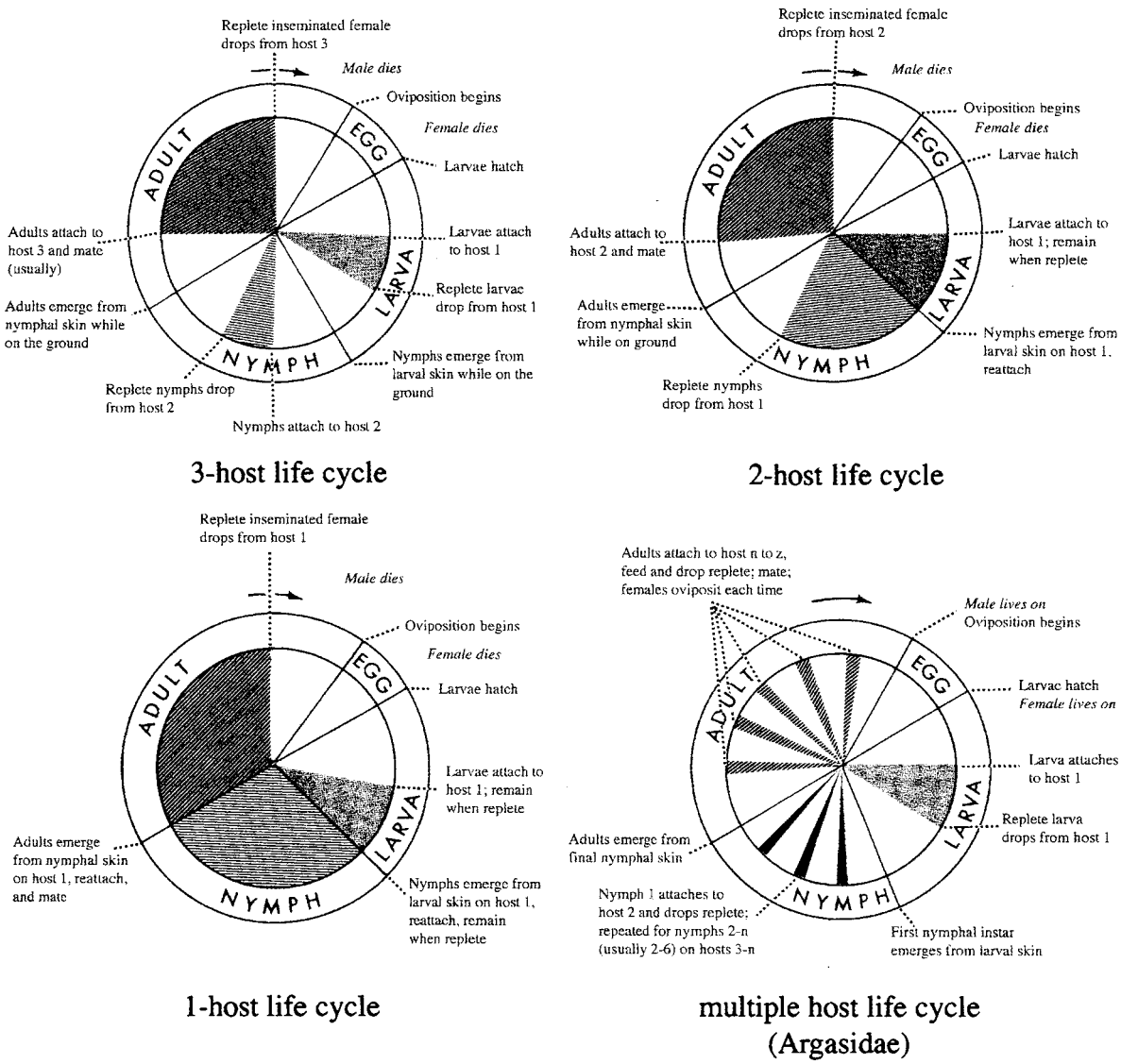

Fig. 2 Overview of life cycles observed in ticks. [Reprinted from Biology of Disease Vectors, 2nd edn, (eds) Marquardt WC, Black IV WC, Freier JE, Hemingway J, Higgs S, James AT, Kondratieff B, Moore C. Chap 4 Ticks, the Ixodida, p 50, Copyright (2005), with permission from Elsevier]

after. The eggs hatch into larvae, which begin active questing for a host. In "three-host" species such as *Ixodes, Amblyomma*, and some species of *Dermacentor*, larvae will attach and feed for 3–7 days. Once fully engorged, the larvae will drop off the host, molt to a nymph, and will then search for a new host. The nymph will feed for 3–8 days, drop off the host, molt to an adult, and seek a new host for a third time. The most common hosts of immature ixodid ticks are small mammals, ground dwelling birds, and lizards. Adult ixodid ticks tend to feed on larger mammals such as deer, livestock, dogs, and humans.

Depending on the species of tick, mating may occur on or off the host post-feeding. Some *Hyalomma* and *Rhipicephalus* species do not drop off after larval feeding, but instead molt on the host. This is considered a two-host life cycle. *Boophilus, Margaropus*, and some species of *Dermacentor* exhibit a one-host life cycle in which all stages of the tick remain on the

host from the first attachment until drop off as mated females.

Once a questing tick finds a host, and a suitable site to feed on the host, hard ticks penetrate the host skin with their chelicera and secrete a cement-like substance that helps to prevent detachment. Ticks imbibe the blood that pools in the wound site created by the mouthparts. Hemostasis is prevented by a mixture of several compounds present in the tick saliva (Ribeiro 1989, 1995), which are injected into the host by alternating cycles of feeding and salivating (Gregson 1967). Tick saliva also contains anti-inflammatory and immunomodulatory compounds that prevent immune reactions from disrupting the feeding process (Wikel 1999; Wikel and Alarcon-Chaidez 2001; Francischetti et al. 2005). While host immunity to salivary components may inhibit pathogen transmission, it has also been observed that the pharmacologic effects of tick saliva can enhance pathogen transmission (Gillespie et al. 2000).

8.1.4 Current Research Trends

Control of human tick transmitted diseases is difficult due to the lack of vaccines (Walker 1998; Dennis and Piesman 2005) and reliance on protective clothing, repellents, and tick checks (Ginsberg and Stafford 2005). Acaricides are the primary method for protecting livestock from tick infestation and tick-borne pathogens. However, the widespread development of acaricide resistance poses a serious challenge to effective control (Mitchell 1996; George et al. 2004). Development of novel control strategies depends on in-depth knowledge of tick biology and tick-host-pathogen interactions. While much progress has been made, significant gaps still exist in our understanding of many of these important and fundamental processes. An overview of the trends of current tick research is provided below, including specific areas that could likely benefit from advances in genetic and physical mapping.

We currently know very little of the mechanisms that allow one tick species to be a permissive vector and yet another refractory. Further elucidation of the molecular interactions that occur between the disease-causing agent and the tick during arthropod infection is needed. There has been considerable emphasis on the interaction between tick and vertebrate host, although surprisingly, not on the genetic basis for host preference and selection. Numerous tick salivary components have been characterized to identify pharmacologically active molecules (Ribeiro et al. 2006) as well as novel transmission blocking and anti-tick vaccine targets (Labuda et al. 2006). *Ixodes scapularis* saliva has been the most intensely studied producing a large annotated catalog of salivary transcripts (Ribeiro et al. 2006), and several specific proteins have been thoroughly described in function and structure. The proteins studied include enzymes, enzyme inhibitors, host protein homologs, immunoglobulin-binding proteins, amine-binding lipocalins, receptor agonist/antagonists, calcium-binding components, and cement cytokine components and an excellent review of 50 of these proteins is provided by Steen et al. (2006). An aspartic protease and a troponin-I-like molecule with anti-angiogenesis properties have been described in *Haemaphysalis longicornis*, a vector for a wide range of pathogens in East Asia and Australia (Boldbaatar et al. 2006; Fukumoto et al. 2006). Histamine release factors, which are critical to feeding, have been identified for *D. variabilis*, *D. andersoni*, *R. microplus*, and *A. americanum* (Mulenga and Azad 2005). Several tick proteins, including a histamine binding protein and a recombinant complement inhibitor are currently in clinical or preclinical human trials.

In recent years, the gene silencing technique of RNA interference (RNAi) has also been applied to understand the function of tick genes. Some of the first studies to silence expression of tick salivary gland transcripts using RNAi were demonstrated by Aljamali et al. (2002) and Narasimhan et al. (2004). More recently, RNAi has proved a rapid and cost-effective tool for screening large numbers of cDNAs to identify potential tick protective antigens (de la Fuente et al. 2005). Silencing of the anti-complement protein, *isac* in *I. scapularis* nymphs negatively affected tick feeding by reducing the fed tick weight by 40% (Soares et al. 2005). The causative agent of human granulocytic anaplasmosis, *Anaplasma phagocytophilum*, induces expression of the *salp16* gene in *I. scapularis* salivary glands during tick feeding. RNAi-induced silencing of *salp16* expression reduced the survival of *A. phagocytophilum* in infected mice (Sukumaran et al. 2006).

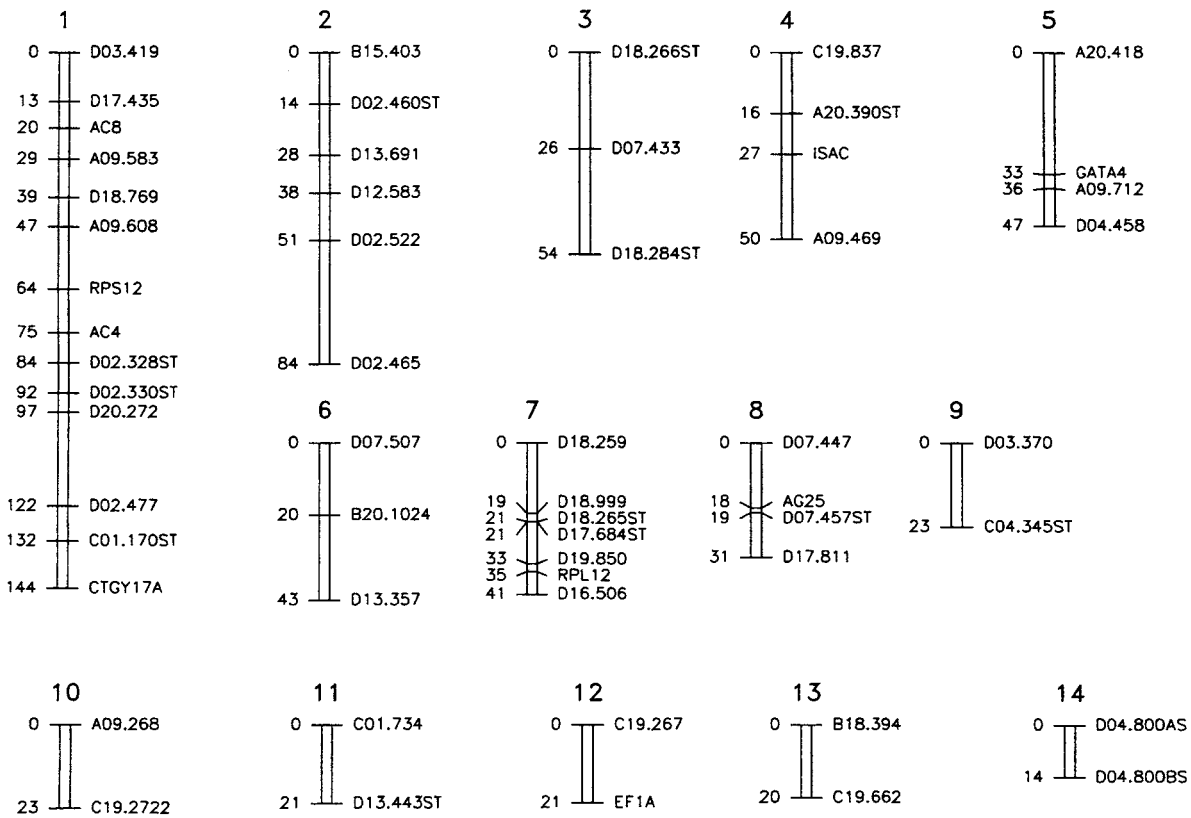

Fig. 3 Preliminary linkage map of the genome of *I. scapularis*. Corresponding mapped markers and accession numbers are shown in Table 2. [Reprinted from Insect Molecular Biology, vol 12, AJ Ullmann, J Piesman, MC Dolan, WC Black IV, A preliminary linkage map of the hard tick, *Ixodes scapularis*, pp 201–210, Copyright (2003), with permission from Blackwell]

Considerable research effort has also been focused on understanding the mechanisms of tick resistance to organophosphate (OP), synthetic pyrethroid, and amitraz acaricides. Both target site insensitivity and metabolic detoxification have been implicated as mechanisms of acaricide resistance in *R. microplus* (Jamroz et al. 2000). High frequencies of a phenylalanine to isoleucine amino acid substitution in domain III of a *para*-like sodium channel have been found in pyrethroid resistant strains of *R. microplus* (Guerrero et al. 2001). Several reports suggest the existence of multiple forms of acetylcholinesterase (AChE) with varying degrees of OP sensitivity (Reich et al. 1978; Wright and Ahrens 1988; Pruett 2002), but studies of putative AChE cDNAs cloned by a degenerate RT-PCR approach have failed to identify any resistance-associated sequence differences (Baxter and Barker 1998; Hernandez et al. 1999; Temeyer et al. 2004). Reciprocal cross experiments with resistant and susceptible *R. microplus* suggest that amitraz resistance in this species is inherited as an incomplete recessive trait (Li et al. 2005). An improved understanding of acaricide resistance in *R. microplus* and other tick pests is needed to delay the development of resistance to existing acaricides and to identify new acaricides with novel modes of action.

8.1.5 Classical Mapping Efforts

Many areas of tick research would benefit from classical mapping studies. However, one of the biggest impediments to the production of linkage maps for the Ixodida is the extremely long life cycle of some tick species, even when cultured under optimal laboratory conditions. As a consequence, the production of

Table 2 List of molecular markers and corresponding accession number (when available) mapped to the *Ixodes scapularis* genome

RAPDs	STARs	GenBank accession number	Microsatellites	GenBank accession number	cDNAs	GenBank accession number
A09.268	A20.390ST	BZ592382	ISAC4	AF331739-AF331739	EF1A	AF378368
A09.469	C01.170ST	BZ385517	ISAC8	AF331740	ISAC	AF270496
A09.583	C04.345ST	BZ385520	ISAG25	AF331742	RPL12	AAH8230
A09.608	D02.328ST	BZ385392	ISCTGY17A	AF331745-AF331747	RPS12	AF470687
A09.712	D02.330ST	BZ385393	ISGATA4	AF331753		
A20.418	D02.460ST	BZ385394				
B15.403	D04.800AST	BZ85433-BZ85434				
B18.394	D04.800BST	BZ85435-BZ85436				
B20.1024	D07.457ST	BZ385404				
C01.734	D13.443ST	BZ385415				
C19.267	D17.684ST	BZ385419				
C19.2722	D18.265ST	BZ385420				
C19.662	D18.266ST	BZ385421				
C19.837	D18.284ST	BZ385422				
D02.465						
D02.477						
D02.522						
D03.370						
D03.419						
D04.458						
D07.433						
D07.447						
D07.507						
D12.583						
D13.357						
D13.691						
D16.506						
D17.435						
D17.811						
D18.259						
D18.769						
D18.999						
D19.850						
D20.272						

a suitable F_1 backcross population is time consuming, laborious, and expensive. For example, *I. scapularis* has a two-year life cycle in the field although this can be shortened to approximately 9 months under optimal laboratory conditions. Another limiting factor is that few markers are available for map generation. To date, the only linkage map published for ticks is a preliminary map for *I. scapularis* developed by Ullmann et al. (2003) (Fig. 3).

8.2 Construction of Genetic Maps

The linkage map of Ullmann et al. (2003) was constructed based on segregation amongst 127 loci (Table 2). These included 84 random amplified polymorphic DNA (RAPD) markers, 32 sequence-tagged RAPD (STAR) markers, 5 cDNAs,

and 5 microsatellites in 232 F_1 intercross progeny from a single P_1 female collected from Bridgeport, Connecticut. Fourteen linkage groups were found, possibly reflecting the haploid number of chromosomes in *I. scapularis*. A preliminary map of 616 cM was generated with one marker every 10.8 cM. Given the genome size of *I. scapularis* (approximately 2.1 – 2.3 Gbp), the relationship of physical to genetic distance is estimated to be approximately 663 kb/cM (2.1×10^3 Mbp/3,166 cM).

Sequence-tagged RAPD markers were the most useful of all the markers analyzed in the Ullmann et al. (2003) study. Of the 65 primer sets designed for STARs, 52 were polymorphic with 37 conforming to mendelian ratios. Markers derived from simple sequence repeats (SSR) or microsatellites are highly desirable because they are based upon DNA polymorphisms and are co-dominant. Unfortunately, microsatellites were not as productive for generating markers in *I. scapularis*. The reasons for this are still unclear but may be associated with the organization of short sequence repeats in the genome (see discussion below). Characterization of microsatellites, or the lack thereof, in *I. scapularis* has been described in earlier work (Fagerberg et al. 2001).

From the 20 RAPD primers identified by Ullmann et al. (2003), a total of 63 markers were mapped to the *I. scapularis* genome (Table 2). This is in stark contrast to 94 markers generated with 10 RAPD primers in *Aedes aegypti* (Antolin et al. 1996) and once again, may reflect the organization of repetitive DNA in the genome. The advantage of RAPD-PCR is that it requires no sequence information and is thus especially useful in the absence of genomic sequence. In addition, each RAPD-PCR primer can potentially reveal several usable loci. A drawback is that RAPD-PCR generates dominant markers which hinder phase determination, or identification of the origins of a genotype in the F_2 and some backcross genotypes. Since phase determination is essential to estimate recombination frequencies, dominant markers are of limited utility in linkage mapping. STARs were used for *I. scapularis* to address the phase determination problem because this method converts dominant into co-dominant bands (Bosio et al. 2000).

Tick research would greatly benefit from linkage maps for a range of tick species. Additional markers are also needed to develop a second generation, high-resolution map for *Ixodes*. Such maps would have enormous utility for map-based (positional) cloning of genes of interest, particularly as so few tick genes have been characterized to date. The drawback is the large number of markers that will be required to adequately map these organisms, especially given the genome size of many ixodid ticks (see below). One benefit of tick genomic sequence will be the opportunity to identify additional, potentially polymorphic markers such as microsatellites and single nucleotide polymorphisms (SNPs) in *Ixodes* and other ticks. cDNA sequence can also be used to generate polymorphic markers (Fulton et al. 2001) and could potentially provide a solution to this problem; however, large introns in *I. scapularis* and possibly other tick species may make this methodology horribly expensive and restrictive.

8.3 Efforts in Forward Genetics

The fact that ticks are not genetically tractable has undoubtedly proven a massive impediment to forward genetics studies of the Ixodida. Methods for tick mutagenesis and genetic screening have not been developed and there are currently no phenotypic and few molecular markers available for ticks. The majority of these have been developed in *Ixodes* and *Rhipicephalus* species (reviewed by Navajas and Fenton 2000). To date, the only approach to investigate gene function in ticks is the reverse genetics method employing RNAi (reviewed above).

8.4 Mapping of Quantitative Trait Loci

High-density linkage mapping of a genome provides an opportunity to identify the genes which affect quantitative traits. There is a real need to identify quantitative trait loci (QTL) associated with phenotypes such as tick vector competence, host seeking and specificity, and acaricide resistance. Currently, QTL mapping is not possible for any tick species, due to the lack of high-density linkage maps. Unfortunately, tick QTL mapping is also limited by the lack of tick populations that are either permissive or refractory for a specific phenotype of interest. However, with

a tick genome sequence in hand for *Ixodes*, it is now incumbent on the tick research community to develop the necessary markers and strains for QTL mapping in this and other tick species of medical and veterinary importance. The development of *Ixodes* strains that are refractory and susceptible to key pathogens, or that differ in their preference for a vertebrate host as a blood meal source would be an important investment for scientists. Strains of *R. microplus* with resistance to one or several classes of acaricides are available and a number of these are maintained by the United States Department of Agriculture (USDA); such populations may find application in the mapping of resistance traits.

8.5 Advanced Work

8.5.1 Physical Mapping Efforts

The ultimate goal of any genome sequencing project is the development of a physical map of the genome in which the order of every base within the genome and its location on chromosomes is known. Physical maps provide the framework needed to identify expressed, non-expressed, repetitive, unique non-coding, structural, and regulatory sequences from raw sequence data. Unfortunately, with the exception of rudimentary karyotyping by Oliver (1977) and Hilburn et al. (1989), nothing regarding tick chromosome structure and genome organization is known. Until recently, physical mapping techniques such as fluorescence in situ hybridization (FISH), widely used in many genome efforts (Adams et al. 2000; Holt et al. 2002; Hong et al. 2003) to assign and orient scaffolds on individual chromosomes, have not been developed for the Ixodidae.

The chromosomal organization of the *R. microplus* genome is currently being resolved by applying FISH to testicular chromosome preparations (C. Hill personal communication). Unfortunately, despite analysis of numerous tissues, researchers have failed to identify polytene chromosomes in the ticks. However, *R. microplus* cells in meiosis I provide a resolution that is sufficient for detailed observations. A combination of FISH, chromosome morphology, and relative chromosome length has made it possible to identify the X chromosome and specific autosomes (2–10) in

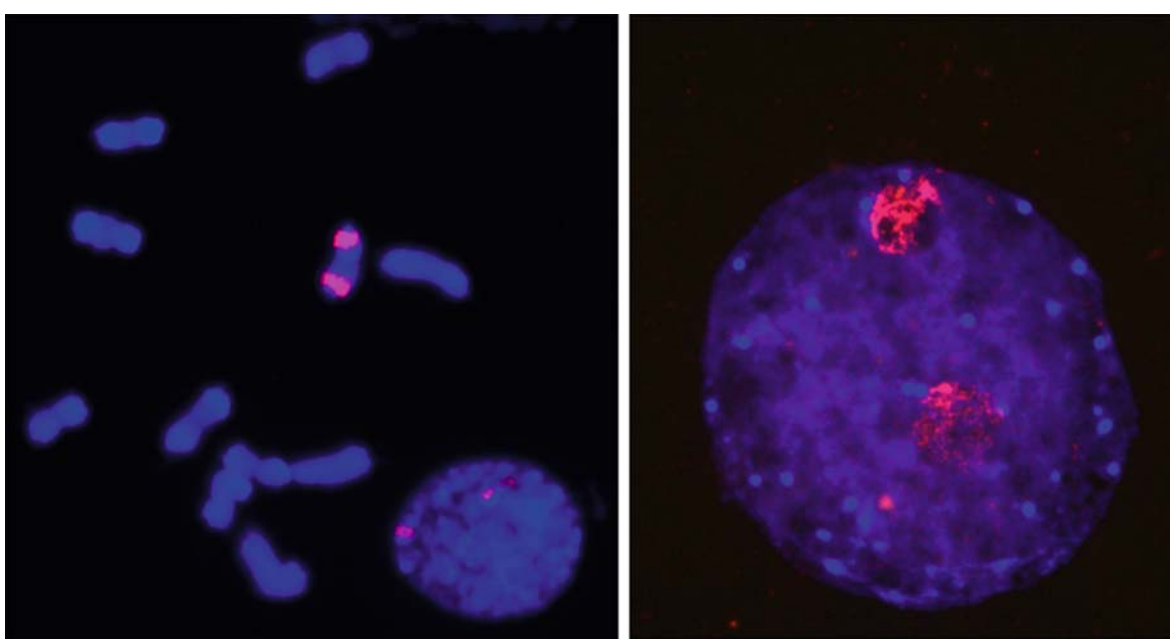

Fig. 4 Fluorescence in situ hybridization (FISH) mapping of an rDNA probe (red label) to *R. microplus* meiotic chromosomes (*left*) and diploid interphase nucleus (*right*)

metaphase I preparations from *R. microplus* (Fig. 4, left panel). The chiasmata associated with each autosome pair are visible in preparations as the chromosomes take the end-to-end formation typical of holocentric chromosomes. Hybridization of the ribosomal DNA (rDNA) repeat on chromosome 6 confirms this orientation and holocentric nature. Heterochromatic termini have also been discovered containing highly repetitive DNA sequence. These knobs are visible in interphase cells as condensed spots (Fig. 4, right panel) that are likely chromomeres. Tandem repeats of approximately 150 bp have been localized to these possibly telomeric regions (N. Geraci unpublished). The quantity of hybridization of these sequences on each chromosome is used as an aid in chromosome (bivalent) identification.

This work represents an important advance for tick genomics and genetics. Such techniques must now be established for *I. scapularis* in order to assemble and mine the genome for sequences of interest. Physical mapping will also permit invaluable studies of tick chromosome biology and population genetics. Physical mapping techniques and resources such as bacterial artificial chromosome (BAC) libraries must now be developed for other tick species of economic importance.

8.5.2 Sequencing Projects: ESTs and Whole-genome Shotgun Sequencing

Historically, expressed sequence tag (EST) sequences have proven invaluable for tick gene discovery, especially in the absence of genomic sequence. A review of the NCBI dbEST database reveals approximately 64,000 EST sequences for both pro- and metastriate species including *Amblyomma, Boophilus, Rhipicephalus, Dermacentor*, and *Ixodes* species. Libraries produced from tick salivary gland, midgut and ovary tissues have proved popular (Santos et al. 2004; Ribeiro et al. 2006) as have studies of tick expression profiles pre-, during, and post-blood meal and pre- and post-infection with various pathogens. The most comprehensive *R. microplus* EST study to date was undertaken by Guerrero et al. (2005) who identified 8,270 unique tentative consensus (TC) and singleton sequences from 20,417 EST sequencing reads of an *R. microplus* pooled tissue library. The Institute for Genomic Research (TIGR) auto-annotation pipeline was employed to assign putative function to these TCs and to create a gene index for several tick species (http://www.compbio.dfci.harvard.edu/tgi).

Expressed sequence tags will continue to be an invaluable tool for tick research and genome annotation especially. Firstly, the evolutionary distance between the subphylum Mandibulata and Chelicerata (Klompen et al. 1996) will likely restrict the identification of tick genes based on homology to insect sequences. The fact that approximately 45% of tick ESTs have no matches in sequence databases (Hill and Gutierrez 2000; Nene et al. 2002; Valenzuela et al. 2002; Guerrero et al. 2005) illustrates this point. Secondly, tick genome annotation will likely be complicated by large introns and significant amounts of repetitive DNA (discussed below).

Several studies have provided an insight into the size and organization of tick genomes and are an important precursor to large-scale genome sequencing. Genome size is an important consideration for sequencing projects because it determines the amount of sequencing that must be undertaken and thus ultimately the cost of a genome project. Reassociation kinetics has been used to determine the genome size of three species of hard ticks. Ullmann et al. (2005) estimated the genomes of *I. scapularis* and *R. microplus* to be approximately 2.15 pg (2.1 Gbp) and 7.5 pg (7.1 Gbp), respectively. The genome of *A. americanum* was reported as 1.08 pg (1.04 Gbp) (Palmer et al. 1994). In other work, an extensive analysis of genome size in multiple species of Ixodida was conducted using the technique of flow cytometry (N. Geraci personal communication). Results suggest a haploid genome size of >1,000 Mbp for all Ixodida examined, with a mean of 1,252 Mbp (1.28 pg) for the Argasidae and 2,610 Mbp (2.67 pg) for the Ixodidae. Estimates for *I. scapularis* compared favorably with that of Ullmann et al. (2005). It would appear that the hard and soft tick species examined to date have genomes that are significantly larger than any sequenced invertebrate. Large genome size in the Ixodida is likely due to the accumulation of non-coding, repetitive DNA. The reasons for this are unclear but could involve the accumulation of transposable elements, segmental duplications and simple sequence repeats, an increase in intron size, and possibly one or multiple polyploidy events.

The above-mentioned studies suggest that moderately repetitive sequences constitute a greater percentage of the *R. microplus*, *I. scapularis*, and *A. americanum* genomes than either highly repetitive or unique sequences (Palmer et al. 1994; Ullmann et al. 2005). Furthermore, *R. microplus* and *I. scapularis* both exhibit a mix of short and long period interspersion (Ullmann et al. 2005), a characteristic of large vertebrate genomes. Additionally, highly repetitive sequences in the *R. microplus* and *I. scapularis* genomes were reported to be of relatively low complexity (Ullmann et al. 2005), suggesting the presence of many simple sequence repeats. These findings have important implications for tick genomics research; extensive sequencing of these and possibly other ixodid ticks will likely be necessary in order to identify unique, presumably coding sequence. Furthermore, significant amounts of repetitive DNA may present a challenge for genome assembly and annotation.

Several tick species have been considered for a genome sequencing effort. Large-scale sequencing projects are underway for both a pro- and a metastriate species. Foremost amongst these is the *Ixodes scapularis* Genome Project (IGP), the goal of which is to sequence the genome of a medically significant tick. The IGP was approved by the National Institutes of Health (NIH) in 2004 (Hill and Wikel 2005). This project will sequence the *I. scapularis* genome to a 6× level of coverage; it is the first to sequence a tick and a member of the subphylum Chelicerata. The project is a partnership between the NIH, the tick research community, the J. Craig Venter Institute (JCVI) and the Broad Institute. As of September 2007, over 19 million trace reads representing approximately 10.4×10^{10} bp of *I. scapularis* DNA have been generated, the equivalent of more than 6× coverage of the genome. A 10× *I. scapularis* BAC library has been generated to facilitate genome assembly and BAC-end sequencing of these clones is ongoing. Over 200,000 *I. scapularis* ESTs have also been sequenced for gene discovery and annotation. Genome assemblies and automated annotations will be made available through public databases maintained by the National Center for Biotechnology Information (NCBI), the JCVI, and Broad sequencing centers as well as VectorBase (http://www.vectorbase.org/index.php; Lawson et al. 2007), the NIH-supported bioinformatics database for invertebrate vectors of human disease.

There is also considerable interest in an *Rhipicephalus microplus* genome sequencing project and USDA scientists have developed a number of resources toward such an effort. Over 40,000 ESTs have been generated and a 1× BAC library is also available. In preliminary work, investigators have been able to assemble a 120-kbp insert BAC from BAC shotgun reads (F. Guerrero personal communication) suggesting that assembly of regions of the *R. microplus* genome will likely be feasible. More recently, a whole-genome shotgun sequencing project was approved for another member of the subclass Acari, namely the two-spotted spider mite *Tetranychus urticae* (M. Navajas personal communication). This project may be of particular advantage to the *I. scapularis* genome project. At 79 Mbp, the *T. urticae* genome is estimated to be one of the smallest invertebrate genomes and, as such, may be an extremely useful model for assembly and annotation of larger tick genomes. Furthermore, comparative analyses between haematophagous tick vectors and the plant phytophagus *T. urticae* may aid the identification of genes associated with parasitism and pathogen transmission.

The *I. scapularis* and *R. microplus* genomes represent a tremendous resource for tick and tick-borne disease research. In addition to informing on many aspects of tick biology, sequence data may permit the identification of new targets for vaccine and acaricide development. These genomes will also facilitate comparative genomic analyses with a plethora of sequenced organisms, revealing fundamental differences in the genomes of tick and insect disease vectors as well as between the pro- and metastriate ticks. As such, they offer the opportunity to understand the genetic level differences associated with aspects of tick biology such as host preference, reduction in number of hosts, and vector competence between three- and one-host ticks. They will also offer insights into the architecture, organization, and regulation of large, repetitive tick genomes and may help to resolve questions of Ixodida phylogeny and evolution.

8.6 Integration of Genome Information and Future Work

Tick genomics will present scientists with unprecedented opportunities and unique challenges. Some

of these opportunities are outlined above and many more will likely become apparent as scientists begin to interrogate genomic sequence. Unfortunately, the size of tick genomes and their repetitive DNA content may be problematic, particularly for genome assembly and annotation. Given the imminent release of the assembled *Ixodes* genome, scientists must be prepared to address such challenges in order to fully exploit the *Ixodes* sequence and ultimately advance tick genomics research.

Physical mapping will be essential for assembly of the *Ixodes* genome as well as for population genetics studies. FISH mapping techniques developed for *R. microplus* must now be expanded to *Ixodes* and a range of other ticks that impact human and animal health. The IGP will generate the first physical map of a tick and possibly a chelicerate genome. As such it will provide an insight into the nature and organization of coding and non-coding DNA in the genome. The automated and manual annotation of genes based largely on sequence homology will not be sufficient to catalog the entire repertoire of *Ixodes* genes and transcripts or to identify the genes associated with specific phenotypes of interest. Additional markers and a high-resolution genetic map of *I. scapularis* will be essential for integration of the *Ixodes* genetic and physical maps as well as to facilitate QTL mapping in this vector.

Repetitive DNA and divergence between ticks and insects may necessitate the development of specific assembly and gene prediction software for tick genomics. Scientists will also need to acquire expertise in the manual annotation of tick sequence. Additional EST sequences from tissue-specific libraries and from a range of tick and chelicerate species will be invaluable for the identification of low abundance transcripts, genome annotation, and comparative analyses. Microarrays, widely used for transcription profiling in a number of invertebrates, are an essential tool that must be developed by the tick research community. RNAi of several tick genes has proved successful and this technique must now be expanded for functional analysis of predicted genes.

Genome size and sequencing costs may prohibit the sequencing of additional tick species of medical and veterinary importance in the immediate future. Consequently, comparative analyses between tick species will likely prove invaluable until such time as genome sequencing becomes more cost-effective. In the interim, the development of genetic and physical maps and associated resources is essential in order to realize the full potential of available and anticipated genomic data.

Acknowledgement. We thank F. Guerrero, J. Pagel VanZee, and J. Walling for *R. microplus* FISH images.

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