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TEMPORAL GENETIC STRUCTURE OF FERAL HONEY BEES (HYMENOPTERA: APIDAE) IN A COASTAL PRAIRIE HABITAT OF SOUTHERN TEXAS: IMPACT OF AFRICANIZATION

A Dissertation

by

MARIA ALICE DA SILVA PINTO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Entomology

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Approved to the style and content by:

J. Spencer Johnston (Co-Chair of Committee)	Robert N. Coulson (Co-Chair of Committee)
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December 2003

Major Subject: Entomology

ABSTRACT

Temporal Genetic Structure of Feral Honey Bees (Hymenoptera: Apidae) in a Coastal Prairie Habitat of Southern Texas: Impact of Africanization. (December 2003)

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Co-Chairs of Advisory Committee: Dr. J. Spencer Johnston
Dr. Robert N. Coulson

The goal of this study was to examine the impact of Africanization on the genetic structure of the Welder Wildlife Refuge feral honey bee population by scoring mtDNA and microsatellite polymorphisms**.** Adult honey bee workers, collected between 1991 and 2001, were screened for mtDNA using the cytochrome *b*/*Bgl*II, ls rRNA/*Eco*RI, and COI/*Hinf*I PCR-based assays. The procedure allowed identification of four mitotypes: eastern European, western European, *A. m. lamarckii*, and *A. m. scutellata*. The relative frequencies of the four mitotypes changed radically during the 11-year period. Prior to immigration of Africanized honey bees, the resident population was essentially of eastern European maternal ancestry. The first colony of *A. m. scutellata* mitotype was detected in 1993. Between 1995 and 1996 there was a mitotype turnover in the population from predominantly eastern European to predominantly *A. m. scutellata*. From 1997 onward, most colonies (69 %) were of *A. m. scutellata* mitotype.

The temporal change in mtDNA was paralleled by nuclear DNA. The 12 microsatellite loci analyzed indicated (1) the mechanism of Africanization of the Welder

population involved both maternal and paternal bi-directional gene flow (hybridization) between European and Africanized honey bees; and (2) the resident panmitic European population was replaced by panmitic asymmetrical admixtures of *A. m. scutellata* and European genes. The steepest increase in the proportion of introgressed *A. m. scutellata* nuclear alleles occurred between 1994 and 1997. The post-Africanization gene pool was composed of a diverse array of recombinant classes with a substantial European genetic contribution (mean proportion of European-derived alleles was 37 % as given by m_R estimator or 25 % as given by m_Y estimator, for 1998-2001). If European genes continue to be retained at moderate frequencies, then the Africanized population is best viewed as a “hybrid swarm” instead of “pure African”.

The most radical change in the genetic structure of the Welder Wildlife Refuge feral honey bee population (observed between 1995 and 1997) coincided with arrival of the parasitic *Varroa* mite. We suggest that *Varroa* likely hastened the demise of European honey bees and had a major role in restructuring the Welder Wildlife Refuge feral honey bee population.

DEDICATION

To João and Gabriela for your patience and support throughout this long journey.

To the memory of my father.

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My sincere gratitude to my advisory committee: Dr. Spencer Johnston, Dr. Robert Coulson, Dr. William Rubink, Dr. Tanya Pankiw, and Dr. John Gold for their guidance and support throughout this research project. Special thanks to Dr. Spencer Johnston and Dr. Robert Coulson, who served as co-Chairs, for their advice and support, and for always being available when help was needed. Special thanks, also, to Dr. Rubink for having the vision and persistence to gather such a unique and valuable collection of feral honey bees.

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CHAPTER I

INTRODUCTION

The western honey bee, *Apis mellifera* L., is of important economic and ecological value as pollinators of commercial crops and native flowering plants, and as honey producers. In recent years, separate events have threatened both the honey bee and beekeeping. Arrival of the *Varroa* mite in 1987 (De Jong 1990), and Africanized honey bees in 1990 (Sudgen and Williams 1990; Rubink et al. 1996) into the United States, are probably the most important of these events.

Honey bees were first introduced into the Americas by settlers. Honey bees are naturally distributed throughout Africa, Europe, and the Middle East, where they have evolved into 24 distinct subspecies (Ruttner 1988). Over the past 400 years, a subset of these subspecies has been introduced into the New World, first by settlers and later by beekeepers (Morse et al. 1973; Sheppard 1989a,b). Most of the introductions were from Europe. In the United States, until early 1990's, managed and feral honey bees were typically derived from eastern (*A. m. ligustica*, *A. m. carnica*, and *A. m. caucasia*) and western (*A. m. mellifera*, and *A. m. iberiensis*) European subspecies (Schiff et al. 1994; Schiff and Sheppard 1995, 1996; McMichael and Hall 1996), reflecting early introductions and the history of beekeeping. Because of their predominant origin in Europe, the United States honey bee is referred to as European. In 1990, honey bees

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This dissertation follows the style and format of Evolution.

descending from a sub-Saharan African subspecies, *A. m. scutellata*, arrived in the United States (Hidalgo, South Texas; Sudgen and Williams 1990; Rubink et al. 1996). The migrants have since spread through Texas, New Mexico, Arizona, Nevada, and California; contributing new genes to the southwestern United States honey bee gene pool.

In tropical regions of Brazil, the temperate-evolved European subspecies were poor honey producers (Kerr 1967; Gonçalves et al. 1991). Thus, in an attempt to create a tropical-adapted and more productive honey bee, queens of *A. m. scutellata* were brought from South Africa into Brazil in 1956, to be crossed with European honey bees (Kerr 1967). In 1957, 26 queens escaped from the quarantine apiary, giving rise to the Africanization process (Nogueira-Neto 1964; Kerr 1967). After the accidental release, queens from the remaining imported stock were reared and systematically distributed to beekeepers (Spivak et al. 1991). In less than 35 years, their descendents expanded through South and Central America and established large feral populations in regions where feral European honey bees were scarce (Michener 1975). The spread of Africanized honey bees throughout most of South America, Central America, and the southwestern United States, in such a short time, is perhaps the most remarkable ecological event of the last decades (Rinderer 1988). Mechanisms underlying this notorious expansion and the genetic composition of Africanized bees in the Neotropics are well documented but not fully understood. Indeed, two conflicting views, later described, have emerged from studies conducted in the Neotropics.

It has been suggested that long term studies (encompassing time periods prior to, during and after Africanization) of co-occurring distribution patterns of mitochondrial and nuclear DNA are critical for a better understanding of the Africanization process (Page 1989, Smith 1991). Such studies would permit documentation of the pattern and time course of gene flow between the resident European population and the migrating Africanized population (Smith 1991). In the present study, mitochondrial DNA (mtDNA) and nuclear DNA (microsatellite) markers are used to assess temporal changes in the genetic structure of a feral honey bee population from southern Texas (Welder Wildlife Refuge, San Patricio County) undergoing Africanization. The study spans a continuous period of 11 years, encompassing pre, during, and post-Africanization.

The goal of this study was to investigate the impact of Africanization on the genetic structure of the Welder Wildlife Refuge feral honey bee population, by scoring mtDNA and microsatellite polymorphisms on honey bees collected for 11-years. The specific objectives were:

1) Examine the mechanism of Africanization in the Welder Wildlife Refuge feral population. The following questions were addressed: (a) did Africanization occur by maternal gene flow, paternal gene flow, or both? (b) was paternal gene flow, if any, between resident and migrant honey bees unidirectional or bi-directional?

2) Examine the genetic composition of the WWR population over time. The following questions were addressed: (a) if Africanization involved hybridization, have European mitochondrial and nuclear genes persisted over time? (b) is the contribution of mitochondrial and nuclear genes symmetrical?

This dissertation is divided into six Chapters. Chapter I introduces the goal and objectives of the dissertation. Chapter II provides background information that pertains to: (1) Old World subspecies and respective evolutionary lineages; (2) United States honey bee colonization events; (3) views of the Africanization process; and (4) molecular markers that have been used to document Africanization. In Chapter III, validation of a rapid polymerase chain reaction (PCR)-based assay (cytochrome *b*/*Bgl*II), using a large collection of Old World honey bee subspecies and pre-Africanized southern United States feral honey bees, is reported. Chapter IV examines the temporal changes in frequencies of mtDNA haplotypes (mitotypes) in the Welder Wildlife Refuge population and utilizes the cytochrome *b*/*Bgl*II PCR-based assay to discriminate non-*A. m. scutellata* from *A. m. scutellata* maternal lineages. Chapter V examines the temporal changes at the nuclear level (microsatellite loci) in the Welder Wildlife Refuge population. In this Chapter mitochondrial and nuclear DNA data are combined and the mechanism of Africanization is discussed. Estimates of nuclear admixture proportions are also presented in this Chapter. The objectives of the dissertation are addressed in Chapter IV and Chapter V. Chapter VI provides an overall summary of the conclusions from each Chapter.

CHAPTER II

BACKGROUND

Old World Honey Bees: Evolutionary Lineages

Honey bees are naturally distributed throughout Africa, Europe, and the Middle East. In this broad range they have evolved into 24 subspecies, which have been grouped into three (Ruttner et al. 1978) or four (Ruttner 1988) distinct evolutionary branches. Based upon morphology, Ruttner et al. (1978) proposed the following three branches: A, which included subspecies from Africa (A. *m. lamarckii*, A. m. *yemenitica*, *A. m. litorea*, *A. m. scutellata*, *A. m. adansonii*, *A. m. monticola*, *A. m. capensis*, and *A. m. unicolor*); M, which included subspecies from northern Europe (*A. m. mellifera*), Portugal and Spain (*A. m. iberiensis*), and northern Africa (*A. m. intermissa,* and *A. m. saharensis*); and C, which included subspecies from eastern Europe, the northern Mediterranean, and the Middle East. Later on, Ruttner (1988) divided Branch C into two groups: the subspecies *A. m. sicula*, *A. m. carnica*, *A. m. ligustica*, *A. m. cecropia*, and *A. m. macedonica* were maintained in Branch C while other subspecies (*A. m. anatolica*, *A. m. adami*, *A. m. cypria*, *A. m. syriaca*, *A. m. meda*, *A. m. caucasia*, and *A. m. armeniaca*) were placed into a new branch named O. Additional global support of the Ruttner et al. (1978) classification was provided by data from microsatellites (Estoup et al. 1995; Franck et al.1998) and from mtDNA (Smith et al. 1991; Garnery et al. 1992, 1993, 1995; Franck et al.1998). Discrepancies, revealed by the mtDNA data, pertained to inclusion of *A. m. intermissa, A. m. saharensis* (Garnery et al. 1992), and some

samples of *A. m. iberiensis* in Branch A (Smith et al. 1991), and *A. m. lamarckii* in Branch O (Franck et al. 2000b).

New World Honey Bees

Honey bees were first introduced into the Americas by settlers. In the United States, two honey bee colonization events have been described (Sheppard 1989a,b). The first pertained to introduction of *A. m. mellifera* and probably *A. m. iberiensis* by English and Spanish settlers in the early to mid 1600's. The second event concerned a period of 63 years (1859-1922) when bee breeders actively imported seven more subspecies: *A. m. intermissa*, *A. m. lamarckii, A. m. cypria*, *A. m. syriaca*, *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*. Although the first four of these subspecies were tried in the 1880's and 1890's, they proved to be less popular than the latter three among beekeepers of the time (Sheppard 1989b).

The genetic composition of managed and feral European honey bee populations currently found in the United States largely reflects the aforementioned introductions and the history of beekeeping. Indeed, allozyme and mtDNA analysis revealed a commercial population constituted predominantly of *A. m. ligustica* and *A. m. carnica* (Schiff and Sheppard 1995, 1996). In the feral population, other than the subspecies also found within the managed population, Schiff et al. (1994) reported an unexpected significant contribution (about 35%) of *A. m. mellifera*.

In 1956 an African race, *A. m. scutellata*, was introduced into the Americas. The geneticist Warwick E. Kerr brought 47 queens from the savannah of eastern and

southern South Africa to Brazil to provide a more productive honey bee (Kerr 1967). One year later, 26 queens escaped from the quarantine apiary (Nogueira-Neto 1964; Kerr 1967) and the Africanization process started. After the accidental release, queens from the remaining imported stock were reared and systematically distributed to beekeepers (Spivak et al. 1991). The descendents of those queens have since spread throughout South and Central America at the rate of 300-500 km per year until reaching Mexico (Taylor 1985). The first Africanized swarm in the United States was captured in Hidalgo (Texas), only 34 years after the accidental release in Brazil (Sugden and Williams 1990; Rubink et al. 1996). Presently, populations of Africanized honey bees exist from Argentina to the southwestern United States, where they are found in both managed and feral habitats.

Views of The Africanization Process in the Neotropics

The mechanism by which Africanized honey bees have spread throughout two continents, and their genetic composition are controversial subjects. Genetic and morphometric analyses of Neotropical feral and managed colonies have suggested two different views. One states that hybrid swarms, resulting from paternal gene flow from Africanized drones into European colonies, have expanded throughout the Neotropics, leaving behind a hybrid feral population (Rinderer 1986). Evidence of varying degrees of hybridization has been suggested in independent studies of mtDNA (Rinderer et al. 1991; Sheppard et al. 1991a; Moritz and Meusel 1992; Lobo 1995; Quezada-Euán and Hinsull 1995; Quezada-Euán et al. 1996; Quezada-Euán and Medina 1998; Quezada-

Euán 2000; Clarke et al. 2001; Diniz et al. 2003), microsatellites (Clarke et al. 2002), allozymes (Lobo et al. 1989; Del Lama et al. 1988, 1990; Sheppard et al. 1991a,b; Lobo and Krieger 1992; Lobo 1995; Diniz et al. 2003), and morphometry (Lobo et al. 1989; Rinderer et al. 1991; Lobo 1995; Quezada-Euán and Hinsull 1995; Diniz-Filho and Malaspina 1995, 1996; Diniz-Filho 1996; Rubink et al. 1996; Quezada-Euán and Medina 1998) of feral and/or managed honey bees from Brazil, Uruguay, Costa Rica , Mexico, and South Texas. The term "Africanized bees" reflects this interpretation of the Africanization process.

The other view claims that Africanized honey bees have spread by maternal migration of essentially pure African swarms and that feral populations have retained an African genetic integrity (Taylor 1985; Taylor 1988; Hall and Muralidharan 1989; Smith et al. 1989; Hall 1990; Hall and Smith 1991). Thus, these authors suggest that these bees should be called "African" rather than "Africanized". Results obtained from mtDNA (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Sheppard et al. 1991b), nuclear DNA (Hall 1990; McMichael and Hall 1996; Suazo et al. 1998), and allozymes (Spivak et al. 1988; Smith et al. 1989) of bees from Brazil, Venezuela, Costa Rica, and Mexico support this view.

Molecular Markers Used to Document Africanization

Several molecular markers have been used to document genetic interaction between European and Africanized honey bees. Allozymes (alone or in conjunction with morphometrics) and mtDNA analyses have been the most widely used. Nuclear

restriction fragment-length polymorphisms (nRFLP), has probably been the second most applied category of nuclear markers in the study of Africanization (Hall 1990; Hall 1991; McMichael and Hall 1996; Suazo et al. 1998). Sheppard and Smith (2000) summarized the advances, important findings, limitations, advantages and potential use of nRFLP's and other molecular markers (microsatellites, DNA fingerprinting, randomly amplified polymorphic DNA - RAPD, amplified fragment-length polymorphisms - AFLP), in identification and study of population genetics of New World honey bees.

Mitochondrial DNA

In animals, including insects, mtDNA is typically maternally inherited and does not recombine during sexual reproduction. As a result, for honey bees, mtDNA is transmitted intact from queen to offspring, so that one honey bee represents the entire colony. The maternal inheritance and relatively rapid evolution of this marker have led to its widespread use in studies of matrilineal gene flow and the dynamics of hybrid zones (Moritz et al. 1987). Thus, mtDNA markers have proven to be particularly useful in the study of Africanization process in the New World (Hall and Muralidharan 1989; Smith et al. 1989; Rinderer et al. 1991; Sheppard et al. 1991a,b, 1999; Lobo 1995, Quezada-Euán and Hinsull 1995; Quezada-Euán 2000; Clarke et al. 2001; Diniz et al. 2003).

Restriction fragment analysis of the entire mtDNA molecule has been performed in feral and managed honey bees collected in Africanized areas of South and Central America (Smith and Brown 1988; Hall and Muralidharan 1989; Smith et al. 1989;

Rinderer et al. 1991; Sheppard et al. 1991a, b, 1999) and in non-Africanized areas of the southern United States (Schiff et al. 1994; Schiff and Sheppard 1993, 1995, 1996). Because this analysis is not suited for rapid testing of large numbers of samples (Hall and Smith 1991; Garnery et al. 1993), PCR-based tests have been developed. They consist of amplification of sections of the large ribosomal subunit gene (ls rRNA), cytochrome oxidase I gene (COI) (Hall and Smith 1991; Nielsen et al. 2000), cytochrome *b* gene (Crozier et al. 1991), or COI-COII intergenic space (Hall and Smith 1991; Garnery et al. 1993) followed by digestion with restriction enzymes (see Chapter III and IV). These PCR-based assays have been used in mtDNA analysis of feral and managed bees collected in the United States, Central and South America (Hall and Smith 1991; Lobo 1995; Quezada-Euán and Hinsul 1995; Nielsen et al. 1999, 2000; Clarke et al. 2001; Diniz et al. 2003). These amplified regions do not contain known polymorphisms that discriminate *A. m. scutellata* from *A. m. intermissa* and from *A. m. iberiensis* with intermissa-like mtDNA (Hall and Smith 1991). Early RFLP analysis of the Africanized honey bee mtDNA may also have failed in making such distinctions. This may have resulted in an overestimation of *A. m. scutellata* mitochondria-type in some Africanized populations of countries where honey bees from the Iberian peninsula were presumably brought into the New World by Spanish and Portuguese settlers. Sheppard et al. (1999) after re-analysis of Argentinean honey bees, previously classified as originating from introduced *A. m. scutellata*, found that over 25% of African mtDNA was derived from non-*A. m. scutellata* sources. Using the same sample collected in Yucatan, three years after the arrival of Africanized honey bees, Clarke et al. (2001)

reported only 5.3% of mitotypes originating in Africa or western Europe, whereas Rinderer et al. (1991) had attributed *A. m. scutellata* ancestry to 30% of the colonies.

Another PCR-based test, which consists of amplification of COI-COII intergenic region, was developed by Hall and Smith (1991) and Garnery et al. (1993). This intergenic region contains an important length and *Dra* I restriction site polymorphisms (Garnery et al. 1993). Using the "*Dra* I test" Garnery et al. (1993) analyzed 302 colonies, from Africa and Europe, belonging to 12 subspecies. The authors reported 21 distinct haplotypes: 10 exhibited by branches A and M, and 1 by Branch C. In spite of the amount of variability (in branches A and M) provided by this simple test, it may not also distinguish *A. m. scutellata* from *A. m. intermissa* or from *A. m. iberiensis* because they share some haplotypes. This test was recently used in a temporal survey of mitotypes of honey bees from Yucatan (Clarke et al. 2001). In a sample collected before the arrival of Africanized honey bees, the authors found a small frequency of a haplotype (A1) shared by bees from South Africa and the Iberian peninsula. The presence of the A1 haplotype was attributed to honey bees from Spain presumably brought by Spanish settlers. Thus, it is conceivable that a small proportion of the 61% of African mitotypes reported 12 years after Africanization are of Iberian origin.

Since there are no PCR-based diagnostic tests available, to be certain that a particular African mitotype descended from *A. m. scutellata* rather than from *A. m. iberiensis* or *A. m. intermissa*, one can survey local honey bees for mtDNA types before the arrival of Africanized honey bees (Sheppard and Smith 2000). Therefore, with the purpose of establishing a genetic baseline for future studies of Africanization process,

Schiff et al. (1994) conducted a mtDNA survey on feral colonies across non-Africanized areas of the southern United States. Out of the 692 colonies scored, 12 exhibited an African haplotype. Further analysis of these 12 colonies revealed a typical *A. m. lamarckii* haplotype. A subset of the 692 colonies were re-examined in Chapter III using the cytochrome *b*/*Bgl*II PCR-based assay.

Microsatellites

Microsatellites are a class of DNA markers that consist of tandem repeats of very short nucleotide motifs (1-6 bp long) that are densely distributed throughout eukaryotic genomes. Due to their high mutation rate, generally attributed to polymerase slippage at DNA replication (Levinson and Gutman 1987), these markers often have a large number of alleles (Estoup et al. 1995). Microsatellites have proved particularly useful for insect population genetic studies, as they are numerous, highly polymorphic, codominant, provide high quality data, and are easy to use (Queller et al. 1993).

As in other animals, microsatellites are abundant in honey bees. In early studies, a set of 52 $(CT)_n$ and 23 $(GT)_n$ microsatellites were reported by Estoup et al. (1993) in honey bees. In the *Apis* species, microsatellites typically have been used to determine levels of polyandry and intra-colony relatedness (Estoup et al. 1994; Oldroyd et al. 1995b, 1996, 1997, 1998; Rinderer et al. 1998; Haberl and Tautz 1999). At the population level, microsatellites have mostly been used in phylogenetic studies of honey bees from Old World (Estoup et al. 1995; Franck et al. 1998, 2000b; 2001). These studies have reported no fixed alleles for any population, many shared alleles among the

three branches, and allele frequency variation among populations within a branch. Based on this information, and anticipating the potential application of microsatellites to the New World honey bees, Sheppard and Smith (2000) stated that Estoup's et al. (1994, 1995) microsatellites may not be suitable for assessing the ancestry of individual colonies. However, since some loci (for example A43, A88, A28; Estoup et al. 1995) exhibit important allele frequency differences between branches, they are suitable in tracking temporal changes that would occur as a result of Africanization (Estoup et al. 1995; Sheppard and Smith 2000), as showed by a temporal study of Africanization in the Yucatan peninsula of Mexico (Clarke et al. 2002).

CHAPTER III

VALIDATION OF A PCR-BASED ASSAY FOR MTDNA IDENTIFICATION*

Introduction

The history of honey bee colonization of the United States can be divided into the period prior to and the period following Africanization. Historical records indicate that during the pre-Africanization period several races were introduced from their native range in Europe, north Africa, and Middle East into the United States. In the early to mid 1600's, *A. m. mellifera* L. and probably *A. m. iberiensis* were brought by English and Spanish settlers (Sheppard 1989a, b). Between 1859 and 1922, beekeepers imported seven more subspecies: *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica* (Europe), *A. m. intermissa*, *A. m. lamarckii* (north Africa), *A. m. cypria*, and *A. m. syriaca* (Middle East) (Sheppard 1989a, b). The latter four subspecies were only tried briefly whereas *A. m. carnica, A. m. caucasia* and *A. m. ligustica* have remained available as commercial strains from the United States queen producers (Sheppard 1989b). The genetic composition of pre-Africanized managed and feral United States honey bee populations largely reflects the aforementioned introductions and historical preferences of beekeepers. Indeed, mitochondrial DNA (mtDNA) analyses of the United States commercial queen breeding population have detected a high frequency of the mitotype

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common to *A. m. ligustica, A. m. carnica, A. m. caucasia* (96 %), while only 4 % of the colonies exhibited the mitotype common to *A. m. mellifera* (Schiff and Sheppard 1995, 1996). In feral populations, the frequency of the *A. m. mellifera* mitotype was much higher (37 %) and a low frequency of maternal descendents of the Egyptian bee *A. m. lamarckii* was also found (Schiff and Sheppard 1993; Schiff et al. 1994; Nielsen et al. 2000). Nonetheless, because the pre-Africanized gene pool of the United States population has been derived predominantly from European subspecies, these bees have been traditionally referred to as being of European origin.

In 1990, with the arrival of Africanized bees in Texas (Sugden and Williams 1990), a new period of honey bee colonization began. Africanized honey bees are descendants of a sub-Saharan African subspecies, *A. m. scutellata*, introduced into Brazil from South Africa in 1956 (Kerr 1967). Since their accidental release in 1957 (Nogueira-Neto 1964; Kerr 1967), they established a self-sustaining feral population that expanded throughout the neotropics with differential levels of genetic introgression from European genes (Hall and Muralidharan 1989; Lobo et al. 1989; Smith et al. 1989; Hall 1990; Hall and Smith 1991; Rinderer et al. 1991; Sheppard et al. 1991a, b; Quezada-Euán et al. 1996; Clarke et al. 2001, 2002). In the ensuing 46 years, Africanized bees colonized a broad area encompassing most of South America, Central America, Mexico and the southwestern states of the United States (Texas, New Mexico, Arizona, Nevada, and California).

Prior to the arrival of Africanized bees in the United States, various molecular and non-molecular methods were developed for the detection and identification of

Africanized bees and used either for population genetic studies of New World bee populations or for regulatory purposes (reviewed by Sheppard and Smith 2000). Among the molecular markers, the non-recombining and maternally inherited mtDNA has been one of the most widely used markers. Insights into the mechanisms of expansion and maternal genetic composition of the Africanized population were provided by restriction fragment analysis of the entire mtDNA molecule of honey bees from South and Central America (Smith and Brown 1988; Hall and Muralidharan 1989; Smith et al. 1989; Rinderer et al. 1991; Sheppard et al. 1991a, b, 1999). A similar molecular approach was used in mtDNA surveys of feral and commercial colonies from non-Africanized areas of the southern United States (Schiff and Sheppard 1993, 1995, 1996; Schiff et al. 1994). Although restriction analysis of the entire mitochondria proved to be very informative, widespread use of the procedure in screening programs is unlikely due to the expense and time requirements. The anticipated necessity of screening large number of colonies during the post-Africanization period, either for research or regulatory purposes, led to the development of rapid polymerase chain reaction (PCR)-amplified mtDNA assays (Crozier et al. 1991; Hall and Smith 1991; Garnery et al. 1993; Nielsen et al. 2000). These methods, summarized in Table 1, consist of PCR-amplification of a given fragment followed by restriction enzyme digestion. PCR-amplified mtDNA assays have been used in studies of the Africanization process in neotropical feral and managed populations (Hall and Smith 1991; Lobo 1995; Quezada-Euán and Hinsull 1995; Clarke et al. 2001), and have been adopted, in conjunction with morphometric analysis, to

identify Africanized bees for regulatory purposes in California (Nielsen et al. 1999, 2000).

The PCR-based assays that have been used for maternal identification of bees in California, originally developed by Hall and Smith (1991) and improved by Nielsen et al. (2000), require PCR-amplification of two genes (Ls rRNA and COI) followed by three restriction enzyme (*Eco*RI, *Hinc*II, and *Hinf*I*)* digestions. Crozier et al. (1991) designed primers, within the cytochrome *b* gene, flanking a seemingly diagnostic *Bgl*II restriction site for Africanized bees (Table 1). If this assay were shown to be highly discriminatory, then the advantage of the technique is that a single enzyme digestion of a PCR-amplified segment would be sufficient for maternal identification of Africanized bees. Considering the small sample size (15 Africanized and 10 non-Africanized) and the origin (New World, country not stated) of the colonies used by Crozier et al. (1991) as a source of baseline data, further studies are needed to ascertain the robustness of the cytochrome *b*/*Bgl*II assay so it could be used for Africanized honey bee identification. In this study, we report the results of a survey for *Bgl*II variation in the cytochrome *b* gene (Crozier et al. 1991) within 211 Old World colonies, representing all races known to have been introduced to the New World, and 451 colonies from non-Africanized areas of the southern United States. The survey allowed us to address whether cytochrome *b*/*Bgl*II is a reliable test for discriminating maternal descendents of *A. m. scutellata* from bees derived from non-*A. m. scutellata* ancestors in the United States.

Table 1. PCR-based assays that have been used to identify honey bee matrilineal origins.

PCR-generated DNA fragment	Restriction enzyme	Mitotype cleaved fragment	Mitotype uncleaved fragment	Authors
Cytochrome *b*	*Bgl*II	Non-Africanized	Africanized	Crozier et al. (1991)
Ls rRNA	*Eco*RI	Eastern European[a] (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*)	Western European (*A. m. mellifera* and *A. m. iberiensis* with *A. m. mellifera*-like mtDNA)	Hall and Smith (1991)
COI	*HincII*	Western European (*A. m. mellifera* and *A. m. iberiensis* with *A. m. mellifera*-like mtDNA)	Eastern European[a] (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*)	Hall and Smith (1991)
COI	*Xba*I	Eastern European[a] (*A. m. ligustica*, *A. m. carnica*, *A. m. caucasia*)	Western European (*A. m. mellifera* and *A. m. iberiensis* with *A. m. mellifera*-like mtDNA)	Hall and Smith (1991)
COI	*Hinf*I	*A. m. lamarckii*	Non-*A. m. lamarckii*	Nielsen et al. (2000)
COI-COII intergenic region[b]	*Dra*I			Garnery et al. (1993)

[a] Also named eastern Mediterranean.
[b] This mtDNA region contains length and *Dra*I restriction site polymorphisms. More than 60 distinct mitotypes have been reported for honey bee populations (Garnery et al. 1993, 1995; Franck et al. 1998, 2000a, b, 2001). Therefore, a simple allocation in two mitotype categories, as given in Table 1, is not possible.

Material and Methods

Sample Collection

A collection of 662 Old World and United States colonies was screened for *Bgl*II variation in the amplified cytochrome *b* gene region (Crozier et al. 1991). The Old World collection comprises 211 colonies from the 10 subspecies known to have been

introduced into the United States. These colonies include representatives from various mitochondrial lineages (Garnery et al. 1992, 1995; Arias and Sheppard 1996; Smith et al. 1997; Franck et al. 2000b, 2001), as shown in Table 2. With the exception of *A. m. caucasia*, all subspecies were morphometrically identified using standard discriminant analyses procedures (Ruttner, 1988). The United States collection (n = 451) was obtained from feral colonies (as defined by Schiff and Sheppard 1993) throughout non-Africanized areas in the southern states (Table 2).

Analysis of Mitochondrial DNA

DNA extraction

DNA of 54 colonies from southern Texas was extracted from the thorax of a single adult worker per colony using a QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. DNA samples of *A. m. caucasia* were provided by D.R. Smith, University of Kansas. All other Old and New World DNA samples were from single bee extractions made with a phenol-chloroform method (Sheppard and McPheron 1991) in the laboratory of W.S.S.

Restriction site analysis

A 485-bp section of the cytochrome *b* gene was amplified using the primers 5'-TAT GTA CTA CCA TGA GGA CAA ATA TC-3' and 5'-ATT ACA CCT CCT AAT TTA TTA GGA AT-3' developed by Crozier et al. (1991). PCR amplifications were performed in 5 µl total volume containing 0.5 X *Taq* DNA polymerase buffer

Table 2. Description of the Old World and United States sample collection. Location, sample size, number of mitotypes, and mitochondrial lineages of each subspecies are indicated.

Origin	Subspecies (mitochondrial lineage)	Number of colonies	Number of mitotypes	
			Two-band	One-band
Old World				
France	*A. m. mellifera* (western European[a])	18	17	1
Italy	*A. m. ligustica* (eastern Mediterranean[a])	22	22	
Portugal, Spain	*A. m. iberiensis* (western European and African[a,b])	28	10	18
Syria	*A. m. syriaca* (Middle Eastern[c])	20	20	
Cyprus	*A. m. cypria*[d]	20	20	
Egypt	*A. m. lamarckii* (Middle Eastern[e])	24	24	
Morocco	*A. m. intermissa* (African[a])	16		16
Kenya	*A. m. scutellata* (African[a])	33		33
Turkey	*A. m. caucasia* (eastern Mediterranean [a, f])	11	11	
Germany, Slovenia, Austria	*A. m. carnica* (eastern Mediterranean[a])	19	19	
United States				
Alabama[g]		7	7	
Arizona[g]		146	146	
Georgia[g]		31	30	1
Louisiana[g]		51	51	
Mississippi[g]		3	3	
New Mexico[g]		58	57	1
North Carolina[g]		6	6	
Oklahoma[g]		3	3	
South Carolina[g]		15	15	
Texas[g, h]		131	130	1

a. Garnery et al. 1992, Arias and Sheppard 1996, Franck et al. 2001.
b. Data on mtDNA revealed the coexistence in the Iberian peninsula of mitotypes belonging to the mitochondrial African and western European lineages (Smith et al. 1991; Garnery et al. 1992, 1993, 1995; Franck et al. 1998; Clarke et al. 2001).
c. Franck et al. 2000b.
d. Mitochondrial lineage is not reported in the literature. Based on morphometric data this subspecies belongs to the Middle Eastern lineage (Ruttner 1988).
e. Arias and Sheppard 1996, Franck et al. 2000b, 2001.
f. Smith et al. 1997.
g. Feral colonies tested previously by restriction analysis of the entire mitochondria (Schiff and Sheppard 1993; Schiff et al. 1994).
h. 54 colonies, collected in southern Texas prior to Africanization, are newly investigated.

(Promega), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 pM of each primer, 0.5 µl of template DNA, and 0.25 U of *Taq* DNA polymerase (Promega). The PCR temperature profile was 94º C for 3 min followed by 30 cycles of 94º C for 15 s, 50º C for 15 s, and 68º C for 5 s. After the final cycle, an additional 10-min at 72º C was performed. Following DNA amplification, samples were digested with *Bgl*II, (Promega) restriction enzyme using the temperature and buffer conditions recommended by the supplier. The total digestion volume was then electrophoresed on a 2 % agarose/TBE gel, stained with ethidium bromide, and visualized under UV light. The restriction site was scored as present (PCR product cut) or absent (PCR product not cut), based on the visualization of a two-band pattern or one-band pattern on the gel, respectively (Fig. 1).

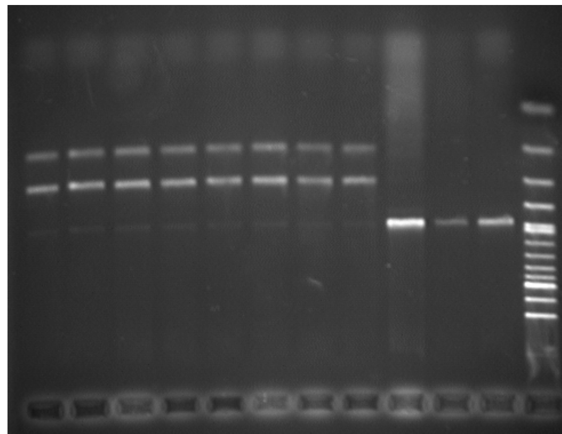

Fig. 1. Samples of mtDNA from Old World honey bee subspecies PCR-amplified, using cytochrome *b* primers developed by Crozier et al. (1991), and digested with restriction enzyme *Bgl*II. Lane 1-8: 291-bp and 194-bp fragments (PCR product cut) obtained for *A. m. mellifera*, *A. m. ligustica*, *A. m. syriaca*, *A. m. cypria*, *A. m. lamarckii*, *A. m. caucasia*, *A. m. carnica*, *and A. m. iberiensis*. Lane 9-11: 485-bp fragment (PCR product not cut) obtained for *A. m. iberiensis*, *A. m. intermissa*, *and A. m. scutellata*. Lane 12: size standard 100-bp ladder (New England Biolabs).

Results

Two mitotypes (Fig. 1), as described by Crozier et al. (1991), were observed among the 211 Old World and the 451 United States colonies screened for *Bgl*II variation in the amplified cytochrome *b* gene region (Table 2). All 52 colonies sampled from the eastern Mediterranean mitochondrial lineage (*A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*), and all 64 from Middle Eastern lineage (*A. m. lamarckii, A. m. cypria*, *A. m. syriaca*) exhibited a two-band mitotype. All 33 *A. m. scutellata* and 16 *A. m. intermissa* colonies examined from the African mitochondrial lineage shared the one-band mitotype. Colonies of morphologically identified *A. m. mellifera* and *A. m. iberiensis* displayed both mitotypes. Of the 18 sampled colonies of *A. m. mellifera* the two-band mitotype was observed in 17 and one-band mitotype in one colony from Ille-sur-Tet (southern France, close to the border with Spain). Finally, among *A. m. iberiensis* colonies, both mitotypes were relatively common, with 10 two-band and 18 one-band patterns observed. Sequence data from the cytochrome *b* gene (M.A.P., unpublished data) of the *A. m. mellifera* colony from Ille-sur-Tet, revealed the typical "African" mitochondrial DNA type common in *A. m. iberiensis*, suggesting that the colony likely represents introgression between both western European subspecies (Smith et al. 1991).

The restriction analysis of the colonies from the southern tier of the United States revealed a highly asymmetric frequency of the two mitotypes. The non-African (two-band) mitotype was present in a frequency higher than 99% (448 of the 451 colonies examined) whereas only three colonies (one from New Mexico, one from Texas, and one from Georgia) exhibited the African (one-band) mitotype. Because A. *m. intermissa* and

probably *A. m. iberiensis* were introduced in the United States, the presence of African mitotypes prior to the arrival of Africanized bees was not unexpected.

Discussion

The baseline information developed here from the Old World colonies indicates that overall, the cytochrome *b*/*Bgl*II assay discriminated the mitochondrial African lineage (one-band mitotype) from the mitochondrial western European, eastern Mediterranean, and Middle Eastern lineages (two-band mitotype). Either one-band or two-band mitotypes were detected for all geographical regions but southern Europe. In the Iberian peninsula we detected the coexistence of both African and non-African mitotypes. This result is concordant with previous studies conducted in Spain and Portugal, which have reported a mixture of western European and African mitotypes (Smith et al. 1991; Garnery et al. 1992, 1993, 1995; Franck et al. 1998; Clarke et al. 2001).

The amplified cytochrome *b* region does not contain *Bgl*II polymorphisms that discriminate *A. m. scutellata* from *A. m. intermissa* and from some *A. m. iberiensis*. The same findings have been reported for Old and New World colony screening using other rapid PCR-based assays as Ls rRNA/*Eco*RI, COI/ *Hinc*II, COI/*Xba*I (Hall and Smith 1991), and COI/*Hinf*I (Nielsen et al. 2000). The COI-COII/*Dra*I test, which has been widely used in Old World (Garnery et al. 1992, 1993, 1995; Moritz et al. 1994; Franck et al. 1998, 2000a, b, 2001) and New World (Clarke et al. 2001) population genetic and phylogenetic studies, has produced more than 60 distinct mitotypes in bees sampled

from all mitochondrial lineages. Many mitotypes are subspecies-specific but some are shared, occurring at different frequencies in different subspecies. This is the case of the A1, A2, and A4 mitotypes present in *A. m. scutellata*, *A. m. intermissa* and *A. m. iberiensis* populations (Garnery et al. 1993; Clarke et al. 2001). Therefore the COI-COII/*Dra*I assay is not *A. m. scutellata*-diagnostic either. RFLP analysis of Africanized honey bees using the entire mtDNA and 6-base recognizing enzymes also present limitations in making subspecies distinctions. For example, the procedure was found to overestimate the proportion of *A. m. scutellata* by approximately 25% in bees from Argentina (Sheppard et al. 1999) and 5.3 % in bees from the Yucatan peninsula of Mexico (Clarke et al. 2001). These non-*A. m. scutellata* African mitotype frequencies are much higher than that found for the United States (< 1%) in this study.

The restriction data shown in this paper reports, for the first time, the presence of relictual maternal descendents of probable *A. m. intermissa* and/or *A. m. iberiensis* origin in the United States. The strength of this association assumes that no other African mitotypes were introduced prior to the arrival of Africanized bees in 1990 and that all the variation for *Bgl*II in non-African bees was sampled. This assertion can be further confirmed by sequencing the three United States colonies carrying the "African" restriction pattern. However, given that the frequency of African non-*A. m. scutellata* mtDNA mitotypes in the southern United States is very low (< 1%), the probability of misidentifying the matrilineal origin of African colonies is consequently quite low. Therefore, cytochrome *b*/*Bgl*II can be used to identify maternally Africanized bees with a high degree of reliability and very low estimated error rate.

Previous mtDNA surveys on feral colonies reported *A. m. lamarckii* as the only African type mitochondria present in the United States before Africanization (Schiff and Sheppard 1993; Schiff et al. 1994; Nielsen et al. 1999, 2000). Our baseline data indicate that cytochrome *b*/*Bgl*II variation can discriminate *A. m. lamarckii*, and the other two Middle Eastern subspecies (*A. m. syriaca* and *A. m. cypria*), from African *A. m. scutellata*, *A. m. intermissa* and some *A. m. iberiensis*, but not from the eastern Mediterranean *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*, and western European A. *m. mellifera and* some *A. m. iberiensis*. Therefore, if the purpose of honey bee screening is merely identification of maternal descendants of *A. m. scutellata* then the cytochrome *b*/*Bgl*II assay is as accurate as the two genes (ls rRNA and COI)/three enzyme digestion (*Eco*RI, *Hinc*I, and *Hinf*I) assay used by the California Department of Food and Agriculture (Nielsen et al. 1999, 2000).

The cytochrome *b*/*Bgl*II assay, developed by Crozier et al. (1991) and further validated here, is a simple, rapid, and relatively inexpensive test suited for screening large number of samples. Indeed this assay, consisting of low volume (5 μl) PCR reactions performed in less than an hour and digested in one hour, produces very distinctive and easily identifiable mitotypes (Fig. 1). Because mtDNA provides information about maternal ancestry, for proper identification this marker should be used in conjunction with either morphometrics, as in California (Nielsen et al. 1999), or nuclear markers. With the anticipated sequencing of the complete honey bee genome there will be opportunities for further refinement of identification methods to include nuclear markers.

CHAPTER IV

AFRICANIZATION INFERRED FROM MTDNA

Introduction

What happens when previously allopatric and genetically divergent populations are brought together by secondary contact? The outcome is an experiment in evolution. Are the gene pools combined to produce a simple mixture of the different constituent populations, or is one gene pool replaced by the other? Additionally what happens when parasites are introduced along with the populations? Are these parasites major determinants of the eventual outcome? The western honey bee (*Apis mellifera* L.) is a good system in which to address these evolutionary questions. Prior to human-assisted global dissemination, honey bees were confined to Africa, Europe, and the Middle East where they have differentiated into four evolutionary lineages (Ruttner 1988). The introduction of genetically divergent honey bee populations to the New World has provided opportunities to study artificial contact zones in new environments (Rinderer et al. 1991; Sheppard et al. 1991a; Oldroyd et al. 1995a; Clarke et al. 2001, 2002).

The United States honey bee populations descended from several subspecies introduced from Europe, North Africa, and the Middle East by settlers, in the 17th century, and by beekeepers, between 1859 and 1922 (Sheppard 1989a, 1989b). Despite the varied geographical origins of introduced subspecies, mitochondrial DNA (mtDNA) and allozyme surveys of commercial and feral populations, conducted prior to the arrival of Africanized honey bees (also known as neotropical African honey bees), revealed an

admixed gene pool predominantly derived from eastern (*A. m. ligustica*, *A. m. carnica*, and *A. m. caucasia*) and western (*A. m. mellifera*, and *A. m. iberiensis*) European subspecies (Schiff et al. 1994; Schiff and Sheppard 1995, 1996). A negligible maternal contribution of the Egyptian subspecies *A. m. lamarckii* was also found in the feral population (Schiff and Sheppard 1993; Schiff et al. 1994). The pre-Africanized United States (and South and Central America) honey bees have been referred to as European because feral and managed populations were predominantly derived from stocks imported from Europe.

Africanized honey bees were first recorded in the United States (Hidalgo, Texas) in 1990 (Sudgen and Williams 1990; Rubink et al. 1996). These bees derive from a founder population of a sub-Saharan African subspecies, *A. m. scutellata*, introduced into Brazil from South Africa in 1956 (Kerr 1967). Since their accidental release in 1957 (Nogueira-Neto 1964; Kerr 1967), the descendents of *A. m. scutellata* have dispersed in the neotropics at an astonishing rate of 300-500 km per year (Taylor 1985) and have established large feral populations in regions where European honey bees did not thrive (Michener 1975). By 2003 populations of Africanized honey bees were distributed from Argentina to the southwestern United States (Texas, New Mexico, Arizona, Nevada, and California).

MtDNA markers, alone or in conjunction with bi-parentally inherited markers, have been used to document the mechanisms of this remarkable dispersion and the genetic composition of Africanized feral populations. Surveys of mitochondrial haplotypes (mitotypes) of feral honey bee populations from tropical South and Central

America revealed a predominantly *A. m. scutellata* origin (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Sheppard et al. 1991b). This evidence suggested that in the neotropics honey bees expanded and colonized new territories by migrating swarms that retained an African maternal genetic integrity (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Hall 1992). Various hypotheses were evoked to explain the paucity of European mtDNA markers in the neotropical Africanized population. The most favored ones were (1) natural selection against feral European matrilines due to reproductive advantages and high viability of Africanized honey bees (Michener 1975; Rinderer 1988; Taylor 1988), and (2) the small size of feral European population in the neotropics (Taylor 1985, 1988; Page 1989; Rinderer et al. 1991). If these hypotheses were correct then the contribution of European matrilines in the Africanized gene pool would increase as Africanized honey bees moved into regions with a more temperate climate and/or with a large European population (Taylor 1985, 1988). The hypotheses were tested in more temperate regions of Argentina (Sheppard et al. 1991a), and in the tropical Yucatan peninsula of Mexico (Rinderer et al. 1991; Quezada-Euán and Hinsull 1995; Clarke et al. 2001), which supported one of the highest densities of managed European colonies in the World prior to Africanization (Quezada-Euán and Hinsull 1995). As predicted, a greater frequency of European mitotypes was observed in feral populations from those regions (Sheppard et al. 1991a; Quezada-Euán and Hinsull 1995; Clarke et al. 2001).

In the United States, where both conditions are met, it has been assumed that greater gene flow from the resident feral European to the immigrant Africanized

population would occur (Taylor 1985, 1988). However, the nearly coincidental arrival in the United States in 1987 (De Jong 1990) of one of the most serious pests of honey bees worldwide, the parasitic mite *Varroa destructor*, might have compromised this assumption. Although more than 10 years have passed since the arrival of both organisms in the United States, there have been no studies addressing this issue. Herein we report the results of a temporal mtDNA survey of a feral honey bee population from southern Texas. The study spans a continuous period of 11 years, encompassing pre-, during, and post-Africanized honey bee and the parasitic *Varroa* mite invasions.

Material and Methods

Study Site and Sample Collection

This study was conducted from 1991 to 2001 on the Welder Wildlife Refuge (28° N latitude), located in San Patricio County, approximately 60 km north of Corpus Christi, Texas. The Welder Wildlife Refuge lies in a transitional zone between the South Texas Plains and the Gulf Prairies and Marshes ecoregions (Drawe et al. 1978). Chaparral brushland, scattered mesquite, open grassland, and live oak mottes comprise 80 % of the vegetational cover found in the Welder Wildlife Refuge (Blankenship 2000). The climate is humid, subtropical with hot summers and cool winters (Blankenship 2000).

The Welder Wildlife Refuge harbors a large population of feral honey bees with most (85 %) colonies occurring in natural cavities of live oak trees (Baum 2003). Cavities occupied by honey bee colonies (active cavities) were located through searches

of a 6.25 km^2 study area during periods of high honey bee activity. A limited level of surveys was done initially. As time progressed more exhaustive searches were conducted and greater numbers of active cavities were discovered. Once discovered a cavity would be examined in all subsequent surveys. A total of 112 tree cavities were included in the study area (Table 3).

Table 3. Number of cavities surveyed, new and established colonies from cavities, and colonies from swarm traps.

Year	Cavities surveyed[a]	Established colonies from cavities	New colonies from cavities	Colonies from Swarm traps
1991	10		10	
1992	15	9	4	15
1993	34	8	22	
1994	70	19	44	24
1995	89	39	40	16
1996	89	19	13	6
1997	89	12	22	13
1998	89	23	16	1
1999	96	30	31	
2000	111	51	27	5
2001	112	46	15	11

[a.] Some cavities were occupied by more than one colony within a year and some were inactive for variable periods of time. Monitoring of five cavities was discontinued in 1993. Two cavities were in man-made structures (discovered in 1991) and three were in tree cavities (discovered in 1993) located outside of the study area.

Adult honey bee workers (partially sterile females) were sampled yearly from cavities in early (February-May) and late (July-December) seasons, except in 1996 and 1998 when only late season sampling was made. In most years, honey bee workers were

also collected from colonies caught in swarm traps (Schmidt and Thoenes 1987) (Table 3). Once sampled, swarm trap colonies were sacrificed. Honey bees from sampled cavities and swarm traps were cryogenically preserved on dry ice and transported back to the laboratory where they were stored at -80° C until DNA extraction.

Based on presence/absence of colonies, cavities were scored as active or inactive multiple times per year as follows: two in 1991, two in 1992, three in 1993, six in 1994, six in 1995, two in 1996, three in 1997, one in 1998, three in 1999, two in 2000, and two in 2001. These scores were used to separate colonies into two categories: new and established. The new category included colonies from newly discovered cavities and from recolonization of inactive cavities. The established category included colonies that have survived at least one winter after the cavity was discovered. The 11-year collection comprises 500 honey bees from colonies in cavities and 91 from swarm traps (Table 3). Colonies from swarm traps were included in the new category.

Mitochondrial DNA Analysis

Mitochondrial DNA, which is shared by all individuals in a colony, was extracted from the thorax of a single worker per colony per sampling season using a QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA). After extraction, template DNA was stored at –20° C until analyzed.

One to three regions of the mitochondrial genome were analyzed through polymerase chain reaction (PCR) in a stepwise fashion (Fig. 2). We used primers that amplify a 485-bp section of the cytochrome *b* gene (Crozier et al. 1991), a 738-bp

section of the large ribosomal subunit (ls rRNA) gene (Hall and Smith 1991), and a 1028-bp section of the cytochrome oxidase I (COI) gene (Nielsen et al. 2000). Single PCR amplifications were performed in 5 μl total volume containing 0.5X *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 pM of each primer, 1 μl of template DNA, and 0.25 U of *Taq* DNA polymerase (Promega, Madison, WI). The PCR temperature profile was 94º C for 3 min followed by 30 cycles of 94º C for 15 s, 50º C for 15 s, and 68º C for 5 s for all three pairs of primers. After the final cycle, an

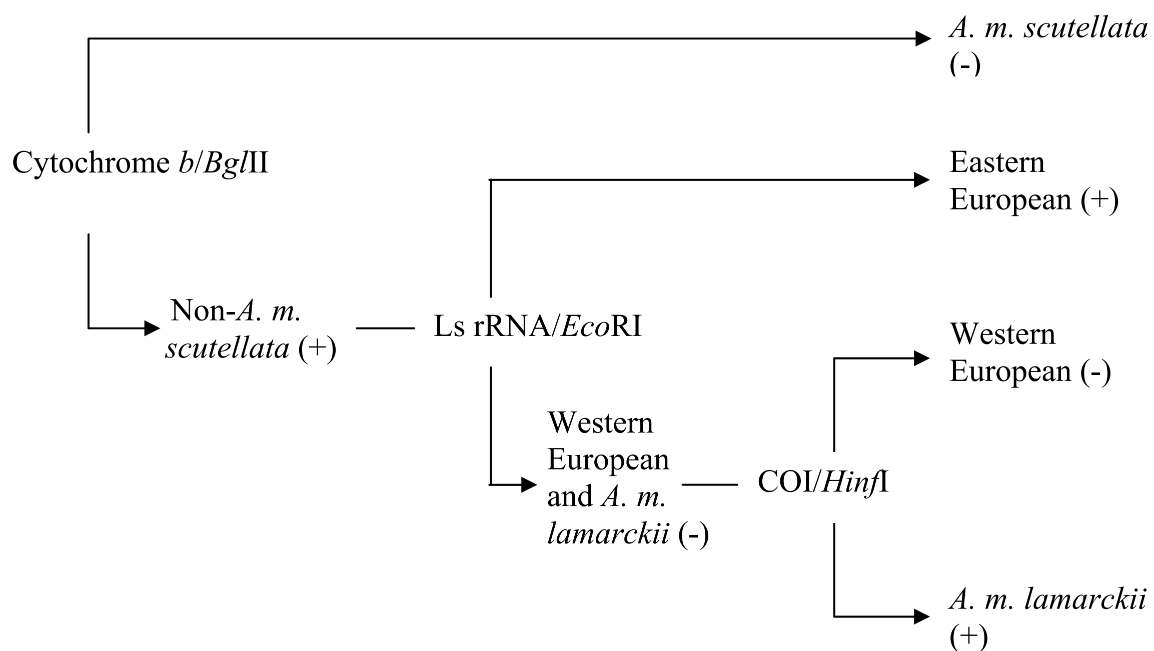

Fig. 2. Flow chart of mitotype determination. The (+) sign indicates presence of a restriction site, (-) indicates its absence.

additional 10-min at 72º C was performed. Following PCR, samples amplified with cytochrome *b*, ls rRNA, and COI primers were digested with the restriction enzymes *Bgl*II, *Eco*RI, and *Hinf*I (Promega, Madison, WI) respectively, using the temperature and buffer conditions recommended by the supplier. The total digestion volume was then electrophoresed on a 2 % agarose/TBE gel, stained with ethidium bromide, and visualized under UV light.

In animals, mtDNA is typically maternally inherited and does not recombine during sexual reproduction. Therefore, Africanized honey bee colonies maternally descending from *A. m. scutellata* carry *A. m. scutellata*-type mitochondria. The same circumstance applies to eastern European, western European, and *A. m. lamarckii* (non-*A. m. scutellata*) sources. Because this study focuses on the maternal component of the Africanization process, colonies will be referred to as *A. m. scutellata*, eastern European, western European, and *A. m. lamarckii* throughout this article. Colony mitotypes were scored as shown in Figure 2. Colonies determined to carry *A. m. scutellata* mitochondria following *Bgl*II digestion of the cytochrome *b* PCR-amplified fragment (Crozier et al. 1991; Pinto et al. 2003) were not analyzed further. The Egyptian A. *m. lamarckii* is not discriminated from eastern and western European honey bees using the cytochrome *b*/*Bgl*II assay (Pinto et al. 2003). Colonies that exhibited a non-*A. m. scutellata* mitotype were then PCR-amplified for ls rRNA and digested with *Eco*RI. This assay discriminates colonies of eastern European maternal ancestry from colonies of western European maternal ancestry (Hall and Smith 1991). Baseline data developed from Old World colonies revealed that *Eco*RI polymorphism does not discriminate western

European subspecies from *A. m. lamarckii* (M. A. Pinto, unpublished data). Thus, colonies exhibiting the lack of an *Eco*RI restriction site were further PCR-amplified for COI and digested with *Hinf*I (Nielsen et al. 2000). *A. m. lamarckii* was identified as having a unique *Hinf*I restriction site within COI (Nielsen et al. 2000).

Statistical Analysis

The homogeneity of mitotype distributions was tested for all pairwise comparisons between years by Fisher's exact test using GENEPOP 3.3 (Raymond and Rousset 1995). Multiple comparisons were corrected for type I error by sequential Bonferroni correction using a global significance level (α) of 0.05 (Rice 1989). The monotonic association between year and mitotype frequency was tested using Spearman's rank order correlation coefficient (r_s) computed using SPSS version 11.0 (Norušis 1993).

Results

Colonies from Cavities

The mtDNA analysis of the 11-year honey bee collection from cavities is shown in Figure 3. Maternal descendents of eastern European, western European, *A. m. lamarckii*, and *A. m. scutellata*, subspecies were each represented in the Welder Wildlife Refuge feral population. The first colony carrying *A. m. scutellata* mitochondria was detected in 1993. Since then the frequency of *A. m. scutellata* mitotype increased significantly over time (r_s = -0.98, P = 0.000). Between 1991 and 1993 there were no

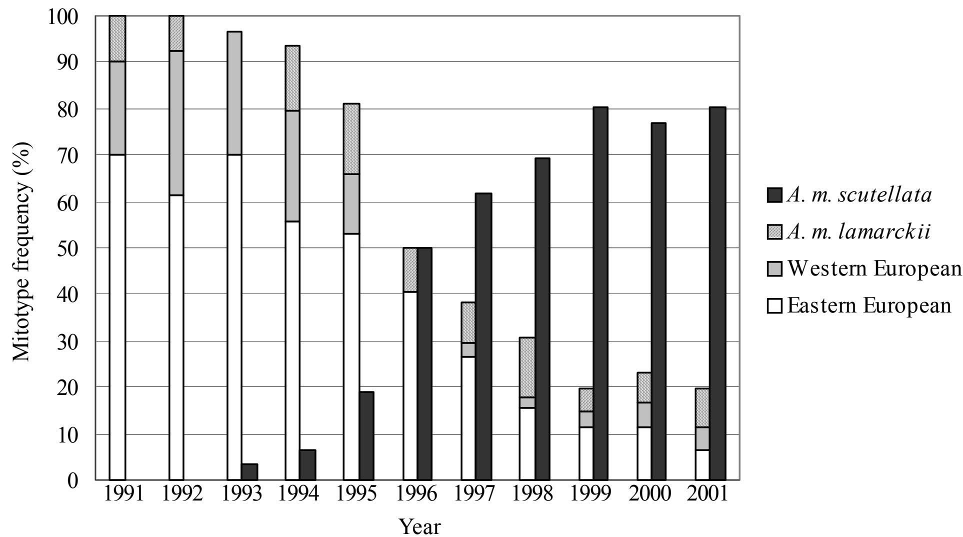

Fig. 3. Temporal mitotype frequency distributions of colonies (new and established) from cavities on the Welder Wildlife Refuge.

significant differences in mitotype distributions of the Welder Wildlife Refuge population (Fisher's exact test, $P \geq 0.129$). During this period the maternal composition of the Welder Wildlife Refuge population was 68.0 % ± 5.7 (mean ± SD for 1991-1993) eastern European, 26.1 % ± 5.5 western European, and 5.9 % ± 5.2 *A. m. lamarckii*. However, between 1994 and 1996 the mitotype composition of the population changed radically and the predominantly eastern European population became predominantly *A. m. scutellata* in maternal origin (Fig. 3). Over the 11-year study, the greatest frequency change occurred between 1995 and 1996 (Fisher's exact test, $P = 0.000$).

As the Africanization process progressed the mitotype proportions also changed among non-*A. m. scutellata* colonies. There was a marginal decrease ($r_s = -0.69$, $P = 0.018$ is non-significant following sequential Bonferroni correction; $\alpha = 0.05$ and $k = 3$)

in mitotype frequency of eastern European colonies and a corresponding increase (r_s = 0.92, P = 0.000) in the mitotype frequency of *A. m. lamarckii* colonies.

The Welder Wildlife Refuge population appeared to have reached stability in maternal composition between 1998 and 2001, because there were no significant changes in mitotype frequency distributions (Fisher's exact test; significant differences at $P < 0.003$ following Bonferroni correction, $\alpha = 0.05$ and k = 55). During this period, 76.7 % ± 5.2 (mean ± SD) of the colonies from cavities exhibited the *A. m. scutellata* mitotype. Among the 23.3% non-*A. m. scutellata*, the mitotype distribution was 47.9 % ± 10.5 eastern European, 18.1 % ± 7.3 western European, and 34.0 % ± 8.9 *A. m. lamarckii*.

The temporal mitotype distribution of colonies from cavities is shown in Figure 4. The largest number of cavities occupied by colonies of non-*A. m. scutellata* maternal descent was observed in 1995. Between 1995 and 1996, most (53.2 %) cavities became inactive (Fig. 5). During this period, the number of colonies carrying non-*A. m. scutellata* mitochondria dramatically decreased from 64 to 16. In contrast, the number of colonies of *A. m. scutellata* origin increased from 15 to 16 (Fig. 4). This abrupt decrease in the frequency of active cavities observed between 1995 and 1997 (Fig. 4) coincided with the arrival of the parasitic *Varroa* mite into the Welder Wildlife Refuge early in 1995 (Rubink et al. 1995). Following Welder Wildlife Refuge population

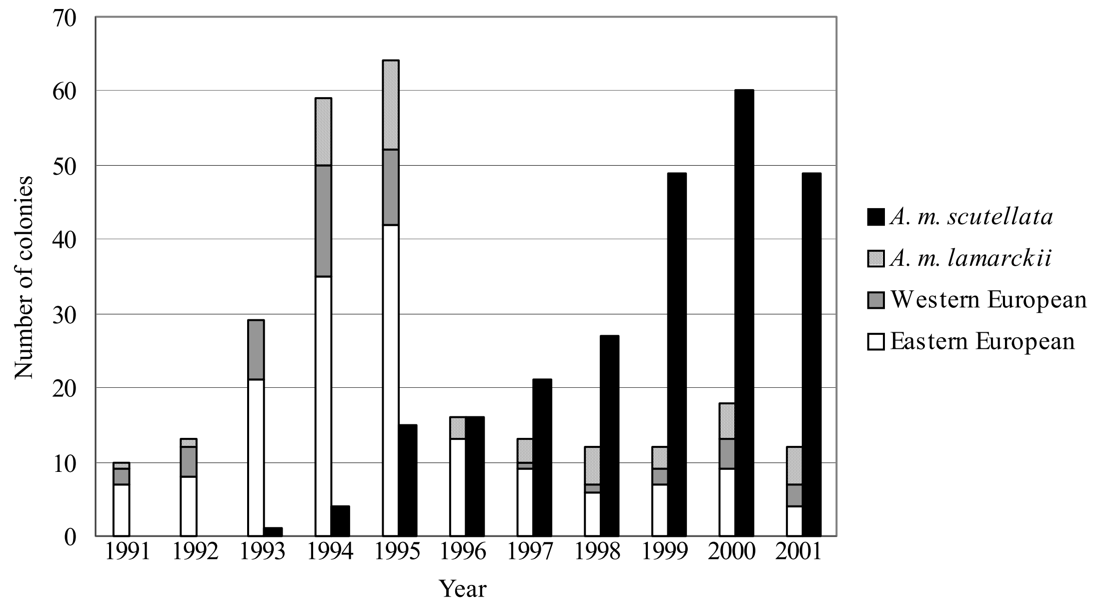

Fig. 4. Temporal mitotype distributions of colonies (new and established) from cavities on the Welder Wildlife Refuge.

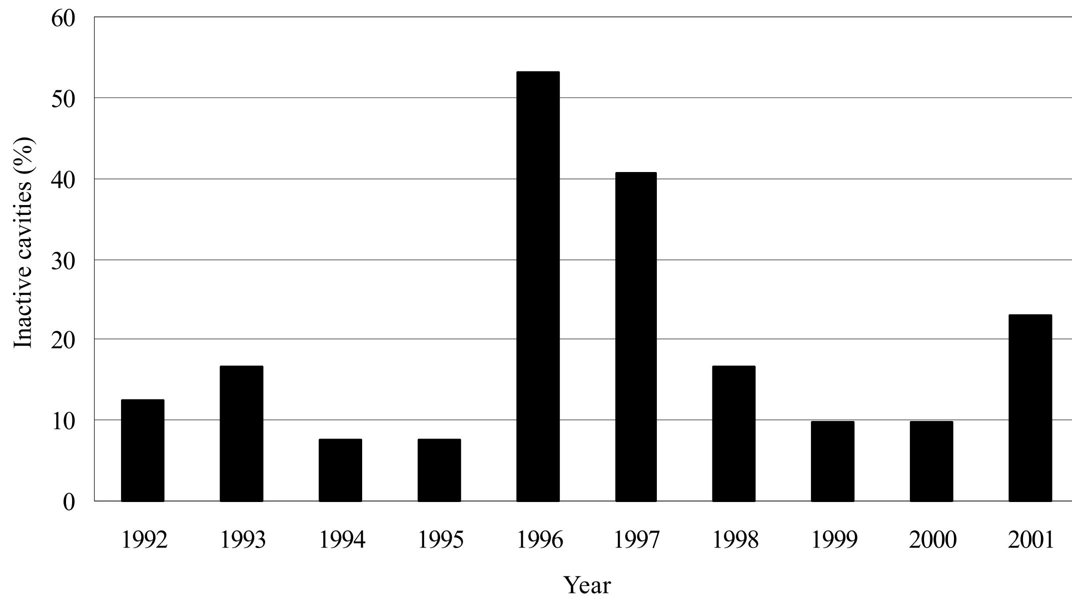

Fig. 5. Temporal frequency of inactive cavities on the Welder Wildlife Refuge. Calculated by comparing the number of active cavities in a given year with that of the respective cavities the following year. A cavity was considered active in a given year if it was occupied at least in one of the sampling periods. To insure consistency across years, we only compared cavity score recorded during both sampling seasons, except in 1996 when only a single sampling was made. Since in this year only the late season sampling was carried out, cavity score recorded during early season was taken into account.

collapse, there was a surplus of vacant cavities that were gradually reoccupied (Fig. 6) predominantly by colonies of *A. m. scutellata* maternal descent (Fig. 4).

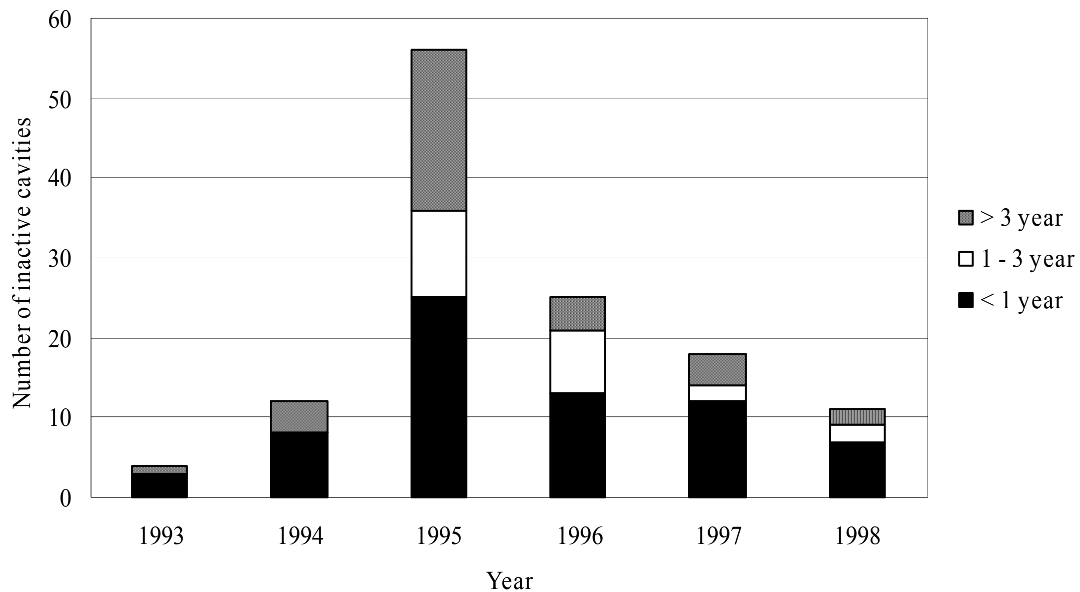

Fig. 6. Recolonization rates of inactive cavities on the Welder Wildlife Refuge. Twelve cavities in 1995, three in 1996, four in 1997, and two in 1998 have never been reoccupied (they were included in the > 3 years class).

New Colonies from Cavities and Swarm Traps

The mitotype composition of the Welder Wildlife Refuge shown in Figure 3 was generated from colonies representing overlapping generations, which acts to delay expression of yearly-realized gene flow within a population undergoing a profound evolutionary change. The results for new colonies represent the best estimate of yearly gene flow.

Given the surveillance schedule, the number of new colonies from cavities (Table 3) may be underestimated. A colony could die or abscond and have its cavity reoccupied between surveys. Such colonies would be classified as established instead of new. Between 1993 and 2000, 79 out of 148 (53.4 %) inactive cavities were reoccupied in less than a year. Data obtained for 1993-1998 period suggests a slow reoccupation rate of the remaining vacant cavities (Fig. 6). Seeley (1978) found a lower reoccupation rate in a 3-year study of a feral honey bee population of New York State. The relatively rapid rate of cavity reoccupation observed in Welder Wildlife Refuge suggests that recolonizations between surveys could have occurred. An additional source of error could result from colony usurpation. However, a low rate (5 - 9 %) of colony usurpation of European colonies by Africanized swarms has been reported for managed colonies (Vergara et al. 1989; Danka et al. 1992). If a new colony carried a mitotype different from a replaced colony, then an undetected reoccupation or usurpation would be later identified through mtDNA analysis. Therefore, this error of colony misclassification could be lowered through colony mitotype screening. MtDNA analysis revealed 17 undetected recolonizations and/or colony usurpations out of 244 (7 %) new colonies over the 11-year study, suggesting a low rate of colony misclassification. However, as the frequency of *A. m. scutellata* mitotype increased the power of identifying colony turnover by mtDNA decreased due to an increase in the probability of *A. m. scutellata* by *A. m. scutellata* replacements. In this case the frequency of *A. m . scutellata* would be underestimated and the mitotype frequency distribution would be biased. If this were true then the frequency of colonies maternally derived from *A. m. scutellata* that were

captured in swarm traps would be consistently higher than in cavities. However, comparisons of mitotype frequencies between swarm traps and cavities showed no significant differences (Table 4), suggesting that colony misclassification was likely negligible and mitotype frequencies were not biased. Because mitotype frequency distributions did not differ between new colonies from swarm traps and cavities, we combined both sub-samples and estimated mitotype frequencies over time (Fig. 7).
A highly contrasting pattern in mitotype composition is exhibited by the new colonies prior to and after 1996 (Fig. 7). The pre-1996 period is characterized by a temporal heterogeneous mitotype composition with *A. m. scutellata* mitotype rapidly increasing in frequency since detection of first colony in 1993. During this period, swarms were produced predominantly by colonies of eastern European maternal ancestry, except in 1992 when most (13 of 23; 58 %) new colonies exhibited the western European mitotype. This result was unexpected because (1) for the years before1996 and most years (all except 1998) after 1996 period eastern European was the most frequent mitotype among non-*A. m. scutellata* colonies and (2) a predominantly eastern European ancestry was found in a large scale mtDNA survey of feral populations from the southern United States (Schiff and Sheppard 1993; Schiff et al. 1994).

Stability appeared to be reached during the post-1996 period. Between 1997 and 2001 mitotype frequency distributions were homogeneous (Fisher's exact test;

Table 4. Mitotype frequencies of new colonies from cavities and swarm traps for different years. *P*-values (Fisher's exact test) are also included.

Mitotype	1994		1995		1996		1997		2000		2001	
	Cavity	Swarm trap	Cavity	Swarm trap	Cavity	Swarm trap	Cavity	Swarm trap	Cavity	Swarm trap	Cavity	Swarm trap
Eastern European	47.7	41.4	42.5	37.5	30.8	33.3	27.2	15.4	18.5	20.0	6.7	18.2
Western European	25.0	10.3	15.0	18.8	0	0	4.6	15.4	11.1	0.0	6.7	9.1
A. m. lamarckii	20.4	31.0	12.5	18.8	7.7	0	9.1	0	7.4	20.0	13.3	9.1
A. m . scutellata	6.8	17.2	30.0	25.0	61.5	66.7	59.1	69.2	63.0	60.0	73.3	63.6
P-value	0.06		0.74		1.00		0.13		0.44		0.69	

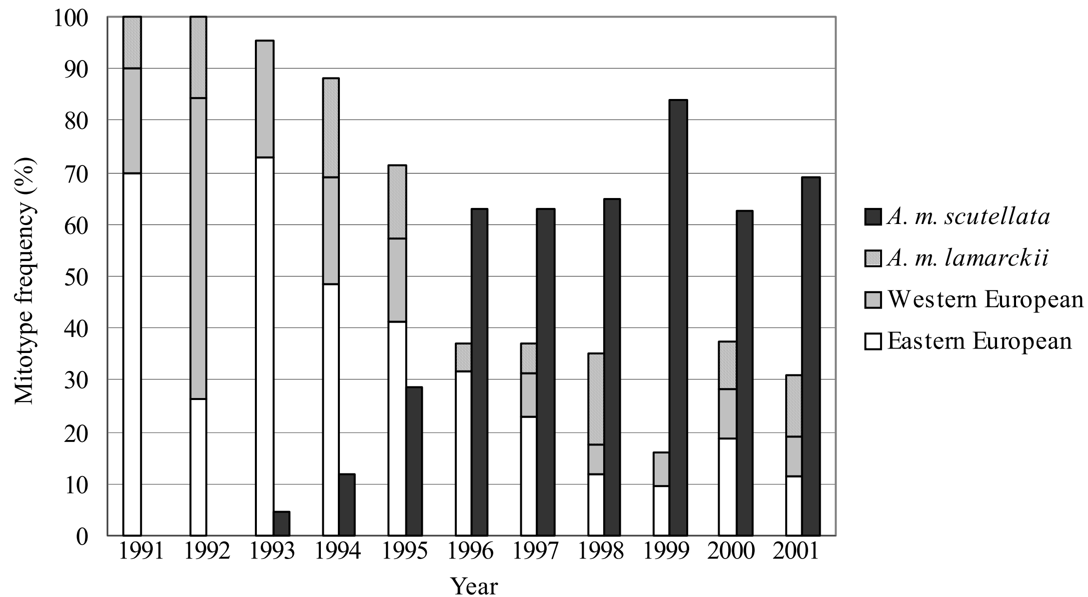

Fig. 7. Temporal mitotype frequency distributions of new colonies (from cavities and swarm traps) on the Welder Wildlife Refuge.

significant differences at $P < 0.003$ following Bonferroni correction, $\alpha = 0.05$ and k = 55), with most swarms (68.6 % ± 8.9 SD) being produced by colonies maternally derived from *A. m. scutellata*. Among the 31.4 % of colonies of non-*A. m. scutellata* maternal ancestry eastern European mitotype was the most frequent (48.5 % ± 12.8) whereas both western European (26.0 % ± 8.6) and *A. m. lamarckii* (26.0 % ± 19.3) mitotypes exhibited similar frequencies.

Discussion

The 11-year continuous sequence of mtDNA frequencies indicated a dramatic evolutionary change in the Welder Wildlife Refuge feral honey bee population over a

very short period of time. Introduction of *A. m. scutellata* mitotypes started at least in 1993. Since then, an increase in the frequency of *A. m. scutellata* mitotypes occurred as more Africanized queens immigrated and/or the recently arrived residents reproduced. The most impressive change on the mitotype composition occurred between 1995 and 1996. In this period, there was a matriline turnover from a predominantly eastern European to a predominantly *A. m. scutellata* population. This evolutionary event was coincidental with *Varroa* mite arrival into the Welder Wildlife Refuge. Although *Varroa* is considered one of the most serious pests of honey bee colonies worldwide, it has been shown that losses are less severe in Africanized than in European colonies (Moretto et al. 1991; 1993; Message and Gonçalves 1995; Guzman-Novoa et al. 1996, 1999). Therefore, the finding that between 1995 and 1996 the number of colonies carrying non-*A. m. scutellata* mitochondria dramatically decreased whereas *A. m. scutellata* ones slightly increased, suggests that *Varroa* mite may have played a role in the change of the mitotype composition of the Welder Wildlife Refuge population.

In addition to colony usurpation and gene flow into the resident European population, Africanized honey bees may expand by winning in the competition for food resources and nest sites (Michener 1975). In the tropics of South and Central America feral European colonies were scarce (Taylor 1988; Roubik 1989; Roubik and Boreham 1990). In contrast, the sub-tropical United States has supported a large well-adapted European feral population (Taylor 1988; Rubink et al. 1990). This situation was the case of the Welder Wildlife Refuge where a density of 11.7 colonies per km^2 was found for 1995 (Baum 2003), and where only 19 % of the colonies were maternal descendents of

A. m. scutellata. In comparing the Welder Wildlife Refuge results with those from other feral honey bee studies, Baum (2003) suggested that this is the highest estimated colony density for feral honey bees ever reported for a contiguous study area including both suitable and unsuitable habitat. It is thus conceivable that the collapse suffered by the non-*A. m. scutellata* colonies facilitated invasion of Africanized honey bees into the Welder Wildlife Refuge by reducing competition pressure for environmental resources. As non-*A. m. scutellata* colonies would succumb, cavities and food resources would be available to be utilized by immigrating and recently arrived resident *A. m. scutellata* derived colonies. This likely resulted in accelerated and increased rates of Africanization in the Welder Wildlife Refuge feral population.

MtDNA surveys of honey bees collected from Brazil to Mexico have revealed low levels of European mitotypes (between 0 and 4 %) in feral neotropical populations (Hall and Muralidharan 1989; Smith *et al.* 1989; Hall and Smith 1991; Sheppard et al. 1991b; Clarke et al. 2001). A greater frequency of colonies of European maternal ancestry was found in a feral Africanized population living in the tropical but densely honey bee populated Yucatan. In that region, European mitotype frequencies of 20 % (Quezada-Euán and Hinsull 1995) and 13 % (Clarke et al. 2001) were reported 5 and 12 years after the arrival of Africanized honey bees, respectively. In the Welder Wildlife Refuge, the proportion of non-*A. m. scutellata* mitotypes appears to have stabilized at about 31 %, similar to estimates (30 %) reported for feral populations from transitional temperate-subtropical regions of Argentina (Sheppard et al. 1991a) and for managed (39 %) populations from tropical Yucatan (Clarke et al. 2001). The moderate frequency of

non-*A. m. scutellata* mitotypes, persisting eight years after detection of first Africanized honey bees in the Welder Wildlife Refuge, is an unusual condition given that (1) the Welder Wildlife Refuge lies in the Africanized saturation zone proposed by Taylor and Spivak (1984) and (2) there was a coincidental severe loss suffered by non-*A. m. scutellata* colonies with expansion of the Africanization front into the Welder Wildlife Refuge. Whether or not the frequency of non-*A. m. scutellata* matrilines in the post-Africanization period would be greater had the Welder Wildlife Refuge feral population not collapsed is an unanswerable question. Nonetheless, as predicted for the United States, the observed frequency of non-*A. m. scutellata* mitotypes in the Welder Wildlife Refuge population was greater than that reported for feral neotropical populations. Only future surveys will tell whether the Africanization process is complete. However, the apparent stability reached in the last years of the study suggests that non-*A. m. scutellata* mitotypes are unlikely to be driven to extinction.

The frequency of eastern European, western European, and *A. m. lamarckii* mitotypes found in the pre-Africanization Welder Wildlife Refuge population, was similar to previous estimates from large-scale surveys of the southern United States feral population (Schiff et al. 1994). In both studies, colonies of eastern European matrilineal ancestry were the most common and colonies of *A. m. lamarckii* matrilineal ancestry were present in a low frequency. Interestingly, as Africanization progressed in the Welder Wildlife Refuge, a decrease in the relative mitotype frequency of eastern European and an increase in A. *m. lamarckii* were observed. During the post-Africanization period, colonies carrying *A. m. lamarckii* mitochondria became as

abundant as those of western European. Further investigation is needed to ascertain whether the observations represent a local effect.

The results shown herein indicate that the non-*A. m. scutellata* mitochondrial genomes have not been replaced by that of *A. m. scutellata*. Instead, a mixture of mitotypes was present in the Welder Wildlife Refuge, eight years after initial secondary contact between immigrant and resident populations. We recognized that evidence of mtDNA addresses only the maternal component of the Africanization process. A more complete understanding into the nature of the genetic interaction between the two populations requires further investigation at the nuclear DNA level.

CHAPTER V

AFRICANIZATION INFERRED FROM MTDNA AND NUCLEAR DNA

Introduction

The evolutionary significance of natural hybridization has been debated for decades. At one extreme, it has been argued that natural hybridization is an evolutionary dead end due to formation of inviable and/or infertile hybrids. At the other extreme, it has been suggested that natural hybridization may lead to new evolutionary lineages due to formation of relatively fit hybrids that expand into novel habitats. A third potential evolutionary outcome (and one likely associated with an invading species) is expansion of an intermixed form within the native progenitor's habitat, in which case the degree of mixing between hybridizing forms may range from formation of a hybrid swarm to genetic assimilation of one form by the other (reviewed and extensively referenced in Barton and Hewitt 1989; Harrison 1993; Arnold 1992, 1997; Arnold and Hodges 1995; Rhymer and Simberloff 1996). Our study of hybridization builds on one of the most publicized and feared examples of a biological invasion in the Americas, that of the Africanized honey bees. The study spans a continuous period of 11 years, encompassing pre-, during, and post-secondary contact between migrant Africanized and resident European honey bees. To our knowledge this is the only study reporting genetic interaction between migrant and resident populations at the exact moment of secondary contact.

The western honey bee, *Apis mellifera* L., is naturally distributed throughout Africa, Europe, and the Middle East, where it has evolved into 24 distinct subspecies (Ruttner 1988). Over the past 400 years, a subset of these subspecies has been introduced into the New World, first by settlers and later by beekeepers (Morse et al. 1973; Sheppard 1989a,b). Most of the introductions were from Europe. In the United States, until early 1990's, managed and feral honey bees were typically derived from eastern (*A. m. ligustica*, *A. m. carnica*, and *A. m. caucasia*) and western (*A. m. mellifera*, and *A. m. iberiensis*) European subspecies (Schiff et al. 1994; Schiff and Sheppard 1995, 1996; McMichael and Hall 1996), reflecting early introductions and the history of beekeeping. Because of their predominant origin in Europe, the United States honey bees are referred to as European. In 1990, honey bees descending from a sub-Saharan African subspecies, *A. m. scutellata*, arrived in the United States (Hidalgo, South Texas; Sudgen and Williams 1990; Rubink et al. 1996). The migrants have since spread through Texas, New Mexico, Arizona, Nevada, and California, contributing new genes to the southwestern United States honey bee gene pool. The descendants of *A. m. scutellata* have been referred to as either "neotropical African" or "Africanized", depending on the underlying perspective of the Africanization process. Because the latter term has been the most used and known, herein we will adopt it to refer to the descendants of *A. m. scutellata*, regardless of the level of introgressed European alleles.

In tropical regions of Brazil, the temperate-evolved European subspecies were poor honey producers (Kerr 1967; Gonçalves et al. 1991). Thus, in an attempt to create a tropical-adapted and more productive honey bee, queens of *A. m. scutellata* were

brought from South Africa into Brazil in 1956, to be crossed with European honey bees (Kerr 1967). In 1957, 26 queens escaped from the quarantine apiary, giving rise to the Africanization process (Nogueira-Neto 1964; Kerr 1967). After the accidental release, queens from the remaining imported stock were reared and systematically distributed to beekeepers (Spivak et al. 1991). In less than 35 years, their descendents expanded through South and Central America and established large feral populations in regions where feral European honey bees were scarce (Michener 1975). During their range expansion they have been displacing resident European honey bees (Michener 1975, Taylor 1985, 1988). Biological, behavioral, and demographic factors have contributed to the rapid spread and notorious establishment of strong feral Africanized honey bee populations in the American tropics (reviewed and extensively referenced in Michener 1975; Winston et al. 1983; Winston 1992). Africanized honey bee workers have a shorter development period, shorter life span, and start foraging at younger ages than European honey bees. Requirements of cavities for nesting are less stringent in Africanized honey bees. In fact, in contrast with European honey bees, Africanized honey bees frequently construct exposed nests, which are smaller and store a lower amount of honey. Differences in reproductive biology are also striking with swarming rates and extent of drone production considerably larger in Africanized than in European honey bees. In addition to swarming, Africanized honey bees have spread in the neotropics due to absconding. Africanized colonies may promptly abandon their nest when disturbed or when food resources are scarce. Absconding swarms may travel as far as 160 km or more. Finally, in what is their best known and publicized characteristic,

Africanized honey bees tend to exhibit a higher level of defensive behavior than do European honey bees. When disturbed they tend to respond faster and with a larger number of stinging bees than is typical of European bees. However, these are not the only factors driving displacement of European honey bees. Genetic mechanisms involving hybridization have also been implicated (Rinderer et al. 1991; Sheppard et al. 1991a,b; Lobo et al. 1989; Clarke et al. 2002).

Mechanisms underlying Africanization, and the genetic composition of the honey bee population left behind the expanding front have been well documented (Hall and Muralidharan 1989; Lobo et al. 1989; Smith et al. 1989; Hall 1990; Rinderer et al. 1991; Sheppard 1991a,b; Diniz et al. 2003) but not fully understood in the American tropics. Whether Africanization has occurred through replacement of the resident European honey bees by *A. m. scutellata*, with little or no introgression of European genes (Taylor 1985; Hall and Muralidharan 1989; Smith et al. 1989; Hall 1990), or through extensive hybridization (Rinderer 1986; Rinderer et al. 1991; Sheppard et al. 1991a) has been intensively debated. Most surveys of mtDNA of feral honey bees in tropical regions of South and Central America have revealed a fixed or nearly-fixed African-type mitochondria (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Sheppard et al. 1991b; Diniz et al. 2003). However, surveys of allozymes, RFLP and RAPD markers showed either significant (Lobo et al. 1989; Sheppard et al. 1991b; Lobo and Krieger 1992; Diniz et al. 2003) or limited introgression predominantly from western European subspecies (Smith et al. 1989; Hall 1990; Muralidharan and Hall 1990; McMichael and Hall 1996; Suazo et al. 1998; Hall and McMichael 2001).

Suggested hypotheses to explain the apparent asymmetric introgression of nuclear and cytoplasmatic markers into the gene pool of neotropical Africanized bees include: 1) low fitness of hybrids due to incompatibility of European mitochondria and African-derived nuclear DNA (Hall and Muralidharan 1989; Harrison and Hall 1993), 2) natural selection against European matrilines due to reproductive advantages and high fitness of Africanized honey bees (Michener 1975; Rinderer 1988; Taylor 1988), and 3) small size of the European population (Taylor 1985, 1988; Page 1989; Rinderer et al. 1991).

Prior to arrival of Africanized honey bees, the Yucatan peninsula (Mexico) sustained one of the largest densities of managed European colonies in the world (Rinderer et al. 1991). Temporal post-Africanization morphometrical, allozymic, and DNA (nuclear and mitochondrial) surveys of the feral and managed Yucatecan honey bees revealed a persisting and greater contribution of European genes in the Africanized population than that reported for other neotropical populations (Rinderer et al. 1991; Quezada-Euán and Hinsull 1995; Quezada-Euán et al. 1996, Quezada-Euán and Medina 1998; Quezada-Euán 2000; Clarke et al. 2001, 2002). The large size of the pre-existing European population was suggested as the most plausible account of the results, implying that the third hypothesis (limited numbers of European honey bee colonies) could partially explain the paucity of European alleles in neotropical Africanized honey bees.

Mirroring the expectation in temperate North America, a considerable contribution of European genes to the Africanized honey bee's gene pool has been

reported in subtropical-temperate boundary regions in South America. In Uruguay and Brazil, north-south clines showing a gradual increase in frequency of allozyme and mtDNA markers typical of European honey bees have been reported (Lobo et al. 1989; Lobo and Krieger 1992; Diniz-Filho and Malaspina 1995; Diniz et al. 2003). In Argentina, a zone of complex hybridization between European and Africanized honey bees was also reported (Sheppard et al. 1991a). In the more temperate central and southern Argentinean regions, honey bee colonies were almost exclusively European; whereas in the more tropical northern regions there was little or no introgression of European genes in the Africanized gene pool. In the transitional zone, however, both European and African mitotypes were associated with a range of morphometric and allozymic phenotypes (Sheppard et al. 1991a). Given these findings, it has been predicted that extensive dilution of *A. m. scutellata* genes would occur as Africanized honey bees expand through the more temperate and densely European-honey bee populated southern United States. However, although more than 10 years have passed since arrival of Africanized honey bees into the United States, the genetic interaction between migrant and resident European honey bees, using concurrently nuclear and mitochondrial DNA markers, has not been documented. In the present study we used mitochondrial DNA (Chapter IV) and nuclear DNA (microsatellites) markers to assess temporal changes in the genetic structure of a feral honey bee population from southern Texas undergoing Africanization. The mechanisms underlying the Africanization process and the genetic composition of the feral population are described.

Materials and Methods

Sample Collection

A total of 335 honey bee workers (partially sterile females) was sampled from feral colonies at the Welder Wildlife Refuge (28° N latitude, San Patricio County, Texas, USA) between 1991 and 2001 (see Chapter IV for details on sampling procedure). The 11-year sample collection comprised honey bees from new colonies obtained from tree cavities (244 samples) and swarm traps (91 samples).

Honey bee workers of two additional populations (one European-derived and one *A. m. scutellata*-derived) were sampled for use as reference populations. The European-derived population (referred to as reference European, Eur., throughout this Chapter) was sampled from 50 feral colonies caught in swarm traps prior to Africanization. The swarm traps were deployed across an east-west 120 km long transect, established in the lower Rio Grande Valley (between the cities of Brownsville and La Joya) of South Texas, USA (Rubink et al. 1996). European ancestry of the sampled honey bees was confirmed by mitochondrial DNA (mtDNA) analysis (Pinto et al. 2003). The *A. m. scutellata*-derived population (referred to as reference Brazilian, Braz., throughout this chapter) was sampled from 43 colonies kept in the apiary of University of São Paulo (Ribeirão Preto, São Paulo, Brazil) in 2002. The sampled colonies, for the most part, originated as feral swarms collected in Luiz Antonio area (approximately 100 km North from the initial release site of *A. m. scutellata* in Rio Claro). The swarms were maintained without subsequent queen management practices, and therefore were considered representative of the local feral population (D. De Jong,

pers. comm. 2002). MtDNA analysis was performed on the Brazilian honey bees; and only the individuals exhibiting the African mitotype (Pinto et al. 2003) were selected for the purpose of this study. We assumed that the sample from South Texas was representative of the pre-Africanized Welder Wildlife resident population. The sample from Brazil was our best representative of the *A. m. scutellata*-derived founder population, and was regarded as the source of the migrants.

The honey bees were either frozen on dry ice (samples from South Texas and Welder Wildlife Refuge) or stored in 95% ethanol (samples from Brazil) and transported back to the laboratory where they were stored at -80° C and 4° C respectively until DNA extraction.

Microsatellite Analysis

Total DNA was extracted from the thorax of a single worker per colony using a QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA) and stored at –20° C until analyzed. A total of 428 honey bee samples (Reference Brazilian = 43; Reference European = 50; Welder Wildlife refuge = 335), each representing a different colony, were scored at 12 microsatellite loci (A14, A7, A88, A107, A113, A35, A28, A79, A43, A8, IM, and ED1; see Estoup et al. 1994, 1995, Oldroyd et al. 1995b, and Rowe et al. 1997 for primer sequences). Single polymerase chain reaction (PCR) amplifications were performed in 8 µl total volume containing 0.5X *Taq* DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 pM of each primer, 1 µl of template DNA, and 0.4 U of *Taq* DNA polymerase (Promega, Madison, WI) for all primer pairs. The

forward primer for each marker was labeled with one of three fluorescent dyes (HEX, 6-FAM, or NED; Applied Biosystems, Foster City, CA). The PCR conditions consisted of an initial 3 min denaturation step at 94º C followed by 30 cycles of 94º C for 15 s, annealing temperature at 55º C (except for A79, 60º C) for 15 s, and 72º C for 5 s, plus a final extension at 72º C for 10-min in a Perkin Elmer 9600 thermal cycler GeneAmp PCR system. The PCR products were loaded on Long Ranger polyacrylamide gels (BioWhittaker Molecular Applications, Rockland, ME) and then run on an ABI Prism 377 automated DNA sequencer using HD Rox 400 (Applied Biosystems) as the internal size standard. The software Genotyper® 2.5 (Applied Biosystems) was used for allele identification and comparison.

Statistical Analysis

Genetic diversity was assessed using unbiased estimates of gene diversity (Nei 1987) and allelic richness (Petit et al. 1998) for each microsatellite locus in each sample. The rarefaction method of Petit et al. (1998) allowed the estimation of allelic richness that would be obtained if all samples were of equal size. Allele frequencies, number of alleles (A), proportion of heterozygotes (H_p), unbiased gene diversity (H_d), and allelic richness (R_s) per locus and population sample were computed using FSTAT version 2.9.3. package (Goudet 2001). In FSTAT, the sample size for allelic richness estimation is fixed as the smallest number of individuals typed for a locus in a sample, which in this study was 10 (Welder Wildlife Refuge population sample of 1991). Differences in average unbiased gene diversity and allelic richness between pairs of samples were

assessed by Wilcoxon's signed rank test (Snedecor and Cochran 1978) using SPSS version 11.0 (Norušis 1993).

Departure from Hardy-Weinberg equilibrium (HWE) was tested for each locus and population sample using the probability test. A global probability value over all loci was obtained for each population sample using Fisher's method (Raymond and Rousset 1995b). Heterozygote excess and deficiency were tested for each locus and across loci for each sample using the score test (U test) according to Rousset and Raymond (1995). Values of F_{IS} were calculated for each locus and sample as in Weir and Cockerham (1984), and tested by permutation using FSTAT. Fisher's exact tests were performed to assess genotypic linkage disequilibrium. The null hypothesis tested was that genotypes at one locus were independent from genotypes at the other locus. The test was performed for all locus pairs within each population sample (Raymond and Rousset 1995b). Population sample pairwise comparisons were performed to test for homogeneity of allelic and genotypic distributions (genic and genotypic differentiation) across loci; the null hypotheses tested were that the allelic and genotypic distributions were identical across populations. An unbiased estimate of the *P*-value of the probability test or Fisher's exact test (Raymond and Rousset 1995a), and the *P*-value of a log-likelihood (G) based exact test (Goudet et al. 1996) were performed to test for genic and genotypic differentiation, respectively. Exact tests for HWE, genotypic linkage disequilibrium, heterozygote deficiency, heterozygote excess, and population differentiation (genic and genotypic) were tested by the Markov chain method (Guo and Thompson 1992) using GENEPOP version 3.4 (Raymond and Rousset 1995b). The

Markov chain parameters were as follows: 10,000 dememorization steps, 1000 batches, and 10,000 iterations per batch.

Reference and Welder Wildlife Refuge population sample pairwise values for F_{ST} were computed according to Weir and Cockerham (1984) using GENEPOP version 3.4 and then departures from 0 were tested by 1000 permutations using GENETIX version 4.04 (Belkhir et al. 2002). Proportion of shared allele distance (D_{PS}, Bowcock et al. 1994) between pairs of population samples were estimated using the program MSAT. These distances were used to build a neighbor-joining tree (Saitou and Nei 1987) using PAUP version 4.0b 10 (Swofford 2002). Where applicable, throughout the analysis, statistical significance levels were adjusted for multiple comparisons using the sequential Bonferroni procedure to correct for type I error (Rice 1989).

The European and Brazilian reference population samples were used as parentals to compute the relative proportions of introgressed nuclear alleles in the Welder Wildlife Refuge population over time using the estimators m_R (Roberts and Hiorns 1965) and m_Y (Bertorelle and Excoffier 1998). The least-squares estimator m_R is based on the comparison of allele frequencies between parental and admixed populations and assumes identity by descent (Roberts and Hiorns 1965). The estimator m_Y is based on a coalescent approach that takes into account molecular information as well as gene frequencies. This estimator, which assumes the single stepwise mutation model, was computed using the squared difference in allele size as molecular distance (Bertorelle and Excoffier 1998). Both estimators were computed using the program ADMIX version 1.0 (Bertorelle and Excoffier 1998). The bootstrap average and bootstrap

standard deviation of the admixture coefficients were estimated over 1000 replications. Differences in m_R and m_Y over time were assessed by a paired t-test using SPSS.

The European and Brazilian reference samples were also used as parentals to simulate 100 sets of F_1, European backcross, and Brazilian backcross population samples of 50 individuals each. The multilocus genotype of each simulated individual was generated by randomly taking one allele from each of the parental populations according to their frequencies using a program written by J. B. Patton (pers. comm. 2003). The simulated F_1 and reciprocal backcrosses were used as additional reference samples to build the above-mentioned neighbor-joining tree and to classify individual honey bees from Welder Wildlife Refuge over time using GENECLASS (Cornuet et al 1999). GENECLASS is a software package that assigns individuals of unknown origin to a set of reference samples using either likelihood (frequency or Bayesian) or distance-based methods. To compare the performance of frequency, Bayesian, Cavalli-Sforza and Edwards (1967) chord distance, and $(\delta\mu)^2$ Goldstein et al. (1995) distance methods, multilocus genotypes of 1000 European, 1000 Brazilian, 1000 F_1, 1000 European backcross, and 1000 Brazilian backcross individuals were randomly generated by crossing European x European, Brazilian x Brazilian, European x Brazilian, F_1 x European, and F_1 x Brazilian respectively. Using one random set (among 100) of reference samples, each simulated individual was assigned and the percentage of misclassification was calculated for each method. The most accurate method was examined over all 100 sets of simulated reference samples and then used to classify individual honey bees from the Welder Wildlife Refuge. Assignment scores for each

Welder Wildlife Refuge honey bee were computed 100 times, using the 100 sets of simulated reference samples. Classification of individual honey bees was based on the predominant assignment given for the 100 replications.

The simulated reference samples represented a subset of a vast array of possible recombinant classes. It is important to emphasize that individuals possessing recombinant genotypes other than F_1 and reciprocal backcrosses would be assigned to one of the five reference populations because the Bayesian method always designates a population of origin.

Results

Genetic Diversity

Allele frequencies, number of alleles, allelic richness, proportion of heterozygotes, and gene diversity are given for each locus and population sample in Table 5. All loci showed high variability with the total number of alleles ranging from 11 (A8) to 38 (A7), the mean number of alleles ranging from 5.8 (Welder Wildlife Refuge 1991) to 15.1 (Welder Wildlife Refuge 1995), the mean allelic richness ranging from 5.833 (Welder Wildlife Refuge 1991) to 9.168 (Welder Wildlife Refuge 2000), the mean proportion of heterozygotes ranging from 0.700 (Welder Wildlife Refuge 1994) to 0.876 (Welder Wildlife Refuge 1999), and finally the mean gene diversity ranging from 0.681 (Welder Wildlife Refuge 1991) to 0.874 (Brazilian), across all population samples.

Genetic diversity was significantly higher in the Brazilian reference sample (R_s = 8.890; H_d = 0.874; Table 5) than in the European (R_s = 5.934; H_d = 0.693; Table 5)

Table 5. Loci, number of sampled chromosomes (n), allele frequencies, number of alleles (A), allelic richness (R_s), proportion of heterozygotes (H_p), and unbiased gene diversity (H_d) per locus and reference (Braz. - Brazilian from São Paulo, Brazil; Eur. - European from South Texas, USA) and Welder Wildlife Refuge (from 1991 to 2001) honey bee population samples.

Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A7	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n = 38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	096	0.140	0	0	0	0	0.007	0.018	0.053	0.100	0.176	0.145	0.156	0.096
	098	0.035	0	0	0	0	0.007	0.018	0.026	0.043	0.029	0.032	0.016	0.019
	099	0.012	0	0	0	0	0	0	0.026	0	0	0.016	0.031	0
	100	0.035	0	0	0	0	0.007	0.018	0.026	0.029	0.059	0.016	0.031	0.058
	101	0.035	0	0	0	0	0	0.009	0.026	0.014	0	0	0	0.038
	102	0	0.010	0	0.105	0.091	0.037	0.018	0.026	0.014	0	0.016	0.016	0.019
	103	0	0	0	0	0	0	0	0	0	0	0.016	0	0
	104	0.163	0	0	0	0	0	0.009	0	0.043	0	0.032	0.047	0.038
	105	0.023	0.150	0.100	0.105	0.182	0.176	0.116	0.184	0.143	0.118	0.097	0.141	0.135
	106	0.116	0	0	0	0	0	0.009	0	0.014	0.029	0.016	0.047	0.058
	107	0.093	0	0	0	0	0.007	0.018	0	0.029	0.029	0.032	0.078	0
	108	0.035	0	0	0	0	0	0	0	0	0	0.016	0	0
	109	0.151	0.360	0.550	0.395	0.364	0.294	0.259	0.132	0.243	0.235	0.274	0.156	0.154
	110	0.128	0	0	0	0	0	0.027	0.105	0.043	0.029	0.065	0.078	0
	112	0.023	0	0	0	0.045	0.044	0.089	0.053	0.057	0.059	0.032	0.062	0.038
	114	0.012	0.140	0	0.053	0.136	0.074	0.071	0.026	0.057	0.088	0.032	0.047	0.038
	116	0	0.100	0.050	0.053	0.045	0.125	0.054	0.026	0.057	0.118	0.081	0.031	0.135
	118	0	0.130	0.100	0.132	0.045	0.103	0.116	0.079	0.057	0	0.016	0.016	0.058
	120	0	0.010	0.050	0	0.023	0.007	0.009	0.026	0	0	0	0.016	0.038
	122	0	0	0	0	0	0.007	0	0	0	0	0	0	0
	124	0	0.010	0	0	0	0	0	0	0	0	0	0	0
	126	0	0	0.050	0.026	0	0.022	0.009	0	0	0	0	0	0

Table 5. Continued.

Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A7	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n = 38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	128	0	0	0	0	0	0.007	0.018	0	0	0	0	0	0
	130	0	0.020	0	0	0	0	0	0	0	0	0	0	0
	132	0	0	0.050	0	0	0	0.018	0	0.014	0	0	0	0
	134	0	0.010	0	0.026	0	0.015	0.036	0.079	0.029	0	0	0	0.019
	136	0	0	0	0	0	0	0	0	0	0	0.016	0	0
	138	0	0	0	0	0	0	0	0	0	0	0	0	0
	140	0	0	0	0	0	0.007	0	0	0	0	0	0	0
	142	0	0.020	0.050	0.053	0.023	0.015	0.018	0.053	0.014	0	0.016	0	0
	150	0	0	0	0	0	0	0.009	0	0	0	0	0	0.019
	152	0	0	0	0	0	0	0	0	0	0	0.016	0	0
	156	0	0	0	0	0	0.007	0	0	0	0	0	0	0
	158	0	0.010	0	0	0.023	0.007	0.018	0	0	0	0	0	0
	164	0	0	0	0	0	0	0	0.026	0	0.029	0.016	0	0.038
	166	0	0	0	0	0	0	0.009	0	0	0	0	0	0
	170	0	0.020	0	0.053	0.023	0.015	0.009	0.026	0	0	0	0.031	0
	172	0	0.010	0	0	0	0.007	0	0	0	0	0	0	0
A		14	14	8	10	11	22	25	18	18	12	21	17	17
R_s		9.218	7.085	8.000	8.082	7.841	8.639	10.613	12.356	10.718	9.510	10.832	10.862	11.479
H_p		0.814	0.860	0.700	0.895	0.773	0.838	0.875	0.789	0.886	0.882	0.774	0.938	0.885
H_d		0.898	0.807	0.700	0.811	0.819	0.852	0.893	0.939	0.902	0.893	0.892	0.918	0.929
Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A88	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	136	0.023	0	0	0	0	0	0.009	0	0	0	0.016	0.031	0.019

Table 5. Continued.

Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A88	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	138	0.081	0.010	0	0	0	0	0.009	0.026	0.043	0.118	0.016	0.047	0.019
	139	0.012	0.120	0.200	0.079	0.136	0.081	0.054	0	0.014	0.059	0.016	0.016	0.058
	140	0.070	0.010	0	0	0	0.007	0.054	0.053	0.086	0.118	0.145	0.141	0.096
	142	0.326	0.320	0.250	0.500	0.500	0.382	0.375	0.237	0.171	0.147	0.210	0.219	0.250
	143	0	0	0	0	0	0	0	0	0	0	0	0.016	0
	144	0.012	0	0	0	0	0	0.009	0	0.029	0	0	0.047	0.096
	145	0	0	0	0	0	0.015	0.009	0.053	0.029	0	0.032	0.016	0.019
	146	0.023	0	0	0	0	0	0	0.026	0.014	0	0	0.016	0.019
	148	0.035	0.020	0.150	0.079	0	0.081	0.116	0	0.029	0	0.065	0.016	0.038
	149	0.081	0	0	0	0	0.007	0	0.026	0.014	0.118	0.016	0.062	0.038
	150	0.128	0.510	0.350	0.342	0.341	0.360	0.259	0.395	0.400	0.353	0.274	0.203	0.231
	151	0	0	0	0	0	0	0.018	0.079	0.014	0	0.065	0.016	0.019
	152	0.047	0.010	0.050	0	0.023	0.059	0.036	0.053	0.057	0	0.032	0.062	0.019
	153	0.151	0	0	0	0	0.007	0.045	0.053	0.086	0.088	0.113	0.094	0.077
	155	0.012	0	0	0	0	0	0.009	0	0.014	0	0	0	0
A		13	7	5	4	4	9	13	10	14	7	12	15	14
R_s		8.187	3.904	5.000	3.807	3.435	5.122	6.740	7.611	8.044	6.705	7.898	9.216	9.046
H_p		0.791	0.700	0.900	0.737	0.409	0.706	0.750	0.789	0.771	0.824	0.871	0.844	0.885
H_d		0.842	0.628	0.783	0.635	0.634	0.712	0.776	0.789	0.799	0.825	0.849	0.882	0.868
Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A43	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	124	0.163	0	0	0	0	0.015	0.054	0.053	0.129	0.088	0.032	0.125	0.154
	125	0	0.030	0.050	0.026	0.045	0.066	0.054	0.053	0	0	0.016	0	0.019

Table 5. Continued.

Locus A43	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	126	0.279	0.220	0.350	0.237	0.182	0.235	0.277	0.211	0.257	0.294	0.290	0.375	0.269
	128	0	0.010	0	0	0	0	0	0	0	0	0	0	0
	130	0.035	0	0	0	0	0.015	0.018	0.026	0	0	0.016	0	0
	132	0.035	0	0	0	0	0	0.036	0.026	0.014	0	0.097	0.031	0.019
	133	0.023	0	0	0	0	0	0	0.026	0.014	0	0	0.031	0.019
	134	0	0	0	0	0	0	0	0.053	0.029	0.059	0.032	0.031	0
	136	0.035	0	0	0	0	0	0.018	0.053	0	0	0	0.031	0.038
	137	0.035	0	0	0	0.023	0.015	0.027	0.132	0.014	0.059	0.032	0.047	0.019
	138	0.035	0.050	0.050	0.079	0.136	0.081	0.036	0.079	0.043	0.088	0.065	0.078	0.154
	139	0.023	0.570	0.550	0.447	0.432	0.412	0.295	0.184	0.300	0.206	0.194	0.078	0.192
	140	0.023	0	0	0	0	0	0.018	0	0.029	0	0.048	0	0
	141	0	0.120	0	0.211	0.182	0.088	0.134	0.026	0.014	0.029	0	0.031	0.019
	142	0.116	0	0	0	0	0.007	0	0	0.029	0.029	0.016	0.047	0.038
	143	0	0	0	0	0	0.022	0	0.053	0	0	0	0	0
	144	0.081	0	0	0	0	0.007	0.009	0	0.014	0.029	0	0	0.019
	146	0	0	0	0	0	0	0.009	0	0	0	0	0	0
	148	0.105	0	0	0	0	0.029	0.018	0.026	0.086	0.118	0.161	0.078	0.038
	150	0.012	0	0	0	0	0	0	0	0	0	0	0	0
	152	0	0	0	0	0	0.007	0	0	0.029	0	0	0.016	0
A		14	6	4	5	6	13	14	14	14	10	12	13	13
R_s		8.934	4.312	4.000	4.428	5.135	6.590	7.574	10.426	7.881	8.296	7.993	8.863	8.153
H_p		0.907	0.640	0.700	0.632	0.909	0.721	0.804	0.842	0.829	0.824	0.903	0.813	0.885
H_d		0.866	0.615	0.594	0.713	0.739	0.760	0.816	0.906	0.826	0.858	0.845	0.829	0.852

Table 5. Continued.

Locus A107	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	140	0	0	0	0	0	0	0.009	0	0	0	0	0	0
	142	0.093	0.020	0.050	0.026	0	0.015	0.098	0.026	0.057	0	0.145	0.094	0.077
	144	0.035	0	0	0	0	0.007	0.018	0	0	0	0	0	0
	146	0.012	0.010	0	0	0	0.022	0	0	0.014	0.088	0	0	0
	148	0.023	0	0	0	0	0	0.027	0	0.043	0	0	0	0.038
	150	0.047	0	0	0	0	0.015	0.009	0	0.014	0	0.032	0	0.019
	152	0.058	0.030	0.050	0.053	0.023	0.007	0.018	0.132	0.043	0.176	0.129	0.078	0.058
	154	0.058	0.010	0	0	0	0.007	0.045	0.079	0.071	0.118	0.113	0.062	0.096
	156	0.070	0	0.050	0	0.045	0.037	0.054	0.079	0	0.029	0.032	0.062	0.077
	158	0.081	0	0.050	0.053	0.068	0.074	0.054	0.053	0	0	0.032	0.078	0.058
	160	0.035	0	0.050	0.053	0.023	0.022	0.009	0	0.043	0.029	0.065	0.031	0.038
	162	0.012	0.090	0.050	0.079	0	0.066	0.036	0.026	0.100	0.059	0.048	0.047	0.019
	163	0	0	0	0	0	0	0.009	0	0	0	0	0	0
	164	0.070	0.040	0.100	0	0.136	0.088	0.071	0.053	0.071	0.029	0.048	0.078	0.019
	166	0.128	0.030	0.050	0.053	0.091	0.059	0.125	0.079	0.029	0.029	0	0.141	0.173
	167	0	0.020	0	0	0	0.015	0	0.053	0.029	0	0.016	0.031	0
	168	0.012	0.040	0	0.132	0.068	0.103	0.089	0.026	0.100	0.059	0.032	0.047	0.019
	169	0	0.010	0	0	0	0.007	0	0	0	0	0	0	0.019
	170	0	0.080	0.100	0.026	0.091	0.081	0.036	0.026	0.043	0.029	0.032	0.031	0.019
	171	0	0.110	0	0.026	0.023	0.022	0.009	0	0.043	0.029	0.016	0.016	0
	172	0.093	0.020	0.050	0	0.068	0.015	0.036	0	0.043	0	0	0.016	0
	173	0	0.010	0	0	0	0	0	0	0	0	0	0	0
	174	0.023	0.100	0.050	0.211	0.068	0.140	0.045	0.158	0.086	0.029	0.048	0.047	0.058
	175	0	0.120	0	0.026	0.045	0.059	0.098	0.079	0.043	0.059	0.032	0.016	0.038

Table 5. Continued.

Locus A107	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	176	0.023	0	0	0	0.023	0.015	0.018	0.105	0	0.029	0.016	0.016	0.096
	177	0	0	0	0	0.068	0	0	0	0.014	0.029	0.032	0	0
	178	0	0.090	0.100	0	0.045	0.059	0.036	0.026	0.057	0	0	0	0.019
	179	0	0	0	0	0.023	0	0.009	0	0	0	0	0	0
	180	0.047	0.030	0.100	0.211	0	0.022	0.018	0	0.029	0.029	0.048	0.078	0.038
	182	0.012	0.060	0	0	0	0.015	0	0	0.029	0.059	0.048	0.016	0.019
	184	0.070	0.010	0	0	0	0	0	0	0	0.088	0.016	0.016	0
	188	0	0.070	0.150	0.053	0.091	0.029	0.027	0	0	0	0.016	0	0
A		20	21	14	13	17	25	25	15	21	18	21	20	20
R_s		12.098	11.677	14.000	9.901	12.379	12.125	12.567	11.527	13.181	13.088	12.596	12.397	12.479
H_p		1.000	1.000	0.900	1.000	0.909	0.868	0.911	0.684	0.829	1.000	1.000	0.875	0.846
H_d		0.940	0.933	0.967	0.892	0.947	0.937	0.943	0.942	0.955	0.943	0.939	0.944	0.941
Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A14	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	209	0.035	0	0	0	0	0	0	0	0	0	0	0	0
	217	0.035	0.360	0.250	0.289	0.455	0.375	0.268	0.105	0.171	0.088	0.161	0.047	0.154
	218	0.023	0	0	0	0	0	0.018	0	0.014	0	0.016	0.031	0.019
	219	0	0.180	0.200	0.105	0.182	0.162	0.071	0.079	0.057	0	0.065	0.047	0.077
	220	0.070	0.010	0	0	0.023	0.029	0.018	0.053	0.114	0.059	0.065	0.078	0.096
	221	0	0	0	0.026	0	0	0.027	0	0	0	0.016	0	0
	222	0.012	0.090	0.050	0.132	0.068	0.103	0.107	0.132	0.057	0.029	0.048	0.031	0.058
	223	0.058	0.040	0.050	0.026	0	0.029	0.027	0.026	0.014	0	0	0.031	0
	224	0	0	0	0.026	0	0.015	0.018	0.053	0	0	0.016	0.016	0
	225	0.081	0.020	0	0	0	0	0	0	0.029	0	0	0.031	0.038

Table 5. Continued.

Locus A14	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	226	0	0.010	0	0.079	0.023	0.022	0.009	0.026	0	0	0	0	0
	227	0.035	0	0	0	0.023	0	0.018	0.053	0.043	0.059	0.048	0.016	0.038
	228	0	0	0	0	0	0.007	0	0	0	0.029	0	0	0.019
	229	0.186	0	0	0	0	0.007	0.045	0.053	0.114	0.353	0.226	0.156	0.096
	230	0.081	0.010	0.050	0	0	0	0.036	0.105	0.043	0.029	0.032	0.062	0.096
	231	0.035	0	0	0	0	0	0.027	0.053	0.029	0	0.016	0.047	0.019
	232	0.070	0.080	0	0.105	0.114	0.088	0.080	0.026	0.086	0.088	0.113	0.141	0.115
	233	0	0.020	0	0	0	0	0	0.079	0	0	0.016	0	0
	234	0.012	0.130	0.350	0.105	0.068	0.103	0.045	0.026	0.071	0.118	0.032	0.062	0.058
	235	0.058	0	0	0	0	0.007	0.071	0.053	0.029	0.029	0.016	0.094	0.019
	236	0	0.040	0	0.026	0	0.015	0.009	0	0	0.029	0.016	0	0
	241	0.023	0	0	0	0	0.007	0.009	0	0	0	0	0	0
	243	0.012	0	0	0	0	0	0.027	0.026	0.014	0	0	0	0
	245	0	0	0	0	0	0	0	0	0	0	0.016	0	0.019
	247	0.070	0.010	0.050	0.079	0.045	0.029	0.071	0.026	0.029	0.059	0.016	0.062	0.019
	251	0.047	0	0	0	0	0	0	0	0	0	0	0	0.019
	253	0	0	0	0	0	0	0	0	0	0.029	0.016	0	0
	255	0.058	0	0	0	0	0	0	0.026	0.071	0	0.048	0.047	0.038
	257	0	0	0	0	0	0	0	0	0	0	0	0	0
	262	0	0	0	0	0	0	0	0	0.014	0	0	0	0
A		19	13	7	11	9	15	20	18	18	13	20	17	18
R_s		11.748	7.383	7.000	8.770	6.723	7.594	10.627	13.085	11.299	9.900	10.933	11.557	11.697
H_p		0.884	0.840	0.900	0.947	0.818	0.838	0.875	0.947	0.857	0.882	0.935	0.906	0.885
H_d		0.930	0.810	0.800	0.871	0.750	0.806	0.894	0.952	0.927	0.855	0.904	0.931	0.935

Table 5. Continued.

Locus A35	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	095	0.058	0.080	0.100	0.105	0.068	0.066	0.036	0.053	0.071	0	0.032	0.062	0.058
	096	0.023	0	0	0.053	0.023	0.029	0.080	0.053	0.129	0.176	0.145	0.078	0.038
	097	0	0.010	0	0	0	0	0.018	0	0.014	0	0.016	0.031	0.019
	098	0.209	0	0	0	0.023	0.007	0.045	0.053	0.029	0.059	0.129	0.047	0.154
	099	0.081	0	0	0	0	0	0.027	0.026	0.014	0.088	0.065	0.125	0.038
	100	0.267	0.010	0	0	0	0.007	0.018	0	0.086	0.029	0.048	0.125	0.212
	101	0.047	0.080	0.050	0	0.068	0.022	0.036	0.105	0.029	0.029	0	0	0.019
	103	0.081	0	0	0	0	0.007	0.018	0.132	0.114	0.147	0.145	0.062	0.096
	104	0	0	0	0	0	0	0	0	0	0	0.016	0	0
	105	0.012	0	0	0	0	0	0	0	0	0	0	0	0
	107	0	0	0	0	0	0	0	0.026	0.043	0	0.016	0.016	0
	111	0	0	0	0	0	0.007	0.009	0	0	0	0	0	0
	112	0	0	0	0	0	0	0	0	0.014	0	0.016	0.047	0
	113	0	0.040	0.050	0.079	0.182	0.074	0.062	0.105	0.014	0.059	0	0.031	0.038
	114	0.081	0.590	0.700	0.579	0.455	0.610	0.482	0.316	0.229	0.206	0.177	0.172	0.173
	115	0.058	0.110	0	0.026	0	0.007	0.098	0.079	0.071	0.088	0.113	0.125	0.077
	116	0	0.040	0.050	0.079	0.114	0.081	0.036	0	0.057	0.029	0	0.047	0
	118	0	0.010	0	0	0	0	0	0	0	0	0	0	0
	119	0.047	0.020	0	0	0.023	0.059	0.018	0.026	0.029	0.029	0.016	0	0.038
	121	0.012	0	0	0	0	0.007	0	0	0.029	0.059	0.032	0.031	0
	123	0	0	0	0.026	0	0.007	0.009	0.026	0.014	0	0.016	0	0.038
	125	0.023	0.010	0.050	0.053	0.045	0.007	0	0	0.014	0	0.016	0	0
	128	0	0	0	0	0	0	0.009	0	0	0	0	0	0
A		13	11	6	8	9	15	16	12	18	12	16	14	13

Table 5. Continued.

Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A35	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
R_s		8.659	5.969	6.000	6.382	6.723	6.182	8.072	9.256	10.507	9.735	9.701	10.251	9.440
H_p		0.698	0.600	0.600	0.474	0.682	0.603	0.643	0.842	0.829	1.000	0.935	0.906	0.808
H_d		0.864	0.630	0.511	0.656	0.753	0.611	0.747	0.867	0.905	0.899	0.901	0.914	0.894
Locus	Allele	Braz.	Eur	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A8	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	165	0.174	0.290	0.100	0.368	0.341	0.257	0.259	0.184	0.257	0.412	0.242	0.344	0.327
	167	0.023	0	0	0	0	0	0.036	0	0	0.059	0.048	0.031	0.077
	169	0.128	0	0	0	0	0	0.018	0.026	0.043	0.206	0.129	0.062	0.135
	171	0.267	0	0	0	0	0.037	0.036	0.184	0.171	0.206	0.113	0.156	0.135
	173	0.256	0.020	0.100	0.053	0.091	0.074	0.125	0.079	0.157	0.059	0.081	0.109	0.077
	175	0.047	0.170	0.300	0.263	0.295	0.184	0.143	0.105	0.100	0	0.129	0.078	0.038
	176	0.012	0	0	0	0	0.007	0.009	0.026	0.043	0	0	0.016	0
	177	0.058	0.140	0.100	0.026	0	0.066	0.107	0.053	0.114	0	0.129	0.094	0.038
	179	0.012	0.220	0.150	0.158	0.205	0.221	0.196	0.132	0.071	0.059	0.113	0.062	0.154
	181	0.023	0.160	0.150	0.132	0.068	0.147	0.062	0.211	0.043	0	0.016	0.031	0.019
	183	0	0	0.100	0	0	0.007	0.009	0	0	0	0	0.016	0
A		10	6	7	6	5	9	11	9	9	6	9	11	9
R_s		6.637	5.288	7.000	5.285	4.767	6.379	7.365	7.674	7.581	5.512	7.672	7.945	7.307
H_p		0.791	0.800	0.900	0.632	0.864	0.750	0.857	0.789	0.857	0.706	0.903	0.844	0.808
H_d		0.819	0.801	0.867	0.773	0.756	0.825	0.847	0.874	0.858	0.759	0.870	0.833	0.835
Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A113	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	198	0	0	0	0.053	0.023	0	0.027	0	0	0	0	0	0
	202	0.058	0.070	0.150	0.105	0.136	0.125	0.134	0.184	0.129	0.088	0.065	0.047	0.038

Table 5. Continued.

Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A113	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=52)
	206	0	0	0	0	0	0	0	0.053	0	0	0	0	0
	208	0.070	0.010	0	0	0	0.007	0.018	0	0.014	0.059	0	0	0
	210	0.012	0	0	0	0	0	0	0	0	0	0.016	0	0.019
	212	0.058	0.010	0	0	0	0.007	0.027	0.053	0.043	0.029	0.065	0.047	0.154
	214	0.081	0.120	0.300	0.211	0.159	0.118	0.152	0.105	0.143	0.176	0.113	0.172	0.115
	216	0.279	0.020	0	0.026	0	0.022	0.054	0.105	0.200	0.176	0.177	0.250	0.173
	218	0.035	0.010	0	0.053	0.023	0.007	0	0	0	0.029	0	0.016	0.038
	220	0.174	0.570	0.400	0.447	0.523	0.566	0.402	0.342	0.286	0.324	0.290	0.359	0.346
	222	0.105	0.080	0.100	0.026	0.045	0.022	0.045	0.105	0.057	0.059	0.048	0.016	0.019
	224	0.035	0	0	0	0	0.037	0.045	0.026	0.014	0.029	0.097	0.062	0.058
	226	0.093	0.050	0.050	0	0.023	0.037	0.045	0.026	0.029	0	0.097	0.016	0.038
	228	0	0.050	0	0.079	0.045	0.044	0.045	0	0.057	0.029	0.016	0	0
	232	0	0.010	0	0	0.023	0.007	0	0	0.014	0	0	0	0
	234	0	0	0	0	0	0	0.009	0	0	0	0	0	0
	236	0	0	0	0	0	0	0	0	0.014	0	0.016	0.016	0
A		11	11	5	8	9	12	12	9	12	10	11	10	10
R_s		8.285	6.111	5.000	6.478	6.207	5.961	7.571	7.490	7.706	7.963	8.029	6.389	7.357
H_p		0.837	0.640	0.900	0.684	0.591	0.662	0.750	0.947	0.886	0.765	0.871	0.750	1.000
H_d		0.862	0.650	0.744	0.753	0.695	0.649	0.792	0.827	0.843	0.842	0.855	0.782	0.817
Locus	Allele	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A79	(bp)	(n=86)	(n=100)	(n=20)	(n=38)	(n=44)	(n=136)	(n=112)	(n=38)	(n=70)	(n=34)	(n=62)	(n=64)	(n=50)
	089	0.035	0	0	0	0	0	0.045	0.079	0.057	0.059	0.048	0.109	0.020
	091	0	0	0	0	0	0.015	0	0	0	0	0	0.031	0
	093	0.105	0.310	0.400	0.342	0.364	0.478	0.304	0.316	0.243	0.118	0.177	0.125	0.260

Table 5. Continued.

Locus A79	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=50)
	097	0.140	0.010	0	0	0	0	0.009	0.026	0.029	0.059	0.048	0.047	0.020
	099	0.093	0	0	0.026	0	0.007	0.045	0.105	0.029	0	0.065	0.062	0
	101	0.105	0.010	0	0	0	0.015	0.036	0.026	0.057	0.088	0.048	0.031	0.080
	103	0.128	0.080	0	0.026	0.091	0.037	0.080	0.026	0.043	0	0.065	0.031	0.080
	105	0	0.240	0.350	0.316	0.205	0.162	0.152	0.132	0.100	0.059	0.065	0.125	0.080
	107	0.023	0.110	0.150	0.079	0.114	0.118	0.116	0.105	0.071	0.147	0.065	0.047	0.080
	109	0.047	0.110	0.100	0.105	0.068	0.059	0.080	0.053	0.114	0.088	0.065	0.125	0.160
	111	0.023	0	0	0.053	0.045	0.015	0.036	0.079	0.057	0.059	0.032	0.031	0
	113	0	0.030	0	0.026	0	0.029	0.009	0	0.029	0	0	0	0
	115	0.209	0	0	0	0.023	0.037	0.036	0.026	0.129	0.147	0.145	0.125	0.120
	117	0.058	0.040	0	0	0.045	0.007	0	0	0	0.088	0.129	0.062	0.040
	119	0	0.060	0	0.026	0.045	0.022	0.045	0.026	0.014	0	0.032	0.031	0.020
	121	0.012	0	0	0	0	0	0.009	0	0.014	0	0	0	0.020
	123	0.023	0	0	0	0	0	0	0	0.014	0.088	0.016	0.016	0.020
A		13	10	4	9	9	13	14	12	15	11	14	15	13
R_s		9.079	6.930	4.000	6.749	7.307	6.722	8.678	9.120	9.869	10.072	10.433	10.681	9.120
H_p		0.721	0.860	0.700	0.842	0.864	0.691	0.768	0.737	0.743	0.941	0.677	0.656	0.720
H_d		0.894	0.817	0.722	0.779	0.811	0.729	0.857	0.868	0.897	0.925	0.919	0.927	0.884

Locus A28	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	123	0	0	0	0	0	0	0	0	0.014	0	0.048	0.016	0.019
	125	0.151	0	0	0	0	0	0.054	0.053	0.143	0.206	0.129	0.125	0.096
	128	0.116	0	0	0	0	0.015	0.027	0.053	0.057	0.088	0.113	0.047	0
	129	0.023	0	0	0	0	0.015	0.009	0.158	0.114	0.088	0.113	0.094	0.173

Table 5. Continued.

Locus A28	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	130	0.209	0.240	0.300	0.342	0.136	0.221	0.214	0.158	0.286	0.176	0.210	0.219	0.212
	132	0	0	0	0	0	0	0.009	0.026	0	0	0	0	0
	133	0.314	0.030	0	0.053	0.023	0.066	0.089	0.053	0.114	0.029	0.129	0.188	0.173
	134	0	0	0	0	0	0	0.009	0.026	0.029	0	0	0.047	0
	135	0.012	0	0	0	0	0	0	0	0	0.029	0	0.016	0
	136	0.058	0.730	0.700	0.605	0.841	0.662	0.554	0.447	0.229	0.294	0.210	0.203	0.231
	138	0.058	0	0	0	0	0	0.009	0	0	0	0.016	0.016	0.038
	139	0	0	0	0	0	0.007	0.018	0.026	0.014	0.088	0.032	0.031	0.058
	140	0.035	0	0	0	0	0.007	0	0	0	0	0	0	0
	142	0	0	0	0	0	0.007	0	0	0	0	0	0	0
	144	0.023	0	0	0	0	0	0.009	0	0	0	0	0	0
A		10	3	2	3	3	8	11	9	9	8	9	11	8
R_s		7.011	2.490	2.000	2.782	2.435	3.756	5.238	6.913	6.671	6.991	7.378	7.686	6.690
H_p		0.814	0.380	0.600	0.526	0.182	0.559	0.536	0.684	0.800	0.765	0.839	0.813	0.885
H_d		0.822	0.413	0.433	0.528	0.282	0.512	0.642	0.762	0.827	0.842	0.864	0.859	0.843

Locus ED1	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	140	0.070	0.020	0	0.053	0.068	0.044	0.036	0.079	0.029	0.029	0.145	0.078	0.058
	142	0.221	0.730	0.850	0.763	0.591	0.676	0.652	0.474	0.386	0.412	0.355	0.422	0.404
	143	0	0	0	0	0	0	0	0	0	0.029	0	0	0
	144	0.116	0.010	0	0.053	0.045	0.037	0.116	0.079	0.129	0.118	0.226	0.219	0.077
	146	0.174	0.010	0	0	0	0.029	0.054	0.105	0.129	0.059	0.016	0.078	0.173
	148	0.058	0	0	0	0	0.007	0	0	0.057	0	0.048	0	0
	150	0.174	0.020	0	0	0	0.015	0.045	0.053	0.086	0.088	0.065	0.094	0.135

Table 5. Continued.

Locus ED1	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	152	0.012	0	0	0	0	0	0	0	0.014	0	0	0	0
	154	0.023	0.030	0	0.079	0.045	0.022	0.027	0	0.029	0.088	0.032	0.016	0.058
	156	0	0	0	0	0	0	0	0	0	0.029	0.016	0.016	0.038
	160	0	0	0	0	0	0	0	0.026	0.014	0.029	0.032	0.016	0
	166	0	0.010	0	0	0	0	0	0	0	0	0	0	0
	168	0	0.060	0.100	0.053	0.182	0.096	0.018	0.079	0.043	0.029	0.032	0.031	0.019
	170	0.047	0.030	0	0	0	0.029	0.027	0.053	0.029	0	0	0.016	0.019
	172	0.070	0.060	0	0	0.068	0.037	0	0.026	0.029	0.059	0.016	0.016	0
	174	0	0.020	0.050	0	0	0.007	0.009	0	0	0	0	0	0
	176	0	0	0	0	0	0	0.009	0.026	0	0	0	0	0.019
	182	0.035	0	0	0	0	0	0.009	0	0.014	0.029	0	0	0
	186	0	0	0	0	0	0	0	0	0.014	0	0.016	0	0
A		11	11	3	5	6	11	11	10	14	12	12	11	10
R_s		8.129	5.164	3.000	4.250	5.107	5.517	5.580	7.812	8.306	9.062	7.396	6.712	7.165
H_p		0.977	0.500	0.300	0.421	0.545	0.485	0.571	0.842	0.857	0.882	0.903	0.719	0.769
H_d		0.868	0.461	0.278	0.414	0.620	0.530	0.558	0.756	0.813	0.811	0.803	0.764	0.789

Locus IM	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	174	0.070	0	0	0	0	0	0.036	0.026	0.071	0	0	0	0.019
	176	0.035	0	0	0	0	0.015	0	0.026	0.029	0.059	0.016	0.016	0.038
	178	0.221	0.240	0.150	0.158	0.273	0.169	0.196	0.237	0.186	0.265	0.290	0.344	0.346
	179	0.105	0	0	0	0	0.007	0.062	0	0.043	0.118	0.129	0.016	0.154
	180	0.105	0	0	0	0	0	0	0	0.014	0	0.065	0.078	0
	182	0.070	0	0	0	0	0	0.009	0	0.029	0	0.065	0.047	0.019

Table 5. Continued.

Locus IM	Allele (bp)	Braz. (n=86)	Eur. (n=100)	1991 (n=20)	1992 (n=38)	1993 (n=44)	1994 (n=136)	1995 (n=112)	1996 (n=38)	1997 (n=70)	1998 (n=34)	1999 (n=62)	2000 (n=64)	2001 (n=52)
	184	0.140	0.030	0.050	0.053	0.068	0.022	0.071	0.132	0.086	0.147	0.161	0.156	0.173
	186	0.128	0.310	0.200	0.158	0.159	0.228	0.241	0.184	0.314	0.235	0.161	0.109	0.173
	188	0.035	0.310	0.400	0.395	0.318	0.338	0.223	0.237	0.114	0.147	0.065	0.109	0.038
	190	0	0.080	0.200	0.184	0.114	0.191	0.143	0.132	0.100	0.029	0	0.094	0.019
	192	0.070	0.020	0	0.053	0.068	0.029	0.018	0.026	0.014	0	0.048	0.031	0.019
	194	0.023	0	0	0	0	0	0	0	0	0	0	0	0
	202	0	0.010	0	0	0	0	0	0	0	0	0	0	0
A		11	7	5	6	6	8	9	8	11	7	9	10	10
R_s		8.699	4.895	5.000	5.549	5.646	5.246	6.571	6.542	7.755	6.390	7.355	7.462	6.761
H_p		0.907	0.740	0.900	0.737	0.864	0.676	0.786	0.789	0.886	0.647	0.903	0.781	0.731
H_d		0.886	0.750	0.767	0.776	0.793	0.773	0.830	0.841	0.839	0.844	0.845	0.829	0.809
Mean ± SE		Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
A		13.250	10.000	5.833	7.333	7.833	13.333	15.083	12.000	14.417	10.500	13.833	13.667	12.917
		± 0.496	± 0.671	± 0.980	± 0.694	± 0.802	± 0.653	± 0.726	± 0.807	± 0.642	± 0.804	± 0.828	± 0.571	± 0.745
R_s		8.890	5.934	5.833	6.039	6.142	6.653	8.100	9.151	9.127	8.602	9.018	9.168	8.891
		± 0.246	± 0.325	± 0.980	± 0.487	± 0.534	± 0.257	± 0.293	± 0.508	± 0.330	± 0.504	± 0.322	± 0.349	± 0.399
H_p		0.845	0.713	0.750	0.711	0.701	0.700	0.760	0.807	0.836	0.843	0.876	0.820	0.842
		± 0.014	± 0.024	± 0.060	± 0.043	± 0.049	± 0.014	± 0.016	± 0.019	± 0.008	± 0.027	± 0.015	± 0.015	± 0.016
H_d		0.874	0.693	0.681	0.717	0.717	0.725	0.800	0.860	0.866	0.858	0.874	0.868	0.866
		± 0.006	± 0.022	± 0.061	± 0.032	± 0.035	± 0.016	± 0.015	± 0.016	± 0.008	± 0.012	± 0.007	± 0.011	± 0.010

reference sample (Wilcoxon's signed rank test: $P = 0.002$ for both allelic richness and gene diversity). Pairwise comparisons between the reference and Welder Wildlife Refuge samples showed first that mean allelic richness and mean gene diversity were significantly lower in the European ($R_s = 5.934$; $H_d = 0.693$; Table 5) than in the Welder Wildlife Refuge after 1994 ($R_s = 6.653 - 9.168$; Table 5) and 1995 ($H_d = 0.800 - 0.874$; Table 5), respectively ($P \leq 0.005$); and second that mean allelic richness and mean gene diversity were significantly higher in the Brazilian ($R_s = 8.890$; $H_d = 0.874$; Table 5) than in the Welder Wildlife Refuge ($P \leq 0.006$) prior to 1994 ($R_s = 5.833 – 6.653$; Table 5) and 1995 ($H_d = 0.681 - 0.800$; Table 5), respectively. No significant differences in genetic diversity were found for the remaining reference-Welder Wildlife Refuge sample pairwise comparisons ($P \geq 0.060$). During the 11-year study, the Welder Wildlife Refuge population experienced a striking increase in gene diversity and allelic richness, with the greatest increment observed between 1994 and 1996 (Fig. 8).

Hardy-Weinberg Equilibrium and Genotypic Linkage Equilibrium

The proportion of observed and expected homozygotes, proportion of observed and expected heterozygotes, F_{IS} estimates and respective significance, *P*-values for HWE, *P*-values for heterozygote deficiency, and *P*-values for heterozygote excess and their standard errors per locus and population sample are shown in Table 6.

In the reference European sample, no significant departures from HWE were detected for any locus and across loci (Fisher's exact test; $0.085 \leq P_{single\ locus} \leq 0.963$;

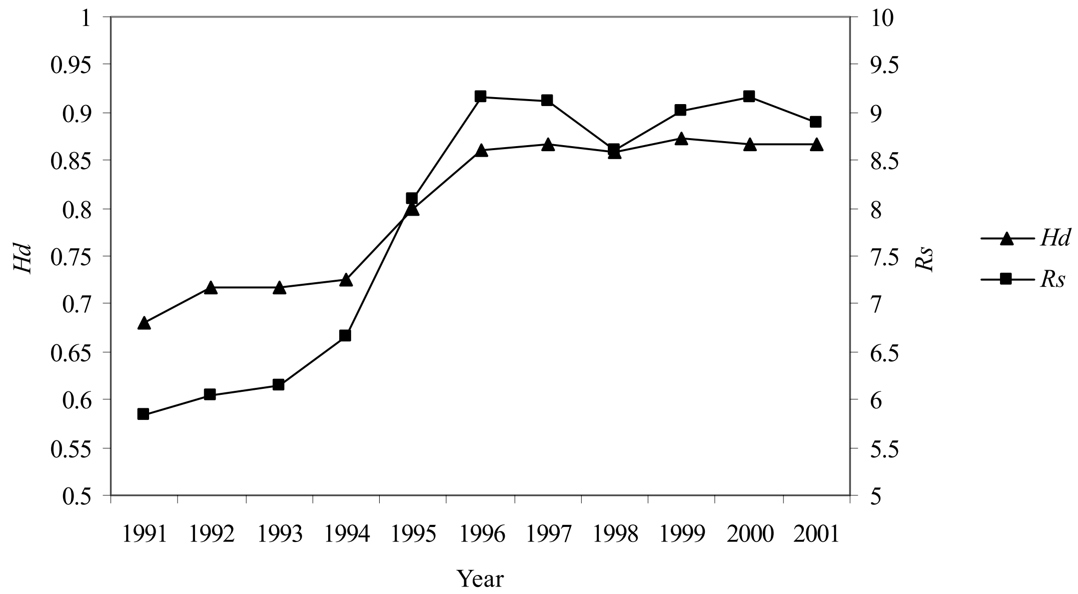

Fig. 8. Temporal pattern of unbiased gene diversity (H_d) and allelic richness (R_s) in the feral honey bee population of the Welder Wildlife Refuge.

Table 6. Expected homozygotes (Exp. Hm.), observed homozygotes (Obs. Hm.), expected heterozygotes (Exp. Ht.), observed heterozygotes (Obs. Ht.), F_{IS} estimate, significance of F_{IS} estimate (P F_{IS}), exact test probability of Hardy-Weinberg equilibrium (P HWE), U test probability of heterozygote deficiency (P Ht. Def.), U test probability of heterozygote excess (P Ht. Exc.), and respective standard errors (SE) obtained for reference (Braz. - Africanized from Brazil; Eur. - European from South Texas) and Welder Wildlife Refuge (from 1991 to 2001) honey bee population samples.

Locus A7	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	4.435	9.616	3.000	3.541	4.000	10.067	6.018	1.243	3.449	1.818	3.410	2.619	1.863
Obs. Hm.	8	7	3	2	5	11	7	4	4	2	7	2	3
Exp. Ht.	38.565	40.384	7.000	15.460	18.000	57.933	49.982	17.757	31.551	15.182	27.590	29.381	24.137
Obs. Ht.	35	43	7	17	17	57	49	15	31	15	24	30	23
F_{IS}	0.093	-0.066	0.000	-0.103	0.057	0.016	0.020	0.159	0.018	0.012	0.132	-0.021	0.048
P (F_{IS})	0.075	0.909	0.708	0.935	0.358	0.428	0.393	**0.021**	0.478	0.573	**0.026**	0.754	0.283
P (HWE)	0.474	0.963	0.492	0.527	0.091	0.968	0.103	**0.021**	0.088	0.112	0.112	0.172	0.291
± SE	± 0.003	± 0.001	± 0.003	± 0.003	± 0.002	± 0.002	± 0.006	± 0.002	± 0.004	± 0.002	± 0.006	± 0.004	±0.005
P (Ht. Def.)	0.161	0.862	0.711	0.947	0.611	0.146	0.164	**0.032**	0.087	0.152	0.081	0.318	**0.023**
± SE	± 0.003	± 0.003	± 0.003	± 0.001	± 0.004	± 0.006	± 0.007	± 0.002	± 0.003	± 0.003	± 0.004	± 0.005	± 0.001
P (Ht. Exc.)	0.836	0.137	0.718	0.200	0.486	0.854	0.844	0.973	0.909	0.884	0.918	0.689	0.978
± SE	±0.003	± 0.003	± 0.003	± 0.002	± 0.004	± 0.006	± 0.007	± 0.002	± 0.003	± 0.002	± 0.005	± 0.005	± 0.002
Locus A88	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	6.812	18.566	2.105	6.892	8.163	19.563	12.541	4.000	7.044	2.970	4.672	3.794	3.431
Obs. Hm.	9	15	1	5	13	20	14	4	8	3	4	5	3
Exp. Ht.	36.188	31.434	7.895	12.108	13.837	48.437	43.460	15.000	27.957	14.030	26.328	28.206	22.569
Obs. Ht.	34	35	9	14	9	48	42	15	27	14	27	27	23
F_{IS}	0.061	-0.115	-0.149	-0.161	0.355	0.009	0.034	0.000	0.035	0.002	-0.026	0.043	-0.020
P (F_{IS})	0.171	0.930	0.912	0.896	**0.021**	0.505	0.361	0.617	0.407	0.622	0.732	0.321	0.702
P (HWE)	0.607	0.631	0.929	0.132	**0.005**	0.299	0.186	0.584	0.561	0.780	0.326	0.367	0.163 ±
± SE	± 0.004	± 0.002	± 0.000	± 0.001	± 0.000	± 0.003	± 0.004	± 0.003	± 0.006	± 0.001	± 0.003	± 0.005	0.003

Table 6. Continued.

Locus A88	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
P (Ht. Def.)	**0.029**	0.932	0.914	0.219	0.097	0.360	0.071	0.605	0.346	0.257	0.479	0.175	0.748
± SE	± 0.001	± 0.002	± 0.001	± 0.001	± 0.001	± 0.004	± 0.002	± 0.004	± 0.005	± 0.001	± 0.004	± 0.004	± 0.004
P (Ht. Exc.)	0.975	0.082	0.253	0.788	0.905	0.637	0.926	0.544	0.704	0.776	0.539	0.834	0.327
SE	± 0.001	± 0.002	± 0.001	± 0.001	± 0.001	± 0.004	± 0.002	± 0.004	± 0.004	± 0.001	± 0.004	± 0.004	± 0.004
Locus A43	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	5.729	19.253	4.000	5.487	5.651	16.333	10.333	1.811	6.102	2.424	4.771	5.476	3.824
Obs. Hm.	4	18	3	7	2	19	11	3	6	3	3	6	3
Exp. Ht.	37.271	30.748	6.000	13.514	16.349	51.667	45.667	17.189	28.899	14.576	26.230	26.524	22.177
Obs. Ht.	39	32	7	12	20	49	45	16	29	14	28	26	23
F_{IS}	-0.047	-0.041	-0.178	0.115	-0.230	0.052	0.015	0.071	-0.004	0.041	-0.069	0.020	-0.038
$P(F_{IS})$	0.896	0.735	0.865	0.279	0.994	0.230	0.464	0.260	0.605	0.459	0.903	0.465	0.762
P (HWE)	0.964	0.264	0.373	0.502	0.752	0.120	0.314	**0.044**	0.822	0.851	0.561	0.208	0.818
± SE	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.003	± 0.005	± 0.002	± 0.003	± 0.002	± 0.003	± 0.004	± 0.003
P (Ht. Def.)	0.774	0.091	0.855	0.197	0.996	0.228	0.280	0.343	0.072	0.213	0.910	0.726	0.515
± SE	± 0.003	± 0.001	± 0.001	± 0.001	± 0.000	± 0.004	± 0.004	± 0.005	± 0.002	± 0.002	± 0.002	± 0.004	± 0.005
P (Ht. Exc.)	0.240	0.908	0.199	0.825	**0.025**	0.777	0.731	0.659	0.927	0.813	0.114	0.347	0.500
± SE	± 0.004	± 0.001	± 0.001	± 0.001	± 0.000	± 0.004	± 0.004	± 0.005	± 0.002	± 0.002	± 0.002	± 0.004	± 0.005
Locus A107	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	2.565	3.293	0.368	2.000	1.186	4.311	3.225	1.243	1.652	0.939	1.853	1.825	1.588
Obs. Hm.	0	0	1	0	2	9	5	6	6	0	0	4	4
Exp. Ht.	40.435	46.707	9.632	17.000	20.814	63.689	52.775	17.757	33.348	16.061	29.148	30.175	24.412
Obs. Ht.	43	50	9	19	20	59	51	13	29	17	31	28	22
F_{IS}	-0.064	-0.071	0.069	-0.121	0.040	0.074	0.034	0.273	0.132	-0.060	-0.065	0.073	0.101
$P(F_{IS})$	1.000	1.000	0.320	1.000	0.327	**0.023**	0.223	0.001	**0.005**	1.000	1.000	0.100	0.066

Table 6. Continued.

Locus A107	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
P (HWE)	0.490	0.430	0.408	0.625	0.745	0.088	0.394	0.001	0.094	0.863	0.979	**0.032**	0.256
± SE	± 0.005	± 0.006	± 0.006	± 0.004	± 0.004	± 0.004	± 0.007	± 0.000	± 0.003	± 0.004	± 0.001	± 0.002	± 0.006
P (Ht. Def.)	1.000	1.000	0.188	1.000	0.279	0.002	0.061	0.001	**0.009**	1.000	1.000	**0.024**	0.070
± SE	± 0.000	± 0.000	± 0.005	± 0.000	± 0.005	± 0.001	± 0.004	± 0.000	± 0.001	± 0.000	± 0.000	± 0.002	± 0.004
P (Ht. Exc.)	0.074	**0.036**	0.950	0.108	0.802	0.998	0.945	1.000	0.994	0.354	0.131	0.974	0.932
± SE	± 0.003	± 0.002	± 0.003	± 0.002	± 0.005	± 0.001	± 0.003	± 0.000	± 0.001	± 0.007	± 0.005	± 0.002	± 0.003
Locus A14	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	3.024	9.485	1.947	2.405	5.465	13.163	5.973	0.919	2.594	2.455	2.951	2.222	1.726
Obs. Hm.	5	8	1	1	4	11	7	1	5	2	2	3	3
Exp. Ht.	39.976	40.515	8.053	16.595	16.535	54.837	50.027	18.081	32.406	14.545	28.049	29.778	24.275
Obs. Ht.	38	42	9	18	18	57	49	18	30	15	29	29	23
F_{IS}	0.050	-0.037	-0.125	-0.087	-0.091	-0.040	0.021	0.005	0.075	-0.032	-0.035	0.027	0.054
P (F_{IS})	0.183	0.795	0.904	0.946	0.892	0.835	0.380	0.606	0.102	0.789	0.826	0.391	0.239
P (HWE)	**0.034**	0.886	1.000	0.850	0.607	0.746	0.557	0.514	0.225	0.682	0.057	0.808	0.198
± SE	± 0.002	± 0.002	± 0.000	± 0.002	± 0.003	± 0.005	± 0.007	± 0.006	± 0.004	± 0.004	± 0.003	± 0.003	± 0.005
P (Ht. Def.)	**0.043**	0.797	0.903	0.943	0.713	0.892	0.300	0.353	**0.039**	0.338	0.863	0.104	0.306
± SE	± 0.002	± 0.004	± 0.001	± 0.001	± 0.002	± 0.003	± 0.006	± 0.007	± 0.002	± 0.004	± 0.005	± 0.003	± 0.006
P (Ht. Exc.)	0.959	0.232	0.295	0.211	0.329	0.110	0.713	0.705	0.965	0.748	0.226	0.898	0.713
± SE	± 0.002	± 0.004	± 0.002	± 0.003	± 0.003	± 0.003	± 0.006	± 0.007	± 0.002	± 0.004	± 0.006	± 0.003	± 0.006
Locus A35	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	5.918	18.535	4.842	6.622	5.465	26.489	14.216	2.541	3.362	1.667	3.066	2.746	2.804
Obs. Hm.	13	20	4	10	7	27	20	3	6	0	2	3	5
Exp. Ht.	37.082	31.465	5.158	12.378	16.535	41.511	41.784	16.460	31.638	15.333	27.934	29.254	23.196
Obs. Ht.	30	30	6	9	15	41	36	16	29	17	29	29	21

Table 6. Continued.

Locus A35	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
F_{IS}	0.193	0.047	-0.174	0.278	0.095	0.012	0.140	0.029	0.085	-0.113	-0.039	0.009	0.096
P (F_{IS})	0.004	0.314	1.000	**0.022**	0.249	0.496	**0.012**	0.478	0.100	1.000	0.837	0.543	0.146
P (HWE)	**0.018**	0.143	1.000	**0.029**	0.665	0.497	**0.012**	0.054	0.137	0.580	0.269	0.343	0.480
± SE	± 0.001	± 0.004	± 0.000	± 0.001	± 0.003	± 0.009	± 0.001	± 0.002	± 0.004	± 0.003	± 0.004	± 0.003	± 0.004
P (Ht. Def.)	0.000	0.430	1.000	**0.017**	0.208	0.097	0.205	0.619	0.075	1.000	0.759	0.226	0.203
± SE	± 0.000	± 0.004	± 0.000	± 0.001	± 0.002	± 0.003	± 0.004	± 0.004	± 0.003	± 0.000	± 0.004	± 0.003	± 0.003
P (Ht. Exc.)	1.000	0.590	0.349	0.983	0.816	0.902	0.797	0.459	0.922	0.160	0.259	0.792	0.803
± SE	± 0.000	± 0.004	± 0.002	± 0.001	± 0.002	± 0.003	± 0.004	± 0.004	± 0.003	± 0.002	± 0.004	± 0.003	± 0.003
Locus A8	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	7.788	9.950	1.316	4.378	5.302	11.933	8.541	2.432	4.957	4.121	4.000	5.333	4.314
Obs. Hm.	9	10	1	7	3	17	8	4	5	5	3	5	5
Exp. Ht.	35.212	40.051	8.684	14.622	16.698	56.067	47.460	16.568	30.044	12.879	27.000	26.667	21.686
Obs. Ht.	34	40	9	12	19	51	48	15	30	12	28	27	21
F_{IS}	0.035	0.001	-0.039	0.183	-0.142	0.091	-0.012	0.097	0.002	0.070	-0.038	-0.013	0.032
P (F_{IS})	0.442	0.572	0.791	0.106	0.934	0.069	0.651	0.216	0.571	0.384	0.798	0.665	0.419
P (HWE)	0.069	0.085	0.798	0.116	0.287	0.065	**0.040**	0.344	0.495	0.710	0.882	0.636	0.177
± SE	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.002	± 0.002	± 0.001	± 0.001	± 0.003	± 0.002
P (Ht. Def.)	0.329	0.299	0.780	0.130	0.863	0.001	0.507	0.240	0.428	0.482	0.647	0.646	0.373
± SE	± 0.003	± 0.001	± 0.001	± 0.001	± 0.001	± 0.000	± 0.003	± 0.002	± 0.002	± 0.001	± 0.002	± 0.003	± 0.002
P (Ht. Exc.)	0.671	0.703	0.513	0.888	0.159	0.999	0.494	0.773	0.575	0.646	0.391	0.373	0.645
± SE	± 0.003	± 0.001	± 0.002	± 0.001	± 0.001	± 0.000	± 0.003	± 0.002	± 0.002	± 0.001	± 0.002	± 0.003	± 0.002
Locus A113	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	5.965	17.495	2.474	4.730	6.767	23.874	11.649	3.216	5.478	2.727	4.492	6.984	4.667
Obs. Hm.	7	18	1	6	9	23	14	1	4	4	4	8	0
Exp. Ht.	37.035	32.505	7.526	14.270	15.233	44.126	44.351	15.784	29.522	14.273	26.508	25.016	21.333

Table 6. Continued.

Locus A113	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Obs. Ht.	36	32	9	13	13	45	42	18	31	13	27	24	26
F_{IS}	0.028	0.016	-0.209	0.091	0.150	-0.020	0.054	-0.145	-0.051	0.092	-0.019	0.041	-0.224
P (F_{IS})	0.367	0.501	0.958	0.304	0.146	0.683	0.226	0.985	0.836	0.274	0.707	0.401	1.000
P (HWE)	0.220	0.774	0.485	0.376	0.220	0.966	0.389	0.584	0.605	0.345	0.956	0.889	0.447
± SE	± 0.002	± 0.004	± 0.001	± 0.002	± 0.003	± 0.001	± 0.004	± 0.002	± 0.003	± 0.003	± 0.001	± 0.002	± 0.003
P (Ht. Def.)	0.557	0.631	0.954	0.427	0.134	0.632	0.474	0.983	0.336	0.325	0.597	0.469	1.000
± SE	± 0.003	± 0.004	± 0.000	± 0.002	± 0.002	± 0.005	± 0.003	± 0.001	± 0.004	± 0.003	± 0.003	± 0.004	± 0.000
P (Ht. Exc.)	0.452	0.394	0.189	0.638	0.902	0.391	0.533	0.097	0.680	0.729	0.414	0.536	0.003
± SE	± 0.003	± 0.004	± 0.001	± 0.002	± 0.002	± 0.005	± 0.004	± 0.001	± 0.004	± 0.003	± 0.003	± 0.003	± 0.000
Locus A79	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	4.659	9.121	2.790	4.162	4.140	18.452	8.063	2.568	3.696	1.273	2.623	2.476	2.980
Obs. Hm.	12	7	3	3	3	21	13	5	9	1	10	11	7
Exp. Ht.	38.341	40.879	7.211	14.838	17.861	49.548	47.937	16.432	31.304	15.727	28.377	29.524	22.020
Obs. Ht.	31	43	7	16	19	47	43	14	26	16	21	21	18
F_{IS}	0.193	-0.052	0.031	-0.081	-0.065	0.052	0.104	0.152	0.172	-0.018	0.263	0.292	0.186
P (F_{IS})	0.004	0.839	0.565	0.861	0.844	0.230	**0.034**	0.085	**0.009**	0.736	0.000	0.000	**0.014**
P (HWE)	0.002	0.794	0.594	**0.040**	0.534	0.667	0.242	0.125	**0.028**	0.264	**0.005**	0.000	0.182
± SE	± 0.000	± 0.002	± 0.001	± 0.001	± 0.002	± 0.005	± 0.004	± 0.003	± 0.001	± 0.002	± 0.000	± 0.000	± 0.003
P (Ht. Def.)	0.000	0.272	0.429	0.336	0.558	0.110	0.078	**0.028**	0.001	0.492	0.000	0.000	**0.015**
± SE	± 0.000	± 0.002	± 0.001	± 0.003	± 0.003	± 0.002	± 0.002	± 0.001	± 0.000	± 0.003	± 0.000	± 0.000	± 0.001
P (Ht. Exc.)	1.000	0.733	0.639	0.669	0.455	0.893	0.922	0.981	1.000	0.602	1.000	1.000	0.987
± SE	± 0.000	± 0.002	± 0.001	± 0.003	± 0.003	± 0.002	± 0.002	± 0.001	± 0.000	± 0.003	± 0.000	± 0.000	± 0.001
Locus A28	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	7.671	29.364	5.579	8.973	15.837	33.170	20.099	4.568	6.058	2.727	4.230	4.524	4.059
Obs. Hm.	8	31	4	9	18	30	26	6	7	4	5	6	3

Table 6. Continued.

Locus A28	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Ht.	35.329	20.636	4.421	10.027	6.163	34.830	35.901	14.432	28.942	14.273	26.771	27.476	21.941
Obs. Ht.	35	19	6	10	4	38	30	13	28	13	26	26	23
F_{IS}	0.009	0.080	-0.385	0.003	0.356	-0.092	0.166	0.102	0.033	0.092	0.029	0.055	-0.049
P (F_{IS})	0.471	0.330	1.000	0.594	0.059	0.895	**0.028**	0.259	0.407	0.286	0.425	0.308	0.790
P (HWE)	0.934	0.236	0.480	1.000	0.057	0.781	**0.026**	0.087	0.622	0.116	0.512	0.513	0.952
± SE	± 0.001	± 0.001	± 0.000	± 0.000	± 0.000	± 0.003	± 0.001	± 0.002	± 0.002	± 0.001	± 0.002	± 0.003	± 0.001
P (Ht. Def.)	0.554	0.342	1.000	0.589	0.056	0.427	**0.020**	0.325	0.557	0.103	0.464	0.389	0.756
± SE	± 0.003	± 0.001	± 0.000	± 0.001	± 0.000	± 0.004	± 0.001	± 0.003	± 0.002	± 0.001	± 0.002	± 0.003	± 0.001
P (Ht. Exc.)	0.454	0.694	0.347	0.598	0.975	0.615	0.979	0.746	0.473	0.910	0.564	0.620	0.300
± SE	± 0.003	± 0.001	± 0.000	± 0.001	± 0.000	± 0.004	± 0.001	± 0.002	± 0.003	± 0.001	± 0.002	± 0.003	± 0.002
Locus ED1	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	5.600	26.939	7.211	11.135	8.395	31.963	24.721	4.595	6.536	3.182	6.066	7.587	5.490
Obs. Hm.	1	25	7	11	10	35	24	3	5	2	3	9	6
Exp. Ht.	37.400	23.061	2.790	7.865	13.605	36.037	31.279	14.405	28.464	13.818	24.934	24.413	20.510
Obs. Ht.	42	25	3	8	12	33	32	16	30	15	28	23	20
F_{IS}	-0.125	-0.085	-0.080	-0.018	0.120	0.085	-0.023	-0.114	-0.055	-0.088	-0.125	0.059	0.025
P (F_{IS})	0.996	0.944	1.000	0.725	0.233	0.130	0.714	0.949	0.849	0.924	0.971	0.327	0.499
P (HWE)	0.526	0.882	1.000	0.472	0.365	0.100	0.631	0.730	0.559	0.963	0.245	0.382	0.489
± SE	± 0.002	± 0.004	± 0.000	± 0.002	± 0.002	± 0.004	± 0.006	± 0.003	± 0.005	± 0.001	± 0.004	± 0.004	± 0.003
P (Ht. Def.)	0.993	0.936	1.000	0.708	0.336	0.271	0.807	0.952	0.772	0.930	0.910	0.291	0.570
± SE	± 0.000	± 0.003	± 0.000	± 0.002	± 0.002	± 0.003	± 0.004	± 0.001	± 0.004	± 0.002	± 0.002	± 0.004	± 0.003
P (Ht. Exc.)	**0.014**	0.190	0.842	0.684	0.725	0.735	0.259	0.194	0.295	0.277	0.093	0.734	0.487
± SE	± 0.001	± 0.005	± 0.001	± 0.001	± 0.001	± 0.003	± 0.004	± 0.003	± 0.004	± 0.004	± 0.002	± 0.003	± 0.003
Locus IM	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Exp. Hm.	4.894	12.505	2.263	4.270	4.512	15.467	9.532	3.054	5.623	2.758	4.787	5.508	5.000

Table 6. Continued.

Locus IM	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Obs. Hm.	4	13	1	5	3	22	12	4	4	6	3	7	7
Exp. Ht.	38.106	37.495	7.737	14.730	17.488	52.533	46.469	15.946	29.377	14.242	26.213	26.492	21.000
Obs. Ht.	39	37	9	14	19	46	44	15	31	11	28	25	19
F_{IS}	-0.024	0.013	-0.174	0.051	-0.089	0.125	0.054	0.061	-0.056	0.233	-0.069	0.057	0.097
P (F_{IS})	0.800	0.477	0.946	0.427	0.864	**0.041**	0.234	0.369	0.850	**0.040**	0.889	0.271	0.212
P (HWE)	0.702	0.151	0.854	0.734	0.261	0.123	0.057	0.909	0.226	0.394	0.915	0.050	0.428
± SE	± 0.002	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.001	± 0.003	± 0.001	± 0.001	± 0.001	± 0.003
P (Ht. Def.)	0.737	0.580	0.945	0.507	0.637	**0.007**	**0.013**	0.361	0.498	0.052	0.816	0.146	0.136
± SE	± 0.002	± 0.002	± 0.000	± 0.001	± 0.001	± 0.000	± 0.000	± 0.002	± 0.003	± 0.001	± 0.002	± 0.002	± 0.002
P (Ht. Exc.)	0.282	0.426	0.254	0.521	0.372	0.993	0.987	0.684	0.503	0.956	0.198	0.856	0.892
± SE	± 0.002	± 0.002	± 0.001	± 0.001	± 0.001	± 0.000	± 0.000	± 0.002	± 0.003	± 0.001	± 0.002	± 0.002	± 0.002
Across Loci	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
P (HWE)	0.013	0.562	0.994	0.190	0.059	0.212	0.003	0.002	0.109	0.748	0.142	0.005	0.371
P (Ht. Def.)	0.001	0.907	0.971	0.368	0.457	0.000	0.003	0.007	0.000	0.156	0.246	0.000	**0.041**
± SE	± 0.000	± 0.002	± 0.000	± 0.002	± 0.003	± 0.000	± 0.000	± 0.000	± 0.000	± 0.002	± 0.004	± 0.000	± 0.001
P (Ht. Exc.)	0.999	0.091	**0.029**	0.625	0.545	1.000	0.997	0.992	1.000	0.842	0.765	1.000	0.960
± SE	± 0.000	± 0.002	± 0.000	± 0.002	± 0.003	± 0.000	± 0.000	± 0.000	± 0.000	± 0.002	± 0.004	± 0.000	± 0.001

P-values marked in bold are not significant following sequential Bonferroni correction ($\alpha = 0.05$).

Table 6). Individual and multilocus U-tests for heterozygote deficiency and heterozygote excess yielded a single significant value at A107 (P = 0.036 for heterozygote excess; Table 6), which became non-significant following sequential Bonferroni correction (α = 0.05, k = 12). In contrast, the Brazilian reference sample showed a deviation from HWE ($P_{multilocus}$ = 0.013), caused by a deficiency of heterozygotes (U test; $P_{multilocus}$ = 0.001) across all loci. Single-locus P-values obtained with Fisher's exact test, U tests, and/or F_{IS} revealed a deficiency of heterozygotes at A88, A14, A35, and A79 and an excess of heterozygotes at ED1 (Table 6). However, when applying sequential Bonferroni correction (α = 0.05, k = 12) only A35 and A79 exhibited a significant deficiency of heterozygotes ($P_{single\ locus}$ = 0.000; Table 6). Fisher's exact tests for genotypic linkage disequilibrium between loci-pairs produced eight (four for European and four for Brazilian reference sample) significant P-values out of 132 comparisons. None of the loci-pairs were shared by the two populations and none of the P-values was significant following sequential Bonferroni's correction (α = 0.05, k = 66 for each of the reference samples; see Appendix 1 for P-values).

In the Welder Wildlife Refuge population, significant departures from HWE across all loci were detected in 1995, 1996, and 2000 (0.002 ≤ $P_{multilocus}$ ≤ 0.005; Table 6). These deviations were caused by a deficiency of heterozygotes (0.0001 ≤ $P_{multilocus}$ ≤ 0.007; Table 6). In addition to those years, multilocus U tests revealed a deficiency of heterozygotes in 1994, 1997, and 2001 (0.000 ≤ $P_{multilocus}$ ≤ 0.041; Table 6) and an excess of heterozygotes in 1991 ($P_{multilocus}$ = 0.029; Table 6). However, when applying

the sequential Bonferroni procedure neither 1991 nor 2001 Welder Wildlife Refuge population samples deviated from HWE ($\alpha = 0.05$, $k = 11$). At the single locus level, departures from HWE occurred at all loci but ED1 for at least one of the sampling years, as indicated by Fisher's exact test, U tests, and/or F_{IS} P-values (Table 6). The greatest number of significant P-values was observed in 1995 (eight values of $P < 0.05$ given by Fisher's exact test, U tests, and F_{IS}) and 1996 (eight values of $P < 0.05$ given by Fisher's exact test, U tests, and F_{IS}). The majority of the 16 P-values was marginally significant and subsequently became non-significant when corrected for type I error ($\alpha = 0.05$, $k = 12$; Table 6). Across all years, locus A79 exhibited the most consistent deficiency of heterozygotes (deficiency of heterozygotes was detected by Fisher's exact test, U test, and/or F_{IS} in 1995, 1996, 1997, 1999, 2000, and 2001; Table 6). The hypothesis of null alleles at this locus was dismissed because no heterozygote deficiency was detected in the reference European and early Welder Wildlife Refuge population samples. However, it was detected in the reference Brazilian. Because deficiency of heterozygotes seems to be correlated with Africanization, this locus deserves further investigation. Fisher's exact tests for linkage disequilibrium yielded 46 significant values out of 704 comparisons (35 significant values are expected at the 5 % level). Most significant values were in the 1995 (22) and 1994 (9) population samples (Fig. 9). When applying sequential Bonferroni correction ($\alpha = 0.05$, $k = 66$ for all years but 1991 and 1993 when $k = 55$), only five (one in 1994 and four in 1995) of the 46 above-mentioned P-values were significant (see Appendix 2 for P-values). Two pair of loci, A28/ED1 (mapping distances = 23 cM) and A7/A14 ED1 (mapping distances = 22 cM),

are physically linked, while the eight remainder loci were unlinked (M. Solignac, pers. comm. 2003). However, all 12 loci behaved similarly and no single locus pair produced consistent significant values for all years after population mixing (Appendix 2). Across the 11-year study, the mean *P*-value was lowest in 1994 and 1995. *P*-values obtained from 1996 onwards suggested a rapid dissipation of linkage disequilibrium (Fig. 9).

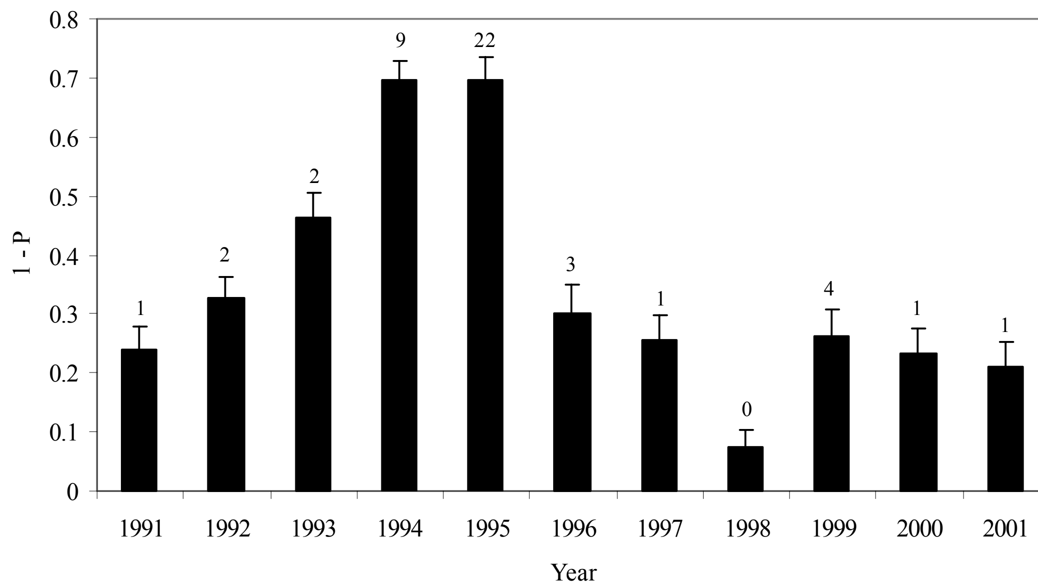

Fig. 9. Temporal pattern of genotypic linkage disequilibrium among 12 microsatellite loci in the feral honey bee population of the Welder Wildlife Refuge. $1 - P$ (where P is the average value of Fisher's exact tests for each locus pair), and their standard errors are given for each sampling year. The number of significant P values (not corrected for type I error), provided by Fisher's exact tests for each locus pair, is shown above the error bars for each sampling year.

Genetic Differentiation

The Brazilian, and European reference population samples and the yearly Welder Wildlife Refuge population samples were compared to ascertain if they were genetically distinct. Multilocus distance measures (D_{PS} and F_{ST}), and tests for homogeneity of allelic and genotypic distributions, showed a high level of differentiation between Brazilian and European reference samples ($P_{multilocus} < 0.000$ for genic differentiation, genotypic differentiation, and F_{ST}; Table 7, Table 8). Pairwise comparisons between reference European and Welder Wildlife Refuge samples revealed an increase in genic and genotypic differentiation over time, with F_{ST} values becoming significantly different from zero since 1995. In contrast, pairwise comparisons between reference Brazilian and Welder Wildlife Refuge population samples showed a decrease in differentiation over time. In spite of the trend, all pairwise comparisons yielded significant values, suggesting that the Welder Wildlife Refuge population was genetically distinct from the Brazilian (Table 7, Table 8). Multilocus distance measures and tests for homogeneity of allelic and genotypic distributions indicated that the Welder Wildlife Refuge population samples could be divided into pre- and post-1996 populations. Between 1991 and 1994 pairwise comparisons for allelic and genotypic distributions were non-significant; and pairwise multilocus F_{ST} values were not significantly different from zero. The same pattern was shown by pairwise comparisons performed between 1997 and 2001 samples. In contrast, pre-1996 versus post- 1996 sample comparisons showed a high level of differentiation suggesting that a profound genetic change occurred in a short time frame (Table 7, Table 8). A neighbor-joining tree confirmed this pattern. Until 1996, the

Table 7. *P*-values for genotypic (above diagonal) and genic (below diagonal) differentiation between pairs of reference (Braz. - Africanized from Brazil; Eur. - European from South Texas) and Welder Wildlife Refuge (from 1991 to 2001) honey bee population samples.

	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Braz.		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Eur.	0.0000		0.2601	**0.0039**	0.0005	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1991	0.0000	0.2204		0.3751	**0.0182**	0.8989	0.2523	0.0011	0.0000	0.0000	0.0000	0.0000	0.0000
1992	0.0000	**0.0028**	0.4668		0.1819	0.7963	**0.0094**	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1993	0.0000	0.0003	0.1010	0.1418		0.8130	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1994	0.0000	0.0001	0.9297	0.8414	0.8660		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1995	0.0000	0.0000	0.8650	0.1644	**0.0045**	0.0000		**0.0282**	0.0000	0.0000	0.0000	0.0000	0.0000
1996	0.0000	0.0000	**0.0156**	0.0000	0.0000	0.0000	0.0005		0.3519	0.0018	0.0001	**0.0262**	**0.0063**
1997	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000	0.0000	0.1379		0.1131	0.0911	0.4959	**0.0219**
1998	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0008	0.1930		0.2181	0.3220	0.1723
1999	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0501	0.4035		0.5132	0.0522
2000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	**0.0046**	0.1776	0.3057	0.4566		0.5245
2001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0012	**0.0079**	0.1371	**0.0314**	0.3170	

P-values marked in bold are not significant following sequential Bonferroni correction ($\alpha = 0.05$, k = 78).

Table 8. Proportion of shared allele distance (above diagonal) and multilocus estimates of F_{ST} (below diagonal) between pairs of reference (Braz. - Africanized from Brazil; Eur. - European from South Texas) and Welder Wildlife Refuge (from 1991 to 2001) honey bee population samples.

	Braz.	Eur.	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
Braz.		0.6670	0.6900	0.6820	0.6520	0.6230	0.4960	0.4850	0.3940	0.4250	0.3660	0.3280	0.3540
Eur.	0.1405		0.2570	0.2340	0.2220	0.1780	0.2780	0.4060	0.4040	0.4910	0.4960	0.5170	0.4850
1991	0.1319	**0.0060**		0.2410	0.2890	0.2600	0.3390	0.4460	0.4770	0.5670	0.5550	0.5510	0.5450
1992	0.1176	**0.0079**	**-0.0014**		0.2210	0.2020	0.2830	0.4100	0.4370	0.5080	0.5100	0.5080	0.4930
1993	0.1213	**0.0088**	**0.0148**	**0.0025**		0.2010	0.2830	0.3950	0.4470	0.4940	0.5070	0.5010	0.4760
1994	0.1225	**0.0053**	**0.0040**	**0.0009**	**0.0017**		0.2160	0.3460	0.3740	0.4710	0.4660	0.4700	0.4470
1995	0.0719	0.0181	**0.0148**	**0.0068**	**0.0118**	0.0079		0.2920	0.2900	0.4020	0.3610	0.3580	0.3550
1996	0.0454	0.0420	**0.0397**	0.0310	0.0326	0.0273	**0.0071**		0.3090	0.3850	0.3630	0.3440	0.3360
1997	0.0277	0.0561	0.0549	0.0464	0.0548	0.0472	0.0191	**0.0025**		0.2940	0.2610	0.2570	0.2810
1998	0.0284	0.0768	0.0796	0.0657	0.0692	0.0686	0.0322	**0.0104**	**0.0001**		0.2770	0.3090	0.3220
1999	0.0199	0.0793	0.0773	0.0654	0.0690	0.0686	0.0304	0.0127	**0.0018**	**-0.0028**		0.2460	0.2730
2000	0.0142	0.0866	0.0811	0.0649	0.0726	0.0722	0.0309	**0.0116**	**0.0047**	**0.0006**	**-0.0006**		0.2570
2001	0.0182	0.0755	0.0776	0.0620	0.0630	0.0617	0.0264	**0.0089**	**0.0038**	**0.0030**	**0.0025**	**-0.0011**	

F_{ST} values marked in bold are not significantly different from zero following sequential Bonferroni correction ($\alpha = 0.05$, k = 78).

Welder Wildlife Refuge samples were placed with the reference European sample. After 1996, the Welder Wildlife Refuge population samples were clustered with the reference Brazilian population (Fig. 10).

Admixture Proportions and Associations of Nuclear and mtDNA Markers

The nuclear genetic contribution of *A. m. scutellata* to the Welder Wildlife Refuge population was estimated by m_R (Roberts and Hiorns 1965) and m_Y (Bertorelle and Excoffier 1998) using the reference European and Brazilian as parental populations. The least-squares estimator m_R compares allele frequencies between parental and admixed populations and assumes identity by descent. The estimator m_Y uses a coalescent approach with the squared difference in allele size equal to molecular distance (Bertorelle and Excoffier 1998). Both estimators revealed a striking change in the admixture proportions of the Welder Wildlife Refuge population over time (Fig. 11). For all years, m_Y estimated a greater contribution of the parental Brazilian to the Welder Wildlife Refuge population than m_R (paired t-test, $P = 0.000$). The proportion of *A. m. scutellata* introgressed alleles in the Welder Wildlife Refuge population increased from 2 - 11 % in 1991 to 63 - 68% in 2001 with m_R and m_Y estimator respectively. The steepest increase in the proportion of introgressed *A. m. scutellata* alleles occurred between 1994 (m_R = 9.6 %; m_Y = 12.1 %) and 1997 (m_R = 52.5 %; m_Y = 63.4 %). From 1998 on, the nuclear proportion of *A. m. scutellata* genes in the Welder Wildlife Refuge population appeared to have stabilized at 62.8 % ± 0.042 (SD) for m_R and 74.7 ± 0.043 (SD) for m_Y. Interestingly, the temporal pattern of introgression of nuclear genes was

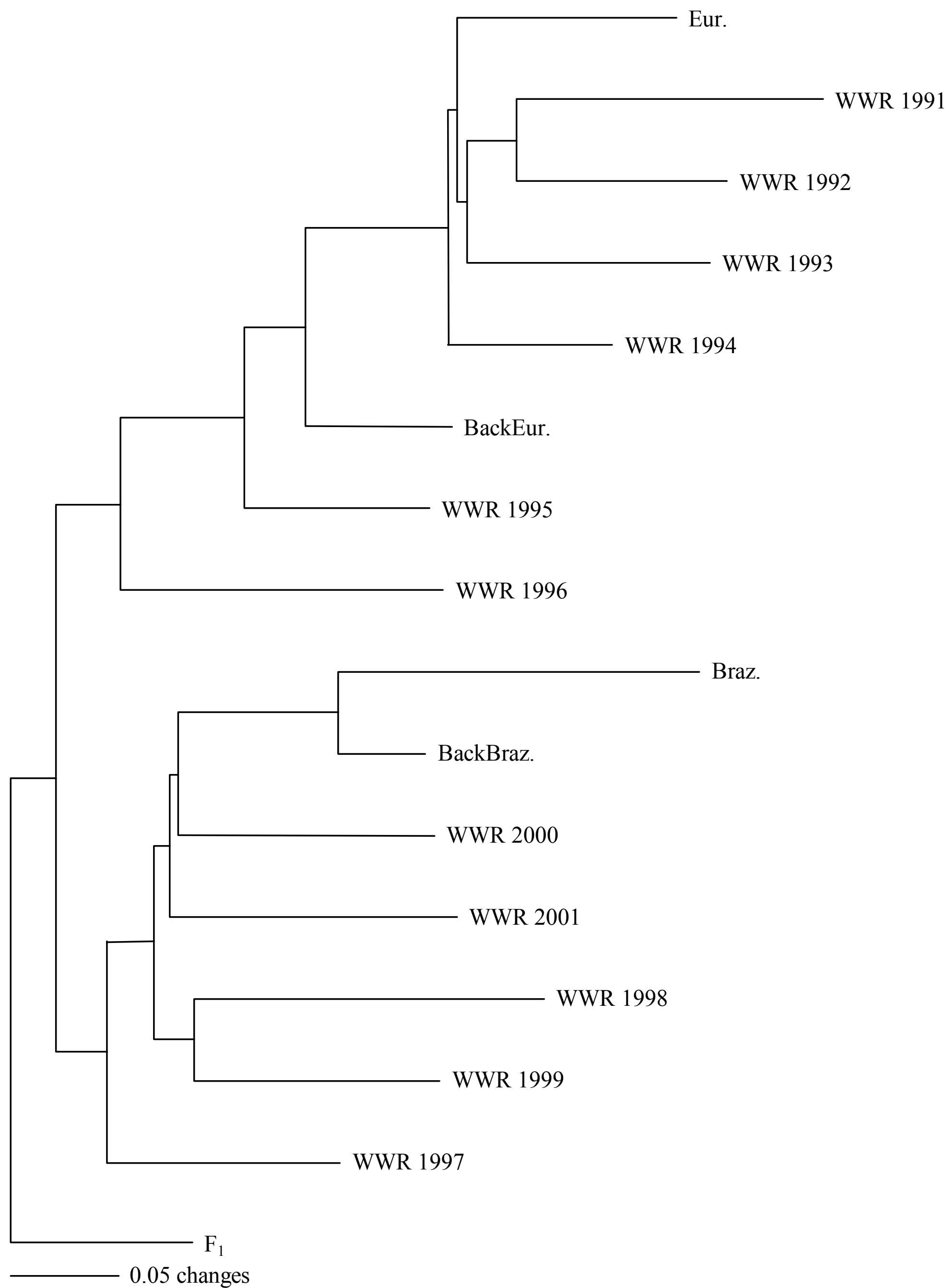

Fig. 10. Rooted neighbor-joining tree constructed from proportion of shared alleles distance for reference and temporal Welder Wildlife Refuge (WWR) population samples. The reference population samples are: European (Eur.), Brazilian (Braz.), simulated backcross with European (BackEur.), simulated F_1, and simulated backcross with Brazilian (BackBraz.). Each simulated reference sample comprises 100 randomly generated individuals.

paralleled by *A. m. scutellata* mitotype (Fig. 11; see Chapter IV for mtDNA).

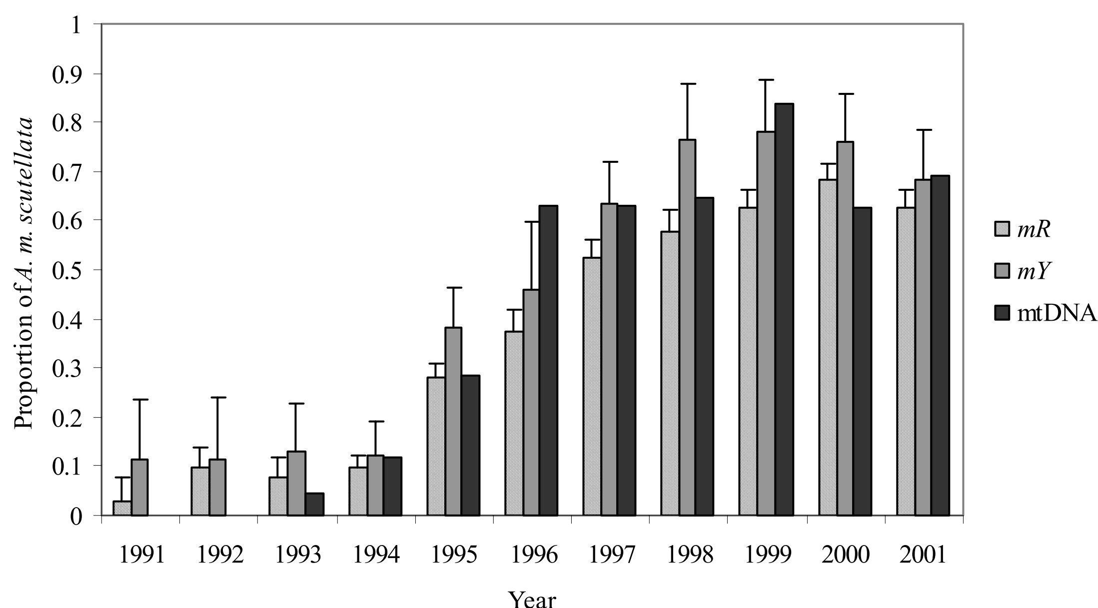

Fig. 11. Proportions of introgressed *A. m. scutellata* nuclear alleles in the Welder Wildlife Refuge population over time. Nuclear admixture bootstrap average and standard deviation were computed over 1000 iterations using the gene frequency-based estimator m_R and the gene frequency and molecular-based estimator m_Y. The temporal frequency of *A. m. scutellata* mitotype (mtDNA) is also shown (see Chapter IV for details).

To better understand the temporal pattern of genetic composition of the Welder Wildlife Refuge population, individual honey bees were classified according to their nuclear genotypes using the Bayesian method available in GENECLASS. Among the assignment methods that were tested, Bayesian showed the best performance with the highest rate (68.3 %) of individuals correctly assigned (Table 9). Cornuet et al. (1999) also found this method to be the most accurate even when the assumptions of HWE and

linkage equilibrium were violated. The percentage of misclassification given by the Bayesian method (and the other methods tested, data not shown) varied across types of simulated individuals (Fig. 12). More than 96 % of the simulated European and Brazilian individuals were correctly assigned whereas less than 50 % of backcross individuals were correctly assigned. Figure 13 shows the average assignment score obtained for simulated individuals. The highest assignment scores were produced by reciprocal backcross (19.04 ± 2.77 for European backcross; 22.15 ± 2.39 for Brazilian backcross) and F_1 (21.11 ± 2.45) individuals whereas the lowest were produced by European (13.64 ± 1.75) and Brazilian (20.03 ± 1.49) individuals. As crosses become more complex, the overlap among scores increases (Fig. 13) and the power of assignment test decreased.

Table 9. Percentage of individuals misclassified using different assignment methods.

Assignment method	% Misclassification
Likelihood	
Bayesian[a]	31.7 %
Frequency[b]	34.4 %
Distance	
Cavalli-Sforza[c]	48.8 %
Goldstein[d]	66.2 %

[a.] Modified by Cornuet et al. (1999) from Rannala and Mountain (1997).
[b.] Modified by Cornuet et al. (1999) from Paetkau et al. (1995).
[c.] Modified by Cornuet et al. (1999) from Cavalli-Sforza and Edwards (1967) chord distance.
[d.] $(\delta\mu)^2$ distance of Goldstein et al. (1995).

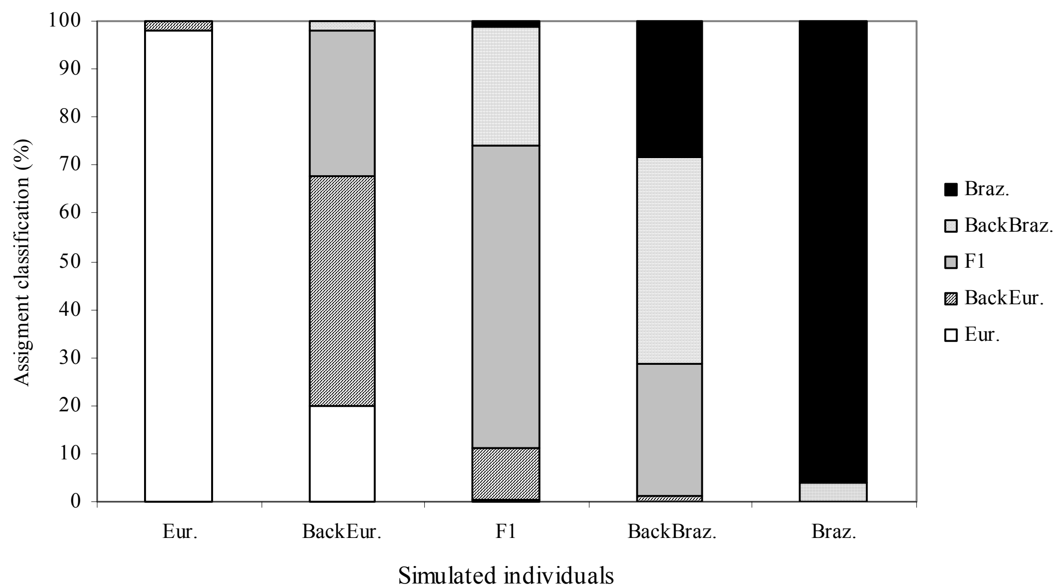

Fig. 12. Proportion of simulated individuals assigned to the reference European (Eur.), Brazilian (Braz.), simulated European backcross (BackEur.), simulated F_1, and simulated Brazilian backcross (BackBraz.) using the Bayesian method. Multilocus genotypes of European, Brazilian, F_1, European backcross, and Brazilian backcross individuals (1000 of each type) were simulated by crossing European x European, Brazilian x Brazilian, European x Brazilian, F_1 x European, and F_1 x Brazilian respectively. Each set of 1000 simulated individuals was then assigned over 100 sets of simulated reference samples.

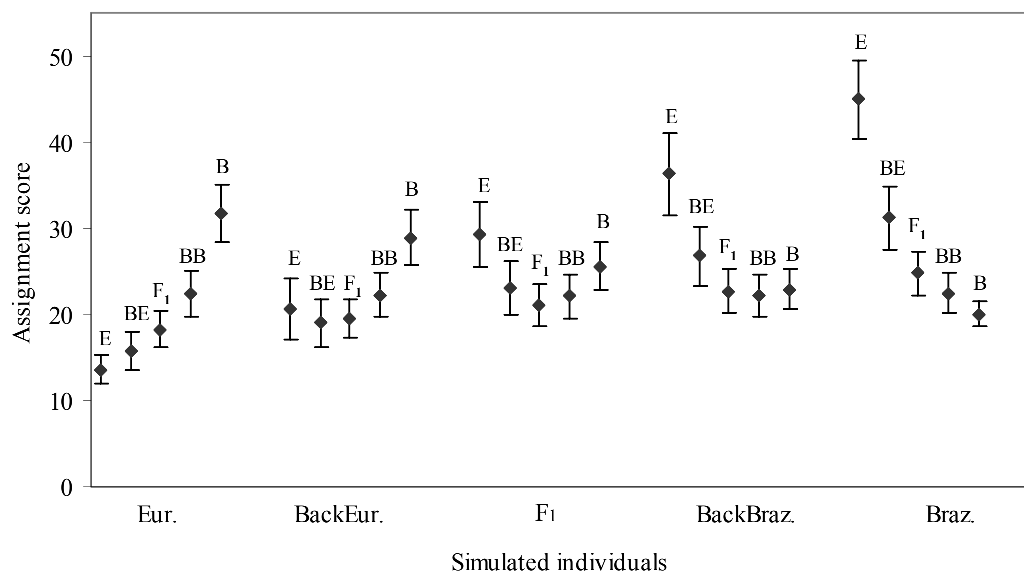

Fig. 13. Average assignment scores and their standard deviations for simulated European (Eur.), European backcross (BackEur.), F_1, Brazilian backcross (BackBraz.), and Brazilian (Braz.) individuals. Assignment scores, given for each reference sample (E – European; BE - backcross with European; F_1; BB - backcross with Brazilian; B – Brazilian), were produced by the Bayesian method. Each individual score was computed over 100 sets of simulated reference samples.

The average assignment score obtained for Welder Wildlife Refuge population over time is shown in Figure 14. A steep increase occurred between 1994 and 1996, suggesting an increase in complexity of multilocus genotypes. After 1997, the average assignment score stabilized at high values. Comparisons of ranges of the assignment scores for simulated (Fig. 13) and Welder Wildlife Refuge individuals (Fig. 14) suggest crosses existed that were more complex than simple backcrosses.

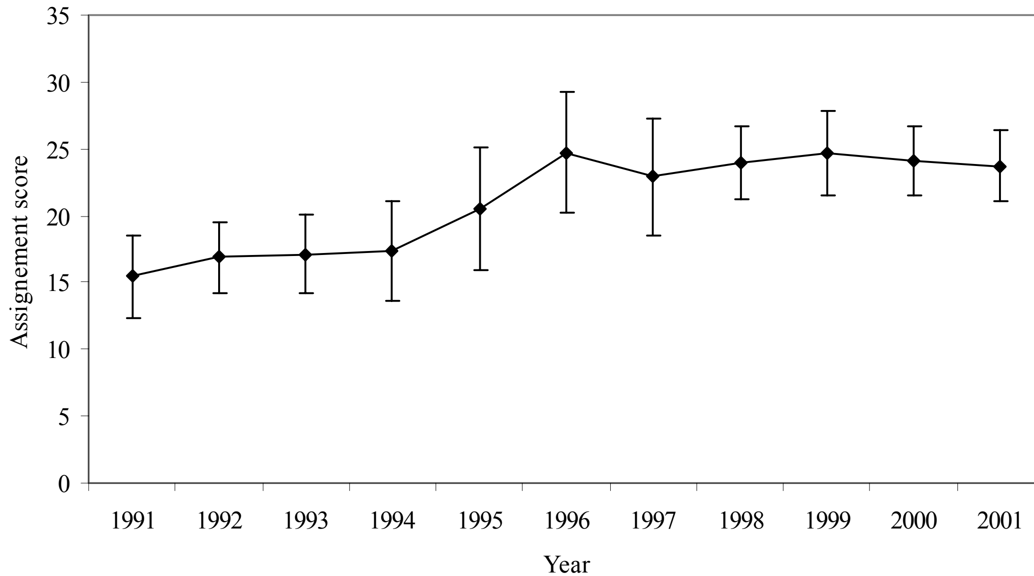

Fig. 14. Average assignment scores and their standard deviations for the temporal Welder Wildlife Refuge population sample. Using the Bayesian method, individual honey bees were assigned to one of the five reference samples (European, Brazilian, simulated European backcross, simulated F_1, and simulated Brazilian backcross). For each individual honey bee, the average value was obtained over 100 data sets of the simulated reference samples of 50 individuals each.

The proportion of individuals of eastern European, western European*, A. m. lamarckii*, and *A. m. scutellata* mitotypes that were genotypically closer to European, Brazilian, F_1, European backcross, or Brazilian backcross reference population changed radically in Welder Wildlife Refuge over time (Fig. 15). Prior to 1994 most individuals exhibited non-*A. m. scutellata* mitotype (see Chapter IV) and were classified either as European or (less frequently) as European backcrosses. Between 1994 and 1996, individuals of non-*A. m. scutellata* maternal descent were genotypically more homogeneous than were those with *A. m. scutellata* mitotype. An association between nuclear and mtDNA genes of non-*A. m. scutellata* origin was apparent (Fig. 15).

Indeed, significant heterogeneity in genic and genotypic distributions between both groups of mitotypes was found in the 3-year period ($0.000 \leq P_{multilocus} \leq 0.005$ when mitotypes of eastern European, western European, and *A. m. lamarckii* origin were pooled to increase our sample size for added statistical power). However, a breakdown of non-*A. m. scutellata* cytonuclear associations appeared to have occurred as no significant differences in allelic and genotypic distributions were found from 1997 onward ($0.117 \leq P_{multilocus} \leq 0.932$; mitotypes of eastern European, western European, and *A. m. lamarckii* origin were pooled). After 1997, most individuals exhibited a mixed ancestry and no observed association between mitotype and nuclear composition.

Discussion

Our mitochondrial and nuclear surveys of the feral honey bee population from Welder Wildlife Refuge showed a rapid and drastic change in the genetic structure of the population over time (see Chapter IV for mtDNA). Within five years following detection of the first migrant colony of *A. m. scutellata* maternal lineage, the resident European honey bees were replaced by panmitic admixtures of *A. m. scutellata* and European genes. We suggest that collapse of the Welder Wildlife Refuge resident population during early stages of Africanization likely hastened the demise of “pure” European bees, and had a major role in restructuring the post-Africanization population.

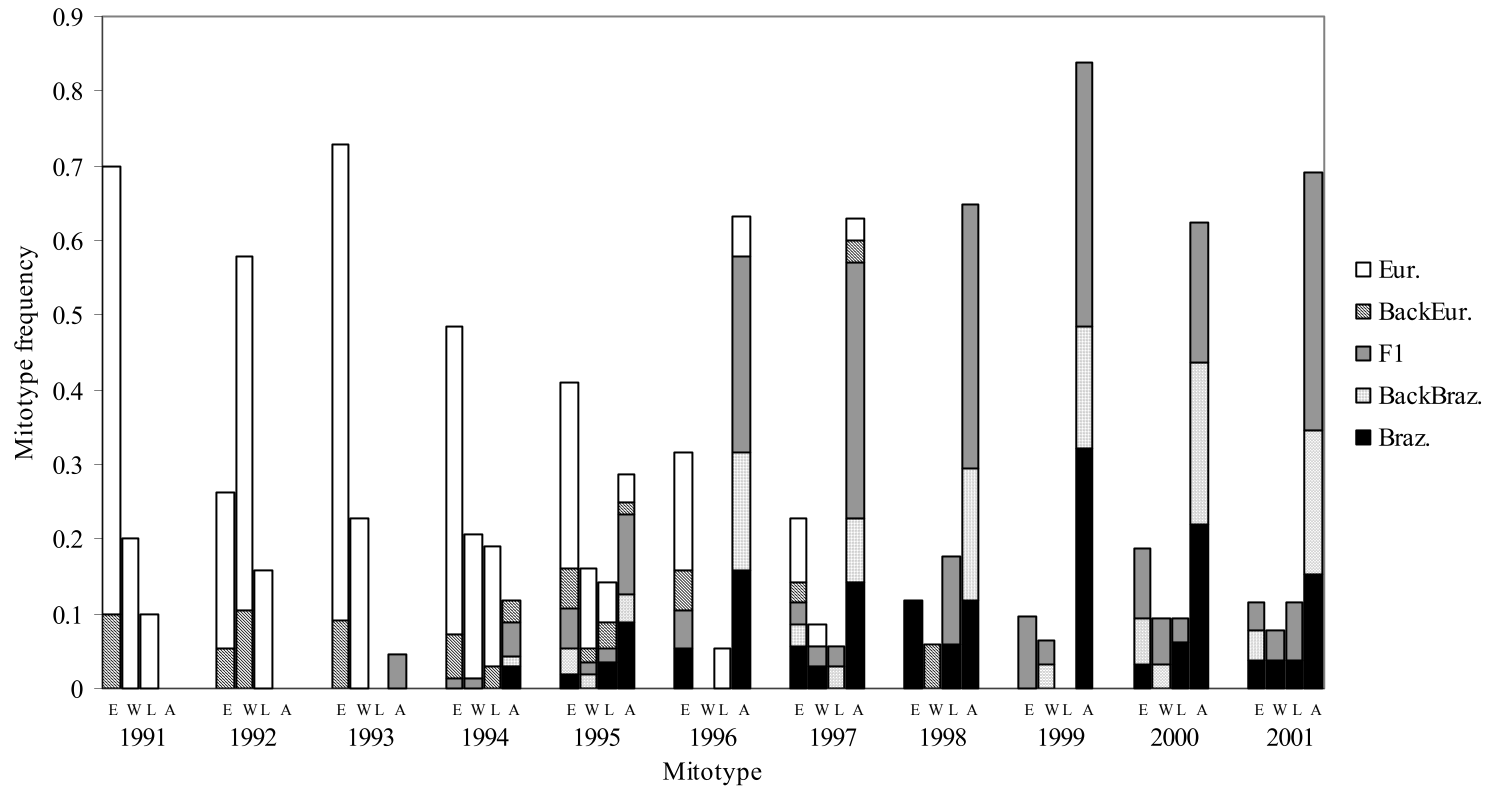

Fig. 15. Associations of nuclear and mitochondrial markers. Proportion of Welder Wildlife Refuge honey bees assigned to the reference European (Eur.), Brazilian (Braz.), simulated backcross with European (BackEur.), simulated F_1, and simulated backcross with Brazilian (BackBraz.) per mitotype (E – eastern European; W – western European; L – *A. m. lamarckii*; A – *A. m. scutellata*; see Chapter IV) across time. Bayesian assignment classification was computed over 100 replications of simulated population samples. The assignment of each individual was based on the predominant assignment given for the 100 replications. The simulated reference samples represent a subset of a vast array of possible recombinant classes. It should be noted that individuals possessing recombinant genotypes rather than F_1 and reciprocal backcrosses would be assigned to one of the five reference populations as the Bayesian method always designates a population of origin.

Mechanism of Africanization

Initial colonization

Mitochondrial data indicated 1993 as the time of Africanization onset in the Welder Wildlife Refuge (see Chapter IV), although estimates of nuclear racial admixture revealed a 6 to 11 % (depending on the estimator) contribution of non-European alleles prior to 1993. Assuming that the estimators of admixture were correct, the result suggests that Africanization could have started earlier, through matings of migrant drones with resident European queens. Alternatively, the African component in the Welder Wildlife Refuge feral honey bees nuclear genome could have been derived from the Egyptian *A. m. lamarckii* introduced into the United States in the 19th century (Schiff and Sheppard 1993; Schiff et al. 1994; McMichael and Hall 1996). Given the presence of colonies of *A. m. lamarckii* maternal ancestry in the Welder Wildlife Refuge population this is a reasonable assumption. Thus, it appears likely that *A. m. lamarckii*-derived genes accounted for the early 6 – 11 % Africanization. In a study of managed colonies from Yucatan a 6 % nuclear African background was also reported prior to Africanization (Clarke et al. 2002). Further, *A. m. scutellata* genes moving into the European Welder Wildlife Refuge population through paternal gene flow should produce a consistent excess of heterozygotes. Even if some paternal gene flow had preceded migration of swarms, considering that significant changes in the nuclear DNA composition were closely paralleled by mitochondrial DNA (Fig. 11), we suggest that range expansion of the Africanized honey bees into Welder Wildlife Refuge occurred

primarily by maternal gene flow. The finding supports the claim that maternal migration is the primary driving force of Africanization in the American tropics (Taylor 1988; Hall and Muralidharan 1989; Smith et al. 1989). However, in contrast with the neotropical virtually "pure African" expanding front (Taylor 1988; Hall and Muralidharan 1989; Smith et al. 1989), migrants arriving at Welder Wildlife Refuge appeared to be of mixed ancestry (Fig. 15).

Genetic disequilibrium

Detection of the first *A. m. scutellata* matrilines was followed by a four-year period of Hardy-Weinberg disequilibrium caused by a strong deficiency of heterozygotes. Hardy-Weinberg disequilibrium was anticipated upon arrival of migrants as mixing of distinct gene pools would cause a Wahlund's effect. While dissipation of the Wahlund effect is expected to occur after one generation of random mating, the persistence of heterozygote deficiency for four years (1994 - 1997) could have been due to: (1) positive assortative mating, (2) continuing influx of genetically distinct migrants, and/or (3) selection. In early reports of Africanization, positive assortative mating, due to asynchrony in mating flight times, was suggested as an explanation for apparent partial reproductive isolation between European and Africanized honey bees (reviewed in Michener 1975). This hypothesis has long been disregarded as findings of associations between European and African mitotypes with a range of morphological and allozymic phenotypes demonstrated interbreeding between the honey bee types (Rinderer et al. 1991; Sheppard et al. 1991a). Although we cannot ascertain whether

mating was random during early stages of Africanization in Welder Wildlife Refuge, at least there is evidence that it was not completely assortative. The pattern depicted in Figure 15 clearly shows that bi-directional gene flow was occurring. As a result of gene exchange, associations between European mitotype and European nuclear classification gradually decayed (Fig. 15). By 1997 there were no nuclear multilocus genotypic and genic differences between non-*A. m. scutellata* and *A. m. scutellata* mitotypes.

The frequency of *A. m. scutellata* mitotype increased between 1993 and 1995 from 4.5 to 28.6 (Chapter IV). This could have been due to "in situ" reproduction of migrants, immigration of additional Africanized swarms, or both. It is likely that movement of additional Africanized colonies occurred during that period further contributing to heterozygote deficiency. Between 1995 and 1997 the Welder Wildlife Refuge honey bee population collapsed (Chapter IV). *Varroa* mite, which is considered one of the most destructive pests of honey bee colonies worldwide, was first detected in Welder Wildlife Refuge in early 1995 (Rubink et al. 1995). Given the coincidence of *Varroa* mite arrival with a drastic decline in number of colonies living in tree cavities, it seems reasonable to assume that the parasite had a major role on the mortality event. Differences in *Varroa* susceptibility between Africanized and European honey bees have been suggested (De Jong and Gonçalves 1981; Moretto et al. 1991; 1993; Message and Gonçalves 1995; Guzman-Novoa et al. 1996, 1999). In a crossbreeding experiment between Africanized (identified through morphometrics and mtDNA) and European bees, Guzman-Novoa et al. (1996) found differential susceptibility of brood and adult workers to *Varroa* mite. The authors reported first that susceptibility to becoming

infested by *Varroa* was least in Africanized brood followed by European, F_1 of European mother, and F_1 of Africanized mother; and second that adult European worker honey bees were more likely to become infested with *Varroa* than were adult Africanized honey bees; whereas hybrid bees were infested at a rate not different from that of the Africanized honey bees. Susceptibility of later generation hybrids was not reported but it is possible that there is differential response according to type of cross. Between 1995 and 1997, at the same time that the total number of colonies of non-*A. m. scutellata* maternal ancestry dramatically decreased, *A. m. scutellata* experienced an increase (Chapter IV). This result suggests that the collapse of the Welder Wildlife Refuge population occurred at the expense of colonies of non-*A. m. scutellata* maternal origin. However, we cannot ignore the fact that the outcome might have resulted not only from the effect of *Varroa* but also from the effect of further immigration. Because discrimination between migrants of first and later generations from migrants recently arrived is not possible, whether the number of *A. m. scutellata*-derived colonies increased as a result of "in situ" reproduction of survivals, immigration, or both is unknown. In the same way, it is unknown whether migrants descending from crosses of European queens with Africanized drones inflated the number of European colonies. In summary, because of differential susceptibility of honey bees to *Varroa* and because the Welder Wildlife Refuge population was hit at an early stage of Africanization, when European and likely F_1 individuals were abundant, we suggest that differential selection pressures possibly combined with ongoing influx of migrants and some assortative mating might have contributed to the observed disequilibrium in 1996 and 1997.

In addition to a one generation Hardy-Weinberg disequilibrium, mixing of divergent populations may cause multilocus or "linkage" disequilibrium. Further, while one generation of random mating is all that is needed to approach Hardy-Weinberg equilibrium, linkage equilibrium typically requires several generations with unlinked loci and many more generations for linked loci. Additionally, the rate of approach is slowed by departure from random mating (Hartl and Clark 1997). The temporal pattern of linkage disequilibrium experienced by the Welder Wildlife Refuge population, as given by the average *P*-values of Fisher's exact test, is shown in Figure 9. Contrary to expectations, linkage disequilibrium decayed more rapidly than heterozygote deficiency. This circumstance is even more striking given that two pairs of loci are physically linked (A28/ED1 and A7/A14; M. Solignac, pers. comm. 2003). This seemingly paradoxical pattern could be an artifact of the analysis. It is possible that Fisher's exact test for linkage disequilibrium is less powerful than the U-test for heterozygote deficiency in detecting small deviations from expectations. Reduced statistical power for the Fisher's exact tests is not unexpected given that European and *A. m. scutellata* subspecies share relatively common alleles at several loci (Table 5 of this study; Estoup et al. 1995, Clarke et al. 2002) and given the small sample size (specially from 1996 onward), which is aggravated by high levels of polymorphism. Nonetheless, given that Hardy-Weinberg proportions were reached in 1998 and linkage disequilibrium was apparently decaying over time, heterozygotes (intermating between migrants and resident honey bees) must have been formed in sufficient numbers for the equilibrium to be approached.

Mechanism of population turnover

In the American tropics, feral colonies of Africanized honey bee that exhibit little or no introgression of European genes are reported to have built up to high densities within two to three years after initial colonization (Taylor 1985). It has been suggested that rapid expansion of a nearly "pure African" population has resulted from (1) high fitness of Africanized honey bees exhibiting virtually no introgression of European alleles (2) reduced competition from a small and poorly adapted European population, and (3) cytonuclear incompatibility in hybrids of European mothers (Taylor 1985, 1988; Rinderer et al. 1991; Sheppard et al. 1991a; Harrison and Hall 1993).

The Welder Wildlife Refuge lies in a subtropical region and supported a large density of European colonies upon the arrival of the first wave of migrant Africanized swarms (11.7 colonies per km^2 in 1995; Baum 2003). Given these conditions, our prediction was that rate and extent of Africanization in the Welder Wildlife Refuge would be lower than in the tropics. We expected (1) superior fitness of European honey bees in more temperate climatic and ecological conditions (2) higher frequency of matings with European honey bees due to the larger resident population size, and (3) superior competition of feral European colonies for limited food and nest resources. Contrary to expectations, in less than four years after initial invasion, European honey bees were replaced by Africanized honey bees exhibiting varying degrees of introgressive hybridization. In the tropical Yucatan peninsula (which also supported a large managed, but not feral, European population prior to Africanization), the managed population was still largely European three to four years after initial colonization

(Rinderer et al. 1991; Quezada-Euán et al. 1996;Clarke et al. 2002). Complete and rapid replacement of resident European honey bees by Africanized honey bees would be expected if the latter exhibited higher fitness than the former. Under this scenario "pure" European honey bees would be extirpated through competitive exclusion and genetic assimilation. Alternatively, if European honey bees were equally or more fit than Africanized honey bees then depletion of European colonies (e.g. through mortality caused by biotic or abiotic factors) could lead to their replacement (Arnold 1997). Whether European honey bees were equally or better fit than Africanized honey bees in the ecological and climatic conditions (pre-*Varroa* mite collapse) of southern Texas is unknown. Regardless of the relative fitnesses in a *Varroa*-free environment, Africanized honey bees were likely more fit than European if *Varroa* mite is taken into account. This would explain rapid Africanization and the rapid recovery of the feral population after collapse (see Chapter IV). Freed of competition for limiting food and nest resources, range expansion of the Africanized population, likely more tolerant to *Varroa* mite, would be greatly facilitated. Eventually, the residual European population would be genetically assimilated by the expanding Africanized population. Therefore, we suggest that the nearly coincidental arrival of *Varroa* mite with expansion of the Africanized front might have hastened the demise of "pure" European honey bees and might have had a major role in restructuring the post-Africanization Welder Wildlife Refuge population. Had the resident European population not collapsed, the rate of Africanization in Welder Wildlife Refuge would have probably been slower. On the other hand, we may hypothesize that hybridization might have prevented loss of

important European polymorphisms if F_1 and later generation hybrids were less tolerant than "pure" European to *Varroa* mite. Although we can only speculate about the role of *Varroa* in shaping the genetic structure of the Welder Wildlife Refuge population and about the pattern of Africanization in absence of *Varroa*, fitness studies would certainly shed some light regarding interaction of the parasite with European and Africanized honey bees.

In contrast with neotropical populations, the nuclear and mitochondrial makeup of the Welder Wildlife Refuge population changed symmetrically over time (Fig. 14). The result does not support the hypothesis of cytonuclear incompatibilities in hybrids of European mothers, as was suggested to explain the paucity of European mitochondria in Neotropical populations (Harrison and Hall 1993). However, given the very recent secondary contact, definitive conclusions cannot be drawn. The long-term evolutionary consequences of nuclear and mitochondrial introgression are unclear because European genes may be disfavored in an *A. m. scutellata* genetic background. If negative selection is occurring then European genes may gradually be eliminated from the population. Alternatively, if selection favors individuals of mixed ancestry, then European contribution will persist. Considering that several decades after initial colonization, Africanized honey bee populations living in subtropical-temperate transitional zones of South America still contain ample genetic remnants of European honey bees (Sheppard et al. 1991a; Lobo et al. 1989; Lobo an Krieger 1992; Diniz-Filho and Malaspina 1995, 1996; Diniz et al. 2003), we suggest that European contribution may persist in the Africanized gene pool of the Welder Wildlife Refuge population over time. However,

only through continued monitoring will it be possible to assess the long-term evolutionary fate of European genes in the Africanized gene pool in southern Texas.

This study shows how critical long-term surveys are for a better understanding of the Africanization process. Had the population not been monitored continuously, population collapse would have likely gone undetected and the drastic genetic change would have been even more perplexing.

Approaching equilibrium

The Welder Wildlife Refuge population reached Hardy-Weinberg equilibrium in 1998, which persisted until 1999. In 2000, it is likely that there was further movement of migrants into the Welder Wildlife Refuge. The migration event would cause the observed deficiency of heterozygotes and linkage disequilibrium detected in A28/ED1 linked loci-pair (see 2000 and 2001 data in Appendix 2). The renewed instability observed in 2000 shows that a very dynamic situation is occurring at Welder Wildlife Refuge, which is typical of hybrid zones. Hardy-Weinberg proportions were reestablished in 2001.

Genetic Composition of the Post-Africanization Population

Within five years following detection of the first migrant colony of *A. m. scutellata* mitotype, a panmitic European population was replaced by panmitic admixtures of African and European subspecies. As for feral neotropical populations, the Welder Wildlife Refuge Africanized gene pool was predominantly of *A. m.*

scutellata origin (m_R = 62.8 %; m_Y = 74.7 %; mtDNA = 69%). However, both mitochondrial and nuclear European markers were more frequent in the Welder Wildlife Refuge population than in feral populations sampled in the American tropics (Hall and Muralidharan 1989; Smith et al. 1991; Hall 1990; Sheppard et al. 1991b; McMichael and Hall 1996; Clarke et al. 2001, 2002). Interestingly, similar nuclear admixture proportions obtained with microsatellites were reported for feral and managed populations from Yucatan (Clarke et al. 2002).

The Welder Wildlife Refuge post-Africanization gene pool was composed of a diverse array of recombinant classes with a substantial European genetic contribution. If European genes continue to be retained at moderate frequencies, then the Africanized population is best viewed as a "hybrid swarm" (Rinderer 1986; Rinderer et al. 1991) instead of "pure African" (Taylor 1985, 1988; Hall and Muralidharan 1989; Smith et al. 1989). Therefore, we suggest that the term "Africanized honey bee" is more appropriate to describe honey bees from southern Texas than the term 'African honey bee".

Microsatellite surveys of Old World honey bees revealed greater gene diversities in populations belonging to the African than to the European evolutionary lineages (Estoup et al. 1995; Franck et al. 2001; Clarke et al. 2002). Given that Africanized honey bees are descendants of an African subspecies, an increase in gene diversity was anticipated upon their arrival into the Welder Wildlife Refuge. As predicted, the temporal increase in *A. m. scutellata*-derived genes in the Welder Wildlife Refuge population was accompanied by an increase in genetic diversity (Fig. 8). The post-Africanization Welder Wildlife Refuge population displayed gene diversity similar to

that of the reference population from Brazil (Table 5) and to populations of *A. m. scutellata* from South Africa (Estoup et al. 1995; Franck et al. 2001; Clarke et al. 2002). Interestingly, despite their putative European origin, the pre-Africanized populations (reference European and Welder Wildlife Refuge; Table 5) exhibited greater gene diversities than any Old World European population (Estoup et al. 1995; Clarke et al. 2002). Similar finding was reported for the pre-Africanized managed population from Yucatan (Clarke et al. 2002). The result can be explained by the fact that the pre-Africanized populations were a mixture of genes derived from subspecies belonging to different evolutionary lineages (Schiff and Sheppard 1993, 1995; Schiff et al. 1994; Clarke et al. 2002). With Africanization we see this same trend continuing. The Africanized honey bees contributed further genetic diversity to the resident feral population, which future breeding programs may use to advantage.

CHAPTER VI

CONCLUSIONS

The goal of this study was to examine the impact of Africanization on the genetic structure of the Welder Wildlife Refuge feral honey bee population. To that end, an 11-year collection of adult honey bee workers was scored for mtDNA and microsatellite polymorphisms. Rapid PCR-based assays were used to identify mtDNA of the Welder Wildlife Refuge honey bee samples. A number of PCR-based assays exist that do not discriminate some *A. m. iberiensis* (lineage M) and *A. m. intermissa* (lineage A) mitotypes from *A. m. scutellata* mitotype. Because both *A. m. iberiensis* and *A. m. intermissa* subspecies were introduced in the United States, failure to distinguish them from *A. m. scutellata* would eventually lead to an overestimation of Africanization. This problem would be alleviated if the pre-Africanized frequency of *A. m. iberiensis* and *A. m. intermissa* could be known. In this study we tested the cytochrome *b*/*Bgl*II assay, which was claimed to be *A. m. scutellata*-diagnostic. To that end, 211 Old World colonies, representing all known introduced subspecies in the United States, and 451 colonies from non-Africanized areas of the southern United States were screened. We found that the *Bgl*II polymorphism discriminated the mitotype of *A. m. scutellata* from that of *A. m. mellifera*, *A. m. caucasia*, *A. m. ligustica*, *A. m. carnica*, *A. m. lamarcki*, *A. m. cypria*, *A. m. syriaca*, and some *A. m. iberiensis,* but not from that of *A. m. intermissa* and some *A. m. iberiensis*. Nonetheless, given the very low frequency (<1 %) of African non-*A. m. scutellata* mitotype present prior to arrival of Africanized bees in the US, we

concluded that cytochrome *b*/*Bgl*II assay could be used to identify maternally Africanized bees with a high degree of reliability.

The 11-year Welder Wildlife Refuge honey bee collection was screened using the cytochrome *b*/*Bgl*II assay in conjunction with ls rRNA/*Eco*RI, and COI/*Hinf*I assays. The procedure allowed identification of four mitotypes: eastern European, western European, *A. m. lamarckii*, and *A. m. scutellata*. The relative frequencies of the four mitotypes changed radically in Welder Wildlife Refuge during the 11-year study period. Prior to immigration of Africanized honey bees, the resident population was essentially of eastern and western European maternal ancestry. The first colony of *A. m. scutellata* maternal origin was detected in 1993. Three years later there was a mitotype turnover in the population from predominantly eastern European to predominantly *A. m. scutellata*. This remarkable change in the mitotype composition coincided with arrival of the parasitic *Varroa* mite to the Welder Wildlife Refuge in early 1995, which was likely responsible for severe losses experienced by colonies of European ancestry. From 1997 onward the population stabilized with most colonies (69 %) being of *A. m. scutellata* maternal origin.

The temporal change in mtDNA was closely paralleled by nuclear DNA. Thus, both mitochondrial and nuclear DNA (microsatellite) markers suggested a rapid and drastic change in the genetic structure of the Welder Wildlife Refuge population over time. Our data suggests that Africanization started in 1993 by maternal migration. *A. m. scutellata* maternal gene flow was accompanied by paternal gene flow. Indeed, our early Africanization data suggested that Africanized colonies produced drones that mated with

European queens. Gene flow from European drones to Africanized queens was also detected. We concluded that there was bi-directional gene flow and therefore Africanization involved hybridization.

The resident panmitic European population was replaced by panmitic asymmetrical admixtures of *A. m. scutellata* and European genes, only five years after detection of the first migrant colony of *A. m. scutellata* maternal ancestry. The post-Africanization population was predominantly of *A. m. scutellata* origin. However, although "pure" European honey bees were extirpated from the feral population there was still a substantial contribution of European genes in the Africanized gene pool, eight years after detection of the first migrants. Therefore, our data indicates that not only Africanization involved hybridization but also European mitochondrial and nuclear genes have persisted over time. The long-term evolutionary consequences of nuclear and mitochondrial introgression are unclear because European genes may be disfavored in an *A. m. scutellata* genetic background. If negative selection is occurring then European genes may gradually be eliminated from the population. Alternatively, if selection favors individuals of mixed ancestry, then European contribution will persist. In subtropical-temperate transitional zones of South America, Africanized honey bee populations still contain ample genetic remnants of European honey bees, several decades after initial colonization. The finding suggests that European contribution may persist in the Africanized gene pool of the Welder Wildlife Refuge population over time. However, only through continued monitoring it will be possible to assess the long-term evolutionary fate of European genes in the Africanized gene pool in southern Texas.

The nearly coincidental arrival of Africanized honey bees and the parasitic *Varroa* mite complicated understanding of the mechanisms underlying Africanization. Whether a greater dilution of *A. m. scutellata* genes would occur had the resident population not collapsed is unknown. However, it is possible that the event hastened the demise of “pure” European honey bees and had a major role in restructuring the post-Africanization population.

The Welder Wildlife Refuge post-Africanization gene pool was composed of a diverse array of recombinant classes with a substantial European genetic contribution (m_R = 37.2 %; m_Y = 25.3 %; mtDNA = 31 %). If European genes continue to be retained at moderate frequencies, then the Africanized population is best viewed as a “hybrid swarm” instead of “pure African”. Therefore, we suggest that the term “Africanized honey bee” is more appropriate to describe honey bees from southern Texas than the term ‘African honey bee”.

LITERATURE CITED

Arias, M. C., and W. S. Sheppard. 1996. Molecular phylogenetics of honey bee subspecies (*Apis mellifera* L.) inferred from mitochondrial DNA sequence. Mol. Phylogenet. Evol. 5:557-566.

Arnold, M. L. 1992. Natural hybridization as an evolutionary process. Ann. Rev. Ecol. Syst. 23:237-261.

Arnold, M. L. 1997. Natural hybridization and evolution. Oxford Univ. Press, New York.

Arnold, M. L., and S. A. Hodges. 1995. Are natural hybrids fit or unfit relative to their parents? Trends Ecol. Evol. 10:67-71.

Barton, N. H., and G. M. Hewitt. 1989. Adaptation, speciation and hybrid zones. Nature 341:497-503.

Baum, K. A. 2003. Feral Africanized honey bee ecology in a coastal prairie landscape. Pp. 182. Entomology. Texas A&M University, College Station.

Belkhir, K. 2002. Genetix 4.04, logiciel sous windows TM pour la génétic des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier, France.

Bertorelle, G., and L. Excoffier. 1998. Inferring admixture proportions from molecular data. Mol. Biol. Evol. 15:1298-1311.

Blankenship, T. L. 2000. Ecological response of bobcats to fluctuating prey populations on the Welder Wildlife Foundation Refuge. Pp. 123. Texas A&M University, College Station.

Bowcock, A. M., A. Ruiz-Linares, J. Tomfohrde, E. Minch, J. R. Kidd, and L. L. Cavalli-Sforza. 1994. High resolution of human evolution with polymorphic microsatellites. Nature 388:455-457.

Cavalli-Sforza, L. L., and A. W. F. Edwards. 1967. Phylogenetic analysis: models and estimation procedures. Am. J. Hum. Genet. 19**:**233–257.

Clarke, K. E., B. P. Oldroyd, J. Javier, G. Quezada-Euán, and T. E. Rinderer. 2001. Origin of honeybees (*Apis mellifera* L.) from the Yucatan peninsula inferred from mitochondrial DNA analysis. Mol. Ecol. 10:1347-1355.

Clarke, K. E., T. Rinderer, P. Franck, J. G. Quezada-Euán, and B. P. Oldroyd. 2002. The Africanization of honey bees (*Apis mellifera* L.) of the Yucatan: a study of a massive hybridization event across time. Evolution 56:1462-1474.

Cornuet, J. M., S. Piry, G. Luikart, A. Estoup, and M. Solignac. 1999. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153:1989-2000.

Crozier, Y. C., S. Koulianos, and R. H. Crozier. 1991. An improved test for Africanized honey bee mitochondrial DNA. Experientia 47:968-969.

Danka, R. G., R. L. Hellmich, and T. E. Rinderer. 1992. Nest usurpation, supersedure and colony failure contribute to Africanization of commercially managed European honey bees in Venezuela. Journal of Apicultural Research 31:119-123.

De Jong, D. 1990. Mites: *Varroa* and other parasites of brood. Pp. 200-218 *in* A. Morse and R. Nowogrodzki, eds. Honey bee pests, predators, and diseases, Cornell University Press, Ithaca, New York.

De Jong, D., and L. S. Gonçalves. 1981. The *Varroa* problem in Brazil. Am. Bee J. 121:186-189.

Del Lama, M. A., R. A. Figueiredo, A. E. E. Soares, and S. N. Del Lama. 1988. Hexokinase polymorphism in *Apis mellifera* and its use for Africanized honeybee identification. Rev. Brazil. Genet. 11:287-297.

Del Lama, M. A., J. A. Lobo, A. E. E. Soares, and D. L. S. N. 1990. Genetic differentiation estimated by isozymic analysis of Africanized honey bee populations from Brazil and from Central America. Apidologie 21:271-280.

Diniz, M. N., A. E. E. Soares, W. S. Sheppard, and M. A. Del Lama. 2003. Genetic structure of honey bee populations from southern Brazil and Uruguay. Genet. Mol. Biol. 26:47-52.

Diniz-Filho, J. A. F. 1996. Clinal morphometric variation in Africanized honey bees under racial admixture hypothesis. J. Apic. Res. 35:104-109.

Diniz-Filho, J. A. F., and O. Malaspina. 1995. Evolution and population structure of Africanized honey bees in Brazil: evidence from spatial analysis of morphometric data. Evolution 49:1172-1179.

Diniz-Filho, J. A. F., and O. Malaspina. 1996. Geographic variation of Africanized honey bees (*Apis mellifera* L.) in Brazil: multivariate morphometrics and racial admixture. Braz. J. Genet. 19:217-224.

Drawe, D. L., A. D. Chamrad, and T. W. Box. 1978. Plant communities of the Welder Wildlife Refuge. Rob and Bessie Welder Wildlife Foundation, Sinton, Texas.

Estoup, A., M. Solignac, and J.-M. Cornuet. 1994. Precise assessment of the number of patrilines and of genetic relatedness in honeybee colonies. Proc. R. Soc. Lond. B 258:1-7.

Estoup, A., L. Garnery, M. Solignac, and J.-M. Cornuet. 1995. Microsatellite variation in Honey Bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. Genetics 140:679-695.

Franck, P., L. Garnery, M. Solignac, and J. M. Cornuet. 1998. The origin of west European subspecies of honeybees (*Apis mellifera*): new insights from microsatellite and mitochondrial data. Evolution 52:1119-1134.

Franck, P., L. Garnery, G. Celebrano, M. Solignac, and J. M. Cornuet. 2000a. Hybrid origins of honey bees from Italy (*Apis mellifera ligustica*) and Sicily (*A. m. sicula*). Mol. Ecol. 9:907-921.

Franck, P., L. Garnery, M. Solignac, and J. M. Cornuet. 2000b. Molecular confirmation of a fourth lineage in honeybees from the Near East. Apidologie 31:167-180.

Franck, P., L. Garnery, A. Loiseau, B. P. Oldroyd, H. R. Hepburn, M. Solignac, and J. M. Cornuet. 2001. Genetic diversity of the honey bee in Africa: microsatellite and mitochondrial data. Heredity 86:420-430.

Garnery, L., J. M. Cornuet, and M. Solignac. 1992. Evolutionary history of the honey bee *Apis mellifera* inferred from mitochondrial DNA analysis. Mol. Ecol. 1:145-154.

Garnery, L., M. Solignac, G. Celebrano, and J. M. Cornuet. 1993. A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. Experientia 49:1016-1021.

Garnery, L., E. H. Mosshine, B. P. Oldroyd, and J. M. Cornuet. 1995. Mitochondrial DNA variation in Moroccan and Spanish honey bee populations. Mol. Ecol. 4:465-471.

Goldstein, D. B., A. Ruiz-Linares, L. L. Cavalli-Sforza, and M. W. Feldman. 1995. Genetic absolute dating based on microsatellites and origin of modern humans. Proc. Natl. Acad. Sci. 92:6723-6727.

Gonçalves, L. S., A. C. Stort, and D. De Jong. 1991. Beekeeping in Brazil. Pp. 359 - 372 *in* M. Spivak, D. J. C. Fletcher and M. D. Breed, eds. The "African" honey bee. Westview Press, Boulder, San Francisco, Oxford.

Goudet, J. 1995. FSTAT (vers. 1.2): a computer program to calculate F-statistics. J. Hered. 86:485-486.

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Updated from Goudet (1995).

Goudet, J., M. Raymond, T. De Meeüs, and F. Rousset. 1996. Testing differentiation in diploid populations. Genetics 144:1933-1940.

Guo, S. W., and E. A. Thompson. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 48:361-372.

Guzman-Novoa, E., A. Sanchez, R. E. Page, and T. Garcia. 1996. Susceptibility of European and Africanized honeybees (*Apis mellifera* L) and their hybrids to *Varroa jacobsoni* Oud. Apidologie 27:93-103.

Guzman-Novoa, E., R. Vandame, and M. E. Arechavaleta. 1999. Susceptibility of European and Africanized honey bees (*Apis mellifera* L.) to *Varroa jacobsoni* Oud. in Mexico. Apidologie 30:173-182.

Haberl, M., and D. Tautz. 1999. Paternity and maternity frequencies in *Apis mellifera sicula*. Insectes soc. 46:137-145.

Hall, H. G. 1990. Parental analysis of introgressive hybridization between African and European honeybees using nuclear DNA RFLPs. Genetics 125:611-621.

Hall, H. G. 1991. Genetic characterization of honey bees through DNA analysis. Pp. 45-72. *in* M. Spivak, D. J. C. Fletcher and M. D. Breed, eds. The "African" honey bee. Westview Press, Boulder, Colorado.

Hall, G. 1992. DNA studies reveal processes involved in the spread of new world African honeybees. Fla. Entomol. 75:51-59.

Hall, H. G., and M. McMichael. 2001. Frequencies of restriction fragment-length polymorphisms indicate that neotropical honey bee (Hymenoptera: Apidae) populations have African and west European origins. Ann. Entomol. Soc. Am. 94:670–676.

Hall, H. G., and K. Muralidharan. 1989. Evidence from mitochondrial DNA that African honey bees spread as continuous maternal lineages. Nature 339:211-213.

Hall, H. G., and D. R. Smith. 1991. Distinguishing African and European honeybee matrilines using amplified mitochondrial DNA. Proc. Natl. Acad. Sci. 88:4548-4552.

Harrison, J. F., and H. G. Hall. 1993. African-European honey bee hybrids have low non-intermediate metabolic capacities. Nature 363:258-260.

Harrison, R. G. 1993. Hybrid zones and the evolutionary process. Oxford Univ. Press, New York.

Hartl, D. L., and A. G. Clark. 1997. Principles of population genetics. Sinauer Associates, Inc., Sunderland, MA.

Kerr, W. E. 1967. The history of the introduction of African bees to Brazil. S. Afr. Bee J. 2:3-5.

Levinson, G., and a. G. A. Gutman. 1987. Slipped-strand mispairing - a major mechanism for DNA sequence evolution. Mol. Biol. Evol. 4:203-221.

Lobo, J. A. 1995. Morphometric, isozymic and mitochondrial variability of Africanized honey bees in Costa Rica. Heredity 75:133-141.

Lobo, J. A., and H. Krieger. 1992. Maximum likelihood estimates of gene frequencies and racial admixture in *Apis mellifera* L. (Africanized honey bees). Heredity 58:441-448.

Lobo, J. A., M. A. Del Lama, and M. A. Mestriner. 1989. Population differentiation and racial admixture in the Africanized honeybee (*Apis mellifera* L.). Evolution 43:794-802.

McMichael, M., and H. G. Hall. 1996. DNA RFLPs at a highly polymorphic locus distinguish European and African subspecies of the honey bee *Apis mellifera* L. and suggest geographical origins of New World honey bees. Mol. Ecol. 5:403-416.

Message, G., and L. S. Gonçalves. 1995. Effect of the size of worker brood cells of Africanized honey bee on infestation and reproduction of the ectoparasitic mite *Varroa jacobsoni.* Apidologie 26: 381-386.

Michener, C. D. 1975. The Brazilian bee problem. Annu. Rev. Entomol. 20:390-416.

Moretto, G., L. S. Gonçalves, D. De Jong, and M. Z. Bichuette. 1991. The effects of climate and bee race on *Varroa jacobsoni* Oud infestations in Brazil. Apidologie 22: 197-203.

Moretto, G., L. S. Gonçalves, and D. De Jong. 1993. Heritability of Africanized and European honey bee defensive behavior against the mite *Varroa jacobsoni.* Rev. Brasil. Genet. 16: 71-77.

Moritz, R. F. A., and M. S. Meusel. 1992. Mitochondrial gene frequencies in Africanized honeybees (*Apis mellifera* L.): Theoretical model and empirical evidence. J. Evol. Biol. 5:71-81.

Moritz, C., T. E. Dowling, and W. M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Ann. Rev. Ecol. Syst. 18:269-292.

Moritz, R. F. A., J. M. Cornuet, P. Kryger, L. Garnery, and H. R. Hepburn. 1994. Mitochondrial DNA variability in South African honeybees (*Apis mellifera* L). Apidologie 25:169-178.

Morse, R. A., D. M. Burgett, J. P. Ambrose, W. E. Conner, and R. D. Fell. 1973. Early introductions of African bees into Europe and the New World. Bee World 54:57-60.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Nielsen, D. I., P. R. Ebert, G. J. Hunt, E. Guzman-Novoa, S. A. Kinnee, and R. E. Page. 1999. Identification of Africanized honey bees (Hymenoptera: Apidae) incorporating morphometrics and an improved polymerase chain reaction mitotyping procedure. Ann. Entomol. Soc. Am. 92:167-174.

Nielsen, D. I., P. R. Ebert, R. E. Page, G. J. Hunt, and E. Guzman-Novoa. 2000. Improved polymerase chain reaction-based mitochondrial genotype assay for identification of the Africanized honey bee (Hymenoptera: Apidae). Ann. Entomol. Soc. Am. 93:1-6.

Nogueira-Neto, P. 1964. The spread of a fierce African bee in Brazil. Bee World 45:119-121.

Norušis, M. J. 1993. SPSS® for windows base system user's guide release 6.0. SPSS Inc., Chicago.

Oldroyd, B. P., J.-M. Cornuet, D. Rowe, T. E. Rinderer, and R. H. Crozier. 1995a. Racial admixture of Apis *mellifera* in Tasmania, Australia: similarities and differences with natural hybrid zones in Europe. Heredity 74:315-325.

Oldroyd, B. P., A. J. Smolenski, J.-M. Cornuet, S. Wongsiri, A. Estoup, T. E. Rinderer, and R. H. Crozier. 1995b. Levels of polyandry and intracolonial genetic relationships in *Apis florea*. Behav. Ecol. Sociobiol. 37:329-335.

Oldroyd, B. P., A. J. Smolenski, J.-M. Cornuet, S. Wongsiri, A. Estoup, T. E. Rinderer, and R. H. Crozier. 1996. Levels of polyandry and intracolonial genetic relationships in *Apis dorsata* (Hymenoptera: Apidae). Ann. Entomol. Soc. Am. 89:276-283.

Oldroyd, B. P., M. J. Clifton, S. Wongsiri, T. E. Rinderer, H. A. Sylvester, and R. H. Crozier. 1997. Polyandry in the genus *Apis,* particularly *Apis andreniformis*. Behav. Ecol. Sociobiol. 40:17-26.

Oldroyd, B. P., M. J. Clifton, K. Parker, S. Wongsiri, T. E. Rinderer, and R. H. Crozier. 1998. Evolution of mating behavior in the Genus *Apis* and an estimate of mating frequency in the *Apis cerana* Hymenoptera: Apidae). Ann. Entomol. Soc. Am. 91:700-709.

Paetkau, D., W. Calvert, I. Stirling, and C. Strobeck. 1995. Microsatellite analysis of population structure in Canadian polar bears. Mol. Ecol. 4:347-354.

Page, R. E., Jr. 1989. Neotropical African bees. Nature 339:181-182.

Petit, R. J., A. El Mousadik, and O. Pons. 1998. Identifying populations for conservation on the basis of genetic markers. Conserv. Biol. 12:844-855.

Pinto, M. A., J. S. Johnston, W. L. Rubink, R. N. Coulson, P. J. C., and W. S. Sheppard. 2003. Identification of Africanized Honey Bee (Hymenoptera: Apidae)

mitochondrial DNA: validation of a rapid PCR-based assay. Ann. Entomol. Soc. Am. 96:679-684.

Queller, D. C., J. E. Strassman, and C. R. Hughes. 1993. Microsatellites and kinship. Trends Ecol. Evol. 8:285-288.

Quezada-Euán, J. J. G. 2000. Hybridization between European and Africanized honeybees in tropical Yucatan, Mexico. II. Morphometric, allozymic and mitochondrial DNA variability in feral colonies. Apidologie 31:443-453.

Quezada-Euán, J. J. G., and S. M. Hinsull. 1995. Evidence of continued European morphometrics and mtDNA in feral colonies of honey bees (*Apis mellifera* L.) from the Yucatan peninsula, Mexico. J. Apic. Res. 34:161-166.

Quezada-Euán, J. J. G., and L. M. Medina. 1998. Hybridization between European and Africanized honeybees (*Apis mellifera* L.) in tropical Yucatan, Mexico. I. Morphometric changes in feral and managed colonies. Apidologie 29:555-568.

Quezada-Euán, J. J. G., C. M. Echazarreta, and R. J. Paxton. 1996. The distribution and range expansion of Africanized honey bees (*Apis mellifera*) in the state of Yucatan, Mexico. J. Apic. Res. 35:85-95.

Rannala, B., and J. L. Mountain. 1997. Detecting immigration by using multilocus genotypes. Proc. Natl. Acad. Sci. 94:9197-9201.

Raymond, M., and F. Rousset. 1995a. An exact test for population differentiation. Evolution 49:1280-1283.

Raymond, M., and F. Rousset. 1995b. Population genetics software for exact test and ecumenism. J. Hered. 86:248-250.

Rhymer, J. M., and D. Simberloff. 1996. Extinction by hybridization and introgression. Ann. Rev. Ecol. Syst. 27:83-109.

Rice, W. R. 1989. Analyzing tables of statistical tests. Evolution 43:223-225.

Rinderer, T. E. 1986. Africanized bees: the Africanization process and potential range in the United States. Bull. Entomol. Soc. Am. 32:222-227.

Rinderer, T. E. 1988. Evolutionary aspects of the Africanization of honey bee populations in the Americas. Pp. 13-28 *in* G. R. Needham, R. E. Page, M. Delfinado-Baker and C. E. Bowman, eds. Africanized honey bees and bee mites. Ellis Horwood, Chichester, England.

Rinderer, T. E., J. A. Stelzer, B. P. Oldroyd, S. M. Buco, and W. L. Rubink. 1991. Hybridization between European and Africanized honey bees in the neotropical Yucatan Peninsula. Science 253:309-311.

Rinderer, T. E., J. A. Stelzer, B. P. Oldroyd, and S. Tingek. 1998. Levels of polyandry and intracolonial genetic relationships in *Apis koschevnikovi*. J. Apic. Res. 37:281-287.

Roberts, D., and R. Hiorns. 1965. Methods of analysis of the genetic composition of a hybrid population. Hum. Biol. 37:38-43.

Roubik, D.W. 1989. Ecology and natural history of tropical bees. Cambridge University Press, New York.

Roubik, D.W., and M.W. Boreham. 1990. Learning to live with Africanized honeybees. Interciencia 15:146-153.

Rousset, F., and M. Raymond. 1995. Testing heterozygote excess and deficiency. Genetics 140:1413-1419.

Rowe, D. J., T. E. Rinderer, J. A. Stelzer, B. P. Oldroyd, and R. H. Crozier. 1997. Seven polymorphic microsatellite loci in honeybees (*Apis mellifera*). Insectes soc. 44:85-93.

Rubink, W. L., W. T. Wilson, J. J. Resendez B., and D. L. Maki. 1990. Pre-Africanized *Apis mellifera* (Hymenoptera: Apidae) swarming dynamics in Northeastern Mexico and Southern Texas. Journal of the Kansas Entomological Society 63:288-297.

Rubink, W. L., W. T. Wilson, and A. M. Collins. 1995. Comparative Africanization rates in feral honey bee populations at three latitudes in northeastern Mexico and southern Texas. Am. Bee J. 135:829-829.

Rubink, W. L., Luevano-Martinez, E. A. Sugden, W. T. Wilson, and A. M. Collins. 1996. Subtropical *Apis mellifera* (Hymenoptera: Apidae) swarming dynamics and Africanization rates in northeastern Mexico and southern Texas. Ann. Entomol. Soc. Am. 89:243-251.

Ruttner, F. 1988. Biogeography and taxonomy of honey bees. Springer, Berlin.

Ruttner, F., L. Tassencourt, and J. Louveaux. 1978. Biometrical-statistical analysis of the geographic variability of *Apis mellifera* L. Apidologie 9:363-381.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-426.

Schiff, N. M., and W. S. Sheppard. 1993. Mitochondrial DNA evidence for the 19th century introduction of African honey bees into the United States. Experientia 49:530-532.

Schiff, N. M., and W. S. Sheppard. 1995. Genetic analysis of commercial honey bees (Hymenoptera: Apidae) from the southeastern United States. J. Econ. Entomol. 88:1216-1220.

Schiff, N. M., and W. S. Sheppard. 1996. Genetic differentiation in the queen breeding population of the western United States. Apidologie 27:77-86.

Schiff, N. M., W. S. Sheppard, G. M. Loper, and H. Shimanuki. 1994. Genetic diversity of feral honey bee (Hymenoptera: Apidae) populations in the southern United States. Ann. Entomol. Soc. Am. 87:842-848.

Schmidt, J. O., and S. C. Thoenes. 1987. Honey bee swarm capture with pheromone-containing trap boxes. Am. Bee J. 129:807-808.

Seeley, T. D. 1978. Life history strategy of the honey bee, *Apis mellifera*. Oecologia 32:109-118.

Sheppard, W. S. 1989a. A history of the introduction of honey bee races into the United States. Part I. Am. Bee J. 129:617-619.

Sheppard, W. S. 1989b. A history of the introduction of honey bee races into the United States. Part II. Am. Bee J. 129:664-667.

Sheppard, W. S., and B. A. McPheron. 1991. Ribosomal DNA diversity in Apidae, pp 89-102 *in* D. R. Smith, ed. Diversity in the genus *Apis*. Westview Press, Inc., Boulder, CO.

Sheppard, W. S., and D. R. Smith. 2000. Identification of African-derived bees in the Americas: a survey of methods. Ann. Entomol. Soc. Am. 93:159-176.

Sheppard, W. S., T. E. Rinderer, J. A. Mazzolli, J. A. Stelzer, and H. Shimanuki. 1991a. Gene flow between African- and European-derived honey bee populations in Argentina. Nature 349:782-784.

Sheppard, W. S., A. E. E. Soares, D. DeJong, and H. Shimanuki. 1991b. Hybrid status of honey bee populations near the historic origin of Africanization in Brazil. Apidologie 22:643-652.

Sheppard, W. S., T. E. Rinderer, L. Garnery, and H. Shimanuki. 1999. Analysis of Africanized honey bee mitochondrial DNA reveals further diversity of origin. Genet. Mol. Biol. 22:21-32.

Smith, D. R. 1991. African bees in the Americas: insights from biogeography and genetics. Trends Ecol. Evol. 6:17-21.

Smith, D. R., and W. M. Brown. 1988. Polymorphisms in mitochondrial DNA of European and Africanized honeybees (*Apis mellifera*). Experientia 44:257-260.

Smith, D. R., O. R. Taylor, and W. M. Brown. 1989. Neotropical Africanized honey bees have African mitochondrial DNA. Nature 339:213-215.

Smith, D. R., M. F. Palopoli, B. R. Taylor, L. Garnery, J. M. Cornuet, M. Solignac, and W. M. Brown. 1991. Geographical overlap of two mitochondrial genomes in Spanish honeybees (*Apis mellifera iberica*). J. Hered. 82:96-100.

Smith, D. R., A. Slaymaker, M. Palmer, and O. Kaftanoglu. 1997. Turkish honey bees belong to the east Mediterranean mitochondrial lineage. Apidologie 28:269-274.

Snedecor, G. W., and W. G. Cochran. 1978. Statistical methods. Iowa State University Press, Ames, IA.

Spivak, M., T. Ranker, O. R. Taylor, W. Taylor, and L. Davis. 1988. Discrimination of Africanized honey bees using behavior, cell size, morphometrics, and a newly discovered isozyme polymorphism. Pp. 313-324 *in* G. R. Needham, R. E. Page, M. Delfinado-Baker and C. E. Bowman, eds. Africanized honey bees and bee mites. Ellis Horwood, Chichester, England.

Spivak, M., D. J. C. Fletcher, and M. D. Breed. 1991. Introduction. Pp. 1-9 *in* M. Spivak, D. J. C. Fletcher and M. D. Breed, eds. The "African" honey bee. Westview Press, Inc., Boulder, CO.

Suazo, A., R. McTiernan, and H. G. Hall. 1998. Differences between African and European honey bees (*Apis mellifera* L.) in random amplified polymorphic DNA (RAPD). Journal of Heredity 89:32-36.

Sugden, E. A., and K. R. Williams. 1990. October 15: the day the bee arrived. Glean. Bee Cult. 119:18-21.

Swofford, D. L., and . 2002. PAUP*. Phylogenetic Analysis Using Parsimony. Version 4.0b 10, Sinauer Associates, Inc. Sunderland, MA.

Taylor, O. R. 1985. African Bees: potential impact in the United States. Bull. Entomol. Soc. Am. 31:14-24.

Taylor, O. R. 1988. Ecology and economic impact of African and Africanized honey bees. Pp. 29-41 *in* G. R. Needham, R. E. Page, M. Delfinado-Baker and C. E.

Bowman, eds. Africanized honey bees and bee mites. Ellis Horwood, Chichester, England.

Taylor, O. R., Jr., and M. Spivak. 1984. Climatic limits of tropical African honeybees in the Americas. Bee World 65:38-47.

Vergara, C., A. Dietz, and A. Perez. 1989. Usurpation of managed honey bee colonies by migratory swarms in Tabasco, Mexico. Am. Bee J. 129:824-825.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

Winston, M. L. 1992. The biology and management of Africanized honey bees. Annu. Rev. Entomol. 37:173-193.

Winston, M. L., O. R. Taylor, and G. W. Otis. 1983. Some differences between temperate European and tropical African and South American honey bees. Bee World 64:12-21.

APPENDIX 1

P-value and standard error (SE) of Fisher's exact tests for each locus pair for genotypic linkage disequilibrium (the null hypothesis tested is that genotypes at one locus are independent from genotypes at the other locus). Reference European and Brazilian population samples were tested.

Population sample	Locus 1	Locus 2	*P*-value	SE
European	A7	A43	0.00159	0.00031
European	A43	A14	0.01338	0.00112
European	A107	A79	0.01399	0.00207
European	A7	A107	0.01624	0.00269
European	A14	IM	0.05685	0.00323
European	ED1	IM	0.06732	0.00309
European	A14	A35	0.08216	0.00479
European	A107	A113	0.08688	0.00594
European	A79	ED1	0.12114	0.00373
European	A28	ED1	0.13251	0.00217
European	A14	ED1	0.13495	0.00549
European	A88	ED1	0.14019	0.00339
European	A88	A8	0.15460	0.00291
European	A8	A28	0.15789	0.00195
European	A7	A88	0.16186	0.00350
European	A43	IM	0.20571	0.00376
European	A88	A79	0.21896	0.00331
European	A107	ED1	0.23294	0.00776
European	A43	A107	0.24371	0.00610
European	A88	IM	0.27924	0.00391
European	A88	A107	0.28658	0.00600
European	A107	A8	0.32542	0.00967
European	A88	A14	0.32714	0.00521
European	A28	IM	0.34550	0.00273

European	A8	ED1	0.34873	0.00654
European	A88	A35	0.39181	0.00550
European	A43	A113	0.41322	0.00646
European	A43	A35	0.41566	0.00601
European	A107	A35	0.41749	0.01055
European	A113	IM	0.46188	0.00854
European	A35	A28	0.46897	0.00382
European	A14	A113	0.48265	0.00985
European	A107	A14	0.48863	0.01258
European	A43	A79	0.50562	0.00449
European	A8	IM	0.50942	0.00574
European	A7	A79	0.51066	0.00762
European	A7	A14	0.52131	0.00905
European	A43	A8	0.54578	0.00446
European	A14	A28	0.55812	0.00342
European	A7	A8	0.56861	0.00711
European	A88	A28	0.57383	0.00213
European	A7	IM	0.60477	0.00672
European	A35	IM	0.61713	0.00706
European	A35	ED1	0.64148	0.00806
European	A79	A28	0.67953	0.00232
European	A88	A113	0.69188	0.00566
European	A88	A43	0.69221	0.00292
European	A43	A28	0.69719	0.00196
European	A8	A113	0.71077	0.00674
European	A35	A8	0.71786	0.00632
European	A7	A113	0.72498	0.00813
European	A113	A28	0.72856	0.00359
European	A7	ED1	0.73818	0.00641
European	A113	ED1	0.74179	0.00784
European	A35	A79	0.76788	0.00562
European	A35	A113	0.81426	0.00747
European	A113	A79	0.83469	0.00541
European	A107	IM	0.83620	0.00666
European	A8	A79	0.87088	0.00341

European	A14	A8	0.88240	0.00428
European	A7	A28	0.88407	0.00175
European	A7	A35	0.89852	0.00449
European	A79	IM	0.91402	0.00266
European	A43	ED1	0.94078	0.00192
European	A14	A79	0.95314	0.00273
European	A107	A28	0.99713	0.00025
Brazilian	A35	IM	0.01999	0.00321
Brazilian	A107	ED1	0.02254	0.00335
Brazilian	A14	A79	0.02751	0.00411
Brazilian	A14	IM	0.03484	0.00478
Brazilian	A79	IM	0.07393	0.00613
Brazilian	A7	A35	0.08009	0.00734
Brazilian	A8	A79	0.11473	0.00524
Brazilian	A28	ED1	0.14352	0.00686
Brazilian	A43	A28	0.19301	0.00860
Brazilian	A43	A14	0.25896	0.01198
Brazilian	A107	A113	0.28154	0.01166
Brazilian	A107	A28	0.32243	0.01187
Brazilian	A14	A113	0.33993	0.01219
Brazilian	A113	A28	0.36534	0.00989
Brazilian	A43	IM	0.36685	0.01255
Brazilian	A14	A28	0.42951	0.01247
Brazilian	A43	A113	0.49622	0.01190
Brazilian	A113	IM	0.49912	0.01186
Brazilian	A88	A43	0.50000	0.01241
Brazilian	ED1	IM	0.53134	0.01175
Brazilian	A79	ED1	0.57004	0.01085
Brazilian	A8	ED1	0.57311	0.00792
Brazilian	A28	IM	0.59653	0.01101
Brazilian	A35	A113	0.67131	0.01096
Brazilian	A88	A35	0.67689	0.01175
Brazilian	A43	A8	0.70820	0.00817
Brazilian	A35	ED1	0.71016	0.01037
Brazilian	A88	A28	0.77552	0.00916

Brazilian	A8	A113	0.84445	0.00542
Brazilian	A88	A8	0.87513	0.00539
Brazilian	A35	A8	0.87671	0.00549
Brazilian	A8	A28	0.92043	0.00350
Brazilian	A7	A88	1.00000	0.00000
Brazilian	A7	A43	1.00000	0.00000
Brazilian	A7	A107	1.00000	0.00000
Brazilian	A88	A107	1.00000	0.00000
Brazilian	A43	A107	1.00000	0.00000
Brazilian	A7	A14	1.00000	0.00000
Brazilian	A88	A14	1.00000	0.00000
Brazilian	A107	A14	1.00000	0.00000
Brazilian	A43	A35	1.00000	0.00000
Brazilian	A107	A35	1.00000	0.00000
Brazilian	A14	A35	1.00000	0.00000
Brazilian	A7	A8	1.00000	0.00000
Brazilian	A107	A8	1.00000	0.00000
Brazilian	A14	A8	1.00000	0.00000
Brazilian	A7	A113	1.00000	0.00000
Brazilian	A88	A113	1.00000	0.00000
Brazilian	A7	A79	1.00000	0.00000
Brazilian	A88	A79	1.00000	0.00000
Brazilian	A43	A79	1.00000	0.00000
Brazilian	A107	A79	1.00000	0.00000
Brazilian	A35	A79	1.00000	0.00000
Brazilian	A113	A79	1.00000	0.00000
Brazilian	A7	A28	1.00000	0.00000
Brazilian	A35	A28	1.00000	0.00000
Brazilian	A79	A28	1.00000	0.00000
Brazilian	A7	ED1	1.00000	0.00000
Brazilian	A88	ED1	1.00000	0.00000
Brazilian	A43	ED1	1.00000	0.00000
Brazilian	A14	ED1	1.00000	0.00000
Brazilian	A113	ED1	1.00000	0.00000
Brazilian	A7	IM	1.00000	0.00000

Brazilian	A88	IM	1.00000	0.00000
Brazilian	A107	IM	1.00000	0.00000
Brazilian	A8	IM	1.00000	0.00000

APPENDIX 2

P-value and standard error (SE) of Fisher's exact tests for each locus pair for genotypic linkage disequilibrium (the null hypothesis tested is that genotypes at one locus are independent from genotypes at the other locus). Welder Wildlife Refuge temporal population samples were tested.

Population sample	Locus 1	Locus 2	*P*-value	SE
1991	A88	A79	0.03128	0.00060
1991	A43	A35	0.08369	0.00069
1991	A14	IM	0.17154	0.00204
1991	A7	A43	0.24633	0.00122
1991	ED1	IM	0.29347	0.00116
1991	A14	A35	0.29618	0.00218
1991	A43	A113	0.32179	0.00129
1991	A35	ED1	0.37661	0.00101
1991	A88	A113	0.42352	0.00199
1991	A88	A35	0.42443	0.00198
1991	A8	A28	0.46689	0.00062
1991	A88	ED1	0.46693	0.00094
1991	A43	A14	0.50630	0.00146
1991	A113	IM	0.51491	0.00216
1991	A35	IM	0.52271	0.00208
1991	A7	ED1	0.59851	0.00107
1991	A35	A79	0.62039	0.00163
1991	A113	A79	0.62081	0.00161
1991	A14	A28	0.71455	0.00049
1991	A35	A113	0.73581	0.00155
1991	A79	A28	0.77085	0.00038
1991	A35	A28	0.77129	0.00040
1991	A113	A28	0.77195	0.00041
1991	A14	ED1	0.77665	0.00092

1991	A43	IM	0.79826	0.00098
1991	A43	ED1	0.83257	0.00053
1991	A79	ED1	0.83330	0.00064
1991	A88	A28	0.84779	0.00035
1991	A113	ED1	0.93339	0.00042
1991	A14	A113	1.00000	0.00000
1991	A14	A79	1.00000	0.00000
1991	A14	A8	1.00000	0.00000
1991	A28	ED1	1.00000	0.00000
1991	A28	IM	1.00000	0.00000
1991	A35	A8	1.00000	0.00000
1991	A43	A28	1.00000	0.00000
1991	A43	A79	1.00000	0.00000
1991	A43	A8	1.00000	0.00000
1991	A7	A113	1.00000	0.00000
1991	A7	A14	1.00000	0.00000
1991	A7	A28	1.00000	0.00000
1991	A7	A35	1.00000	0.00000
1991	A7	A79	1.00000	0.00000
1991	A7	A8	1.00000	0.00000
1991	A7	A88	1.00000	0.00000
1991	A7	IM	1.00000	0.00000
1991	A79	IM	1.00000	0.00000
1991	A8	A113	1.00000	0.00000
1991	A8	A79	1.00000	0.00000
1991	A8	ED1	1.00000	0.00000
1991	A8	IM	1.00000	0.00000
1991	A88	A14	1.00000	0.00000
1991	A88	A43	1.00000	0.00000
1991	A88	A8	1.00000	0.00000
1991	A88	IM	1.00000	0.00000
1991	A107	A113	No information	
1991	A107	A14	No information	
1991	A107	A28	No information	
1991	A107	A35	No information	

1991	A107	A79	No information	
1991	A107	A8	No information	
1991	A107	ED1	No information	
1991	A107	IM	No information	
1991	A43	A107	No information	
1991	A7	A107	No information	
1991	A88	A107	No information	
1992	A7	A88	0.03396	0.00133
1992	A43	A79	0.04890	0.00186
1992	A7	A28	0.11604	0.00155
1992	A88	ED1	0.12035	0.00158
1992	A88	IM	0.16659	0.00268
1992	A28	IM	0.22841	0.00184
1992	A35	A113	0.25760	0.00437
1992	A7	A43	0.26995	0.00359
1992	A35	IM	0.28592	0.00475
1992	A14	A113	0.28710	0.00566
1992	A43	IM	0.31808	0.00351
1992	A107	A113	0.35035	0.00625
1992	A88	A43	0.36379	0.00279
1992	A43	A14	0.38042	0.00444
1992	A88	A79	0.41412	0.00508
1992	A35	A8	0.41795	0.00402
1992	A107	A28	0.41941	0.00266
1992	A7	ED1	0.46321	0.00343
1992	A79	IM	0.49547	0.00574
1992	A7	IM	0.50766	0.00527
1992	A107	ED1	0.51024	0.00342
1992	ED1	IM	0.53728	0.00317
1992	A43	A35	0.54839	0.00353
1992	A88	A113	0.55445	0.00370
1992	A28	ED1	0.56904	0.00161
1992	A79	ED1	0.57188	0.00439
1992	A43	ED1	0.58779	0.00240
1992	A7	A113	0.59047	0.00537

1992	A35	ED1	0.61565	0.00343
1992	A107	A35	0.62363	0.00543
1992	A8	IM	0.63840	0.00369
1992	A43	A28	0.69629	0.00155
1992	A88	A28	0.70392	0.00163
1992	A113	ED1	0.70752	0.00287
1992	A88	A35	0.72119	0.00317
1992	A14	A28	0.76130	0.00198
1992	A88	A14	0.77829	0.00295
1992	A88	A107	0.83124	0.00271
1992	A8	A28	0.83704	0.00118
1992	A7	A79	0.83874	0.00383
1992	A35	A28	0.84209	0.00158
1992	A14	ED1	0.85824	0.00206
1992	A7	A35	0.86024	0.00302
1992	A35	A79	0.88026	0.00332
1992	A113	A79	0.91279	0.00275
1992	A8	ED1	0.92396	0.00106
1992	A113	A28	0.97190	0.00058
1992	A88	A8	0.97255	0.00064
1992	A79	A28	0.99193	0.00035
1992	A107	A14	1.00000	0.00000
1992	A107	A79	1.00000	0.00000
1992	A107	A8	1.00000	0.00000
1992	A107	IM	1.00000	0.00000
1992	A113	IM	1.00000	0.00000
1992	A14	A35	1.00000	0.00000
1992	A14	A79	1.00000	0.00000
1992	A14	A8	1.00000	0.00000
1992	A14	IM	1.00000	0.00000
1992	A43	A107	1.00000	0.00000
1992	A43	A113	1.00000	0.00000
1992	A43	A8	1.00000	0.00000
1992	A7	A107	1.00000	0.00000
1992	A7	A14	1.00000	0.00000

1992	A7	A8	1.00000	0.00000
1992	A8	A113	1.00000	0.00000
1992	A8	A79	1.00000	0.00000
1993	A7	IM	0.01749	0.00160
1993	A14	ED1	0.02039	0.00118
1993	A14	A28	0.06215	0.00091
1993	A35	A79	0.07810	0.00388
1993	A88	A113	0.08492	0.00172
1993	A43	A79	0.13011	0.00263
1993	A88	IM	0.13429	0.00199
1993	A88	A8	0.13774	0.00177
1993	A43	A35	0.15567	0.00288
1993	A14	IM	0.20481	0.00363
1993	A7	A35	0.21268	0.00534
1993	A43	A8	0.23055	0.00274
1993	A8	A28	0.23953	0.00179
1993	A113	A79	0.24209	0.00557
1993	A14	A79	0.27397	0.00484
1993	A8	A79	0.27401	0.00451
1993	A43	A14	0.29443	0.00308
1993	A79	ED1	0.30597	0.00476
1993	A7	A14	0.30860	0.00506
1993	A88	A79	0.33162	0.00288
1993	A35	A28	0.34979	0.00251
1993	A7	A28	0.39505	0.00261
1993	A7	A88	0.40241	0.00304
1993	A79	A28	0.43187	0.00251
1993	A88	A28	0.44591	0.00142
1993	A35	ED1	0.45720	0.00542
1993	A8	A113	0.50010	0.00454
1993	A43	A113	0.55357	0.00358
1993	A35	A8	0.58357	0.00461
1993	A8	IM	0.58652	0.00406
1993	A7	A79	0.61543	0.00611
1993	A14	A113	0.67251	0.00437

1993	ED1	IM	0.68846	0.00411
1993	A43	ED1	0.70432	0.00292
1993	A14	A35	0.74835	0.00451
1993	A14	A8	0.75425	0.00315
1993	A113	IM	0.76214	0.00418
1993	A79	IM	0.78995	0.00430
1993	A8	ED1	0.79263	0.00317
1993	A28	IM	0.79647	0.00176
1993	A35	IM	0.81310	0.00390
1993	A88	A43	0.83127	0.00127
1993	A43	A28	0.85333	0.00102
1993	A7	A43	0.86639	0.00246
1993	A7	A113	0.87517	0.00325
1993	A28	ED1	0.88630	0.00130
1993	A7	A8	0.90352	0.00239
1993	A88	ED1	0.90906	0.00126
1993	A113	A28	0.93253	0.00102
1993	A7	ED1	0.95134	0.00164
1993	A43	IM	0.96520	0.00096
1993	A88	A14	0.96596	0.00066
1993	A113	ED1	0.99741	0.00025
1993	A35	A113	1.00000	0.00000
1993	A88	A35	1.00000	0.00000
1993	A107	A113	No information	
1993	A107	A14	No information	
1993	A107	A28	No information	
1993	A107	A35	No information	
1993	A107	A79	No information	
1993	A107	A8	No information	
1993	A107	ED1	No information	
1993	A107	IM	No information	
1993	A43	A107	No information	
1993	A7	A107	No information	
1993	A88	A107	No information	
1994	A35	ED1	0.00000	0.00000

1994	A7	A14	0.00287	0.00104
1994	A28	IM	0.00308	0.00057
1994	A8	ED1	0.00335	0.00075
1994	A88	A28	0.00921	0.00092
1994	A28	ED1	0.01621	0.00165
1994	A35	A8	0.02042	0.00230
1994	A14	A8	0.02420	0.00274
1994	A43	A8	0.03537	0.00293
1994	A107	A113	0.05229	0.00557
1994	A14	IM	0.05425	0.00364
1994	A43	A35	0.05655	0.00446
1994	A79	A28	0.09263	0.00461
1994	A113	A28	0.09612	0.00469
1994	A88	IM	0.12622	0.00431
1994	A43	A28	0.12721	0.00475
1994	A113	ED1	0.14421	0.00786
1994	A7	A43	0.15010	0.00794
1994	A107	A79	0.15211	0.00952
1994	A107	A28	0.16677	0.00695
1994	A43	IM	0.17627	0.00591
1994	A113	IM	0.18279	0.00660
1994	A43	A79	0.19227	0.00828
1994	A43	ED1	0.19361	0.00819
1994	A8	A28	0.19413	0.00589
1994	A88	A113	0.19444	0.00726
1994	A107	A35	0.19637	0.00966
1994	A8	IM	0.19784	0.00631
1994	A35	A28	0.20031	0.00647
1994	A7	A88	0.20818	0.00825
1994	A7	A28	0.21161	0.00733
1994	A107	A14	0.21233	0.01122
1994	A88	A8	0.22065	0.00690
1994	A88	A14	0.22531	0.00729
1994	A43	A113	0.24475	0.00876
1994	A107	A8	0.25292	0.01155

1994	A8	A79	0.25579	0.00870
1994	A14	ED1	0.26246	0.00938
1994	A14	A35	0.26480	0.00924
1994	A79	ED1	0.26899	0.01022
1994	A35	A79	0.28344	0.00982
1994	ED1	IM	0.29221	0.00803
1994	A7	IM	0.30416	0.00896
1994	A7	ED1	0.31795	0.01127
1994	A14	A28	0.33958	0.00759
1994	A14	A79	0.35131	0.01027
1994	A8	A113	0.35277	0.01001
1994	A7	A113	0.36472	0.01156
1994	A43	A107	0.39857	0.01250
1994	A88	ED1	0.40088	0.00935
1994	A7	A35	0.41990	0.01146
1994	A7	A79	0.44801	0.01234
1994	A113	A79	0.48785	0.01135
1994	A88	A35	0.51718	0.00873
1994	A88	A79	0.56464	0.00916
1994	A35	IM	0.56577	0.00812
1994	A43	A14	0.56957	0.00974
1994	A88	A43	0.62307	0.00738
1994	A107	ED1	0.65889	0.01189
1994	A14	A113	0.65946	0.01013
1994	A79	IM	0.70868	0.00768
1994	A35	A113	0.89392	0.00609
1994	A7	A8	0.89702	0.00648
1994	A88	A107	0.92603	0.00525
1994	A107	IM	1.00000	0.00000
1994	A7	A107	1.00000	0.00000
1995	A88	A28	0.00004	0.00004
1995	A28	ED1	0.00016	0.00009
1995	A43	A79	0.00039	0.00021
1995	A14	A28	0.00070	0.00047
1995	A35	IM	0.00329	0.00097

1995	A43	A8	0.00480	0.00125
1995	A7	ED1	0.00560	0.00150
1995	A35	A28	0.00656	0.00160
1995	A28	IM	0.01284	0.00185
1995	A88	A43	0.01506	0.00199
1995	A43	ED1	0.01817	0.00246
1995	A8	A113	0.02273	0.00282
1995	A43	A113	0.02323	0.00293
1995	A8	A28	0.03132	0.00259
1995	A14	A8	0.03310	0.00411
1995	A7	A28	0.03464	0.00366
1995	A43	A28	0.03660	0.00334
1995	A14	A35	0.03836	0.00506
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1995	A113	ED1	0.08973	0.00563
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1995	A113	IM	0.17327	0.00778
1995	A88	A79	0.17492	0.00790
1995	A35	ED1	0.19599	0.00933
1995	A7	A8	0.20202	0.00923
1995	A35	A113	0.20358	0.01030
1995	A107	A35	0.21835	0.01158
1995	A7	A43	0.26146	0.01119

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VITA

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