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Ecological genetics of Melaleuca quinquenervia (Myrtaceae) : population variation in Florida and its influence on performance of the biological control agent Oxyops vitiosa (Coleoptera: Curculionidae)

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ECOLOGICAL GENETICS OF *MELALEUCA QUINQUENERVIA* (MYRTACEAE): POPULATION VARIATION IN FLORIDA AND ITS INFLUENCE ON PERFORMANCE OF THE BIOLOGICAL CONTROL AGENT *OXYOPS VITIOSA* (COLEOPTERA: CURCULIONIDAE)

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F. Allen Dray Jr.

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To: Dean R. Bruce Dunlap
College of Arts and Sciences

This dissertation, written by F. Allen Dray Jr., and entitled Ecological Genetics of *Melaleuca quinquenervia* (Myrtaceae): Population Variation in Florida and Its Influence on Performance of the Biological Control Agent *Oxyops vitiosa* (Coleoptera: Curculionidae), having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

Jennifer Richards

Suzanne Koptur

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Ray Schnell

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The dissertation of F. Allen Dray Jr. is appr

Dean R. Bruce Dunlap
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Florida International University, 2003

DEDICATION

From the time I was a young boy, I have had a deep fascination with the natural world. Its beauty has often astounded me, its intricacy fascinated me, its peacefulness refreshed me. It has completely captivated me, simultaneously compelling me to strive to understand its workings and to acknowledge the Almighty who is its ultimate source and sustainer.

My parents, Forrest and Eloise Dray, fostered this budding relationship with nature throughout my childhood and encouraged me to educate myself in ways that would further this interest. It was while engaged in this quest that I met Plessy, my bride of 18 years, in the Green Mountains of Vermont. As did my parents before her, Plessy has graciously accepted this calling on my life and thus followed me to the Everglades of southern Florida. Together with my children: Eve, Matthew, Desirée, and Rebecca, she has with patience and understanding endured this final stage of the educational journey my parents started me on decades ago. It has been a long and sometimes arduous journey, and without the prayers, perseverance, and encouragement of my family and friends I could not have completed this momentous task. It is to them, and to my Maker, that I dedicate this work.

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Colleagues at the USDA, ARS Invasive Plant Research Lab in Fort Lauderdale, Florida, have also contributed greatly to my growth as a scientist and to elements of this dissertation. Ted Center suggested *Melaleuca quinquenervia* as a study topic and provided logistical support for my research. Greg Wheeler trained me in the art of extracting and analyzing essential oils, and graciously allowed me to employ techniques he had developed for this purpose. Paul

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ABSTRACT OF THE DISSERTATION

ECOLOGICAL GENETICS OF *MELALEUCA QUINQUENERVIA* (MYRTACEAE): POPULATION VARIATION IN FLORIDA AND ITS INFLUENCE ON PERFORMANCE OF THE BIOLOGICAL CONTROL AGENT *OXYOPS VITIOSA* (COLEOPTERA: CURCULIONIDAE)

by

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Florida International University, 2003

Miami, Florida

Professor Brad Bennett, Major Professor

Melaleuca quinquenervia (Cav.) Blake (Myrtaceae) was imported into Florida from Australia over a century ago as a landscape plant. A favorable climate and periodic wildfires helped *M. quinquenervia* thrive; it now occupies about 200,000 hectares in southern Florida. A biological control (i.e., biocontrol) program against *M. quinquenervia* has been initiated, but not all biocontrol releases are successful. Some scientists have argued that poor biocontrol agent success may relate to genetic differences among populations of invasive weeds. I tested this premise by determining (1) the number and origins of *M. quinquenervia* introductions into Florida, (2) whether multiple introduction events resulted in the partitioning of Florida's *M. quinquenervia* populations into discrete biotypes, and (3) whether *Oxyops vitiosa*, an Australia snout beetle imported to control this weed, might discriminate among putative *M. quinquenervia* biotypes. Careful scrutiny of early horticultural catalogs and USDA plant introduction records suggested at least six

distinct introduction events. Allozyme analyses indicated that the pattern of these introductions, and the subsequent redistribution of progeny, has resulted in geographic structuring of the populations in southern Florida. For example, trees on Florida's Gulf Coast had a greater effective number of alleles and exhibited greater heterozygosity than trees on the Atlantic Coast. Essential oil yields from *M. quinquenervia* leaves followed a similar trend; Gulf Coast trees yielded nearly twice as much oil as Atlantic Coast trees when both were grown in a common garden. These differences were partially explained by the predominance of a chemical phenotype (chemotype) very rich in the sesquiterpene (E)-nerolidol in *M. quinquenervia* trees from the Gulf Coast, but rich in a mixture of the monoterpene 1,8-cineole and the sesquiterpene viridiflorol in trees from the Atlantic Coast. Performance of *O. vitiosa* differed dramatically in laboratory studies depending on the chemotype of the foliage they were fed. Larval survivorship was four-fold greater on the (E)-nerolidol chemotype. Growth was also greater, with adult *O. vitiosa* gaining nearly 50% more biomass on the (E)-nerolidol plants than on the second chemotype. The results of this study thus confirmed the premise that plant genotype can affect the population dynamics of insects released as weed biocontrols.

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Chapter I. General Introduction

Melaleuca quinquenervia (Cav.) Blake (common names: melaleuca, paperbark, punk tree) is a tree in the family Myrtaceae that was imported into Florida about a century ago as a landscape plant (Gifford 1945, Meskimen 1962, Morton 1966). Indigenous to the coastal wetlands of eastern Australia, New Caledonia, and Papua New Guinea (Blake 1968), *M. quinquenervia* is especially well adapted to the flooded, saturated soils of the historical Everglades of southern Florida (Fairchild 1947, Meskimen 1962, Morton 1966, Balciunas 1990, Vardaman 1994). *M. quinquenervia* also successfully invades drier habitats (particularly disturbed areas) when sufficient water is available. Further, *M. quinquenervia* is well adapted to ecosystems periodically swept by fires. Such fires, fuelled by highly volatile oils in melaleuca foliage, kill native plants while *M. quinquenervia* survives due to its thick insulating bark. These fires produce an ash that provides an ideal seed bed for the copious seeds released by the trees following a fire. Dense monocultures (up to 100,000 trees and saplings/acre) of melaleuca are often the result (Hofstetter 1991).

M. quinquenervia stands are of little value to wildlife (O'Hare & Dalrymple 1997). Encroachment by melaleuca into cypress and pine communities of southern Florida threatens the survival of the endangered Big Cypress fox squirrel. It is unacceptable as forage to the white-tailed deer upon which the federally endangered Florida panther feeds. A spring 1980 survey showed that the only birds nesting in *M. quinquenervia* were anhingas (Schortemeyer *et al.* 1981), so

loss of habitat threatens endangered wood storks and snail kites. Many rare orchids, ferns, and palms are also threatened by the melaleuca invasion (personal communication: Dan Austin, Florida Atlantic University, Boca Raton, FL, 1999).

Researchers investigating mechanical and chemical control strategies to mitigate the deleterious effects of *M. quinquenervia* on the Everglades have found that these methods are costly, produce ephemeral results, and are inappropriate for environmentally sensitive areas. Logs, stumps, and chemically treated trees often produce new shoots. Not only do treated trees often regenerate, but massive post-treatment seed release is typical and serves to expand the *M. quinquenervia* population, which already occupies about 200,000 ha in southern Florida (Bodle *et al.* 1994). Consequently, a task force of federal, state, and local land managers and researchers developed a plan for eliminating the threat to the Everglades posed by *M. quinquenervia,* a plan which included as its cornerstone a federal biological control project (Thayer & Bodle 1990; Laroche 1994, 1999).

Establishment of biological control agents, however, can be difficult. Harris (1993) reported that a third of the agents released to control invasive non-indigenous plants fail to establish in the plants' adventive ranges. Another third become established, but fail to stress the plants in any substantive way. Many explanations have been posited to account for these facts. For instance, Harris (1973) suggested that protocols for selecting biocontrol agents favor obtaining safe insects, but may actually interfere with selection of highly damaging agents. Hokkanen and Pimentel (1984) suggested that selection of agents long associated with a target species may limit effectiveness because the host may be adapted to

maximize resistance to the agent. Environmental conditions in the target plant's adventive range may be unsuitable for the proposed biocontrol agent (Harris 1988, 1993), or may produce plants of poor nutritional quality for the insects (Thomas & Room 1985, Wheeler & Center, 1996). Also, some insects may experience a substantial lag time before achieving outbreak densities (Vitelli *et al.* 1996).

One explanation that has received little attention involves biocontrol agent adaptation to specific plant biotypes (but see Gassmann & Schroeder 1995, Hasan *et al.* 1995, Dray & Center 1996, Sobhian *et al.* 2003). Plant species are composed of a heterogeneous mix of genotypes each of which adapts to local environmental conditions over time (Merrell 1981). Populations can therefore differ dramatically in growth habits, nutritive content, resistance to pests and pathogens, etc. (cf. Rhoades 1983, Parker 1992). Adaptive responses to local environmental conditions can become fixed by geographic isolation (Futuyma 1979), resulting in the development of distinct genetic biotypes (i.e., demes). Plant breeders commonly take advantage of this fact to produce resistant strains of row and tree crops (Zobel & Talbert 1984, Parker 1992).

Insect species likewise contain many different genotypes and develop distinct biotypes. This has been most often demonstrated for agricultural pests. For example, Smith (1989) reported that three gall-midge biotypes have developed that are adapted to Asian rice cultivars bred and planted for resistance to gall midges. Cultivated peas harbor four pea-aphid biotypes (Smith 1989). Wheat cultivars in the United States support at least ten Hessian fly biotypes (Smith 1989). Insect biotypes are occasionally so distinct that they have initially been

considered separate species. For instance, the Chinese biotype of the Asian hydrilla fly (*Hydrellia pakistanae* Deonier) was originally thought to be a different species (Gary Buckingham, USDA ARS Invasive Plant Research Lab, Gainesville, Florida: personal communication).

It should not be surprising, then, that insects from different populations might vary in their acceptance of and performance on individual host plants (DeBach & Rosen 1991, Bernays & Chapman 1994). This knowledge has strong implications for weed biocontrol programs. Failure to establish or poor performance by a biocontrol insect may result from poor matching of host and biocontrol agent genotypes. If this is true, then close matching of plant biotypes in native and adventive regions would permit selection of bioagents that are better adapted to target populations, thereby increasing likelihood of establishment and/or improving performance.

Disjunctions occur in the Australian portion of *M. quinquenervia*'s range, particularly in central and northern Queensland. Similar disjunctions in the ranges of *M. alterniflora* and *M. linariifolia* have resulted in the establishment of genetically distinct populations within each species (Butcher *et al.* 1995). Several observations suggest that *M. quinquenervia* populations may also differ genetically, i.e., there may be more than one *M. quinquenervia* biotype. For example, seeds from trees in northern Queensland grow better in soils from that region than in soils from southern Queensland, whereas seeds produced by trees in southern Queensland grow best in soils from the latter region (Balciunas *et al.*, unpublished report). Also, the tree flowers during February-July in Australia, but

only during December in Papua New Guinea (Blake 1968). Further, Ireland *et al.* (2002) reported that *M. quinquenervia* comprises two chemical phenotypes in Australia. Although other factors may explain these observations, they also suggest the possibility that Australia harbors several *M. quinquenervia* biotypes.

M. quinquenervia populations in southern Florida derive from several distinct introduction events (Meskimen 1962), each of which may come from a different portion of the plant's native range. Thus, Florida's populations may represent a suite of different Australian biotypes. Founder effects can strongly influence the population genetics of an introduced species in its adventive range (Barrett & Shore 1989, Hartl & Clark 1989). Also, each Florida population experiences different soils, hydroperiods, and other environmental conditions through which selection may have caused further genetic divergence. Thus, it is quite probable that Florida harbors two or more distinct Melaleuca biotypes.

Objectives

The possibility that Florida harbors more than one biotype of *Melaleuca quinquenervia* has been suggested by numerous researchers (personal communication: Ron Hofstetter, University of Miami, Miami, FL, 1998; personal communication: Francois Laroche, South Florida Water Management District, West Palm Beach, FL, 1998; Meskimen 1962; Wang & Littell 1983; Kaufman 1999). Therefore, determining the number of *M. quinquenervia* biotypes in Florida and identifying their origins (Australian or otherwise) could enhance melaleuca biocontrol efforts by directing researchers to locales where they could find insects

closely adapted to the plant biotypes found in Florida. This could, in turn, enhance the likelihood of establishment success or of improved performance by the biocontrol insects imported into this state. The purpose of this study, then, is to (1) determine the number and origins of *M. quinquenervia* introductions into Florida; (2) determine whether multiple introduction events resulted in the partitioning of Florida's *M. quinquenervia* populations into discrete biotypes; and (3) determine whether *Oxyops vitiosa*, an Australia weevil imported into Florida as a biocontrol agent, discriminates among *M. quinquenervia* populations or biotypes.

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"A few seeds in a letter from far-off Australia, a few trees started, and finally there is a changed landscape. No one can predict what will happen when he drops a seed from foreign parts in a new environment. If it happens to be a weed, he may regret it..."

- John Gifford (1945)

Chapter II. Invasion History of *Melaleuca quinquenervia* (Cav.) S.T. Blake in Florida

The invasion of natural areas by exotic plants is increasingly being recognized as a critical impediment to the preservation of native biodiversity and to ecosystem restoration efforts (Braithwaite *et al.* 1989, D'Antonio & Vitousek 1992, Hughes *et al.* 1991, Malecki *et al.* 1993, Zedler & Rea 1998). While eradication of harmful alien species is desirable it is oftentimes impractical, especially when the invasive species has become widespread. In such instances, a comprehensive regional strategy employing an integrated pest management (IPM) approach to regulating populations of nuisance plants can aid managers in allocating resources and coordinating efforts to optimize results. Recent examples from Florida target the notorious Everglades invader *Melaleuca quinquenervia* (Cav.) S.T. Blake, and the equally pestiferous *Schinus terebenthifolius* Raddi (Laroche 1994, 1999; Ferriter 1997).

A cornerstone of such plans is the call for intensive efforts to develop biological control agents of the weed [i.e., a plant growing in a region where it is not indigenous, and which is having detrimental effects on indigenous species of

that region (Harley & Forno 1992, US Congress 1993)], and to determine approaches for integrating these biocontrols into overall management practices (Laroche 1994, 1999; Ferriter 1997). Such biocontrol projects can be enhanced by an understanding of the introduction history of the invasive species. For example, ecological studies of a target weed in its native range help to identify stages in the plant's life history when biocontrol stresses can be most effective (Julien & White 1997). Weeds often have a broad geographic distribution, however, and many aspects of a species' biology can vary across its range. Thus, information regarding the sources of introduced plants can help focus researchers' attention on those populations in the plant's native range that are most closely related to the populations targeted for control, and thereby improve prospects for success.

Similarly, surveys for natural enemies should be concentrated in a target weed's native range because the greatest number of potential biocontrol agents is generally discovered near the center of diversification of a weed genus (Wapshere 1974, 1981). Individual populations in a plant's adventive range, however, are most susceptible to biocontrol agents from genetically similar populations in its native range (Harley & Forno 1992, Van Driesche & Bellows 1996). Thus, identifying origins of adventive populations allows researchers to concentrate faunal surveys on those populations in the native range that are most closely related to the plants they are trying to regulate. Unfortunately, this important information regarding the introduction and subsequent spread of pest plants is often lacking (Kowarik 1995), thereby impeding management efforts.

Melaleuca quinquenervia (cav.) Blake (traditionally known as the punk tree in Florida; Small 1933, Little 1953, Long & Lakela 1971, Wunderlin 1982) is an alien species that occupies at least 200,000 ha of wetlands in southern Florida (Bodle *et al.* 1994, Laroche 1998). It encroaches upon a variety of habitats: freshwater marshes (including sawgrass prairies), cypress swamps, pine flatwoods, hardwood hammocks, salt marshes, and, occasionally, mangrove forests. *M. quinquenervia* is particularly prominent along ecotones (Myers & Ewel 1990). During early stages of invasion, the presence of *M. quinquenervia* fosters increased structural diversity in herbaceous marsh communities which results in a concomitant increase in wildlife diversity (O'Hare & Dalrymple 1997). The ultimate outcome of invasions, however, is replacement of native habitat by dense punk tree forests with drastically reduced biodiversity (Austin 1978, O'Hare & Dalrymple 1997). Such transformations threaten the biological integrity of Florida's Everglades ecosystem (Mazzotti *et al.* 1997), which has been designated an International Biosphere Reserve (in 1976), a World Heritage Reserve (in 1979), and a Wetland of International Importance (under the Ramsar Convention in 1987) (Maltby & Dugan 1994).

Ongoing efforts to regulate punk tree populations include an ambitious biological control program (Laroche 1994, 1999; Turner *et al.* 1998) which includes studying the ecological genetics of *M. quinquenervia*. In conjunction with these investigations, I examined the origins and invasion history of Florida's *M. quinquenervia* populations.

Melaleuca quinquenervia

Taxonomy

Melaleuca quinquenervia is a member of the Myrtaceae, a widespread family in the tropics and southern temperate zones. The 3,800 species of Myrtaceae are split into two subfamilies: the Myrtoideae, which have fleshy fruits and opposite leaves, and the Leptospermoideae, which have dry fruits and alternate or opposite leaves (Cronquist 1981). *Melaleuca*, along with *Eucalyptus*, *Leptospermum*, *Metrosideros*, and *Callistemon*, belongs to the largely Australasian Leptospermoideae, a paraphyletic taxon (Judd *et al.* 1999).

Melaleuca comprises about 275 taxa, including several species recently transferred from *Callistemon* (personal communications: Lyn Craven, Commonwealth Scientific and Industrial Research Organization, Brisbane, Australia, 2001; see also Dawson 1978, Barlow 1988, Holliday 1989, Craven & Dawson 1998, Craven 1999). It occurs primarily throughout Australia, though a few tropical species extend to New Guinea, New Caledonia, Indonesia, and Malaysia. Linnaeus established the genus in 1767, based on an Amboinan (in present day Indonesia) specimen collected by Rumphius (Blake 1968).

Melaleuca quinquenervia (Cav.) S.T. Blake (Figure II.1) is one of the broad-leaved paperbarks, which includes *M. leucadendra* (L.) L. and closely allied species. It first was described as *Metrosideros quinquenervia* by Cavanilles from specimens collected in April 1793 near Port Jackson, New South Wales (Blake 1968, Craven 1999).

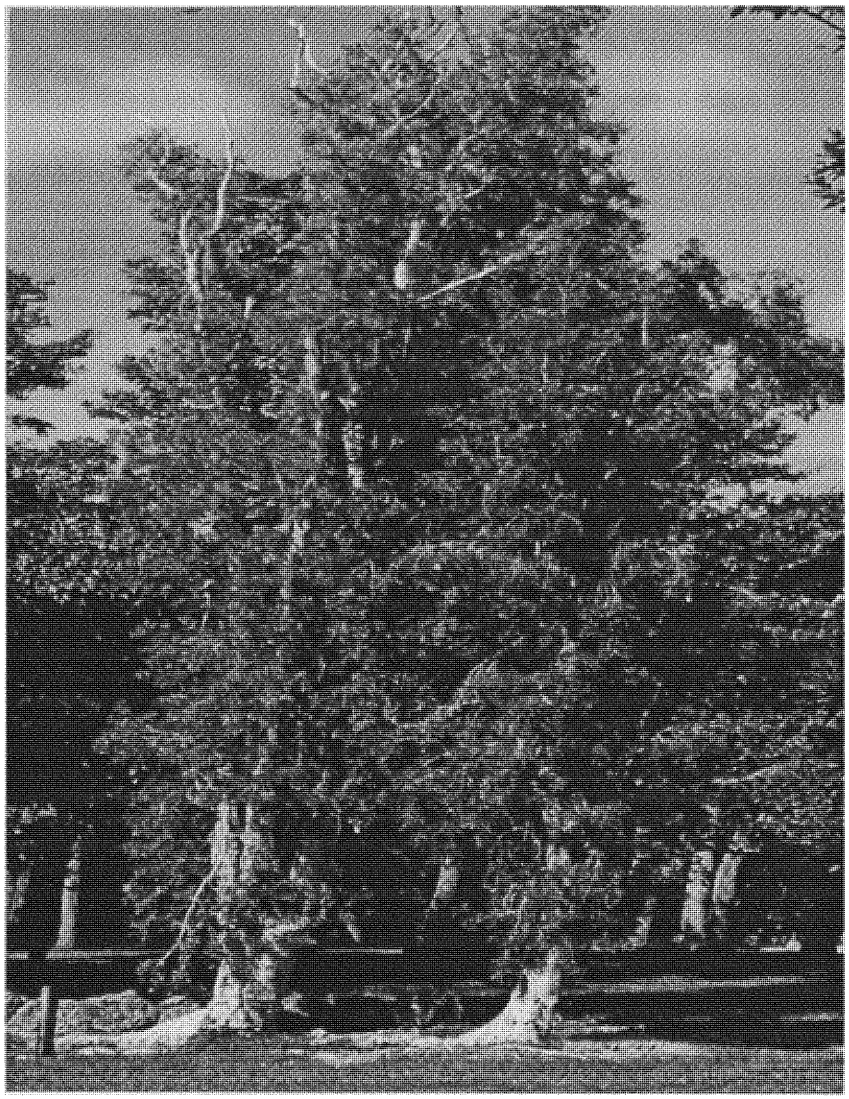

Figure II.1. *Melaleuca quinquenervia* trees in Matheson Hammock Park on Biscayne Bay, Dade County, Florida.

Distinguishing among the broad-leaved paperbacks is difficult. Most early botanists followed Bentham's 1867 treatise and simply classified *Metrosideros quinquenervia* and other broad-leaved paperbacks as varieties of *M. leucadendra* (Blake 1968, Craven 1999). The taxonomy remained confused until Blake's (1968) revision based on floral morphology and indumentum (hair) type

in which he established the new combination *Melaleuca quinquenervia*. Other authors have further clarified relationships and recognized new taxa, so that the broad-leaved paperbark complex now includes 15 species (Barlow 1988, Craven 1999). *Melaleuca leucadendra* is a taxon first described by Rumphius as *Arbor alba* in 1750 (Blake 1968). The binomial has been misapplied to Florida specimens (Wunderlin 1998). Thus, many references to *M. leucadendra*, including much of the early introduction records for the United States, should properly be ascribed to *M. quinquenervia* (Blake 1968).

Melaleuca quinquenervia occurs naturally throughout Queensland, New South Wales, New Caledonia, and southern New Guinea (Blake 1968, Craven 1999). Blake (1968) records cultivated specimens from South America (Guyana, French Guiana), Africa (Uganda, Senegal, Madagascar), and Asia (Hong Kong, Taiwan). It also is cultivated in Benin, Egypt, and the Bahamas (Gifford 1945, Correll & Correll 1982, Aboutabl *et al.* 1991, and Moudachirou *et al.* 1996). Confirmed U.S. collections include specimens from California, Florida, Hawaii, Louisiana, Texas and Puerto Rico (Morton 1966, Blake 1968, Little *et al.* 1974, Kartesz 1999).

Common names for *Melaleuca quinquenervia* in its native range include niaouli, paperbark, broad-leaved paperbark, five-veined paperbark, broad-leaved tea tree, Belbowrie, punk tree, and cajeput (Meskimen 1962, Morton 1966, Blake 1968, Boland *et al.* 1984, Ramey 1996, Craven 1999). Cajeput, however, more properly applies to the related species *M. cajeputi* Powell.

Ecology

In natural habitats, *Melaleuca quinquenervia* primarily occurs in seasonally or permanently inundated freshwater wetlands - both in Florida and in its native Australia. It is also an important component of the riparian vegetation along many Australian rivers and streams, and occurs in the brackish waters of mangrove swamps (Turner *et al.* 1998). *M. quinquenervia* is a fire-adapted species protected by thick, spongy bark. It reproduces copiously (Figure II.2a) and stores its seeds in tough woody capsules (Figure II.2b) that can remain unopened on branches for many years. The canopy in a punk tree forest thus comprises a seed bank containing up to 51 million seeds/tree (Rayachhetry *et al.* 1998). Seeds typically are released only after fire or some other stress interrupts phloem transport thereby causing capsules to dehisce (Rayachhetry *et al.* 1998).

In Florida, the seed rain released by intense fires often results in dense even-aged stands (Figure II.3) estimated to contain from 19,000 to 40,000 saplings/hectare (Meskimen 1962, Hoffstetter 1991). In contrast, Australian woodlands seldom comprise such dense stands, and seedlings and saplings are relatively rare (Balciunas *et al.* 1994). This paucity of young plants may be due to insect herbivory (Balciunas & Burrows 1993, Turner *et al.* 1998), as at least 400 insect species feed on *M. quinquenervia* in Australia (Balciunas *et al.* 1995b). This rich punk tree fauna has been the subject of intense investigation over the past few years as researchers search for organisms (primarily insects) with potential for use as biological controls of *M. quinquenervia* in Florida (Balciunas *et al.* 1993a, 1993b, 1995a, 1995b; Burrows *et al.* 1994, 1996).

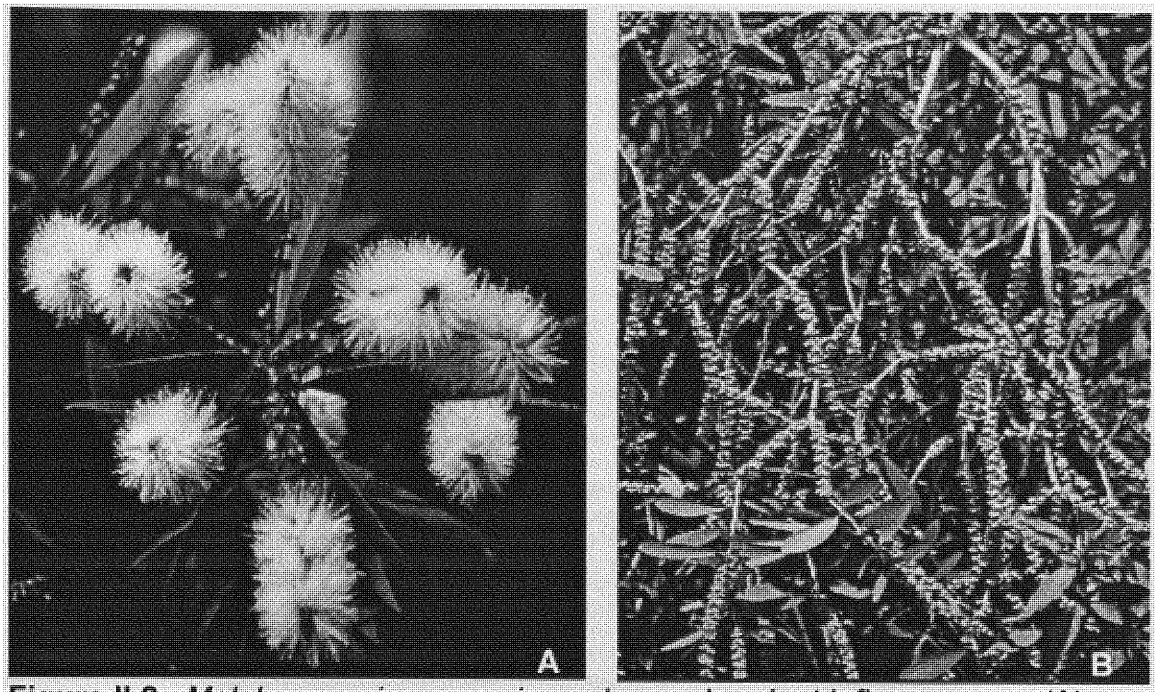

Figure II.2. *Melaleuca quinquenervia* produces abundant inflorescences (A), and stores its seeds in infructescences comprised of many tough woody capsules (B).

Introduction history

Species of *Melaleuca* have long been under cultivation. Blake (1968), for example, cites herbarium specimens of *M. cajeputi* collected in 1777 from a garden in Djakarta, Java (Indonesia). Directors of private and public gardens throughout the world added *Melaleuca* species to their collections because of the plants' colorful bottle brush-shaped flowers and exotic nature. Interest seems to have been particularly strong during the late 19th and early 20th centuries. For example, the Hope Gardens in Jamaica had obtained a specimen of *M. leucadendra* for its collection by 1904 (Blake 1968). The Missouri Botanic Gardens had acquired at least one *M. cajeputi* by 1906 (Blake 1968). Les

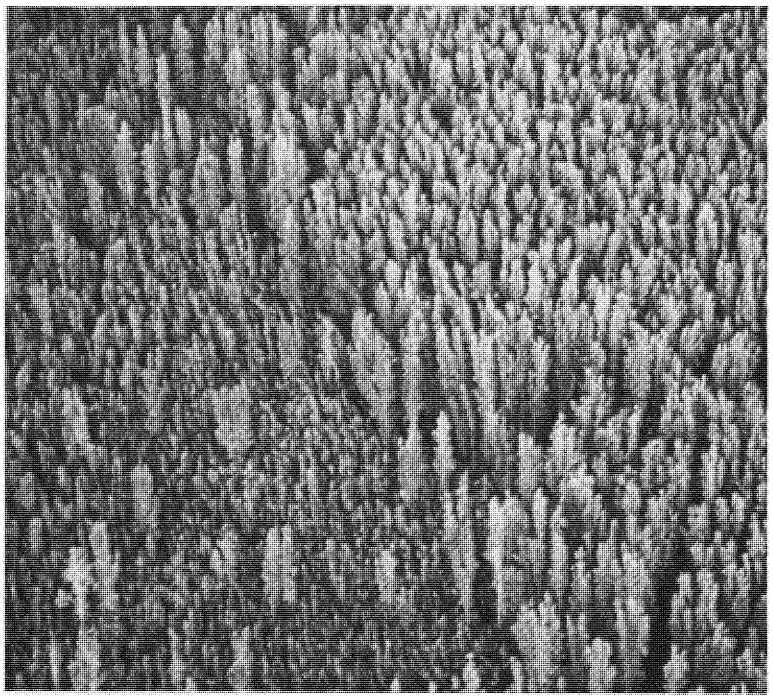

Figure II.3. Aerial view of a dense *Melaleuca quinquenervia* forest in Lake Okeechobee, Florida. (Photograph courtesy of U.S. Department of Agriculture)

Tropiques, the private gardens of Dr. A. Robertson-Proschowsky, in Nice, France, featured several Melaleuca species by the end of the 19th century (USDA 1902, 1905; personal communications: Gabriel Alziar, Curator, Le Jardin Botanique, Nice, France, 1998), as did the nearby La Mortala Gardens (now Giardini Botanici Hanbury at the University of Genova), the private gardens of Sir Thomas Hanbury in Ventimiglia, Itlay (Nehrling 1944; USDA 1913, 1931, 1934). Also, Bailey (1916) lists 14 species of *Melaleuca* grown as ornamental trees in California and Florida during the early part of the 20th century.

The earliest records of cultivated plants now known to be *Melaleuca quinquenervia* come from Uganda, where J.D. Snowden collected specimens in 1815 (Blake 1968). Horticulturists, landscape architects, private homeowners,

and botanical gardens worldwide came to covet the tree (then known as *M. leucadendra*) for its showy, creamy-white flowers, shaggy bark, ease of cultivation, rapid growth, and vigor in a variety of habitats (Maiden 1889, Bailey 1916, Andrews 1930, Nehrling 1933, Gifford 1945). Additionally, this tree was thought to be useful for "subduing malarial vapors" (von Mueller 1888, Gifford 1945, Morton 1966). The thick, spongy bark was used for packing fruit prior to shipping (von Mueller 1888), and as bedding material and insulation (Morton 1966). The heartwood was used for lumber (von Mueller 1888, Gifford 1945). Essential oils distilled from leaves have for centuries been used medicinally (von Mueller 1888, Fairchild 1943, Gifford 1945, Morton 1966), and constitute a principle component in some commercial disinfectants. The identity of the trees producing commonly marketed *Melaleuca* oils is often confused, however, and many uses ascribed to *M. quinquenervia* should actually be referred to *M. alternifolia* or *M. cajeputi* (Blake 1968).

Credit, or blame, for successfully importing *Melaleuca quinquenervia* into Florida's east coast has generally been ascribed to Dr. John Gifford (Fairchild 1943, Schory 1958, Morton 1966, Troop 1979, Ramey 1996, Gordon and Thomas 1997, Laroche 1998) although earlier introductions were known (e.g. Meskimen 1962, Morton 1966, Turner *et al.* 1998). Populations along Florida's west coast have been attributed to plantings made by A.H. Andrews of the Koreshan Unity sect (Rahn 1936, Schory 1958, Meskimen 1962). However, careful scrutiny of early horticultural catalogs and United States Department of Agriculture (USDA) plant introduction records suggests that other importations

contributed at least as strongly to the punk tree's invasion of Florida as did the Gifford and Andrews introductions. Table II.1 presents known introductions of *M. quinquenervia* into Florida (see also Figure II.4). Fifty-six other *Melaleuca* species have been imported into Florida, including three additional members of the broad-leaved paperbark complex *(M. leucadendra* and related species) to which *M. quinquenervia* belongs (Table II.2). Further, the punk tree was introduced into California (as *Metrosideros quinquenervia* by Coronado Nurseries, San Diego) at about the same time as it was in Florida, and was among 16 *Melaleuca* species being sold in the California landscaping trade at the beginning of the 20th century (Hall 1910). The following narrative details the earliest known introductions of *M. quinquenervia* into Florida.

ROYAL PALM NURSERY The first *Melaleuca quinquenervia* recorded in Florida were seedlings offered for sale as *M. leucadendra* by the Royal Palm Nurseries in Oneco, Manatee Co. (Reasoner & Reasoner 1887). Pliny Reasoner moved to Florida and started breaking ground for a nursery in 1881. His earliest plantings were fruits (mostly citrus) and palms (Pinardi 1980), but as the acreage under cultivation increased so did the variety of plants being grown at the Royal Palm Nurseries. Pliny and his brother Egbert searched the world for plants that would thrive in Florida, while accentuating its tropical beauty. Their nursery thus became a portal through which many non-native species, including the punk tree, were first introduced into Florida (Stoutamire 1926). A Manatee County historic marker (Figure II.5) located at the site of the original Reasoner

Table II.1. Introductions of *Melaleuca quinquenervia* (as *M. leucadendra* or *M. viridiflora*) in Florida.

Date	USDA Accession	Source		Imported by	Reference
		Contact	Locale		
1886	none	unknown	unknown	Pliny Reasoner, Royal Palm Nurseries	Reasoner & Reasoner 1887
1898	none	Sir Thomas Hanbury	La Mortala Gardens, Ventimiglia, Italy	Henry Nehrling	Nerhling 1944, Meskimen 1962
30-Apr-1900	5065	A. Robertson-Proschowsky	Les Tropiques, Nice, France	USDA	USDA 1902
13-Jun-1902	8871	A. Robertson-Proschowsky	Les Tropiques, Nice, France	USDA	USDA 1905
24-Oct-1902	9111	A. Robertson-Proschowsky	Les Tropiques, Nice, France	USDA	USDA 1905
1906	none	Dr. J.H. Maiden, Director	Royal Botanical Gardens, Sydney, New South Wales, Australia	John Gifford	Gifford 1972
17-Nov-1908	24166	Dr. J.H. Maiden, Director	Royal Botanical Gardens, Sydney, New South Wales, Australia	John Gifford	USDA 1909
29-Aug-1911	31736	Dr. J.H. Maiden, Director	Royal Botanical Gardens, Sydney, New South Wales, Australia	USDA	USDA 1912

1912	none	B. Harrison, Tweed River, New South Wales	Law, Somner, & Co., Melbourne, Victoria, Australia	A.H. Andrews, Koreshan Unity	Andrews 1930
31-Mar-1916	42357	Eugene Jaegle	Agricultural Station, Ivoloina, Madagascar	USDA	USDA 1919
31-Mar-1917	45510	Eugene Jaegle	Agricultural Station, Ivoloina, Madagascar	USDA	USDA 1922
21-Apr-1930	4104	A.H. Andrews	Estero, FL	A.H. Andrews, Koreshan Unity	unpublished USDA records
18-Dec-1930	90715	G.P Darnell-Smith, Director	Royal Botanical Gardens, Sydney, New South Wales, Australia	USDA	USDA 1932
30-Oct-1943	147097	A.TA. Semple, USDA Soil Conservation Service	Nahampaoma, Madagascar	USDA	USDA 1951
30-Mar-1959	256305	G.H. Spalding, USDA Agricultural Explorer	Northern Territory, Australia	G.H. Spalding, USDA Agricultural Explorer	USDA 1966
30-Mar-1959	256306	G.H. Spalding, USDA Agricultural Explorer	Maryborough, Queensland, Australia	G.H. Spalding, USDA Agricultural Explorer	USDA 1966
10-Apr-1959	256706	G.H. Spalding, USDA Agricultural Explorer	Adelaide Botanic Garden, Adelaide, South Australia	G.H. Spalding, USDA Agricultural Explorer	USDA 1966

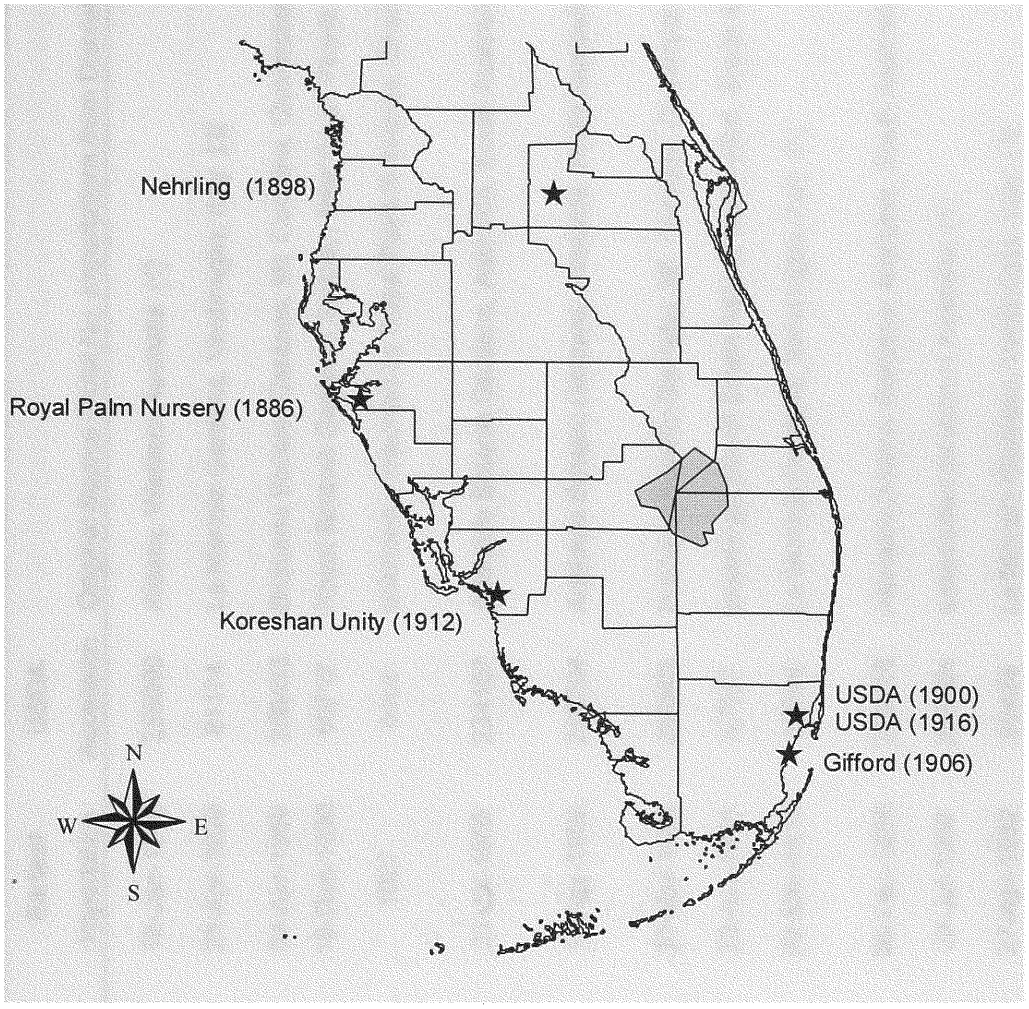

Figure II.4. Locations, dates, and recipients of original *Melaleuca quinquenervia* introductions into Florida.

Table II.2. *Melaleuca* species, other than *M. quinquenervia*, imported into Florida during 1900-1960.

Species[1]	Earliest Importation	USDA Accession	Original Source [Total No. Importations from Discrete Sources]
M. acacioides F. Muell. [as *M. graminea* S. Moore]	16-Jan-1959	255099	Albany, Western Australia [1]
M. acuminata F. Muell.	25-Apr-1929	81174	La Mortola Gardens, Ventimiglia, Italy [3]
M. argentea W. Fitzg.[2]	03-Apr-1959	256463	Brisbane Botanic Gardens, Mt. Coot-tha, Queensland [1]
M. armillaris Smith	18-Dec-1930	90712	Royal Botanic Gardens, Sydney, New South Wales [3]
M. bracteata F. Muell. [as *M. genestifolia* J.E. Smith]	1927	none	unknown - imported by Royal Palm Nurseries, Oneco, FL [2]
M. brevifolia Turcz. [as *M. neglecta* Ewart & B. Wood]	23-Jul-1956	234107	Adelaide Botanic Gardens, Adelaide, South Australia [2]
M. cajeputi Powell[2] [as *M. angustifolia* Gaertn.]	10-Apr-1959	256704	Adelaide Botanic Gardens, Adelaide, South Australia [1]
M. cheelii C. White	30-Mar-1959	256303	Brisbane Botanic Gardens, Mt. Coot-tha, Queensland [2]
M. cordata Turcz.	28-Apr-1926	67077	private garden of Edwin Ashby, Blackwood, South Australia [2]
M. cuticularis Labill. [as *Cajeputi cuticularis* (Labill.) Skeels]	02-Mar-1912	32924	La Mortola Gardens, Ventimiglia, Italy [1]
M. deanei F. Muell.	06-Dec-1955	230416	Adelaide Botanic Gardens, Adelaide, South Australia [2]
M. decussata R. Br. In Ait.	23-Jul-1956	234104	unspecified location in Australia [1]
M. diosmifolia Andr. [as *M. doesmaefolia* Andrews]	27-Apr-1932	99436	La Mortola Gardens, Ventimiglia, Italy [1]

M. eleuterostachya F. Muell. [as *M. adnata* Turcz.]	06-Dec-1955	230414	Adelaide Botanic Gardens, Adelaide, South Australia [1]
M. elliptica Labill.	18-Dec-1930	90713	Royal Botanic Gardens, Sydney, New South Wales [3]
M. ericifolia Smith [as *Cajeputi ericifolia* (Smith) Lyons]	1911	30793	La Mortola Gardens, Ventimiglia, Italy [4]
M. fulgens R. Br.	28-Apr-1926	67079	private garden of Edwin Ashby, Blackwood, South Australia [5]
M. gibbosa Labill.	23-Jul-1956	234106	unspecified location in Australia [1]
M. glomerata F. Muell.	30-Mar-1959	256304	unspecified location in the Northern Territory [1]
M. holosericea Schauer in Lehm.	28-Apr-1926	67080	private garden of Edwin Ashby, Blackwood, South Australia [1]
M. huegelii Endl.	01-Jan-1956	230909	unspecified location in Australia [2]
M. hypericifolia Smith [as *Cajeputi hypericifolia* (Salisb.) Skeels]	1911	30761	La Mortola Gardens, Ventimiglia, Italy [5]
M. incana R. Br.	14-May-1968	330382	Parks and Gardens Section, Canberra, A.C.T. [1]
M. lanceolata Otto [as *Cajeputi pubescens* (Schauer) Skeels]	1911	30795	La Mortola Gardens, Ventimiglia, Italy [6]
M. lateritia A. Dietr.	28-Apr-1926	67081	private garden of Edwin Ashby, Blackwood, South Australia [4]
M. laxiflora Turcz. [as *M. crassifolia* Benth.]	28-Apr-1926	67078	private garden of Edwin Ashby, Blackwood, South Australia [4]
M. leucadendra (L.) L.[2] [as *M. leucadendron* (L.) L.]	19-Nov-1953	210753	unspecified location in Australia [1]
M. linariifolia Smith	27-Apr-1932	99437	La Mortola Gardens, Ventimiglia, Italy [4]
M. macronychia Turcz.	06-Apr-1959	256529	private garden, Blackwood, South Australia [1]

M. megacephala F. Muell.	06-Dec-1955	230420	Adelaide Botanic Gardens, Adelaide, South Australia [1]
M. micromera Schauer in Lehm.	06-Dec-1955	230421	Adelaide Botanic Gardens, Adelaide, South Australia [2]
M. microphylla Smith in Rees	10-Apr-1959	256707	Adelaide Botanic Gardens, Adelaide, South Australia [1]
M. minutifolia F. Muell.	10-Apr-1959	256708	Adelaide Botanic Gardens, Adelaide, South Australia [1]
M. nesophila F. Muell.	27-Apr-1932	99438	La Mortola Gardens, Ventimiglia, Italy [2]
M. nodosa (Gaertn.) Smith	03-Nov-1927	75565	Royal Botanic Gardens, Melbourne, Victoria [1]
M. pauciflora Turcz.	06-Dec-1955	230423	Adelaide Botanic Gardens, Adelaide, South Australia [1]
M. pauperiflora F. Muell.	16-Jul-1952	202095	Dept. of Works, Adelaide, South Australia [2]
M. pentagona Labill.	06-Apr-1959	256530	private garden of Edwin Ashby, Blackwood, South Australia [1]
M. preissiana Schauer in Lehm.	18-Dec-1930	90716	Royal Botanic Gardens, Sydney, New South Wales [1]
M. puchella R. Br. in Ait. [as *Cajeputi pulchella* (R. Br.) Skeels]	02-Mar-1912	32928	La Mortola Gardens, Ventimiglia, Italy [1]
M. pungens Schauer in Lehm.	16-Jan-1959	255100	Popanginning, Western Australia [1]
M. pustulata J.D. Hook	01-Mar-1904	10510	Royal Botanic Gardens, Sydney, New South Wales [1]
M. radula Lindl.	28-Apr-1926	67082	private garden of Edwin Ashby, Blackwood, South Australia [4]
M. scabra R. Br. in Ait.	16-Jan-1959	255101	Geraldton, Western Australia [1]
M. squamea Labill.	08-Apr-1927	73443	Aldenham House Gardens, Elstree, Herts, England [1]
M. squarrosa Donn ex Smith	23-Jul-1956	234109	unspecified location in Australia [2]
M. steedmanii C. Gardner	06-Dec-1955	230425	Adelaide Botanic Gardens, Adelaide, South Australia [6]

M. styphelioides Smith	03-Nov-1927	75567	Royal Botanic Gardens, Melbourne, Victoria [2]
M. subtrigona Schauer in Lehm.	09-Apr-1959	256584	Dripstone, New South Wales [1]
M. teretifolia Endl. In Endl.	09-Apr-1959	256585	Dripstone, New South Wales [1]
M. thymoides Labill.	28-Apr-1926	67083	private garden of Edwin Ashby, Blackwood, South Australia [1]
M. thyoides Turcz.	16-Jan-1959	255103	Murchison, Western Australia [1]
M. uncinata R. Br. In Ait.	28-Apr-1926	67084	private garden of Edwin Ashby, Blackwood, South Australia [2]
M. violacea Schauer in Lehm.	28-Apr-1926	67085	private garden of Edwin Ashby, Blackwood, South Australia [1]
M. websteri S. Moore.	28-Apr-1926	67086	private garden of Edwin Ashby, Blackwood, South Australia [2]
M. wilsonii F. Mueller [as *Cajeputi wilsoni* (Mueller) Skeels]	02-Mar-1912	32929	La Mortola Gardens, Ventimiglia, Italy [3]

[1] Currently accepted names according to Blake (1968), Bailey & Bailey (1976), and Holliday (1989). Names under which these species were originally imported, if different from currently accepted names, are in braces.
[2] Species closely related to *Melaleuca quinquenervia* as members of the *M. leucadendra* complex.

homestead lists some other notable genera introduced by these pioneer horticulturists.

Sales catalogs were published annually by Royal Palm Nurseries beginning in1885, and are the most comprehensive source of information regarding plants imported by the Reasoner brothers. The punk tree first appeared in the catalog in 1887. Unfortunately, the Reasoner's records do not indicate when they first acquired *Melaleuca* seeds, but for seedlings and saplings to be available for sale in 1887 the importation most likely occurred sometime during 1886. A few trees from the earliest *Melaleuca* plantings have survived at the original nursery site (personal communications: Ward Reasoner, Reasoner

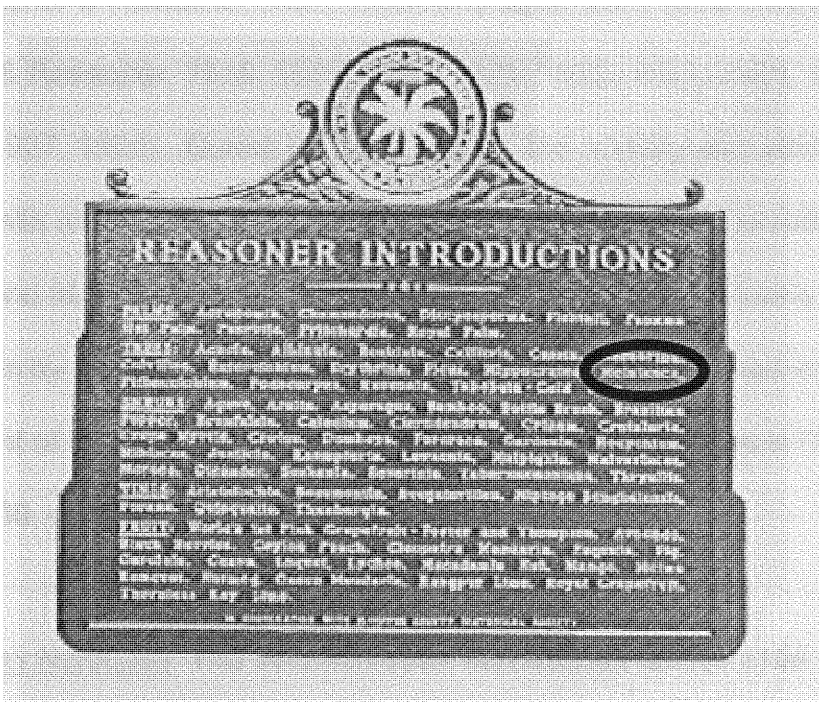

Figure II.5. Historical marker in Oneco, Manatee County, Florida, ascribing priority for introduction of Melaleuca and other exotic plants, many of which are now invasive, to Pliny and Egbert Reasoner of the Royal Palm Nurseries.

Landscaping, Oneco, Forida, 1997) allowing us to confirm their identity as *M. quinquenervia* (personal communications: Lyn Craven, Commonwealth Scientific and Industrial Research Organization, Brisbane, Australia, 2001). The source of these early plants is also unknown, though it is probable that they were acquired from a botanic garden ("Commencing in 1885, ..., exchange arrangements were soon completed with all the then known gardens and nurseries in the tropics and subtropics around the world.", note, p. ii, Reasoner 1927), perhaps even the Sydney Botanic Garden with whom the Reasoners had frequent correspondence (personal communications: Andy Reasoner, Royal Palm Nurseries, Oneco, Florida, 1999). The punk tree was sold at the Royal Palm Nursery from 1887-1889 (Reasoner & Reasoner 1887, 1888) and again from 1919 until 1933 (Reasoner & Reasoner 1918, 1919, 1920, 1921, 1922, 1923, 1924, 1925; Reasoner 1927, 1929). During the latter period, the nursery also sold *M. ericifolia* Smith (during 1922-23), *M. bracteata* F. Muell. (as *M. genistifolia*; during 1928-30), and *M. styphelioides* Smith (during 1930). The punk tree's popularity is attested to by its prominent use by landscape architects involved in the development of Palm Beach and other towns during the 1920s and 1930s (Pinardi 1980, p. 167).

HENRY NEHRLING The second known introduction was in 1898 by Henry Nehrling (Nehrling 1944, Meskimen 1962) who was a teacher by profession, but whose passion was horticulture. Nehrling obtained *Melaleuca* seeds from Sir Thomas Hanbury's La Mortala Gardens and cultivated them at his nursery (Palm

Cottage Gardens) in Gotha, Orange Co., Florida. The site was designated a USDA experimental station for a short period in the early 1900s, and later became a historical site (Old Nerhling Place).

Few of Nehrling's plantings, and none of his *Melaleuca*, have survived at the Gotha site which is now under private ownership (personal communications: Barbara Bichardi, private landowner, Gotha, Florida, 1998). However, a severe frost in 1917 prompted Nehrling to establish in 1919 a second nursery (Tropical Gardens & Arboretum) near Naples, Lee Co., Florida, at which he transplanted punk trees as well as many other cold-sensitive species. This site is currently a privately owned theme park called Caribbean Gardens. The 5-6 tallest melaleuca trees in the Gardens are likely the last remaining trees planted by Nehrling in 1919 (personal communications: Ralph Williams, Assistant Curator, Caribbean Gardens, Naples, Florida, 1998).

Nehrling corresponded and traded plants with the most prominent horticulturists in southern Florida, but there are no records to indicate that he ever distributed *Melaleuca quinquenervia* seeds or seedlings among his friends and acquaintances. Examination of the Caribbean Gardens property indicates, however, that the punk tree escaped cultivation at this site to invade surrounding marshes. Strangely, despite his associations with the Reasoners, David Fairchild, and A.H. Andrews (see below), Nehrling was apparently unaware of others having imported *Melaleuca* and believed himself to be its primary proponent (Nehrling 1933, Nehrling 1944).

The broad-leaved paperbarks at La Mortala Gardens are of unknown origin. In addition to *Melaleuca quinquenervia*, La Mortala Gardens harbored at least six other species of Melaleuca at this time (USDA 1913). Today ten species of *Melaleuca* are maintained in the gardens, but neither *M. quinquenervia* nor *M. leucadendra* is present (personal communications: Giorgio Campodonico, Curator, Giardini Botanici Hanbury, Université degli Studi Genova, Ventimiglia, Italy, 2001).

US DEPT. OF AGRICULTURE Three of the earliest introductions originated from Les Tropiques in Nice, France. This was the private botanical gardens of a physician, Dr. A. Robertson-Proschowsky, about whom very little is known. USDA records indicate that seeds from the first shipment (imported as *Melaleuca viridiflora;* USDA 1902), received during 1900, were successfully cultivated and seedlings distributed to individuals in southeastern Florida. Some of the distributed seedlings apparently were planted in Coconut Grove, Miami-Dade Co., Florida, because David Fairchild observed a 5 m-tall punk tree growing in the Grove during 1906 before John Gifford made his well-documented plantings (Gifford 1972).

USDA (1905) records indicate that additional seeds were imported (also as *Melaleuca viridiflora*) from Les Tropiques twice during 1902, but none of the seedlings germinated from this material survived. Records concerning the origins of the plants at Les Tropiques have been lost as the property changed hands over the years. Le Jardin Botanique (established in 1983) now occupies a

portion of Dr. Robertson-Proschowsky's former gardens (personal communications: Gabriel Alziar, Curator, Le Jardin Botanique, Nice, France, 1998). The flora at Le Jardin includes two species of *Melaleuca* (*M. styphelioides* and *M. preissiana* Schauer in Lehm.), but there are no longer any *M. quinquenervia* in their collections.

Somewhat later (1916) the USDA received seeds from the Ivoloina Agricultural Station near Tamatave, Madagascar (USDA 1919). This 400-hectare forestry station still harbors a thriving *Melaleuca quinquenervia* population (Ramanoelina *et al.* 1994). Both major Australian chemotypes (Boland 1989) are represented in Madagascar (Ramanoelina *et al.* 1994), so the latter trees likely derive from multiple sources in Australia. Unfortunately, records of plant introductions into Ivoloina have been lost (personal communications: Charlie Welch, Madagascar Fauna Group, Ivoloina Park, Tamatave, Madagascar, 2000). Seedlings grown from the Madagascar material were distributed throughout southeastern Florida (USDA unpublished records). Later importations by the USDA, including a second seed shipment from Madagascar, are listed in Table II.1.

JOHN GIFFORD Probably the best known introductions are those of John Gifford. He was apparently unfamiliar with earlier importers of the punk tree, as he believed himself to be the first to import this Australian tree (Gifford 1910, 1945, 1946, 1972). Gifford received *Melaleuca quinquenervia* (as *M. leucadendron*) seeds in November 1906 from Dr. John Maiden, Director of the

Royal Botanical Gardens at Sydney, New South Wales, Australia. It is unclear whether these seeds came from trees in the garden (there are at least two 100+ year old punk trees growing in the garden today; Kaufman 1999) or from the surrounding forests. Gifford first planted his seeds at the USDA Plant Introduction Station on Brickell Avenue in Miami, Miami-Dade Co., Florida, in 1907 with the help of Ed Simmonds (USDA botanist) (Gifford 1972). He later transplanted some of the resulting seedlings at his home along Biscayne Bay in nearby Coconut Grove (Gifford 1972). Additional shipments of seeds were received from Dr. Maiden in 1908 and 1911 (Table II.1).

Seedlings grown from these three shipments attained heights of 3.5-6.0 m, flowered, and produced seed within 12-36 months (USDA 1912, Gifford 1945). So both Gifford and the USDA soon had an abundance of seeds and seedlings to distribute to others. Simmonds frequently gave punk tree seedlings to local visitors at the USDA station (Gifford 1945). One of the earliest recipients was the Davie Experimental Farm, near Fort Lauderdale, which was eventually abandoned (Fairchild 1947). Somewhat later, Frank Sterling established a punk tree nursery in Davie with seed given to him by Gifford (Meskimen 1962, Gifford 1972), and from which he reportedly sold thousands of saplings (Fairchild 1947). Seeds from Gifford's trees were also exported to Louisiana and the Bahamas (Gifford 1946). When the USDA Plant Introduction Station moved to its present Chapman Field site during 1923, Gifford's punk trees were among the first seedlings planted.

JOHN LANGE An early south Florida pioneer, John C. Lange, claimed to have planted the first punk trees in Davie, Broward Co., Florida, around 1900 (Pritchard 1976), an event that is commemorated by a historical marker at what is now Lange Park. The park and surrounding neighborhood in Davie contain several very large punk trees that reputedly represent surviving trees from these plantings. If, as reported, these trees were planted around 1900 then they would most likely represent material derived from the first French introduction of *Melaleuca quinquenervia*. However, immigration of settlers into western Broward County did not begin until 1909 (Wagner 1982). The Lange family didn't arrive in Zona (the original name for Davie) until about a decade later (Wagner 1982; personal communications: Ms. Anna Hammer, daughter of W.A. Griffin who arrived with his parents in Zona during 1909, Davie, Florida, 1998). Thus, Mr. Lange's trees are most likely derived not from the French seeds in 1900, but instead likely represent the plantings of Gifford's material at the Davie Experimental Farm mentioned previously. *Melaleuca* species do not form annual growth rings by which these trees might be aged, but molecular or other techniques may eventually help resolve this issue. For now, it seems unlikely that Lange's trees represent an additional introduction event.

KORESHAN UNITY In 1894, Dr. Cyrus Teed moved to Estero, Lee Co., Forida, to establish a permanent home for the utopian communal society known as Koreshan Unity, which he had founded (Anon. 1994). His followers sought to replace the native pines and cypress they had felled around Estero with one or

more tropical forest species that would grow rapidly, be long-lived, and provide useful timber and other products for their community (Gifford 1946, Meskimen 1962). They tried more than 20 species of *Eucalyptus*, none of which proved suitable, before finally being directed to *Melaleuca quinquenervia* (as *M. leucadendron*) by a nurseryman (Mr. B. Harrison) in Burringbar, Tweed River, New South Wales (Andrews 1930). During 1912, Koreshan horticulturist Allen Andrews received a packet of punk tree seeds from a Melbourne, Australia, seed house (Andrews 1930, Gifford 1946). Since *M. quinquenervia* does not naturally occur near Melbourne, it is likely that the seeds derived from the Burringbar nurseryman who had arranged the shipment. Analysis of the essential oils in *M. quinquenervia* leaves from the Koreshan site in Florida and from Burringbar support this hypothesis (Chapter IV). The trees flourished in the Koreshan Unity nurseries, and seed was soon being spread along the edges of the surrounding swamps (Gifford 1946, Anon. 1966). Later, small saplings were harvested from the swamps and sold by the truckload (personal communications: Evelyn Horn, Koreshan Unity, Estero, Florida, 1998).

W.P. HAZARD Another early pioneer in southwestern Florida, Mrs. W.P. Hazard, in Estero, Lee Co., claimed to have made a separate introduction into this area sometime during 1913-14 (Hazard 1930, 1945). The reported source for the seed was the USDA (Hazard 1930), which would suggest the Hazard plants derived from either the Gifford material or the French material. Andrews (1930) disputed this claim in a letter to the Fort Myers Press, however, asserting

that he had queried the USDA which had no record of shipping material to the Hazards. Our searches of the USDA records confirm this. During a later exchange, Andrews (1945) argued that the Hazard material had been purchased from the nursery at Koreshan Unity. Meskimen (1962) reports that a former Hazard employee believed the seeds originally came from Malaya (present day Malaysia), but the only paperbark found there is *Melaleuca cajeputi* (Blake 1968, Craven 1999). The trees in and around Estero are clearly *M. quinquenervia* (personal communications: Lyn Craven, Australian National Herbarium, Canberra, Australia, 2001), so it seems likely that the Hazard's plants originated either from the Gifford material or the Koreshan Unity material. While molecular techniques may eventually clarify this situation, it is sufficient for the purposes of this paper simply to recognize that it is unlikely that trees on the Hazard property derive from a distinct introduction event.

US ARMY CORPS OF ENGINEERS In an effort to stabilize levees constructed to prevent Lake Okeechobee from flooding nearby agricultural communities and croplands, the U.S. Army Corps of Engineers made extensive plantings of *Melaleuca quinquenervia* during1938-41 (USACE 1959). The proximate source of trees for these plantings was a nursery established by the Corps in Clewiston, Florida, on the southern edge of Lake Okeechobee. We were unable to locate Corps records detailing the seed source for this nursery. However, Norman Reasoner was hired as an Agronomic Consultant by the Corps at about this same time (Pinardi 1980). So it seems likely that the Corps plantings derive from

Reasoner's Tropical Nursery (as it was then called) rather than representing some new importation from overseas.

Invasion of natural areas

Only a small number of alien species[1] escape cultivation to establish self-sustaining populations in their adventive ranges, and even fewer become successful invaders of natural areas (Kowarik 1995, Williamson & Fitter 1996). Determining when in their invasion histories such plants became naturalized is an inexact science. These species are often imported many times and planted in many different locations. Further, the expansion of populations of alien plant species is often interrupted by two discrete lag phases (Kowarik 1995). During the first, the plant is being cultivated but there is no clear evidence that it has begun spreading on its own. During the second, the population has begun spreading on its own, but has not yet achieved an exponential growth phase. Thus, searches of the literature and other historical sources may indicate when a species has been imported and cultivated, and then offer little information about the plant until it "suddenly" appears as a serious weed. This gap in information corresponds to the two lag phases in Kowarik's (1995) model, and is identifiable in the invasion history of *Melaleuca quinquenervia* in Florida.

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[1] "Aliens (non-native, non-indigenous) are species occurring in an area in which they have not evolved since the last Ice Age *and* whose introduction or immigration was supported deliberately or involuntarily by human activities." (Kowarik, 1995)

The earliest flora focused specifically on Florida (Small 1903) was published nearly two decades after the punk tree was first imported, but is drawn primarily from surveys in the southeastern part of the state. Importations preceding this date occurred in the central part of the state, thus it is unsurprising that the genus *Melaleuca* is not mentioned (Small 1903). Prior to publication of the second edition of this flora (Small 1913) *Melaleuca* seeds had been imported both by the USDA and Gifford, and Small had collected specimens from the USDA station at Brickell Avenue in Miami (NY: Small s.n., 1912). Thus, his omission of this species (Small 1913) suggests that *M. quinquenervia* had not yet escaped cultivation and begun to spread in southeastern Florida. This conclusion is supported by Harshberger (1914), who crossed the Everglades in 1911 but did not record the punk tree among the plants encountered during his trip.

The earliest evidence that *M. quinquenervia* was becoming naturalized in Florida appeared in newspaper articles written by Henry Nehrling. A posthumously published compilation of these articles (Nehrling 1933) shows that by the mid-1920s punk trees were spreading from the Koreshan plantings to become naturalized in the cypress swamps near Estero (southwestern Florida). Observations by Simpson (1926) and Small (1933) suggest that *M. quinquenervia* was also becoming naturalized in southeastern Florida during this period. Collections of *M. quinquenervia* (as *M. leucadendra*) by Buswell in the Big Cypress (NY: Buswell s.n., 1930) and by Moldenke at an undisclosed south Florida location (NY: Moldenke 3591, 1927) offer further evidence that the punk

tree escaped cultivation and spread into natural areas during the 1920s. These data show that the duration of the first lag phase in Kowarik's (1995) model was relatively short for *M. quinquenervia*: about a decade in southwestern Florida, and perhaps two decades in southeastern Florida.

No records relating to naturalization of the trees planted at the Royal Palm Nursery or at Nerhling's Palm Cottage Gardens were discovered. There is a small *Melaleuca quinquenervia* forest along a stream on the old Nursery property, but the absence of other large stands in the area suggests that these trees generally failed to escape cultivation. This may perhaps be attributable to the paucity of wetland habitat nearby. The punk tree occurs most commonly in the wetlands and swamps of eastern Australia, a habitat more similar to the southern Florida introduction points than to either the Reasoners' nursery in Oneco or Nehrling's garden in Gotha – both of which are located in well-drained habitat. In any event, trees from these introductions do not appear to have contributed to naturalized punk tree stands at least until after the Reasoners shipped large numbers of trees to Palm Beach from whence they may have escaped.

Nehrling apparently was also the first to report *M. quinquenervia*'s aggressive nature, and to sound an alarm about potential harm to the native environs (Hedwig 1984). Andrews (quoted in Gifford 1946) likewise observed the punk tree's invasive nature during the 1920s, and opined that the tree was more aggressive in Lee County than in Dade (now Miami-Dade) County. A couple of decades later, Fairchild (1943) remarked that early plantings at the

Davie Experimental Station had gone wild and engulfed a nearby abandoned orange grove. Like Gifford, though, he apparently did not foresee the damage this sort of behavior could cause in the Everglades.

Observations by Gifford (1940, 1944) and Fairchild (1943) show that the punk tree continued its spread into the eastern Everglades during the 1930s, but it is difficult to determine how much of the punk tree's present geographical distribution in Florida is attributable to natural dispersal and how much to human efforts. Clearly, dispersal of *Melaleuca quinquenervia* was greatly enhanced by its proponents (Gifford 1940, Pritchard 1976). As noted above, workers at the Koreshan Unity spread seeds along the western edge of the Everglades soon after receiving material from Australia. Meskimen (1962) reports that Hully Sterling scattered seed from an airplane over the eastern edge of the Everglades (in what is now Broward and Miami-Dade counties) during 1936 (see also Gifford 1946, 1972). Further, the Reasoner Brothers landscape architects exported thousands of seedlings to Palm Beach County and other areas (Pinardi 1980).

Human-aided dispersal is a common contributor to success of alien invaders (Pyšek et al. 1995), both by speeding the arrival of alien plants at so-called safe sites (Kowarik 1995, Wiens 1997) and by providing sources for reinvasion when local extinctions occur (i.e., the rescue effect, Gotelli 1995). These factors assist species in overcoming traditional barriers to population establishment: Allee effects (reduced population growth at low densities), demographic stochasticity (random fluctuations in birth and death rates), minimum viable population size (the critical density below which a population is

doomed to extinction), and environmental stochasticity (random environmental changes that drastically alter the suitability of a particular habitat, e.g. prolonged droughts).

Kowarik (1995) observed that 95% of woody invaders in Germany had endured a time lag of more than 50 years before beginning a period of rapid population growth. A study by Laroche and Ferriter (1992) shows that *Melaleuca quinquenervia* has been in such a growth phase since at least 1965 (see also Pritchard 1976). Determining when this exponential growth phase began is problematic, however, as Laroche and Ferriter (1992) represents the only quantitative data available on the subject. The Central Florida Beekeeper's Association issued an alarm in 1955 about the rapid spread of punk trees (Pritchard 1976). Unfortunately, their concern was principally that *Melaleuca* honey is considered undesirable and could spoil the quality of their product. [Ironically, the Florida State Beekeepers Association protested control measures aimed at curbing the spread of this tree two decades later (Pritchard 1976).] Thus, they did not provide any quantitative data in support of their claims.

Conclusion

The invasion history of *Melaleuca quinquenervia* in Florida presents an intriguing irony in that an alien species so vilified today was so esteemed in the past that its earliest importers argued about who deserved credit for its introduction. This present research suggests that extant populations derive from at least six distinct introduction events, the first of which occurred about 1886.

Contrary to popular perception, John Gifford was not responsible for first introducing this plant into Florida. This distinction belongs to Pliny and Egbert Reasoner of the Royal Palm Nurseries. Nor was Gifford the punk tree's primary proponent in southern Florida, he is just the best known. The Reasoner Brothers Landscape Architecture department, for example, planted thousands of punk tree seedlings from their nursery during landscaping work in Tampa and Palm Beach, and probably imported trees into Miami-Dade County as well (Pinardi 1980). Also, Koreshan historians (personal communications: Evelyn Horn, Koreshan Unity, Estero, Florida, 1998) recall bundles of saplings being handed out to settlers from all over southwestern Florida, and the USDA distributed punk tree seedlings for decades (USDA unpublished records).

Melaleuca quinquenervia was imported into this country for a variety of reasons. Henry Nehrling was merely interested in obtaining an attractive exotic tree to add to his personal gardens. The Reasoners and the USDA imported the punk tee as an exotic landscape plant that was well suited to the tropics and that could be readily propagated and sold. Gifford (1945, 1972) originally sought these trees (1) as a landscape species, (2) as a method of combating malaria and yellow fever (a use also advocated by von Mueller, 1888), and (3) to provide a useful forestry crop that could grow successfully on the edges of the Everglades. The Koreshan Unity likewise sought a useful fast-growing forestry crop to replace the pines and cypress they were harvesting from their lands.

For many years, Gifford also advocated using this tree in drainage projects (Gifford 1945). He believed that draining the land naturally (i.e., via

trees) was infinitely superior to the man-made drainage systems (i.e., canals and earthworks) then being initiated (Gifford 1935, 1945, 1972). A natural system comprised of a tropical forest would be limited in scope, permit native species time to adapt to the changing environs, and help conserve water and wildlife. In contrast, a man-made system would drain the land quickly and thereby destroy native species. Despite his advocacy of natural drainage projects, Gifford was an ardent and tireless supporter of the establishment of Everglades National Park (Gifford 1972). It is thus ironic that the tree Gifford was so proud of having introduced poses such a serious threat to the biodiversity of the Everglades ecosystem which he dearly loved (Austin 1978, Mazzotti *et al.* 1997, O'Hare & Dalrymple 1997).

Some of the early introduction events can be traced to discrete Australian sources. Others derive from extra-Australian material. These facts suggest that *M. quinquenervia* in Florida may harbor several distinct biotypes, perhaps as many as six. The relatively close proximity of many original Florida populations (Figure II.4) has likely led to intermingling of these biotypes. Historical distribution records support this hypothesis. Still, the composition of naturalized populations may generally be biased towards the biotype from the closest introduction point. This is currently being investigated, as is the influence that the presence of multiple biotypes may have on biological control agent performance.

The species began pioneering within two decades of introduction, and was naturalized no later than by the 1930s (Nerhling 1933, Gifford 1940). A

related species, *Callistemon viminalis*, started spreading into natural areas in a similarly short period of time (Small 1933), but has thus far failed to rival the menace *Melaleuca quinquenervia* presents to the Everglades. Purposeful seeding of natural areas by the punk tree's proponents may partially explain this discrepancy. Even so, *M. quinquenervia*'s ability to spread unaided, clearly demonstrated by Laroche and Ferriter (1992), has strongly influenced the geographic distribution we see today.

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Chapter III. Allozyme diversity of the Everglades invader *Melaleuca quinquenervia* (Cav.) Blake

Interest in plant invaders has become increasingly intense during recent decades as the effects of invasions in natural and agricultural lands have become ever more apparent (Wilson & Graham 1983, OTA 1993, Zedler & Rea 1998, Cox 1999). Much of the attention has centered on efforts to discern a common suite of attributes that permit colonizing species to become successful invaders (c.f. Zimdahl 1983, Rejmánek 1995, Sakai *et al.* 2001). Life history characteristics, competitive ability, and a lack of natural enemies are among widely cited factors contributing to invasion success (Crawley 1986, Pimm 1989, Massey & Hamrick 1998, Zedler & Rea 1998). Studies aimed at developing methods for lessening the ecological impacts of invaders in their adventive ranges have been equally intensive to those trying to understand invasion success (c.f. Patterson 1983, de Waal *et al.* 1994, Rosen *et al.* 1994, Julien *et al.* 2001). Among the most frequently advocated management tools is release of natural enemies from a weed's native range that have potential to severely impact the plant's population dynamics in its adventive range. Despite the fact that they differ in many respects, these aspects of the invasive plant issue, predicting invasion success and mitigating impacts of invaders, are linked by the common need for better understanding of the ecological genetics of invaders (Burdon *et al.* 1981, Myers & Sabath 1981, Barrett 1982, Scorza 1983, Pimm 1989, Sakai *et al.* 2001, Saltonstall 2002).

Conventional wisdom has long held that founder effects and genetic bottlenecks often result in pioneer populations of invading species which harbor reduced levels of genetic variation relative to source populations (Barrett & Shore 1989, Hartl & Clark 1989). This loss of genetic diversity can restrict the suite of potential responses to environmental variation and stochastic events (Pimm 1989, Barrett 2000). This, in turn, renders small founder populations more susceptible to extinction (Frankel & Soulé 1981, Hanski & Gilpin 1997), which may help explain why many invaders fail to establish persistent populations.

More recently, researchers have recognized that successful invasions commonly are associated with multiple introduction events (Kowarick 1995). Multiple introductions from a broader gene pool would be expected to reduce founder effects and result in substantial genetic differentiation among invasive populations (Hartl & Clark 1989). A number of studies have, in fact, described populations of naturalized, introduced species that exhibit substantial amounts of genetic variation in their adventive ranges (Barrett 1982, Chaboudez *et al.* 1992, Bailey *et al.* 1995). Genetic variation and population differentiation can strongly influence how species will respond to control attempts and to environmental changes in general. Thus, it is increasingly being recognized that understanding the extent of this variation is an important component in the arsenal of information required to properly manage invaders (Scorza 1983, Briese 1993, Kaufman 1999, Roderick & Howarth 1999, Collins *et al.* 2002).

Scientists working on biological control of weeds have been in the vanguard of those calling for increased attention to variation within and among-populations of invasive species (Burdon *et al.* 1981, Myers & Sabath 1981, Ehler & Andres 1983, Gassmann & Schroeder 1995). Crawley (1989), for example, notes that genetic variation among adventive populations of weeds can be an indicator that multiple varieties of natural enemy species may need to be released for some biological control efforts to succeed. Also, Wapshere (1981) suggests that the most virulent biocontrol attacks on invasive plants will likely employ close matching of invader and bioagent strains. Despite this interest at the theoretical level, however, few weed biocontrol projects in the United States have assayed genetic variation among-populations of target weeds (but see Hasan *et al.* 1995, Sobhian *et al.* 2003).

Melaleuca quinquenervia (Cav.) Blake is a large Australian paperbark tree that has invaded 200,000 hectares of Florida's prized Everglades ecosystem, replacing diverse plant communities with dense monocultures that have little value to native species (Bodle *et al.* 1994, O'Hare & Dalrymple 1997). It is particularly well suited for population genetic studies because of its relatively well-documented invasion history (Chapter II) and the natural geographic barriers that occur in its adventive range. Further, *M. quinquenervia* expresses distinctive patterns of chemical profiles in both its native and adventive ranges (Chapter IV, Ireland *et al.* 2001, Wheeler *et al.* 2002, 2003). Also, Kaufman & Smouse (2001) documented a large amount of variation in quantitative traits.

It has long been assumed that *M. quinquenervia* in Florida derived primarily from two sources (Meskimen 1962, Turner *et al.* 1998, Kaufman 1999). Populations along Florida's east coast purportedly arose from seeds imported by John Gifford in 1906 from the Royal Botanical Gardens in Sydney, Australia (Gifford 1972). Populations along the west coast presumably came from seeds imported by A.H. Andrews from a Melbourne, Australia, seed house during 1912. With a small number of founder events, and the likelihood of high levels of inbreeding resulting from a paucity of suitable pollinators, Florida populations have generally been assumed to harbor limited genetic diversity.

More recently, careful scrutiny of early horticultural catalogs and USDA plant introduction records has expanded the number of sources known to have contributed to populations along both coasts (see Chapter II). Also, these same data indicate that progeny from several of these pioneer populations have been widely distributed throughout the state. Further, an influx of indiscriminant pollinators occurred during the second quarter of the 20th century when apiculturists began over-wintering their honey bee colonies in southern Florida (Cutts 1996). Thus, it is conceivable that Florida populations of *M. quinquenervia* harbor greater genetic diversity than previously anticipated.

This study describes the allozyme diversity in 12 populations of *M. quinquenervia* distributed throughout its naturalized range in Florida, and is part of a larger effort to understand factors that may influence biological control agent establishment and efficacy in the state. Based on invasion history, life history characteristics, and observed trait variation, I hypothesized that populations of *M.*

quinquenervia in Florida would harbor substantial genetic diversity, show strong population differentiation, and have experienced modest rates of gene flow.

Methods & Materials

Plant populations

Five of the populations (sites 2, 3, 6, 11, and 12; see Figure 2.1, Table 2.1) selected for sampling were chosen to represent early *Melaleuca quinquenervia* importations in Florida (see Chapter II). A further six sites were selected to ensure that sampled populations were distributed throughout the adventive range of *M. quinquenervia* in southern Florida. One additional site (Estero Bay Preserve) was selected to represent populations that grow in brackish waters (most melaleuca populations in Florida, as in Australia, occur in permanently or seasonally inundated freshwater communities). Voucher specimens from populations included in this study are deposited with Dr. Lyn Craven (Australian National Herbarium, Canberra, Australia) who confirmed the plant identities, and at Fairchild Tropical Gardens, Miami, Florida.

Infructescences were collected from up to 20 open-pollinated individuals at each of these twelve *M. quinquenervia* populations (Table III.1, Figure III.1) to conform to Weir's (1996) recommendations for minimal sample sizes. Some sites, most notably Royal Palm Nurseries, had fewer than 20 reproductive individuals (see Table III.1) in which case all available trees were sampled. Samples were restricted to the third/fourth youngest crops retained on a branch

because viability is highest among seeds taken from these infructescences (Meskimen 1962, Rayachhetry *et al.* 1998, Rayamajhi *et al.* 2002).

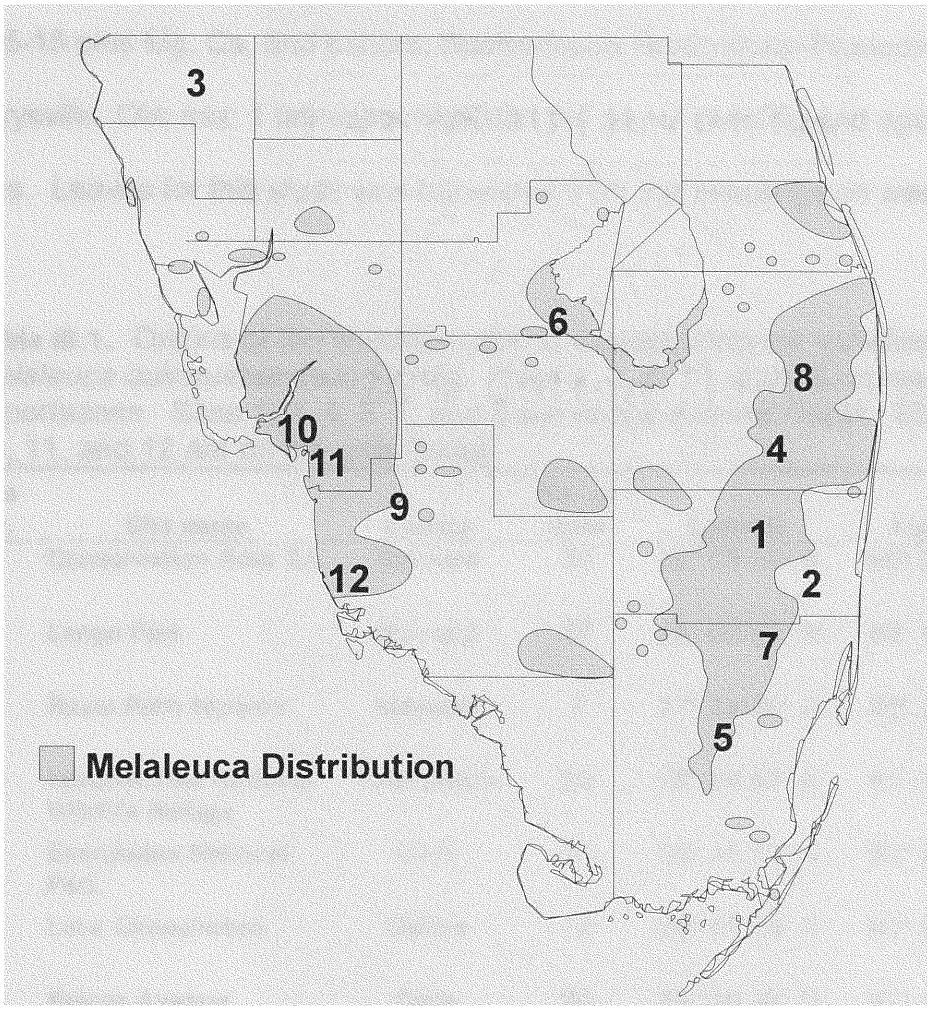

Figure III.1. Distribution of *Melaleuca quinquenervia* in southern Florida, showing populations sampled for this study. Numbers correspond to the sites in Table III.1.

The infructescences from each individual were bagged separately and transported to the laboratory where they were allowed to dehisce. Seeds were planted in germination flats (Figure III.2A) containing ProGro Potting Soil for Starting Seeds (Scotts-Sierra Horticultural Products, Marysville, OH). The flats

were kept in shallow (8 cm) water-filled trays to supply constant moisture. The flats were replaced by 0.5 liter pots into which the seedlings (Figure III.2B) were transplanted after six weeks. Each tray received 1 liter of fertilizer solution (Excel 15-5-15 plus Mg, Ca, and minors; Scotts-Sierra Horticultural Products, Marysville, OH; mix: 1 tablespoon/gal) on the day of planting and again after 90 days. Leaves for this study was harvested from the seedlings on week 20.

Table III.1. Origins of the seedlots used to assess allozyme variation in *Melaleuca quinquenervia* in Florida. Sites 2, 3, 6, 11, and 12 represent early importations. Sites 1, 2, 4, 5, 7, and 8 are on the Atlantic Coast. Sites 3, 9, 10, 11, and 12 are on the Gulf Coast.

Site no.	Site name	County	No. of trees	Latitude	Longitude
1	Conservation Area 2A	Broward	20	26° 09.46' N	80° 21.95' W
2	Lange Park	Broward	20	26° 03.80' N	80° 14.00' W
3	Royal Palm Nursery	Manatee	2	27° 26.80' N	82° 32.77' W
4	Loxahatchee National Wildlife Refuge	Palm Beach	20	26° 29.80' N	80° 16.36' W
5	Everglades National Park	Dade	20	25° 41.33' N	80° 29.83' W
6	Lake Okeechobee	Glades	12	26° 47.04' N	80° 57.14' W
7	Krome Avenue	Dade	20	25° 55.83' N	80° 27.03' W
8	Gramercy Park	Palm Beach	20	26° 45.61' N	80° 07.24' W
9	Corkscrew Wellfields	Collier	20	26° 27.49' N	81° 42.10' W
10	Estero Bay State Buffer Preserve	Lee	20	26° 28.15' N	81° 53.84' W
11	Koreshan State Historic Site	Lee	18	26° 26.13' N	81° 48.71' W
12	Caribbean Gardens	Collier	16	26° 10.20' N	81° 47.28' W

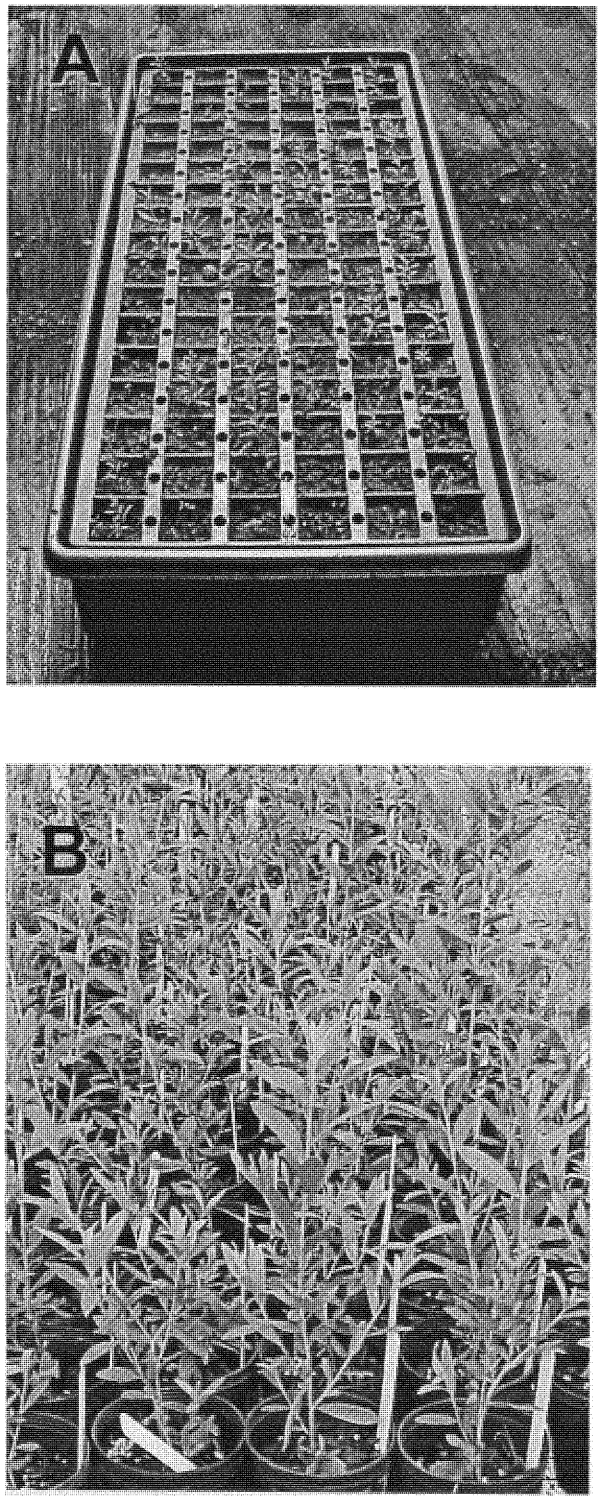

Figure III.2. *Melaleuca quinquenervia* seedlings sprouting in germination trays (A) and at time of harvest (B).

Allozyme analysis

We collected approximately 40 mg of leaf material from each seedling and sampled one seedling per maternal lineage. The leaf material from each seedling was placed in a 1.5 ml microcentrifuge tube, labeled, and stored at -80°C. Following Butcher *et al.*'s (1992) modification of Cheliak and Pitel (1984), an extraction buffer consisting of 3.5 g PVP-40, 0.5 g PVP-360, 25 mg ascorbic acid (Na salt), 85 mg EDTA (disodium salt), 50 mg dithiothreitol, 225 mg DIECA, 20 mg NAD, 10 mg NADP, 190 mg sodium metabisulfite, 400 mg borax (Na tetraborate), 1 mg pyridoxal 5'-phosphate, and 0.5 mg 1% β-mercaptoethanol all dissolved in 50 ml 0.1 M TrisHCl was prepared in advance, adjusted to pH 7.0, and stored at 10°C. Bovine serum albumin and sucrose (at 10mg/ml and 100mg/ml, respectively) were added immediately prior to extraction. To extract the enzymes, the frozen leaf tissue was homogenized in 1 ml of extraction buffer using a chilled pestle and mortar. The resulting slurry was centrifuged for 5 min at 12,000 rpm, and then the supernatant was drawn off and stored at -80°C.

Allozymes were separated using cellulose acetate gel electrophoresis (CAGE), primarily because run times are quite short (less than 1 hour), cellulose acetate is an excellent separation medium, and the gels are easily handled and stored (Acquaah1992, Hebert & Beaton 1993). A Tris-Glycine (TG) continuous buffer system was used in this study, following Hebert & Beaton (1993). The TG buffer consisted of 30 g trizma + 144 g glycine mixed in 1 l distilled water, then diluted at a 1:9 ratio of TG:H_2O. Gel plates (76 mm x 76 mm) were soaked overnight in 0.5x TG buffer prior to use.

Sample extracts were loaded onto the gels using a Super Z-12 applicator kit (Helena Laboratories, Beaumont, Texas) as described in Hebert and Beaton (1993). During preliminary runs, samples loaded into wells 1 and 12 produced irregular banding patterns, as observed by Hebert and Beaton (1993). Thus, only wells 2-11 were loaded with sample during the final study. Gel plates were gently blotted dry prior to sample loading to prevent samples from bleeding into each other, and sample extracts were applied to the gels from 1-3 times depending on the activity exhibited by each particular enzyme being assayed (Hebert & Beaton 1993).

For each run, three gel plates with samples loaded on them were laid Mylar side down on the support rails across the electrophoresis chamber. Both reservoirs of the chamber were filled with sufficient TG buffer to cover the platinum electrodes. Wicks made of Whatman filter paper drew buffer from each reservoir to the anodal and cathodal edges of the plates, respectively. Tank buffer and wicks were replaced as needed. Electrophoresis was carried out at room temperature, and lasted 30-45 minutes at 200 V with a current of 6 mA.

Enzyme activity was detected using stains described in Hebert and Beaton (1993), occasionally modified following Aquaah (1992). Where possible, stain components were prepared in advance and stored frozen until needed (Hebert & Beaton 1993). Stain components were then thawed and mixed while the gels were running, with the exception of photosensitive and labile components (PMS, dye salts, and linking enzymes; see Hebert & Beaton 1993). The latter components were added immediately prior to staining, and 2 ml of

warm (60°C) agar was then added to the mixture. The agar-stain mixture was poured over the gel plate which was covered by a light-proof box for 2 minutes to allow the agar to set. The plates were then transferred to a dark drying oven and incubated at 35°C to accelerate the staining process (Hebert & Beaton 1993). Plates remained in the drying oven until the enzyme bands had clearly resolved. Then the agar was washed off, and the gel was photographed and scored.

Data analysis

Preliminary assays of seven enzyme systems (Table III.2) produced 15 putative loci, 10 of which were consistently scorable across all gels. A locus was defined as a zone of activity that varied independently from other zones. Gels were scored following heuristics described by Richardson *et al.* (1986) to minimize subjectivity in interpretation. Loci within each enzyme system and alleles within each locus were labeled according to their migration rates, with the fastest migrating locus within each enzyme system designated number 1 and the fastest migrating allele in that locus designated as letter "a".

Allele frequencies were calculated from genotype arrays using PopGene (version 1.32; Yeh *et al.* 1999), and were used to parse genetic variation within populations into several components. Allele frequency estimates for the Royal Palm Nursery site (population 3) were excluded from these analyses because only two reproductive trees were available at the site. Allelic richness (*sensu* Brown and Weir 1983) was expressed as the average number of alleles per locus (A) and per polymorphic locus (A_P). Sample sizes were small (20 or fewer

Table III.2. Enzyme systems examined in 12 *Melaleuca quinquenervia* populations from Florida.

Enzyme system	EC code	Gene symbol	Quaternary structure	No. of loci
Aspartate aminotransferase	2.6.1.1	AAT	Dimer	1
Arginine kinase	2.7.3.3	ARK	Monomer	2
Glucose-6-phosphate isomerase	5.3.1.9	GPI	Dimer	2
Malate dehydrogenase	1.1.1.37	MDH	Dimer	1
Mannose-6-phosphate isomerase	5.3.1.8	MPI	Monomer	1
Phosphoglucomutatse	2.7.5.1	PGM	Monomer	2
Trehalase	3.2.1.28	TRE	Monomer	1

individuals per population), so loci were considered polymorphic when the most frequent allele occurred in less than 95% of the individuals in a population (Brown & Weir 1983, Nei 1987). This was expressed as the average proportion of polymorphic loci (P). The effective number of alleles (A_e; Kimura & Crow 1964), observed heterozygosity (H_o) and Nei's (1978) unbiased estimate of expected heterozygosity (H_e) were used to describe allelic evenness (Brown & Weir 1983), which is the equivalent of Nei's (1987) gene diversity. T-tests were used to probe for geographic trends, comparing mean allelic variation of the populations occurring along Florida's Atlantic Coast with those along the Gulf Coast - the principle distributional pattern of *M. quinquenervia* in the state. Genetic structure was examined using the F-statistics developed by Wright (1921, 1969; Nei 1987; Hartl & Clark 1989) as modified by Goudet (2002) in the

software program FSTAT. Overall genetic variation (H_t) was partitioned into gene diversity within subpopulations (H_s) and among subpopulations (D_{st}) using Nei's (1973, 1977) genetic diversity indices. Among-population diversity was expressed relative to overall gene diversity as Nei's (1987) coefficient of gene differentiation ($G_{st} = D_{st} / H_t$). The metrics D_{st} and G_{st} are both dependent on the number of subpopulations (Goudet 2002), which in the present study was only twelve. To compensate for this small sample size, Goudet's (2002) adjusted measures for these metrics were employed.

Among-population differences in allele frequency at each locus were examined using chi-square analysis: $\chi^2 = 2NG_{st}(a-1)$, df=(a-1)(n-1), where N is total number of individuals examined, a is the number of alleles at a locus, and n is the number of populations (Workman & Niswander 1970). Gene flow (N_M) among-populations was estimated as $N_M = 0.25(1 - F_{st})/F_{st}$ (Wright's 1951 method) using PopGene.

Geographic trends in genetic structure were investigated through hierarchical analysis of population structure. For this analysis, G_{st} was calculated for the two regions (Atlantic and Gulf coasts) using FSTAT. Total G_{st} was then parsed into two components: diversity among regions and diversity among-populations within regions, following Butcher *et al.* (1992).

An unweighted pair-group clustering based on arithmetic averages (UPGMA) of Nei's (1972) genetic distances was generated with PopGene (version 1.32; Yeh *et al.* 1999). Clustering programs force objects into clusters whether or not these clusters actually exist, so I tested the goodness of fit of the

clustering by comparing the cophenetic value matrix to the original distance matrix using NTSYSpc (Rohlf 2000). Bootstrap support (10,000 iterations) for the branching patterns within the phenogram was examined using procedures in Phylip (Felsenstein 2002). Genetic distance was also regressed against the natural log of geographic distance using the program SigmaStat (version 3.0; SPSS Inc., Chicago, IL).

Results

Allelic variation

Three of the 36 alleles identified in Florida populations of *Melaleuca quinquenervia* were rare, occurring at frequencies of 0.05 or less for the species as a whole. Interestingly, each of these alleles (PGM-1c, MPI-1c, and GPI-2a) was rare in Atlantic Coast populations but entirely absent from Gulf Coast populations. One additional allele (TRE-2a) that was absent from Gulf Coast populations was relatively common in Atlantic Coast populations. Each of the ten loci included in this study was polymorphic in at least one of the populations surveyed. Overall, these loci were polymorphic (frequency of most common allele < 0.95) 81.6% of the time (Table III.3). There was no difference between Atlantic and Gulf Coast populations in number of monomorphic loci ($\chi^2 = 0.80$, P=0.492).

Allelic richness as measured by mean number of alleles per locus (A) varied from 1.8 to 2.5 (mean=2.00) in Florida, whereas the effective number of

Table III.3. Estimates of genetic diversity for 11 populations of *Melaleuca quinquenervia* in Florida based on 10 allozyme loci. Population 3 was excluded because only two reproductive trees were present at the site. Parameters include the proportion of loci that were polymorphic (%P), number of alleles (A), number of effective alleles (A_e), number of alleles at polymorphic loci (A_p), and the observed (H_o) and expected (H_e) heterozygosities.

Pop.	P(%)	A	A_p	A_e	H_o	H_e
1	90.0	2.50	2.67	1.78	0.280 (0.331)	0.405 (0.196)
2	87.5	2.25	2.43	1.71	0.309 (0.283)	0.359 (0.228)
4	70.0	2.20	2.71	1.68	0.237 (0.351)	0.306 (0.278)
5	70.0	2.40	3.00	1.67	0.310 (0.378)	0.319 (0.275)
6	80.0	2.30	2.62	1.89	0.567 (0.368)	0.418 (0.253)
7	100.0	2.50	2.50	1.67	0.325 (0.310)	0.334 (0.215)
8	60.0	1.80	2.33	1.48	0.290 (0.395)	0.252 (0.251)
9	70.0	2.10	2.43	1.84	0.400 (0.375)	0.365 (0.272)
10	100.0	2.30	2.30	1.77	0.453 (0.296)	0.390 (0.196)
11	90.0	2.30	2.44	1.86	0.371 (0.344)	0.389 (0.229)
12	80.0	2.00	2.00	1.72	0.312 (0.337)	0.374 (0.230)
Regions						
Atlantic	79.6	2.28	2.61	1.67	0.292 (0.341)	0.329 (0.241)
Gulf	84.0	2.20	2.36	1.82	0.421 (0.344)	0.387 (0.236)
Overall	81.6	2.06	2.49	1.73	0.350 (0.343)	0.356 (0.238)

alleles (A_e) varied from 1.48 to 1.89 (mean=1.73). Absence of the rare alleles in Gulf Coast populations contributed to regional differences in A_e (1.82 vs. 1.66 on Atlantic Coast; t=2.85, P=0.019). Polymorphic loci (A_P) averaged 2.49 alleles

(Table III.3). The average diversity (i.e., heterozygosity; Nei 1987) observed within each population did not differ from unbiased estimates of expected diversity (t=0.16, P=0.872). However, mean observed heterozygosity was substantially greater on the Gulf Coast than the Atlantic Coast (Table III.3; t=3.11, P=0.012).

The similarity between observed and expected heterozygosities suggests that when viewed as a single population, *M. quinquenervia* is in Hardy-Weinberg equilibrium. This conclusion is supported by the small fixation index (mean F_{is}=0.044) for the species as a whole in Florida. However, 51 of 96 individual fixation indices were significantly different from zero (P<0.05), which is ten-fold greater than would be expected by chance alone. So, individual loci and populations did not meet Hardy-Weinberg expectations. Fixation indices for MDH-1 were significant, and for MPI-1 non-significant, in all populations. Otherwise, deviations from Hardy-Weinberg expectations presented no discernable pattern across populations and loci.

Population structure

χ^2 analyses showed that allele frequencies were highly (P<0.001) heterogenous among-populations at all ten loci. Mean G_{st} ranged from 0.147 to 0.723, suggesting a substantial *M. quinquenervia* population differentiation in Florida (Table III.4). However, when the 42% of genetic diversity attributable to population differentiation (G_{st}) was parsed into inter- and intraregional components, only about 16% (i.e., 7% of total diversity) resulted from differences

between Gulf and Atlantic Coast populations. The average number of migrants per generation (N_M) was small (0.34), and did not vary between regions (t=-0.153, P=0.880).

Table III.4. Distribution of genetic diversity among *Melaleuca quinquenervia* populations in Florida. Diversity is expressed as total genetic diversity (H_t), within-population diversity (H_s), differentiation among-populations (D_{st}), and the proportion of total diversity attributable to among-population variation ($G_{st}=D_{st}/H_t$) at each of ten loci representing seven enzyme systems.

Locus (alleles)	χ^2 (df)[1]	H_t	H_s	D_{st}	G_{st}
PGM-1	417.7 (22)***	0.46	0.232	0.249	0.517
PGM-2	178.2 (33)***	0.699	0.604	0.104	0.147
MPI-1	254.0 (22)***	0.349	0.241	0.118	0.329
TRE-2	573.7 (33)***	0.707	0.370	0.368	0.498
ARK-1	468.6 (22)***	0.485	0.214	0.296	0.580
ARK-2	499.3 (44)***	0.774	0.549	0.245	0.309
GPI-1	193.1 (22)***	0.569	0.442	0.139	0.239
GPI-2	876.3 (33)***	0.652	0.192	0.501	0.723
AAT-1	497.9 (22)***	0.598	0.224	0.407	0.645
MDH-1	260.4 (33)***	0.625	0.493	0.144	0.226
Atlantic Coast	3631.9 (385)***	0.539	0.328	0.254	0.436
Gulf Coast	1848.1 (341)***	0.565	0.385	0.225	0.368
Overall	5631.4 (385)***	0.592	0.356	0.257	0.419

[1] *** Significant at P<0.001.

Genetic identity (I) among-populations was strongest (0.843) between Loxahatchee Wildlife Refuge (site 4) and Caribbean Gardens (site 12). Trees at

Koreshan Unity (site11) and Estero Bay Preserve (site 10) exhibited similarly high genetic identity (0.830). Royal Palm Nurseries (site 3) and Koreshan Unity (site 11) exhibited the lowest genetic identity (0.324). Genetic identity averaged 0.585 (SD=0.118) overall. Genetic identity between populations in different regions averaged 0.581 (SD=0.111), which was similar to the mean genetic identity within each region (I=0.606, SD=0.109, Atlantic Coast; I=0.574, SD=0.145, Gulf Coast). Geographic distance explained 39% of the variation in genetic distance among Gulf Coast populations (F=8.47, P=0.012). It explained none of the genetic distance among Atlantic Coast populations (R^2=0.001, F=0.008, P=0.932), however, and only 4% of the genetic distance among-populations throughout the state (F = 2.75, P = 0.102).

The dendogram based on Nei's (1972) genetic distances is shown in Figure III.3. Goodness of fit between the original distance matrix and a cophenetic matrix representing the phenogram was marginal (r_{cs}=0.71; Unmack 1999). Bootstrap analysis offered weak (<0.50) support for branching points in the phenogram. Tree architecture is supported by historical (Chapter II) and chemometric (Chapter IV) data, however, so the low cophenetic and bootstrapvalues likely reflect the limited number of loci being examined (Sneath & Sokal 1973, Murphy *et al.* 1996).

Discussion

The invasion history of an exotic plant species has a strong influence on its success as an invader, with invasion success often (but not

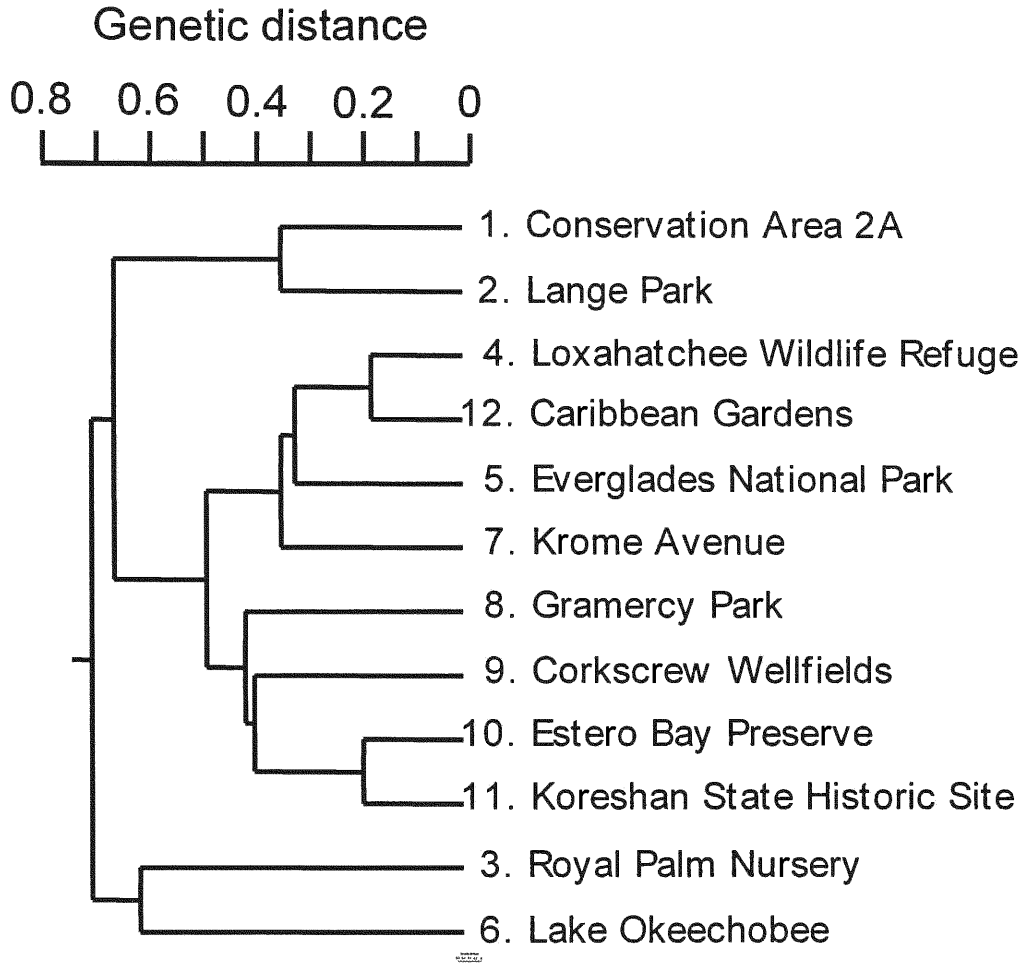

Figure III.3. Cluster analysis of *Melaleuca quinquenervia* populations in Florida based on Nei's (1972) genetic distance and the UPGMA algorithm. Bootstrap analysis offered only weak (<0.50) support for branching points so these values are not included.

exclusively) related to the number of introduction events (Moody & Mack 1988, Kowarick 1995). Multiple introductions increase the likelihood that pioneers represent a diversity of genotypes, which enhances a species' ability to respond both to local environmental conditions and to stochastic events (Mitton 1989, Pimm 1989, Foley 1997). *Melaleuca quinquenervia*'s successful encroachment

into southern Florida was, in part, promoted by more than a dozen introduction events involving at least six different seed sources (Chapter II). One consequence of the plethora of sources has been that this notorious Everglades invader harbors substantial genetic diversity as measured by analysis of ten allozyme loci using cellulose acetate gel electrophoresis. This finding compares favorably with Kaufman (1999, Kaufman & Smouse 2001), who reported substantial variation in several phenotypic traits within both Florida and Australia populations.

M. quinquenervia in Florida exhibits levels of intraspecific genetic variability representative of other species with similar life history traits. For example, a substantial proportion (61%) of allozyme loci tends to be polymorphic in tropical woody plants (Hamrick *et al.* 1979, Hamrick & Loveless 1989), and this was true of *M. quinquenervia* as well (mean P=82%). Likewise, mean number of alleles (A=2.06) in this study falls well within the range (A=1.00-3.12) reported by Hamrick *et al.* (1979) for tropical trees. Further, tropical woody species often harbor substantial heterozygosity (Hamrick *et al.* 1979, Hamrick & Loveless 1989), which was also true of *M. quinquenervia* (mean H_o=0.350). Thus, the gene diversity within Florida's *M. quinquenervia* populations is typical of many large, woody perennial species growing in tropical and subtropical regions of the world.

M. quinquenervia populations in Florida are strongly divergent from one another (G_{st}=0.419), which contrasts sharply with what has been reported for other tropical woody species. Hamrick & Loveless (1989), for example,

examined 14 tropical trees and shrubs and reported levels of interpopulation differentiation (G_{st}=0.022-0.090) an order of magnitude lower than found among Florida's *M. quinquenervia*. High G_{st} values are, however, common among early successional species (mean G_{st}=0.411; Loveless & Hamrick 1984). Successful invaders like *M. quinquenervia* perform like traditional pioneer species when encroaching into new territory, even though they may not fill that niche in their native ranges. Thus, the data from this study suggest that other invasive plant species are likely to exhibit high levels of interpopulation differentiation in their adventive ranges for a considerable time (it has been 100 years since *M. quinquenervia* was imported into Florida) after initial invasion. This prediction is corroborated by North American leafy spurge (*Euphorbia esula* L.) populations, which show a high divergence among populations (G_{st}=0.460; Rowe *et al.* 1997).

The high among-population diversity also relates to *M. quinquenervia*'s distribution in its adventive range. Although the tree has a reasonably restricted geographic range in Florida, the heart of the Everglades ecosystem serves as a natural geographic barrier that has forced a disjunction in the populations here. Butcher *et al.* (1992) demonstrated that a similarly small (<100 km) disjunction in the distribution of *M. alternifolia* populations in Australia has resulted in their separation into two genetically distinct regions. Likewise, the two principle *M. quinquenervia* regions in Florida (occurring along the Atlantic and Gulf coast) are genetically distinct (see also Kaufman 1999, Kaufman & Smouse 2001). The among-population diversity additionally reflects the fact that populations along the Gulf Coast derive primarily from seed sources (Ventimiglia, Italy; Burringbar,

Australia) quite distinct from the sources for populations along the Atlantic Coast (Nice, France; Sydney, Australia; Ivoloina, Madagascar; see also Chapter II).

Despite the evidence for regional differences, only a small proportion (7%) of the total genetic variation present among *M. quinquenervia* populations could be ascribed to the disjunction in this species' adventive range. In contrast, the high level of differentiation among-populations (42%) means that gene flow, even assisted by human agents, has thus far failed to homogenize independent gene pools established a century ago by discrete introduction events (Chapter II). Each introduction consisted of a small envelope of seeds, each of which likely derived from a single maternal seed source (Gifford 1945, Kaufman 1999). Limited numbers of colonization events, each consisting of only a few propagules, is a recipe for founder effects (Hartl & Clark 1989). Nei (1987) says such genetic bottlenecks can temporarily increase estimates of genetic distance, which may help explain the high average G_{st} value for *M. quinquenervia* in Florida.

The heavily infested areas around Lake Okeechobee might have served as a natural bridge between the two regions. However, *M. quinquenervia* seeds are small (Rayamahji *et al.* 2002) and gravity-dispersed, and so are deposited within a short distance (<170 m) of the parent tree (Meskimen 1962, Browder & Schroeder 1981). Hamrick (1989) noted that species with these characteristics typically have low rates of gene flow. Further, Vardaman (1994) reported that honey bees (*Apis mellifera*) were the principle pollinators, and Hamrick *et al.* (1979, see also Levin & Kerster 1967) indicate that efficient social insects

provide limited pollen dispersal. Each of these life history traits restrict the exchange of genetic material, which helps explain the low rate of gene flow (N_M=0.34) observed in this study. Further, historic evidence (Chapter II) suggests that transport of plant material between regions was largely unidirectional, with "truckloads" of saplings being transported from nurseries on the Gulf Coast to developing towns on the Atlantic Coast. Absence in Gulf Coast populations of four alleles that are present in Atlantic Coast populations, and especially lack of the TRE-2a allele (which is common along the Atlantic Coast), supports this interpretation of the historic data.

Interestingly, the origin of the Lake Okeechobee populations has never been clear. The U.S. Army Corps of Engineers (Corps) established a melaleuca nursery on the southern shore of the lake during 1938-41, as a source for trees to be planted along the levee to protect it from wave action (Chapter II). The seeds from which this nursery was established came from an unknown source. However, the close genetic relationship between the Royal Palm plants and those along Lake Okeechobee (Figure 2.3) suggests the Reasoner brothers (see Chapter II) provided the seeds to start the Corps nursery. Further evidence in support of this hypothesis comes from Pinardi (1980) who notes that Norman Reasoner (son of a Royal Palm Nurseries founder) worked as an Agronomic Consultant for the Corps at about this same time.

The *M. quinquenervia* populations included in this study were seldom in Hardy-Weinburg equilibrium. However, this is unsurprising in light of this tree's reproductive biology. Vardaman (1994) reported that *M. quinquenervia* is highly

self-compatible with 70% of artificially-selfed flowers, and 25% of autogamously-pollinated flowers, successfully producing fruit. She also noted, however, that the highest rates of fruit set she obtained were for cross-pollinated flowers, and that *M. quinquenervia* does not appear to be pollinator limited in Florida. Thus, it is likely that *M. quinquenervia* employs a mixed mating system in Florida. Hartl and Clark (1989) indicate that such plants seldom achieve Hardy-Weinburg equilibrium.

Conclusion

In summary, this present study confirms that *Melaleuca quinquenervia* in Florida exhibits the high levels of within-population genetic diversity predicted from examinations of phenotypic traits like caloric content of the wood (Wang & Littell 1983), leaf size, leaf shape, growth rate, biomass (Kaufman 1999, Kaufman & Smouse 2001), and leaf essential oil composition (Chapter IV). This level of diversity is consistent both with *M. quinquenervia*'s life history traits and with its introduction history, which likely includes several independent genetic bottlenecks (Chapter II). Knowledge of the latter might have eased Kaufman and Smouse's (2001) consternation at the level of diversity harbored within Florida populations. Genetic heterogeneity was greater within Gulf Coast populations than Atlantic Coast populations, which is a surprise given the relatively larger number of introduction events (representing a greater number of source gene pools) that occurred in the latter region as compared to the former (Chapter II), and that Gulf Coast populations are predominated by a (E)-nerolidol

chemotype that is less variable than the viridiflorol type which predominates along the Atlantic Coast (Chapter IV).

Substantial among-population differentiation was also observed, which was consistent with observations by Wang and Littell (1983) of marginally significant (P=0.069) differences in caloric content of *M. quinquenervia* tissues from plants in Lee Co. versus Miami-Dade Co. A portion of this differentiation was attributable to regional differences between Gulf and Atlantic Coast populations, which was fostered by *M. quinquenervia*'s life history traits, its introduction history, probable genetic bottlenecks, unidirectional anthropogenic dispersal, and low gene flow. Finally, close genetic identity between plants grown at the Royal Palm Nurseries and trees planted around Lake Okeechobee, coupled with historical data, suggest a solution to the mystery of where the USACE obtained material to start its *M. quinquenervia* nursery.

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Chapter IV. Intraspecific variation in leaf essential oils of *Melaleuca quinquenervia* in Florida

Melaleuca quinquenervia (Cav.) Blake was imported into the US beginning in the late 19th century, primarily for use as a landscape tree (in Florida and California) and in attempts to reclaim south Florida's wetlands by filling them with a useful timber crop (Hall 1910; Gifford 1946; Chapter II). Pre-adapted to thrive in a seasonally flooded habitat that is periodically swept by fire, this tropical evergreen tree quickly spread in Florida so that by 1994 naturalized populations occupied at least 200,000 hectares (Bodle *et al.* 1994). *M. quinquenervia* occurs in at least 21 Florida counties (Wunderlin & Hansen 2000), but naturalized populations are primarily concentrated in two disjunct bands extending from Miami to Stuart along the Atlantic coast and from Naples to Port Charlotte along the Gulf coast. A third, smaller concentration occurs along the southern shore of Lake Okeechobee, and additional populations are scattered throughout the Everglades.

In its native Australia, *M. quinquenervia* occurs in riparian communities and in coastal swamps from southern New South Wales to northern Queensland. It is also native to New Caledonia and southern New Guinea. Several distinct chemical phenotypes (i.e., chemotypes) of the plant have been described based on the composition of the essential oils (primarily terpenoid compounds) in the leaves, although there is disagreement over the exact number. Brophy *et al.* (1989), Brophy and Doran (1996), and Ireland *et al.*

(2002) reported that Australian specimens comprise just two chemotypes – one in which (E)-nerolidol is the primary constituent, and another containing either 1,8-cineole or viridiflorol as the primary constituent. Ramanoelina *et al.* (1992, 1994) suggested that this second chemotype assorts to two distinct types in Madagascar. Trilles *et al.* (1999) reported four additional chemotypes from New Caledonia. There thus may be at least seven chemotypes worldwide.

Little is known of the chemotypes present in the United States. Meskimen (1962) noted an early study concerning the essential oil chemistry of the *Melaleuca* species common in southwestern Florida. Unfortunately, results from this study were never published, and Meskimen (1962) only reported that the oil distilled from leaves on these trees did not meet the specifications for cajeput oil commerce. Doskotch *et al.* (1980), however, reported that ethanol extracts from *M. leucadendra* (probably *M. quinquenervia*) leaves collected in Charlotte Co., Florida, contained the sesquiterpine (*E,S*)-nerolidol.

Recent interest in better understanding the biology of this invasive tree prompted an exploration of *M. quinquenervia* leaf chemistry in its adventive range. In this paper, I characterize the essential oil profiles for a dozen naturalized populations of *M. quinquenervia* in Florida. These are compared to the profiles for plants derived from the same populations but grown in a common garden. Finally, I compare the profiles of Florida trees with other known profiles to ascertain possible origins of the Florida populations.

Methods & Materials

Plant populations

Twelve populations distributed throughout the geographic range of *Melaleuca quinquenervia* in Florida were included in this study (Figure IV.1, Table IV.1). Populations were selected for their proximity to sites of early introductions and to ensure that all invaded habitat types were represented. Locations of early importations of *M. quinquenervia* (as *M. leucadendra*) were obtained through searches of herbarium records, horticultural catalogs, USDA plant introduction records, and personal interviews with descendants of early plant importers (see Chapter II).

Natural stands

Because the essential oil composition of *M. quinquenervia* in Florida had not previously been evaluated, I conducted an initial screening limited to three trees from each of the twelve populations. Samples were collected over a 4-week period in August and September 1999 to minimize possible seasonal effects. Leaf samples were restricted to emergent buds and immature (those on pubescent stems and petioles) leaves as much as possible to minimize physiological age effects. However, sampled trees were of unknown ages and at different phenological stages during the sampling period, so these parameters represent possible confounding effects.

Stem apices 10 cm long were collected from each tree, placed into clear plastic bags, and transported on ice to the laboratory where they were stored at

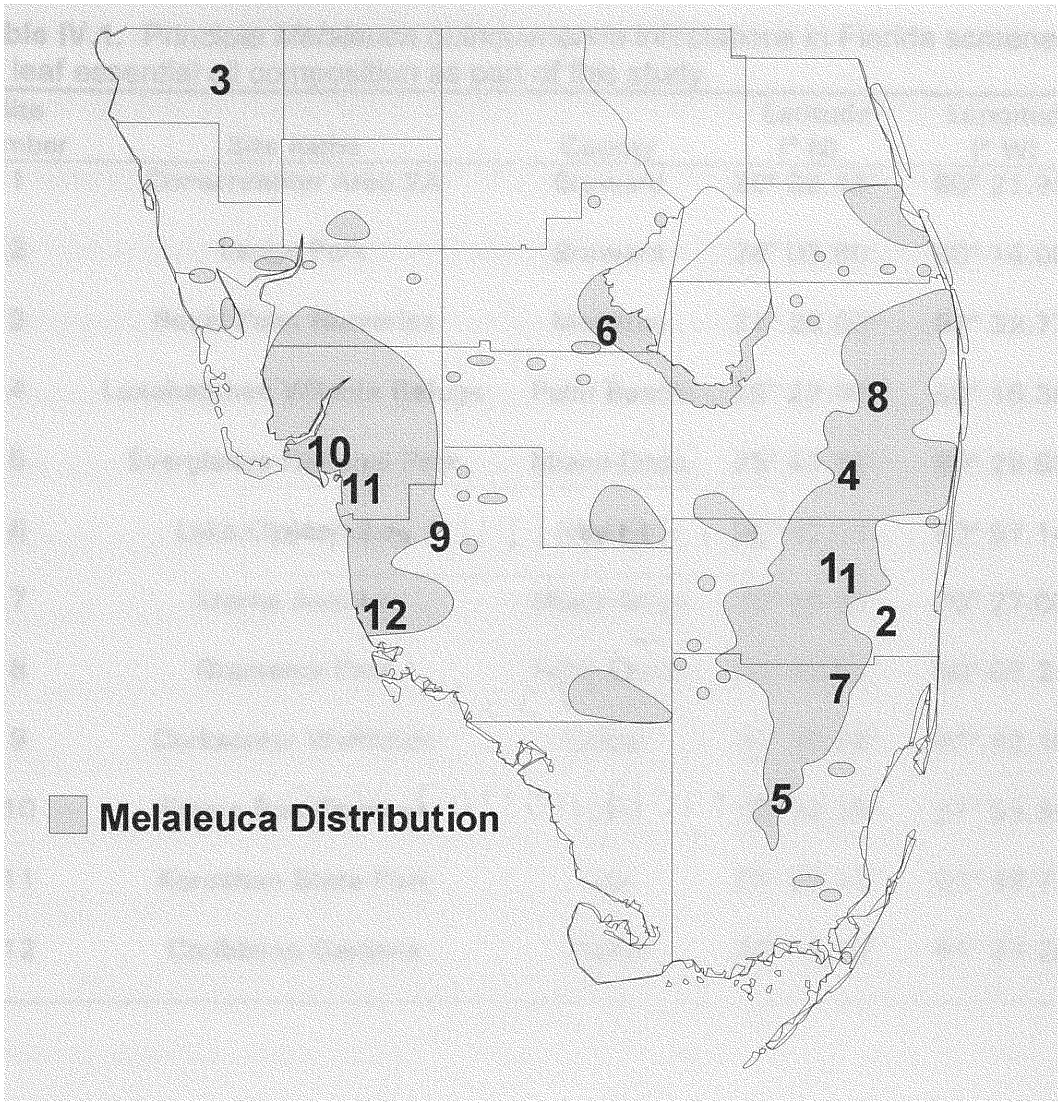

Figure IV.1. Distribution of *Melaleuca quinquenervia* in southern Florida, showing populations sampled for this study. Numbers correspond to the sites in Table IV.1.

-10°C for up to two weeks prior to processing. To extract the essential oils, I excised about 10 g of leaves from each stem, combined the leaves with 50 g crushed ice, 150 ml deionized water, and 10 ml chloroform, and then puréed the mixture in a kitchen blender for 1 minute. The resulting slurry was transferred to a 1000 ml flask and distilled for 3 hours. The distillate was combined in a 100 ml

Table IV.1. Principle *Melaleuca quinquenervia* infestations in Florida screened for leaf essential oil composition as part of this study.

Site number	Site name	County	Latitude (° N)	Longitude (° W)
1	Conservation Area 2A	Broward	26° 09.46'	80° 21.95'
2	Lange Park	Broward	26° 03.80'	80° 14.00'
3	Royal Palm Nurseries	Manatee	27° 26.80'	82° 32.77'
4	Loxahatchee Wildlife Refuge	Palm Beach	26° 29.80'	80° 16.36'
5	Everglades National Park	Miami-Dade	25° 41.33'	80° 29.83'
6	Lake Okeechobee	Glades	26° 47.04'	80° 57.14'
7	Krome Avenue	Miami-Dade	25° 55.83'	80° 27.03'
8	Gramercy Park	Palm Beach	26° 45.61'	80° 07.24'
9	Corkscrew Wellfields	Collier	26° 27.49'	81° 42.10'
10	Estero Bay Preserve	Lee	26° 28.15'	81° 53.84'
11	Koreshan State Park	Lee	26° 26.13'	81° 48.71'
12	Caribbean Gardens	Collier	26° 10.20'	81° 47.28'

flask with sodium chloride, shaken for about a minute, and allowed to stand until the essential oil-containing chloroform layer separated from the rest of the mixture. This layer was then recovered from the flask, filtered through glass wool, and stored at -10°C over anhydrous sodium sulfate until analysis.

Common garden

As noted above, *M. quinquenervia* trees at the twelve study sites in Florida are of indeterminate ages and grow in a variety of soil types, hydrologic

conditions, etc. Such non-genetic factors can confound attempts to classify trees using their chemical composition (Doran & Bell 1994). Consequently, I attempted to minimize the influence of non-genetic factors by comparing the terpenoid profiles of plants grown under uniform conditions in a screen house. Infructescences (Figure IV.2) were collected from up to 20 individuals at each of the twelve study populations. Exceptions were the Royal Palm Nursery site where only two individuals were reproductive, and the Koreshan site which harbored only 18 reproductive individuals. The infructescences from each individual were bagged separately and transported to the laboratory where they were allowed to dehisce. Seeds were germinated in ProGro Potting Soil for Starting Seeds® (Scotts-Sierra Horticultural Products, Marysville, OH), watered daily, and fertilized with Excel 15-5-15 (N-P-K; Scotts-Sierra Horticultural Products, Marysville, OH) at six month intervals.

Ten 15-month old seedlings (representing where possible ten separate, randomly selected, maternal source trees) were sampled from each population. Stem apices (10 cm long) were clipped from each seedling, sealed in clear plastic bags, and stored at -10°C until processed. For each sapling, approximately 100 mg of leaf material (comprising the leaf tips up to the 3rd youngest fully-opened leaf) was excised from the stem. The essential oils were extracted from this foliage using a modified microwave technique (Southwell *et al.* 1995; Degen & Stadler 1998; Gomez *et al.* 1999). In this technique, the thawed leaf tips were placed in sealed glass scintillation vials containing 1 ml of absolute ethanol. The vial contents were then irradiated at 750 W in a

microwave oven for 60 seconds. Equal volumes (500 µl) of the ethanol extract, distilled water, and chloroform were then combined in a 1.5 ml microfuge tube, and this mixture was centrifuged for 10 min at 10,000 rpm. The final samples consisted of 200 µl of the essential oil-containing chloroform layer, to which was added an internal standard constituted to achieve a final concentration of 25 ng/µl each of *n*-tridecane and *n*-eicosane. Samples were dried over anhydrous sodium sulfate and stored at -10°C until analyzed via chromatography.

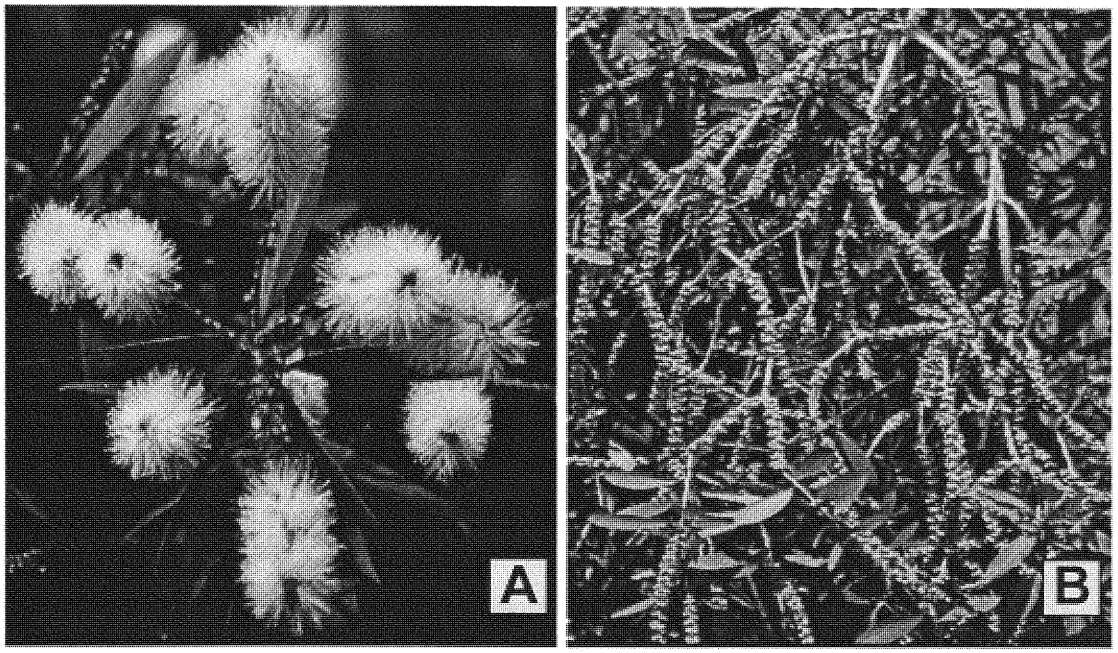

Figure IV.2. *Melaleuca quinquenervia* inflorescences (A) and infructescences (B).

Quantification of compounds

Analytical chromatography was performed on a Hewlett-Packard 6890 gas chromatograph with two fused silica capillary columns (DB5: Hewlett Packard Company, Wilmington, DE; DB17: J&W Scientific, Folsom, CA; both 30 m x 0.32

mm i.d. x 0.25 μm film thickness). The carrier gas (helium) was supplied at a linear flow rate of 37 cm/s. The oven temperature program ramped from 50-250°C at 8°C/min with 2 min initial and 5 min final isothermal periods. The injector and flame ionization detector temperatures were 200°C and 250°C, respectively. Data collection, storage, and analysis were conducted with the ChromPerfect system (Justice Innovations Inc., Mountain View, CA).

The essential oil constituents of the samples were identified by comparison of retention indices (calculated using *n*-paraffins) with purified chemical standards and published descriptions (Adams 1995), and by matching mass spectra with commercial (NIST, Wiley, and Adams) libraries. Compounds were quantified relative to the peak areas of the internal standards, and values were adjusted by calculation of their response factors relative to *n*-tridecane (Debbrecht 1985). Compound identities were confirmed by GC-MS using a Hewlett Packard 6890 instrument fitted with a HP-5MS (30.3 m x 0.25 mm, 0.25 micron film thickness) FSOT column with helium (36 cm/sec) as a carrier gas, injector port (split 1:50) at 250°C, mass selective detector (HP 5973) at 250°C (source) and 150°C (quad) with transfer line 280°C and ion source filament voltage of 70 eV. Quantification of unidentified compounds was accomplished by assuming each had a response factor of 0.5 relative to *n*-tridecane, in a manner similar to that used by Doran and Matheson (1994).

Statistical analysis

There has been controversy regarding appropriate methods for analyzing compositional data. Traditionally, researchers parsing plant samples into discrete classes based on leaf oil profiles have conducted principal components analysis (PCA) using proportional data (Hibbert 1997). But relative (e.g. % peak area) values are highly correlated and sum to unity (i.e., are not independent), which can confound statistical analysis (Aitchison 1983, 1984; Burks & Kanowski 1988). This argues for conducting analyses on quantitative (e.g. µg oil per mg leaf material) rather than relative data. However, despite the fact that test plants for this study were grown in a common garden to eliminate as much environmentally induced variation as possible, potential confounding factors still existed (e.g. shading effects resulting from position in the screen house). Environmental factors can have a strong influence on oil yields (White & Nilsson 1984, Mihaliak *et al.* 1987, Doran & Bell 1994) and thus on the quantities of essential oils recovered from leaves. Thus, it seemed advisable to compare oil yields within each experiment in terms of absolute quantities, but conduct PCA analyses on relative rather than absolute amounts of terpenes in leaves. This approach eliminated the confounding effects of environmental factors and offered the added advantage of permitting us to compare our findings with results from previous studies conducted in vastly different habitats and geographic regions.

Principal components analysis generates a matrix containing the correlations between variables, then re-describes these data in terms of a series

of independent factors (i.e., principal components). These factors represent weighted, linear combinations of the original variables, each of which accounts for some proportion of the overall variation in the data. Large data sets can thus be reduced via PCA to a few (generally 2-3) components that account for the majority of the original variation. These principle components can then be represented by two matrices: a scores matrix that summarizes the contribution of each object (the trees, in this case) to each principal component, and a loadings matrix that summarizes the contribution of each variable (leaf terpenoid compounds).

Several authors (e.g. Hibbert 1997) recommend preprocessing of the data prior to PCA. However, preliminary examinations showed that commonly applied methods produced no appreciable improvement in the discriminatory powers of the analyses (see also Dunlop *et al.* 1995; Adams 2000), so preprocessing of the data included in this study was unwarranted. Principle components derived from this analysis were retained if they accounted for at least 5% of the variation in the original data set (Wulder 2002). Factor loadings of ± 0.30 or greater were considered informative. The PCA analyses were conducted using the PRINCOMP procedure (SAS 1999).

Confirmation of the patterns obtained from PCA was sought using cluster analysis. For this analysis, the original data matrix was transformed into a matrix containing dissimilarity coefficients (Sneath & Sokal 1973) calculated using the average Manhattan distance metric (also called the city block metric) in the SIMINT module of NTSYSpc (v 2.11a; Rohlf 2000). Data in the resulting matrix

were clustered using the weighted option of Saitou and Nei's (1987) neighbor-joining (NJ) algorithm in NTSYSpc. This procedure produced a phenogram rooted at the midpoint of the two most dissimilar clusters.

Use of two different extraction methods over the course of this study caused concern that results from the two experiments would not be comparable. I thus assessed whether rank orders of essential oil constituents (based on average concentrations in µg/mg) differed between the field and screen house samples. This analysis was completed using a non-parametric ANOVA (NPAR1WAY procedure; SAS 1999) because transformations failed to normalize the data. I probed for geographic trends using ANCOVA for yield data (SAS 1999, Littell *et al.* 1991) and a G-test of independence (Sokal & Rohlf 1981) for the proportional data. Sample sizes were small (13 trees/population), so the G-values were adjusted using Williams' correction as recommended by Sokal and Rohlf (1981).

Results

Preliminary screening showed that in Florida, as in New Caledonia and elsewhere (Trilles *et al.* 1999), *M. quinquenervia* leaf essential oils consist of a complex mixture of over 100 compounds. Sampling errors are often large in minor constituents (Simmons & Parsons 1987), however, and only the most abundant terpenes contribute to chemotype differentiation (Southwell 1973, Dunlop *et al.* 1995). So the data set was restricted to those compounds that

constituted 1% or more of the oils in at least 1% of the samples. The resulting data set thus contained twenty compounds: five monoterpenes (α-pinene, β-pinene, limonene, 1-8 cineole, and α-terpineol) and two sesquiterpenes ((E)-nerolidol and viridiflorol) that have been common in previous *M. quinquenervia* essential oil studies (Brophy *et al.* 1989; Aboutabl *et al.* 1991; Ramanoelina *et al.* 1992, 1994; Moudachirou *et al.* 1996; Trilles *et al.* 1999; Ireland *et al.* 2002), and thirteen additional compounds that were consistently present in our samples (Table IV.2).

Oil yield

Plants from natural stands yielded more than double the essential oil of plants in the common garden (means=71.1 and 32.6 µg/mg, respectively; $F=24.03$, $p<0.001$). However, the coefficients of variation differed only marginally between the two experiments ($F=1.53$, $p=0.085$), suggesting the difference in means is likely a statistical artifact resulting from the small sample sizes (n=3) for populations in natural stands as compared to the sample sizes (n=10) for the common garden populations. This interpretation is strengthened by the similarity in ranges of yields between the two experiments (natural stands: 1.7-24.5% fw; common garden: 0.5-24.8% fw).

Despite the fact that plants in the common garden were grown under identical conditions, average yield varied markedly among source populations ($F=6.76$, $p<0.001$). This indicates a strong genetic component to essential oil yield in *M. quinquenervia* (Trilles *et al.* 1999), a portion of which corresponds to

Table IV.2. Terpenoid compounds recovered from *Melaleuca quinquenervia* in Florida. List represents those compounds (of >100 total) that comprised >1% of the essential oils in >1% of the samples (total n=155).

Compound	RT [2] (min)	RI [2]	Samples >1% (no.)	Maximum proportion of essential oils (%)	Mean (±s.e.) concentration (µg/mg)
α-pinene	4.81	987	116	18.7	1.08 (0.14)
β-pinene	5.97	1051	97	5.6	0.32 (0.04)
myrcene	6.16	1061	52	1.6	0.15 (0.02)
limonene	7.04	1108	109	11.4	0.85 (0.11)
1,8-cineole	7.57	1135	104	47.5	2.69 (0.46)
α-terpineol	11.51	1342	95	9.7	0.68 (0.12)
β-caryophyllene	14.72	1527	154	12.9	0.69 (0.09)
α-humulene	15.51	1576	47	1.8	0.08 (0.01)
alloaromadendrene	15.61	1582	79	2.2	0.11 (0.01)
β-selinene [1]	16.16	1617	23	1.6	0.11 (0.01)
α-selinene [1]	16.26	1623	82	4.0	0.35 (0.05)
γ-selinene [1]	16.72	1653	15	1.3	0.08 (0.01)
δ-selinene [1]	16.81	1659	46	2.1	0.14 (0.02)
(E)-nerolidol	17.41	1698	70	96.0	13.52 (2.26)
unknown	17.64	1734	34	4.4	0.05 (0.01)
globulol	18.34	1761	25	4.1	0.05 (0.01)
viridiflorol	18.50	1772	135	48.8	6.78 (0.91)
caryophyllene oxide	18.66	1783	91	17.9	0.43 (0.07)
α-cadinol [1]	19.06	1810	16	1.5	0.10 (0.01)
epi-α-cadinol [1]	19.39	1833	39	2.7	0.13 (0.02)

[1] Tentative identification of compound.
[2] Results from DB17-MS fused silica capillary column on a Hewlett-Packard 6890 gas chromatograph.

differences between the two principle chemical phenotypes (see next section).

In the common garden, trees with an abundance of (E)-nerolidol tended to

produce greater yields than other trees (42.4 vs. 26.2 µg/mg; F=5.48, p=0.021). The strong influence of non-genetic factors in natural stands (White & Nilsson 1984, Doran & Bell 1994) masked differences in yield between the two chemotypes (F=1.99, p=0.170), despite discernable among-population differences (F=2.43, p=0.034).

This influence of non-genetic factors on yield differed geographically, when holding the effects of chemotype constant. Thus, trees growing in natural stands on Florida's Atlantic coast yielded nearly twice the amount of oil on average as trees growing on the Gulf coast (90.8 vs. 51.5 µg/mg; F=8.62, p=0.006). This geographic trend was reversed (23.9 vs. 40.9 µg/mg, Atlantic vs. Gulf coast; F=5.15, p=0.025) for plants grown in the common garden where nutrient availability was uniform. This substantiates our conclusion that environmental factors influence yield, but nonetheless also supports the hypothesis that trees from the two regions differ in terms of essential oil content.

Essential oil composition

The twenty terpenoid compounds included in this study comprised 60-97% of the mono- and sesquiterpenoids extracted in both experiments (Table IV.2, Figure IV.3). Within populations, the rank order of individual oil constituents was consistent between the two experiments (F=1.56, p=0.228), a finding that compares favorably with observations by Doran and Bell (1994) for *Eucalyptus camaldulensis*. This allowed us to combine the two data sets for further analysis of essential oil composition.

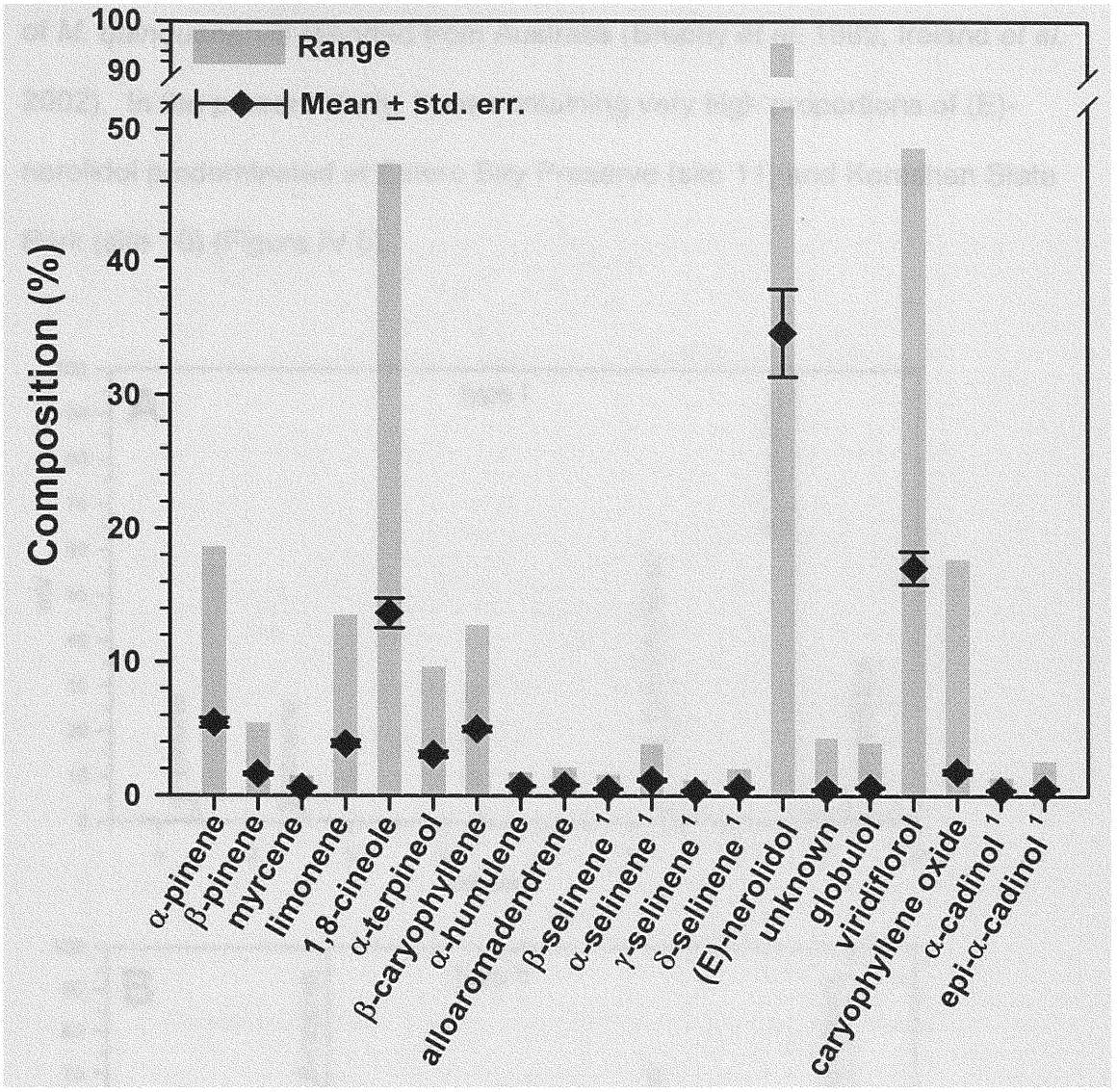

Figure IV.3. The 20 principle terpenoid constituents of essential oils extracted from young leaves of *Melaleuca quinquenervia* in southern Florida. Each constituent comprised at least 1% of the essential oils in 1% or more of the samples. Compounds marked [1] are tentative identifications.

These analyses showed that composition differed qualitatively, both within and between populations. Individual trees produced oils containing primarily either (E)-nerolidol or a combination of viridiflorol and 1,8-cineole (Figures IV.4 and IV.5). These two chemical phenotypes (chemotypes) are also the principle forms

of *M. quinquenervia* reported from Australia (Brophy *et al.* 1989, Ireland *et al.* 2002). In the present study, trees containing very high proportions of (E)-nerolidol predominated at Estero Bay Preserve (site 11) and Koreshan State Park (site 10) (Figure IV.5).

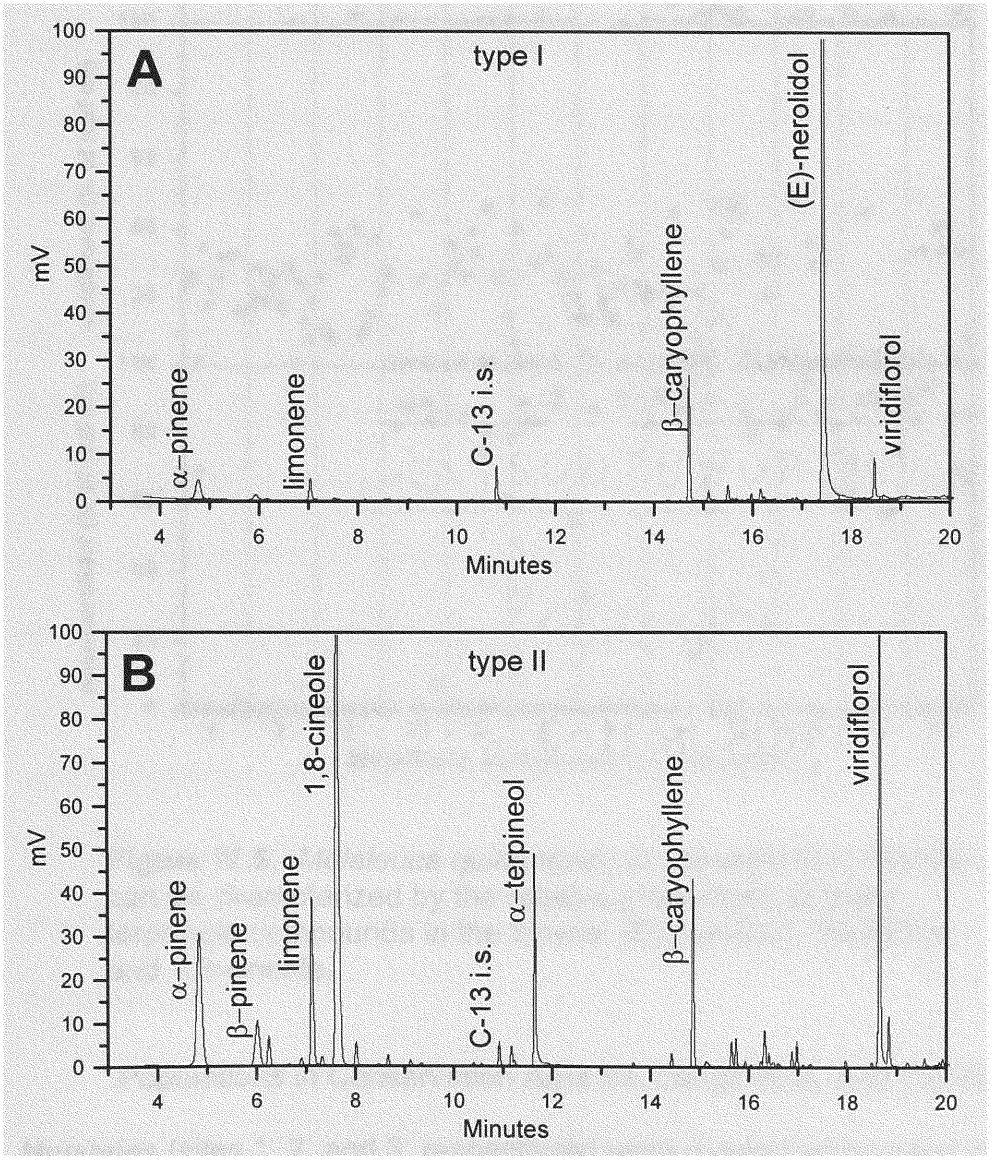

Figure IV.4. Chomatograms of the two chemotypes comprising *Melaleuca quinquenervia* populations in Florida: type I, in which (E)-nerolidol (A) is the principle terpenoid compound in the leaf essential oils, and type II, in which viridiflorol and 1,8-cineole (B) are the principle terpenoid compounds.

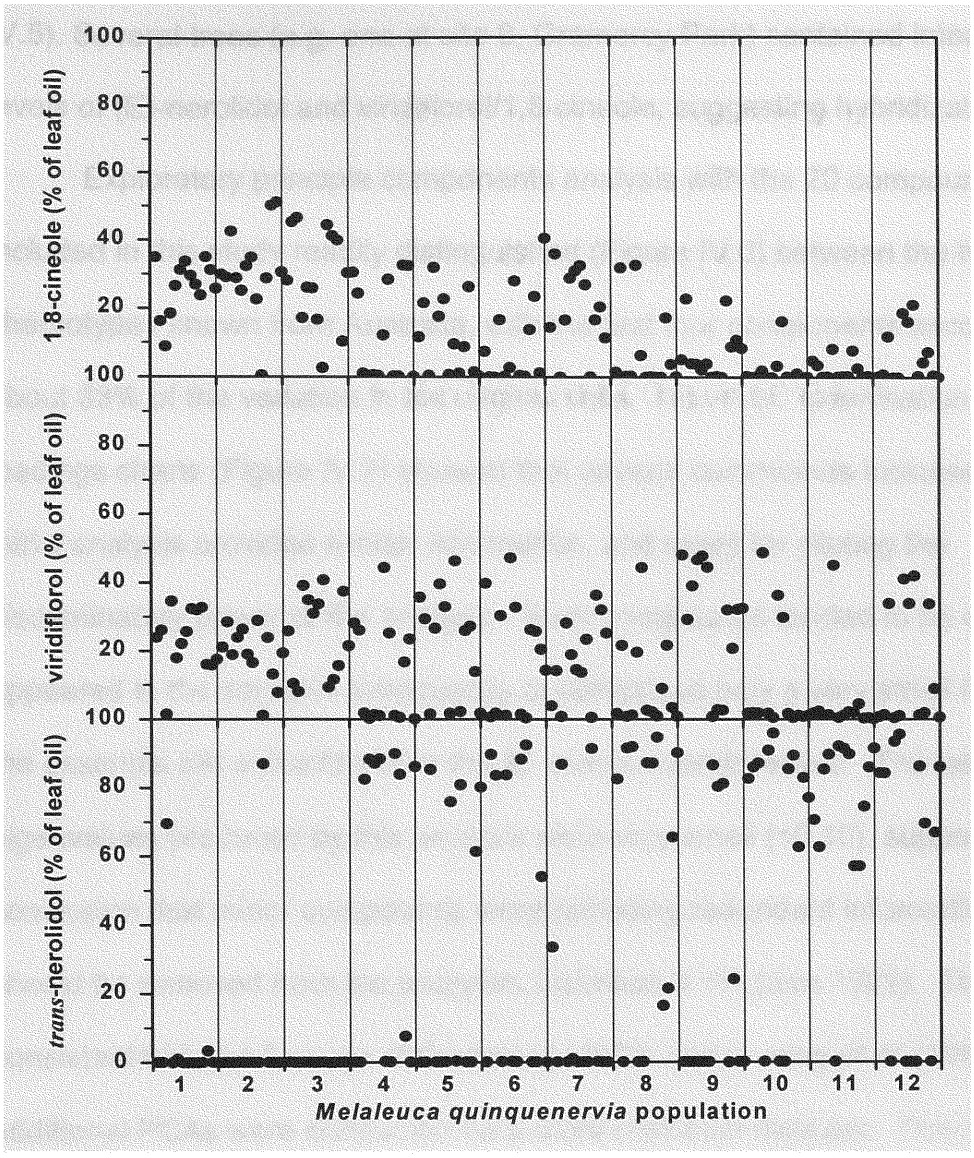

Figure IV.5. *Melaleuca quinquenervia* populations in Florida can be characterized by the relative proportions of three terpenoid compounds in the leaves: (E)-nerolidol, viridiflorol, and 1,8-cineole.

Populations in Conservation Area 2A, Lange Park, and the Royal Palm Nurseries (sites 1, 2, and 3, respectively) were dominated by trees containing a mixture of 1,8-cineole and viridiflorol, but very little (E)-nerolidol. Most of Florida's populations, however, were composed of both chemotypes (Figure

IV.5). Several trees (e.g. one at site 8, Gramercy Park) contained intermediate levels of (E)-nerolidol and viridiflorol/1,8-cineole, suggesting hybridization.

Exploratory principle components analysis with the 20 compounds included in this study readily distinguished (Figure IV.6) between the two primary chemotypes known from Australia, with the first four components accounting for about 83% of the variation in the original data. However, examination of the loadings charts (Figure IV.7) showed that several compounds included in this initial analysis provided similar information, and might be diluting the discriminatory power of the analysis. Such compounds tended to be ones that appeared in the samples infrequently or comprised only a very small fraction of the essential oils extracted from the *M. quinquenervia* leaves. The last eigenvalues produced by this analysis were very small (<0.10), supporting the conclusion that minor compounds were providing redundant information and should be removed from the analyses (Johnson & Wichern 1992). This was consistent with the findings of Southwell (1973) and Dunlop *et al.* (1995), so additional PCAs were conducted on a more restricted data set. This new data set consisted of only those compounds (n=10) that were both common (present in at least 5% of the samples) and abundant (constituted more than 3% of the essential oils).

The first four eigenvalues of the resulting PCA explained 91% of the total variance in the modified data set (Table IV.3). The first principle axis again contrasts (E)-nerolidol against all other constituents, and accounts for 63% of the variation. The second axis, accounting for 13% of the variance, contrasts

α- terpineol and 1,8-cineole with β-caryophyllene (Table IV.3). The third axis primarily contrasts β-caryophyllene with viridiflorol and what is tentatively identified as α-selinene, and explains 8% of the variation. The last axis included in Table IV.3 explains about 7% of the variation and contrasts caryophyllene oxide with α-pinene, β-pinene, and limonene.

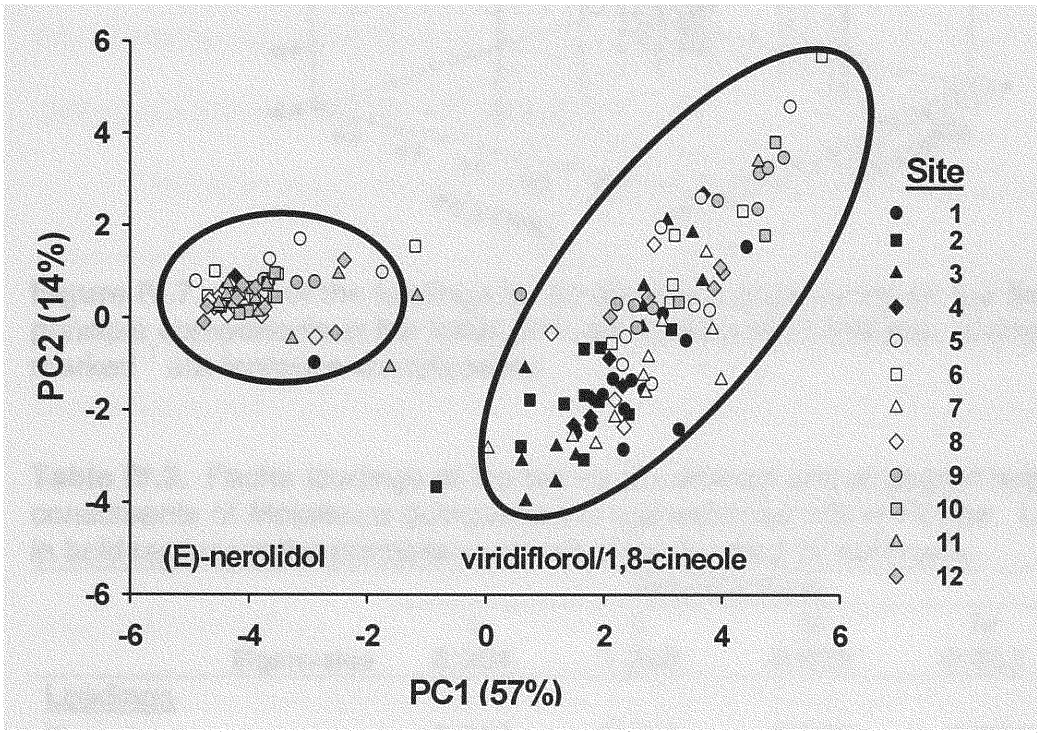

Figure IV.6. Scores of the first two principle components for the 155 trees included in this study distinguish between the two primary chemotypes: one characterized by high concentrations of (E)-nerolidol in the leaf essential oils, the other characterized by high concentrations of viridiflorol and/or 1,8-cineole. Points outside the circles represent potential hybrids - trees containing modest levels of both sets of principle compounds.

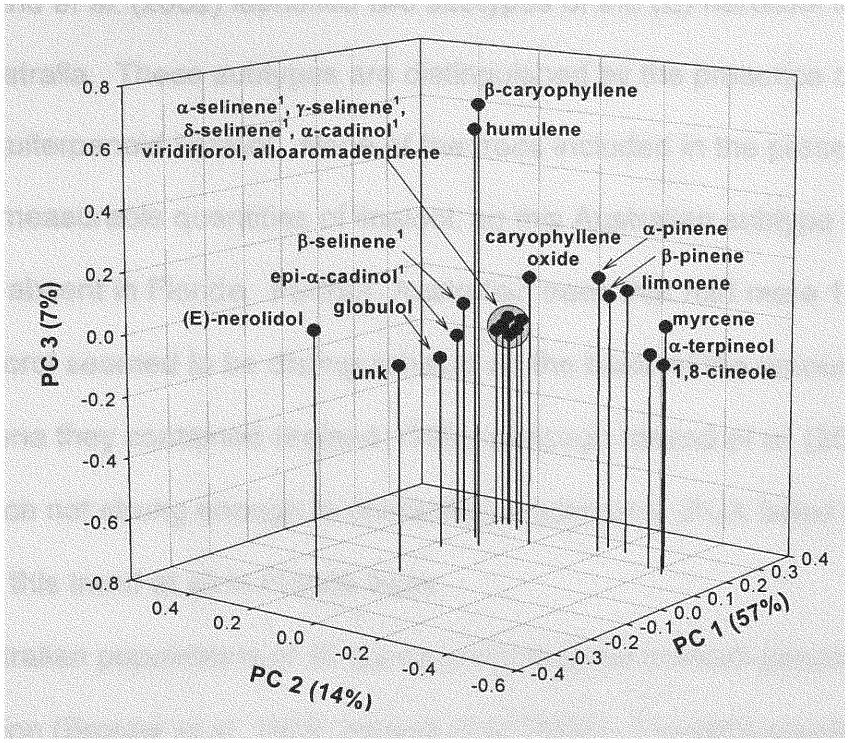

Figure IV.7. Plot of the loadings for 20 terpenoid compounds on the first three principle components in the initial principle components analysis. Compounds marked [1] are tentative identifications.

Table IV.3. Factor loadings of the ten most common and abundant terpenoid constituents of *Melaleuca quinquenervia* leaf essential oils in Florida. Loadings in bold represent the principle contrasts discriminated by each axis.

	Principle Axes			
	I	II	III	IV
Eigenvalue	6.304	1.256	0.829	0.652
Loadings				
% α-pinene	0.333	0.069	0.099	**-0.557**
% β-pinene	0.371	0.038	0.023	**-0.364**
% limonene	0.367	-0.035	0.076	**-0.309**
% 1,8-cineole	0.291	**-0.565**	0.141	0.174
% α-terpineol	0.300	**-0.502**	0.121	0.271
% β-caryophyllene	0.159	**0.449**	**0.754**	0.146
% α-selinene[1]	0.312	0.286	**-0.426**	0.207
% (E)-nerolidol	**-0.390**	0.049	0.097	-0.104
% viridiflorol	0.321	0.289	**-0.411**	0.135
% caryophyllene oxide	0.254	0.227	-0.143	**0.515**

[1] Tentative identification.

Ireland *et al.* (2002) identified two subtypes of the (E)-nerolidol dominated trees in Australia. These subtypes are distinguished by the presence or absence of the sesquiterpenoid linalool. None of the trees included in the present study contained measurable quantities of linalool, so this Australian subtype is apparently absent in Florida. Further, Australian trees that had more 1,8-cineole than viridiflorol seemed to be distinguishable on the basis of the amount of β-caryophyllene they contained (Ireland 1999), although Ireland *et al.* (2002) found this evidence not strong enough to characterize subtypes. PCA failed to distinguish this trend at all in Florida trees.

Australian populations of *M. quinquenervia* show marked geographic differentiation (Brophy *et al.* 1989, Ireland *et al.* 2002). Florida's populations also showed geographic differentiation, though of a less dramatic nature. Both chemotypes occurred throughout Florida, but populations on the west coast tended to be dominated by (E)-nerolidol type trees whereas populations on the east coast tended to be dominated by trees of the viridiflorol chemotype (G_{adj}=5.62, p=0.019).

Results from the cluster analysis confirmed the segregation of Florida's *M. quinquenervia* into chemical varieties based on leaf essential oil composition. The two largest clusters on the phenogram (Figure IV.8) correspond to the viridiflorol and (E)-nerolidol chemotypes (labeled B and C, respectively). In addition, the analysis appeared to distinguish two groups of trees dominated by 1,8-cineole. These were separated by the amount of viridiflorol content. One group (labeled A) contained almost as much viridiflorol as 1,8-cineole. The other

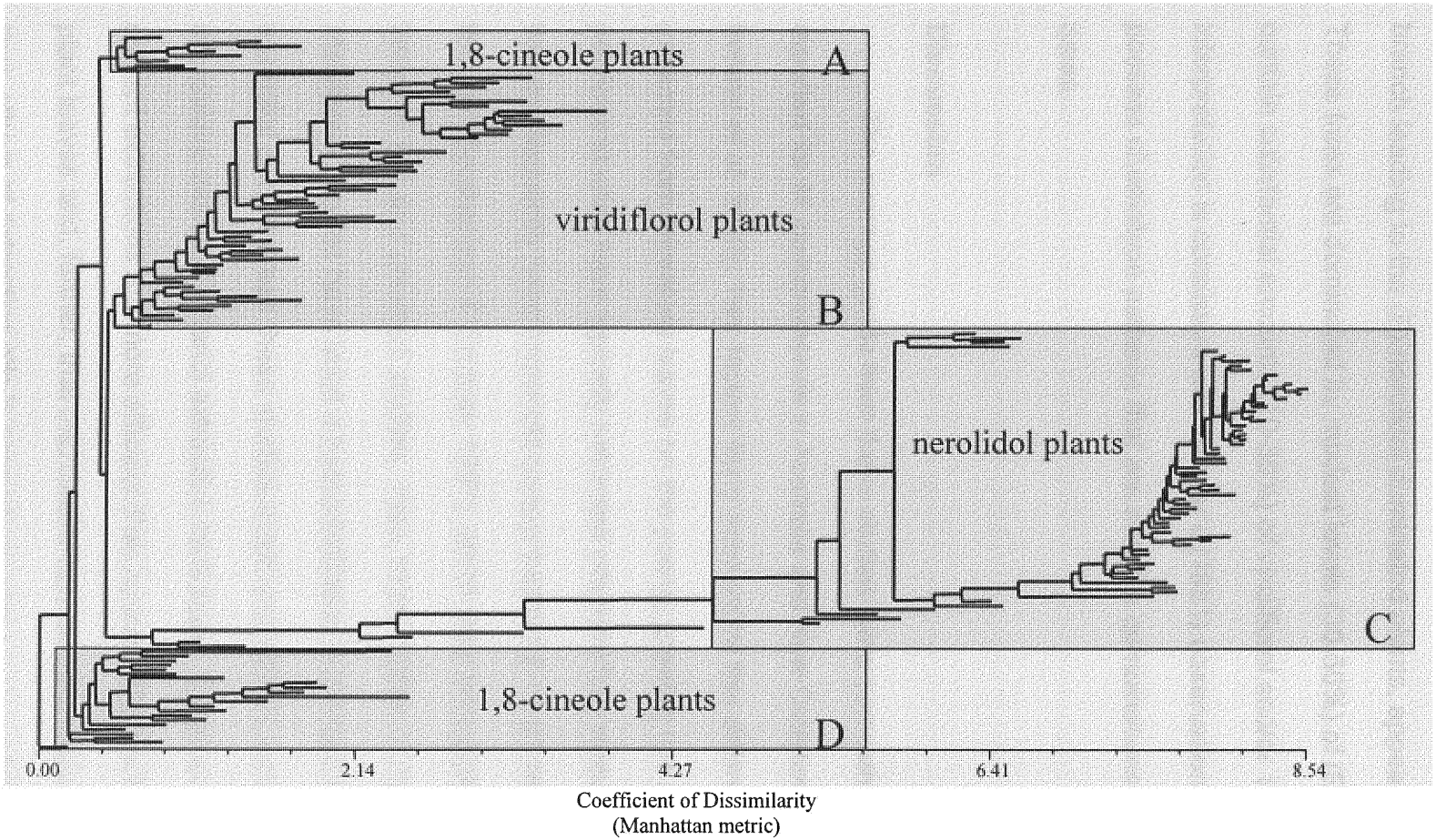

Figure IV.8. Cluster analysis of *Melaleuca quinquenervia* from Florida distinguished four distinct groups: trees dominated by viridiflorol, those dominated by (E)-nerolidol, and two groups dominated by 1,8-cineole.

group (labeled D) contained very little viridiflorol. A fifth group, those along the branch leading to the (E)-nerolidol dominated plants, appear to be hybrids. These trees are dominated by modest levels (30-50%) of (E)-nerolidol, but also contain substantial amounts (10-30%) of viridiflorol and/or 1,8-cineole. This interpretation is consistent with Seigler's (1981) observation that hybrids often possess chemotype patterns that are additive combinations of the parental types.

Discussion

The average yields from these studies were somewhat higher than yields reported for *M. quinquenervia* in Australia and New Caledonia (Trilles *et al.* 1999, Ireland *et al.* 2002). Two factors likely account for this difference. First, oils were extracted from immature leaves on the young, growing tips of plants whereas previous studies have typically extracted oils from fully mature leaves. Young tissues frequently have terpenoid concentrations 2-10 times higher than mature tissues (Gershenzon & Croteau 1991), which may help explain the differences between this and previous studies. Also, most studies of essential oils in *Melaleuca* species have used a hydrodistillation technique for extracting the essential oils, whereas these studies employed solvent extractions – primarily a microwave-assisted ethanol extraction technique. Baker *et al.* (2000) report that solvent extractions are more efficient than hydrodistillation for obtaining oils from plant leaves and, further, that microwave-assisted techniques are more effective than others at extracting oils in proportions closely resembling the original leaf oil

profiles. Thus the differences in yield between this study and those of other workers is likely a reflection of differences in protocols, and not an indication that *M. quinquenervia* trees in Florida produce more essential oil than trees in other regions of the world.

Seasonal differences or effects of fertilization cannot be ruled out as factors potentially influencing essential oil yields in this study, however. For example, Doran and Bell (1994) note that *Eucalyptus camaldulensis* oil yields tend to increase from spring into summer, then decline into winter. Murtagh and Smith (1995) reported similar findings for *M. alternifolia.* The common garden experiment in this present study was harvested during mid-spring (when yields should just be beginning to increase) whereas the natural stands were harvested during late summer (when yields should be at their peak), so oil yield differences between experiments may reflect seasonal effects. Conversely, Gershenzon and Croteau (1991) report that essential oil accumulation generally tends to decrease with N, P, or K fertilization, and our common garden plants were routinely fertilized. Langenheim (1994) notes, however, that data regarding responses to soil nitrogen supplementation have been equivocal, so it is unclear whether fertilization of plants in the common garden suppressed essential oil production.

In a study with *Eucalyptus camaldulensis*, Doran and Bell (1994) found that despite variation in absolute terpenoid yields relating to leaf age, season, and year, individual trees retained their basic chemotype identities. Likewise, Simmons and Parsons (1987) found that seasonal and physiological age-related

variation in essential oil composition did not result in changes in chemotype designation. Thus, despite factors that may have confounded essential oil yields, the results from this study clearly show that Florida harbors at least two chemical phenotypes. The principle constituent of the first is the sesquiterpene (E)-nerolidol, which generally comprises an extremely large (>80%) proportion of the essential oils in this type. The principle constituents of the second are the sesquiterpene viridiflorol and the monoterpene 1,8-cineole. Together these two compounds generally comprise >50% of the essential oils in this second chemotype, but there is a great deal of variation in the ratio with which they occur relative to one another.

The two chemotypes found in Florida represent the two principle chemotypes reported from Australia. Unlike Australia, however, none of the (E)-nerolidol type plants contained linalool (Beylier & Givaudan 1979, Ireland *et al.* 2002) or any other compound that could be used to distinguish sub-types. Further, the cluster analysis seemed to suggest that, in Florida, varieties of the second chemotype might be circumscribed by the amount of 1,8-cineole present in the mixture. However, the principle components analysis failed to support this interpretation.

Ireland *at al.* (2002) chose not to discriminate among varieties of the type II plants in their study, arguing that the various combinations of compounds contained therein merely represented a continuum from high viridiflorol/low cineole plants to low viridiflorol/high cineole plants. This interpretation has been common among Australian workers (Lassak & Southwell 1979, Brophy *et al.*

1989, Brophy & Doran 1996, Brophy 1999). Other workers have been less conservative, however. Ramanoelina *et al.* (1994) suggested that this second chemotype assorts to two distinct types in Madagascar. Trilles *et al.* (1999) reported four additional chemotypes from New Caledonia. Even Ireland's (1999) study contained trees which contained virtually no viridiflorol, 1,8-cineole, or (E)-nerolidol, suggesting that additional chemotypes exist in Australia. The equivocal results from the present study failed to shed any light on this question of total number of worldwide chemotypes, however.

In Australia, *M. quinquenervia* populations are geographically segregated into two distinct groups (Lassak & Southwell 1977, Brophy & Doran 1996, Ireland *et al.* 2002). Those north of latitude 25° S represent non-nerolidol (chemotype II) plants with generally low (<1%) oil yields. Those south of 25° S consist of a mixture of both chemotypes and typically yield more oil (>1%). The generally high yields and the abundance of (E)-nerolidol dominated oils throughout the tree's adventive range suggest the origin of Florida's populations lies in the southern portion of *M. quinquenervia*'s native range. This corresponds well with historical data (Chapter II), which indicate *M. quinquenervia* seeds imported into this country from Australia derive from Sydney and from around the Queensland-New South Wales border. Though certainly not conclusive, data from the present study also suggest that extra-Australian seed sources may ultimately have derived from the same areas in *M. quinquenervia*'s native range. If corroborated by future work, this information would fill an important gap in our understanding of the invasion history of *M. quinquenervia* in Florida.

1989, Brophy & Doran 1996, Brophy 1999). Other workers have been less conservative, however. Ramanoelina *et al.* (1994) suggested that this second chemotype assorts to two distinct types in Madagascar. Trilles *et al.* (1999) reported four additional chemotypes from New Caledonia. Even Ireland's (1999) study contained trees which contained virtually no viridiflorol, 1,8-cineole, or (E)-nerolidol, suggesting that additional chemotypes exist in Australia. The equivocal results from the present study failed to shed any light on this question of total number of worldwide chemotypes, however.

In Australia, *M. quinquenervia* populations are geographically segregated into two distinct groups (Lassak & Southwell 1977, Brophy & Doran 1996, Ireland *et al.* 2002). Those north of latitude 25° S represent non-nerolidol (chemotype II) plants with generally low (<1%) oil yields. Those south of 25° S consist of a mixture of both chemotypes and typically yield more oil (>1%). The generally high yields and the abundance of (E)-nerolidol dominated oils throughout the tree's adventive range suggest the origin of Florida's populations lies in the southern portion of *M. quinquenervia*'s native range. This corresponds well with historical data (Chapter II), which indicate *M. quinquenervia* seeds imported into this country from Australia derive from Sydney and from around the Queensland-New South Wales border. Though certainly not conclusive, data from the present study also suggest that extra-Australian seed sources may ultimately have derived from the same areas in *M. quinquenervia*'s native range. If corroborated by future work, this information would fill an important gap in our understanding of the invasion history of *M. quinquenervia* in Florida.

Finally, predominance of the (E)-nerolidol chemotype among Gulf Coast populations, in contrast to Atlantic Coast populations in which the viridiflorol chemotype predominates, helps explain some of the genetic variation found among *M. quinquenervia* populations in Florida (Chapter III, Kaufman & Smouse 2001). Similar trends toward geographic segregation of essential oil chemotypes have been reported for *M. alternifolia* (Butcher *et al.* 1994, Homer *et al. 2000*). The implications of this variation among *M. quinquenervia* populations on biocontrol efforts directed at this tree in Florida are being investigated.

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Chapter V. Differential survivorship and growth of *Oxyops vitiosa* Pascoe (Coleoptera: Curculionidae) on two *Melaleuca quinquenervia* (Cav.) S.T. Blake (Myrtaceae) chemotypes

Plant populations may comprise a heterogeneous mix of genotypes which differ dramatically in growth habits, nutritional requirements, abundance, and secondary chemistry (Rhoades 1983, Parker 1992). Plant defensive compounds, in particular, can be highly variable. Individuals within populations of *Sanguinaria canadensis* L., for example, exhibit an 18-fold variation in the abundance of the alkaloid sanguinarine (Bennett *et al.* 1990). Likewise, *Eucalyptus miniata* A. Cunn. ex Schauer showed 3-fold and 10-fold differences, respectively, in abundances of its two principle monoterpenes, α-pinene and 1,8-cineole (Ireland 1999). These differences among genotypes have important implications for plant-herbivore interactions, causing some individuals to be less susceptible to herbivores and diseases than others (Denno & McClure 1983, Newton et al. 1999, McIntyre & Whitham 2003). In fact, plant breeders have long taken advantage of this knowledge to produce resistant strains of row and tree crops (Maxwell & Jennings 1980, Gould 1983, Zobel & Talbert 1984, Parker 1992).

In the evolutionary arms race, herbivorous species have had to contend with this variability in plant defensive compounds that confer resistance (Fraenkel 1959, Feeny 1975, Price 1975, Beck & Schoonhoven 1980, Weis & Berenbaum 1989). The strong selective pressures exerted by these variable defenses have

contributed to a diversification of insect adaptive responses, some of which have limited the suite of host plants upon which a particular insect might successfully feed, breed, and develop (Ehrlich & Raven 1964; Feeny 1975; Berenbaum 1981, 1983; Weis & Berenbaum 1989; Boecklen & Mopper 1998). Over time, some species have become so closely adapted to a particular host that they no longer accept other species as developmental habitats (Dethier 1954, Fraenkel 1959, Feeny 1975, Edmunds and Alstad 1978, Alstad and Edmunds 1983, Berenbaum & Zangerl 1997, Alstad 1998). This extreme host fidelity is, in fact, the evolutionary underpinning upon which rests the science of biological control of weeds (Huffaker 1971, DeBach 1974, Harley & Forno 1992, Center *et al.* 1997). As an extension of this host fidelity, some herbivorous species develop distinct biotypes (i.e., host plant races) that vary in their acceptance of and performance on different strains of their host plant (Mitter & Futuyma 1983, DeBach & Rosen 1991, Bernays & Chapman 1994). This knowledge has strong implications for weed biocontrol programs in terms of establishment and efficacy of biocontrol agents.

About one-third of insects released to control exotic weeds have failed to establish persistent populations in their hosts' adventive ranges (Crawley 1989, 1990; Harris 1991, 1993; Cullen 1995; McFayden 2000). Another 40% have become established, but failed to achieve the outbreak levels required for control of the target weed (Harris 1991,1993). Of the remaining agents (25%), those that have established and proven successful, some have differed in efficacy among geographic regions in their target weed's adventive ranges (Crawley 1983).

Explanations for negative outcomes have varied. Persistence in a new locale of any organism, whether biocontrol agent or not, is often dependent on number of introduction incidences (Kowarick 1995, Pyšek *et al.* 1995) and whether the pattern of introductions establishes a metapopulation (Dray *et al.* 2001, Freckleton & Watkinson 2002). Abiotic differences between native and introduced ranges have been cited as a reason for biocontrol agent failures (van den Bosch & Messanger 1973, DeBach 1974, Harley & Forno 1992, Center *et al.* 2000). Other potential contributing factors include predators, parasitoids, disease, and competition (Crawley 1986, 1989).

Some biocontrol researchers (e.g. van den Bosch & Messenger 1973, Crawley 1983, Harris 1989, Mahlberg 1990, Harley & Forno 1992), however, have argued that extreme host fidelity has also contributed to unfavorable outcomes. Their premise is that, in some cases, failure of biocontrol practitioners to carefully match plant genotypes in a weed's adventive range with those in its native range has resulted in release of an inferior biotype of the correct insect species. In other words, some biocontrol insects may be so closely adapted to the plant genotype from which they were collected (i.e., such narrow specialists) that they fail to persist or perform on a different genotype of the same target weed. This occurred, for example, when the rust fungus *Puccinia chondrillina* Bub. & Syd. was released in Australia for control of *Chondrilla juncea* L., but only attacked one form of the weed (Burdon *et al.* 1981). Likewise, gall midge (*Spurgia esulae* Gagné) establishment and impact on leafy spurge (*Euphorbia esula* L.) populations varied by plant genotype (Lym *et al.* 1996).

The forgoing evidence argues that one should be able to anticipate variable post-release performance of a biological control insect by rearing it on different biotypes of its host plant under empirical conditions. If so, such studies could help improve the success rate of weed biological control efforts. The present study examines the growth and development of a biological control agent, *Oxyops vitiosa* (Pascoe) (Coleoptera: Curculionidae), on different biotypes of its host, the notorious Everglades invader *Melaleuca quinquenervia* (Cav.) Blake (Myrtaceae) in a test of this premise.

Study system

Melaleuca quinquenervia (melaleuca, paperbark, punk tree) is an Australian wetlands species that was imported into Florida during the late 19th and early 20th centuries (Meskimen 1962, Chapter II). Today it has encroached on at least 200,000 ha of sawgrass prairies, cypress swamps, pine flatwoods, hardwood hammocks, salt marshes, and mangrove forests (Bodle *et al.* 1994, Laroche 1998). It is particularly prominent along ecotones (Myers & Ewel 1990). Invasion of native plant communities can temporarily increase wildlife diversity through changes in community structure (O'Hare & Dalrymple 1997), but ultimately result in dense punk tree forests with drastically reduced biodiversity and little wildlife value (Austin 1978, O'Hare & Dalrymple 1997). As a consequence, natural resource managers and scientists working in Florida's prized Everglades ecosystems developed a comprehensive integrated pest management plan aimed at reducing deleterious impacts of *M. quinquenervia* in

those systems (Laroche 1994, 1999). The heart of this plan is a biological control program focused on discovering, assessing, and releasing Australian insects that can slow the plant's growth and reproductive success.

Disjunctions occur in the Australian portion of *M. quinquenervia*'s range, particularly in central and northern Queensland. Similar disjunctions in the ranges of *M. alterniflora* and *M. linariifolia* have resulted in the establishment of genetically distinct populations within each species (Butcher *et al.* 1994, 1995). Likewise, several lines of evidence suggest that *M. quinquenervia* populations may differ genetically in its native range, i.e., there may be more than one *M. quinquenervia* biotype. For example, seeds from trees in northern Queensland grow better in soils from that region than in soils from southern Queensland, whereas seeds produced by trees in southern Queensland grow best in soils from the latter region (Balciunas *et al.*, unpublished report). Also, Kaufman and Smouse (2001) found substantial genetic differences in quantitative traits among three Australian populations of *M. quinquenervia*. Further, Ireland *et al.* (2002) reported that there are at least two distinct chemotypes (chemical phenotypes segregated by differences in foliar essential oil content) of *M. quinquenervia* present in Australia.

M. quinquenervia populations in southern Florida derive from many distinct introduction events, several of which came from different portions of the plant's native range and others of which are extra-Australian in origin (Meskimen 1962, Chapter II). This invasion history has produced strong genetic diversity within and among populations in Florida (Chapter III, Wang & Littell 1983, Kaufman 1999). Further, a disjunction in its adventive range between Gulf Coast and Atlantic Coast

populations has combined with *M. quinquenervia*'s life history traits and its short tenure in Florida to restrict gene flow between populations (Chapter III, Kaufman 1999). Both of the predominant chemical phenotypes (chemotypes) present in Australia are represented in Florida (Chapter IV, Wheeler *et al.* 2002, 2003). Taken together, these facts suggest that Florida's populations likely comprise a suite of two or more distinct biotypes.

Oxyops vitiosa is a snout beetle (i.e., weevil) imported into the United States as part of the management effort to control melaleuca in Florida (Purcell & Balciunas 1994, Turner *et al.* 1998, Center *et al.* 2000). The native range of *O. vitiosa* extends from northern Queensland to central New South Wales in Australia (Purcell & Balciunas 1994, Madeira *et al.* 2001), so it is sympatric with *M. quinquenervia* throughout most, but not all, of the plant's native range. Field studies in Australia, and laboratory studies in Australia and the United States, have shown that the host range of this weevil is restricted to (Balciunas *et al.* 1994b; G.R. Buckingham, unpublished report). Larvae and adults feed on young foliage as well as floral and leaf buds (Purcell & Balciunas 1994). The resultant damage leads to reductions in plant growth (Balciunas & Burrows 1993) and flowering incidence (Rayamajhi *et al.* 2002). Adult *O. vitiosa* can live more than one year (Wheeler 2003), and produce about 470 eggs per female (Purcell & Balciunas 1994).

Persistent populations of the melaleuca snout beetle established at nine of thirteen original release sites in Florida (Center *et al.* 2000). All four sites where releases failed to establish persistent *O. vitiosa* populations are located

along Florida's Atlantic Coast. The few Gulf Coast sites inoculated with this biocontrol agent all produced persistent populations. Center *et al.* (2000) note that sites where *O. vitiosa* failed to persist were long hydroperiod sites, and that this habitat characteristic seems incompatible with the snout beetles' pupation requirements. However, the disparity in establishment success between Gulf Coast and Atlantic Coast sites also coincides with the presence of two genetically distinct melaleuca metapopulations in Florida (Kaufman 1999, Kaufman & Smouse 2001, Chapter III).

The snout beetles released in Florida are all progeny of insects that originated from a single *M. quinquenervia* population near Brisbane, Australia (Madeira *et al.* 2001), which is in the southern portion of the insect's range. Released insects thus may not have represented the full suite of genotypes present in the tree's native range. Preliminary genetic analysis has suggested, in fact, that at least two *O. vitiosa* biotypes may be present in Australia (Madeira *et al.* 2001). Therefore, it is possible that differences in *O. vitiosa* performance between Gulf Coast and Atlantic Coast populations of *M. quinquenervia* reflect the importation of a snout beetle biotype better adapted to one melaleuca biotype than others.

Methods & Materials

Plant material

Populations of *Melaleuca quinquenervia* in Florida are composed of two chemical phenotypes (i.e., chemotypes; see Chapter IV). The first of these is

characterized by an essential oil profile dominated by the sesquiterpine (E)-nerolidol (Figure V.1A). The profile of the second chemotype is dominated by a combination of viridiflorol and 1,8-cineole (Figure V.1B). Seeds collected from trees encompassing *M. quinquenervia*'s adventive range in Florida were germinated in ProGro Potting Soil for Starting Seeds® (Scotts-Sierra Horticultural Products, Marysville, OH), watered daily, and fertilized with Peters Excel 15-5-15 Cal-Mag® (N-P-K; Scotts-Sierra Horticultural Products, Marysville, OH) at six month intervals. All plants received equal treatment to eliminate possible confounding effects of environmental variability on insect performance (Wheeler 2001).

Seedlings germinated from the field-collected material were screened for essential oil composition following the procedures described elsewhere (Chapter IV), and plants containing >40% viridiflorol/1-8, cineole or >90% (E)-nerolidol were selected for inclusion in this study. These values were selected because they represented the extreme values observed during studies of essential oil variation among paperbark trees in Florida (Chapter IV). Seedlings were transplanted into 3 gal pots containing a mixture of Florida peat, sand, bark, and micronutrients (Atlas 3002 mix; Atlas Peat & Soil, Inc., Boynton Beach, Florida). They were then grown for 20 months in a screen house and pruned to stimulate luxuriant growth of apical tissues. The resultant flush growth was used to feed the *Oxyops vitiosa* larvae assayed in this study.

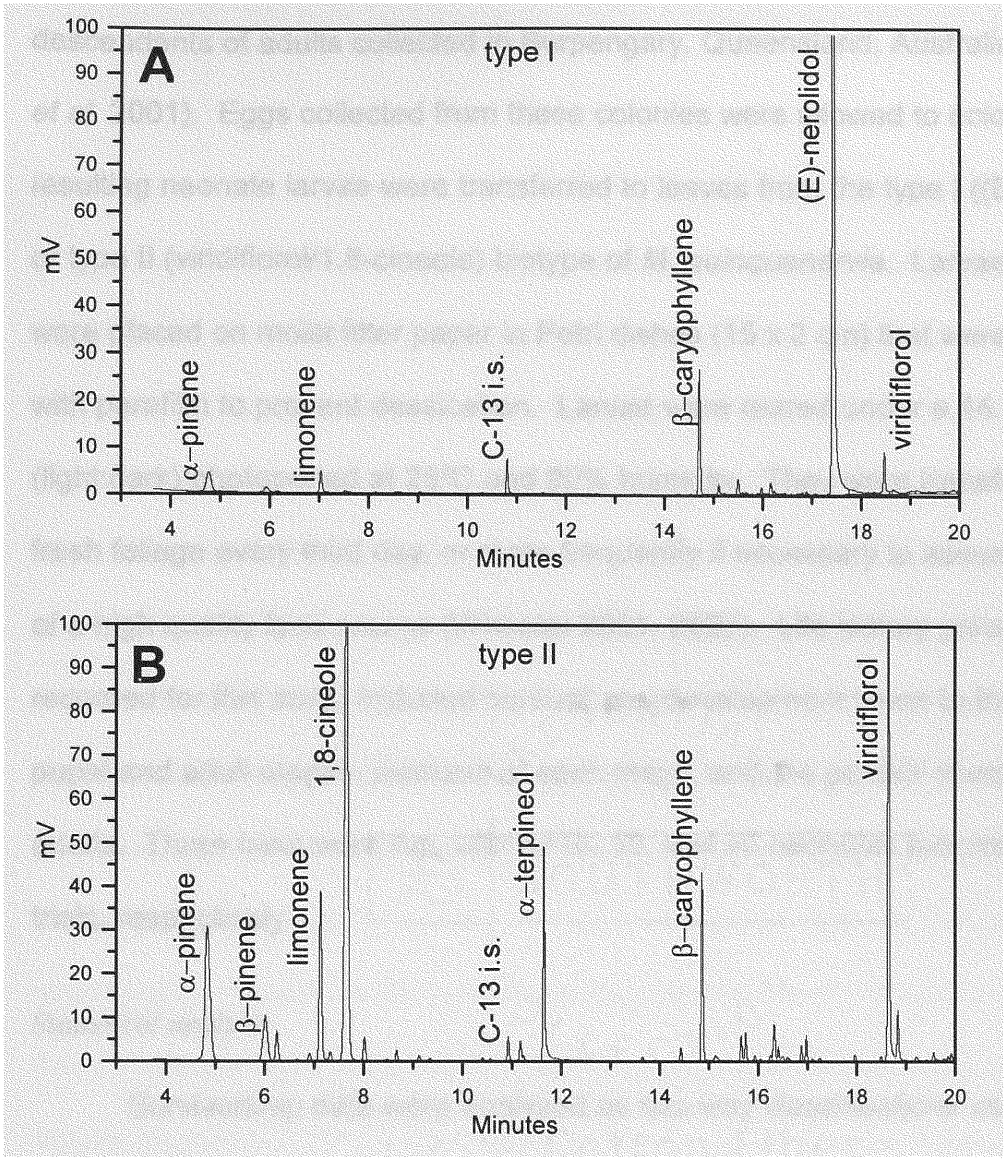

Figure V.1. *Melaleuca quinquenervia* populations in Florida are composed of two chemotypes: one in which (E)-nerolidol (A) is the principle terpenoid compound in the leaf essential oils, and one in which viridiflorol and 1,8-cineole (B) are the principle terpenoid compounds.

Larval growth and development

Oxyops vitiosa included in this study were collected from laboratory colonies at the USDA Agricultural Research Service, Invasive Plant Research

Laboratory in Fort Lauderdale, Florida. These colonies were established from descendants of adults collected in Burpengary, Queensland, Australia (Madeira *et al.* 2001). Eggs collected from these colonies were allowed to eclose, and the resulting neonate larvae were transferred to leaves from the type I ((E)-nerolidol) or type II (viridiflorol/1,8-cineole) biotype of *M. quinquenervia*. Larvae and foliage were placed on moist filter paper in Petri dishes (15 x 2 cm) that were sealed with parafilm to prevent dessication. Larvae were reared under a 14:10 h (light:dark) photoperiod at 25°C and 90% humidity. They were transferred to fresh foliage every third day, or more frequently if necessary to assure availability of a high quality food source (Wheeler 2001, 2003). Life history parameters recorded for this study included survival and development times to the prepupal, pupal and adult stages, biomass at each stage, and the gender of emerging adults. Three trials were run, with n=18, 30, and 90 neonates included in the trials, respectively.

Statistical analysis

Survivorship data were analyzed as two-way classifications using G-tests of independence (i.e., log-likelihood ratio tests; Sokal & Rohlf 1995). Some sample sizes were small (n<30), so the G-values were adjusted using Williams' correction as recommended by Sokal and Rohlf (1995). Growth and development data were analyzed using GLM procedures in the SAS/PC program (SAS 1999). Development was examined using a one-way ANOVA. Growth was analyzed by a two-way ANOVA with interaction, where weevil gender and

plant chemotype were the main effects. Means were compared with the Ryan's Q means comparison test (P=0.05).

Results

Oxyops vitiosa neonate survival to adulthood was much greater (overall G_{adj}=37.684, P<0.001) when the larvae were reared on leaves whose principle terpenoid constituent was (E)-nerolidol (type I) as compared to larvae fed foliage whose principle constituent was a combination of viridiflorol and 1,8-cineole (type II). This trend held true in all three trials (Figure V.2), so the data were combined to provide sufficient numbers of survivors on type II foliage to permit additional statistical analysis. The difference in survivorship was greatest during the larval stage. About two-thirds (38 of 112) of larvae fed type II leaves died prior to pupating, whereas 88% of the larvae (23 of 26) fed type I leaves successfully pupated (G_{adj}=26.820, P<0.001). Pupal mortality also differed, but less dramatically. Nearly all (96%) of the pupae that had been reared on type I leaves emerged as adults, as compared to two-thirds of those reared on type II leaves (G_{adj}=8.323, P=0.004). Chemotype did not influence the sex ratio of adults produced in this study (G_{adj}=0.197, P=0.907), with males and females equally represented on both type I and type II plants.

Surprisingly, the strong relationship between food type and survivorship was poorly reflected in weevil developmental periods. Duration of the larval stage was about 13 days, and was unaffected by the terpenoid content of larval food (Table V.1). Likewise, duration of the prepupal stage (about 7 days) was

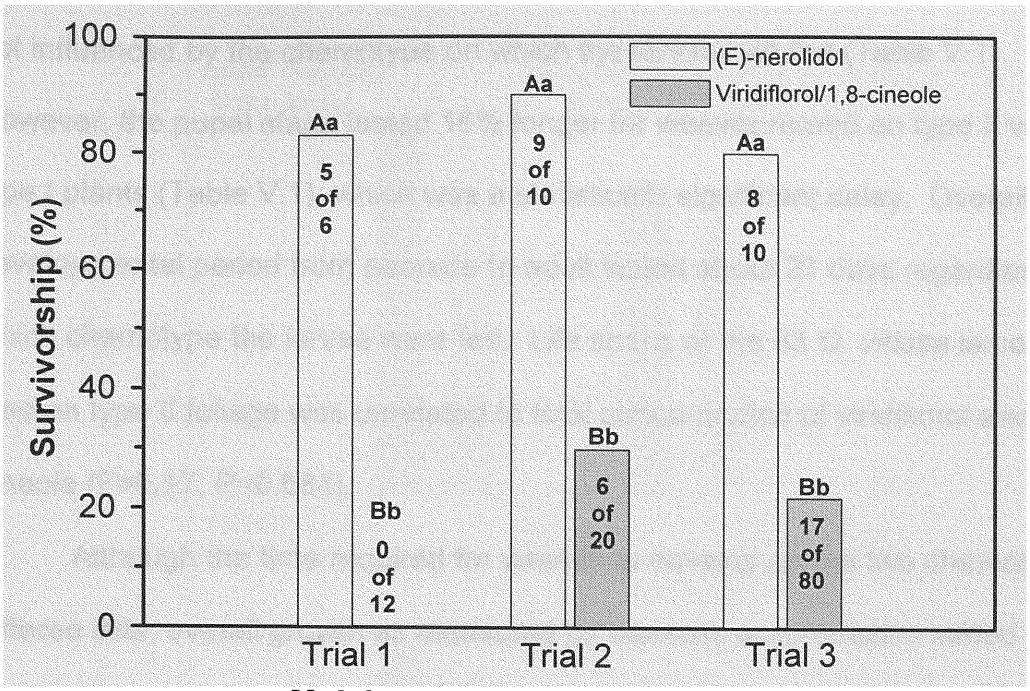

Figure V.2. *Oxyops vitiosa* neonate survival to adulthood was influenced by which terpenoid compound predominated in *Melaleuca quinquenervia* foliage on which the larvae developed. Numbers inside the bars represent the surviving and initial numbers of neonates. Significant ($P<0.05$) differences among trials within chemotypes are indicated by capital letters. Significant ($P<0.05$) differences between chemotypes within trials are indicated by lower case letters.

Table V.1. Differences in *Oxyops vitiosa* development on foliage from two *Melaleuca quinquenervia* chemotypes found in Florida.

	Mean duration in days (s.e.)		
	(E)-nerolidol	viridiflorol	F (P)
Larval stage	13.1 (0.78)	13.1 (0.39)	0.00 (0.995)
Pre-pupal stage	7.4 (0.33)	7.1 (0.29)	0.72 (0.402)
Pupal stage	6.4 (0.21)	7.4 (0.27)	8.93 (0.005)
Overall	27.0 (0.84)	27.7 (0.33)	0.54 (0.467)

not influenced by the chemotype on which the larvae had fed (Table V.1). However, the pupal stage lasted 16% longer for weevils reared on type II versus type I plants (Table V.1), which was a statistically significant delay. Overall, the developmental period from neonate to adult lasted about 27 days regardless of which chemotype the larvae were fed. Life spans of the 83 *O. vitiosa* larvae that died on type II foliage was unrelated to total concentration of viridiflorol and 1,8-cineole ($F=0.17$, $P=0.681$).

Although the time required for weevils to develop on the two chemotypes differed little, overall growth as measured by biomass accumulation varied substantially. Larvae fed on foliage containing an abundance of (E)-nerolidol achieved a greater average biomass (59.4 vs. 50.9 mg) than did larvae fed foliage from type II plants (Figure V.3A). Likewise, final pupal weights of *O. vitiosa* reared on foliage containing an abundance of viridiflorol/1,8-cineole were substantially less (41.2 vs. 51.3 mg) than those of individuals reared on type I plants (Figure V.3B). Similarly, adult biomass was greater (44.5 vs. 32.0 mg) for weevils that developed on foliage with an essential oil profile dominated by (E)-nerolidol as compared to viridiflorol/1,8-cineole (Figure V.3C). Among adults, males tended to be smaller than females (33.2 vs. 42.8 mg; $F=18.2$, $P=0.0001$), but this relationship had no effect on the influence of chemotype on *O. vitiosa* biomass (Figure V.3). When compared on the basis of relative growth rates (RGR), weevils fed type I foliage again performed better than those fed type II foliage. *O. vitiosa* larvae ingesting leaf material defended by an abundance of (E)-nerolidol gained 4.78 mg/d. In contrast, larvae reared on diets rich in

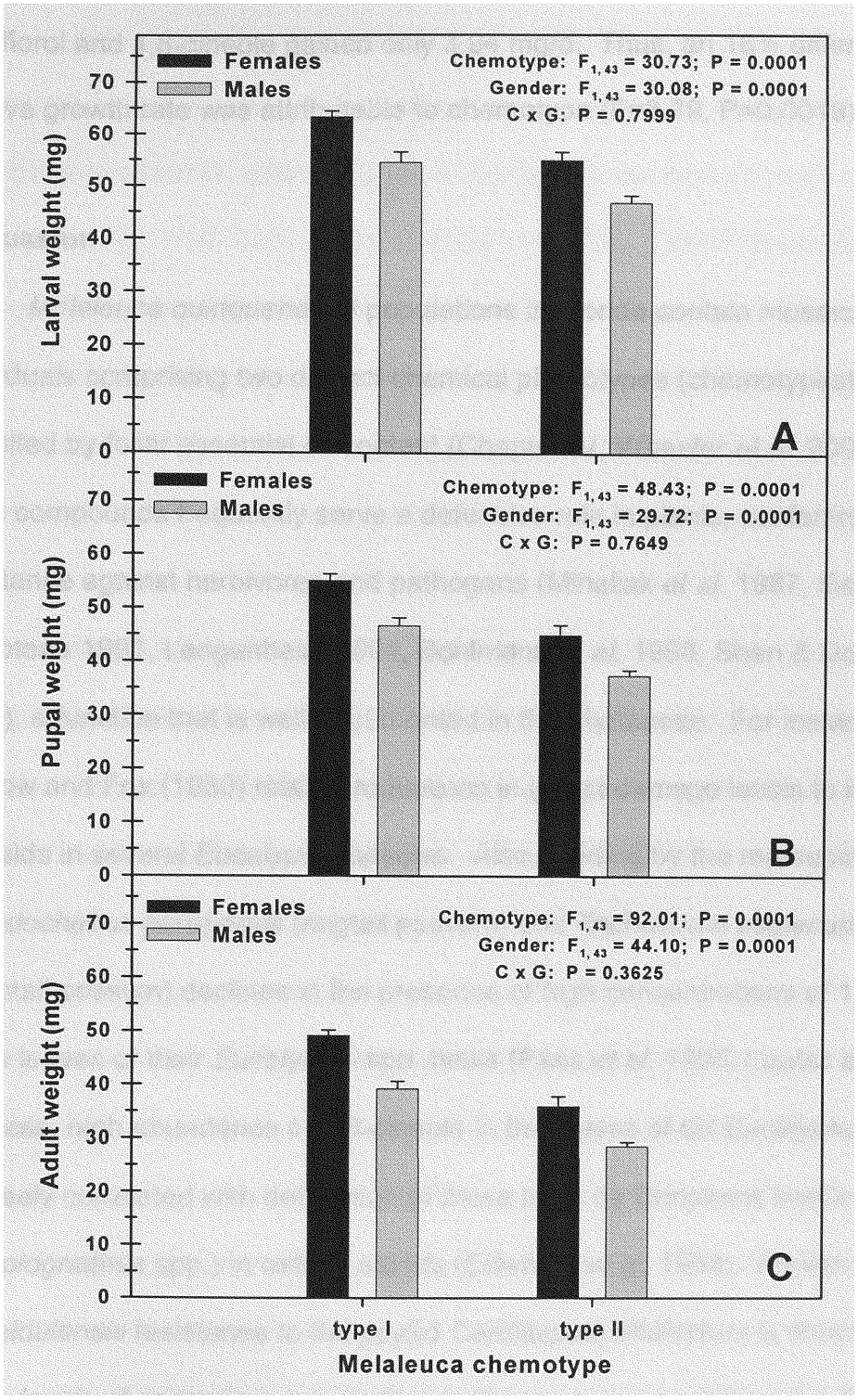

Figure V.3. Biomass accumulation of *Oxyops vitiosa* (n=45) larvae (A), pupae (B), and adults (C) raised on foliage from two *Melaleuca quinquenervia* chemotypes found in Florida.

viridiflorol and 1,8-cineole gained only 3.94 mg/d. Thus, an 18% difference in relative growth rate was attributable to chemotype ($F=8.79$, $P=0.0049$).

Discussion

Melaleuca quinquenervia populations in Florida contain mosaics of individuals comprising two distinct chemical phenotypes (chemotypes) as delimited by foliar essential oil content (Chapter IV, Wheeler *et al.* 2002, 2003). Such compounds frequently serve a defensive role in plants, conferring resistance against herbivores and pathogens (Mihaliak *et al.* 1987, Gershenzon & Croteau 1991, Langenheim 1994, Bohlmann *et al.* 1998, Shen & Dooner 2000), a function that is well-documented in the Myrtaceae. For instance, Morrow and Fox (1980) related reductions in insect damage levels to increasing oil yields in several *Eucalyptus* species. Also, feeding by the marsupial folivores *Pseudocheirus peregrinus* (ringtail possum) and *Trichosurus vulpecula* (common brushtail possum) declines in the presence of high concentrations of 1,8-cineole in the leaves of their *Eucalyptus* spp. hosts (Pass *et al.* 1998, Lawler *et al.* 1999). Likewise, high abundance of 1,8-cineole in the leaves of six *Eucalyptus* spp. was inversely correlated with defoliation of these trees by Christmas beetle (*Anoplognathus* spp.) in natural stands (Edwards *et al.* 1993). Further, *E. camaldulensis* resistence to the psyllid *Cardiaspina albitextura* is directly related to the levels of secondary metabolites in the tree's leaves (Ohmart & Edwards 1991). Among *Melaleuca* species, (E)-nerolidol extracted from *Melaleuca* (identified as *M. leucadendron* but probably *M. quinquenervia*) leaves has been

shown to deter feeding by gypsy moth (*Lymantria dispar*) larvae (Doskotch *et al.* 1980), although the native ranges of tree and moth are not sympatric. Further, the essential oils in *M. alternifolia* have strong antimicrobial properties (Concha *et al.* 1998; Cox *et al.* 2000, 2001; D'Auria *et al.* 2001), as do those in *M. quinquenervia* and other *Melaleuca* species (Harkenthal *et al.* 1999). And larvae of an undescribed *Pergagrapta* sawfly (Hymenoptera: Symphyta: Pergidae) have developed specialized morphological and physiological adaptations to avoid toxic effects from 1,8-cineole in the leaves of its host *M. quinquenervia (*Schmidt *et al.* 2000).

In the present study, the biological control agent *Oxyops vitiosa* was dramatically impacted by the foliar chemistry of its host plant in the tree's adventive range. Larvae fed foliage containing predominantly (E)-nerolidol, i.e., leaves from type I *M. quinquenervia* plants (Chapter IV, Ireland *et al.* 2002), enjoyed low mortality (12%). In contrast, larvae fed leaves from type II *M. quinquenervia* plants, whose foliage contains an abundance of viridiflorol and/or 1,8-cineole but little (E)-nerolidol (Chapter IV, Ireland *et al.* 2002), experienced 88% mortality. This represents a four-fold difference in survivorship that is related to chemotype.

Wheeler (2003) reported obtaining a similar outcome during trials investigating the effects of plant tissue nitrogen levels on *O. vitiosa* larval performance. In his study, two independent trials produced foliage with comparable tissue nitrogen levels but dramatically different (70 vs. 20%) larval mortality. Wheeler (2003) speculated that these differences in survivorship

might have resulted from "genetic differences in leaf chemistry other than nitrogen". Results from the present study support this hypothesis, suggesting that the trial producing low mortality was conducted with type I foliage whereas the trial with high mortality was conducted using type II foliage.

If this interpretation is correct, then the data also suggest that foliar nutritional value and chemotype likely interact to influence *O. vitiosa* performance. This assertion is supported by observations showing that larvae fed nitrogen-poor leaves from (presumably) type II trees experienced twice the mortality as larvae fed nitrogen-rich leaves (Wheeler 2003). Further support is offered by studies showing that 50-70% of larvae died when reared on "poor quality" foliage (Wheeler 2001) collected from sites in southern Broward and northern Dade counties where type II plants predominate (Chapter IV). These plants also differed in leaf toughness (Wheeler 2001), however, so it seems likely that *O. vitiosa* performance is influenced by the synergistic effects of a variety of factors including tissue nitrogen levels, leaf toughness, and types and abundances of principle leaf terpenoid constituents. Investigating these interactions was beyond the scope of the present study.

Interestingly, Purcell and Balciunas (1994) reported substantial mortality (51%) during preliminary studies of this weevil's life history in its native Australia. Their observation suggests that the insects they tested were fed leaf material from type II *M. quinquenervia.* This interpretation is strengthened by the data from Ireland *et al.* (2002) which shows that type II is the only chemotype present

in northern Queensland where Purcell and Balciunas (1994) conducted their studies.

Average developmental periods during the present study were somewhat shorter than previously reported (Purcell & Balciunas 1994; Wheeler 2001, 2003), but were unaffected overall by the essential oil composition of larval diets. This outcome is somewhat surprising because duration of immature stages is generally regarded as a measure of host quality (c.f., Scriber & Feeny 1979, Scriber 1984). The larval stage lasted 1.6 d more in Wheeler's (2003) study and 5.4 d more in Purcell and Balciunas' (1994) study than in the present study. As in Wheeler's (2003) nutritional study, however, diet had no effect on duration of the larval stage. Wheeler (2003) reported a similar duration for the prepupal stage as in the present study, but this stage lasted about 5 d longer in Purcell and Balciunas' (1994) study. Once again, larval diet had no discernable influence on duration of this stage in either the present or Wheeler's (2003) studies. Interestingly, weevils reared on type II foliage remained pupae nearly a day longer than those reared on type I leaves. Even so, the pupal stage was shorter in the present study than in either Wheeler's (2003) or Purcell and Balciunas' (1994) studies. Reasons for these discrepancies remain unclear.

In addition to greater survivorship, larvae fed foliage from type I plants enjoyed superior growth, as measured by either total biomass accumulation or relative growth rate (RGR), when compared to larvae offered leaves from type II plants. Thus, *O. vitiosa* accumulated about 29% more biomass when developing on type I, as compared to type II, plants. This difference was unrelated to

gender, as the sex ratio was 1:1 and females consistently weighed about 25% more than males regardless of the chemotype upon which they were reared. The greater RGR for weevils reared on type I plants versus type II plants derives primarily from the additional biomass accumulation, since insects on both chemotypes required about the same amount of time to develop.

Often, insects faced with poor quality foods will increase their consumption rate or assimilation efficiency (Scriber 1983, 1984; Slansky & Wheeler 1992; Wheeler & Halpern 1999). Wheeler (2003) noted, however, that *O. vitiosa* larvae may be unable to overcome poor quality food by compensatory feeding due to high terpenoid concentrations in their host trees. If, as the present study suggests, Wheeler's larval performance studies were conducted on type I *M. quinquenervia* then growth differences in the present study suggest that the predominant terpenoids in type II plants are more difficult to detoxify than those in type I plants. This interpretation is supported by the absence of evidence for either compensatory feeding or increased food utilization efficiency by *O. vitiosa* (Wheeler 2003). Confirmation of this hypothesis would require the discovery of decreased food utilization efficiencies in type II versus type I plants. Unfortunately, examination of this question was beyond the scope of the present study.

Taken together, data from the present study indicate that type I *M. quinquenervia* (whose foliar essential oils are dominated by (E)-nerolidol) are a superior host to type II plants (whose essential oils are dominated by a combination of viridiflorol and 1,8-cineole). Type I plants produced more and

larger (as measured by biomass) adult *O. vitiosa* than type II plants. Further, Wheeler (personal communication: USDA, ARS Invasive Plant Research Lab, Fort Lauderdale, Florida, 2001) in an extension of the present study, found that adults reared on type I plants not only weighed more, but also had shorter preovipositional periods and were more fecund than adults reared on type II plants. Clearly, type I *M. quinquenervia* plants are a superior host for the *O. vitiosa* weevils released in Florida.

Madeira *et al.* (2001) reported that weevils imported from two different sites in Australia differed consistently in size and tegument color, prompting concern that they might represent sibling species. RAPD (random amplified polymorphic DNA) analysis of these insects showed that while genetic differences were not sufficient to warrant segregating the two populations as different species, or even subspecies, they likely represent different biotypes (Madeira *et al.* 2001). Data from the present study suggest that one explanation for the size differences observed by Madeira *et al.* (2001) might relate to the *M. quinquenervia* chemotype upon which these weevils developed. Ireland's (1999) data lend credence to this hypothesis, in that not all trees classified as type II in central and northern Queensland have essential oils characterized by high abundances of 1,8-cineole and/or viridiflorol. For instance, the present study shows that *O. vitiosa* grew larger in the absence of these terpenoids. The larger weevils in the Madeira *et al.* (2001) study were collected near Bundaberg, Queensland, Australia. Some of the type II trees in Ireland's (1999) study that lacked substantial quantities of 1,8-cineole and/or viridiflorol were collected at

Hervey Bay (near Bundaberg). So it seems likely that the large *O. vitiosa* biotype was from a population growing on plants in which these terpenoids were largely absent. Foliage containing these terpenoids produced smaller *O. vitiosa* in the present study. The smaller insects reported in Madeira *et al.* (2001) were collected at Burpengary, Queensland, Australia. Trees around Brisbane (about 20 km from Berpengary) are characterized by high concentrations of 1,8-cineole and viridiflorol (Ireland 1999), which suggests that these terpenoids could have been present in the plants at Burpengary. These data are far from conclusive, and other explanations could account for the weevil size differences observed by Madeira *et al.* (2001). Nonetheless, the interpretation that the weevils examined by Madeira *et al.* (2001) differed in size because they developed on different chemotypes is consistent with experimental data from the present study coupled with Ireland's (1999) *M. quinquenervia* chemotype distribution data.

Chemotype differences may also help explain field observations suggesting that *O. vitiosa* populations generally established more readily and increased more rapidly along Florida's Gulf Coast than along the Atlantic Coast. Most *M. quinquenervia* populations along the Gulf Coast tend to be dominated by plants producing (E)-nerolidol (Chapter IV, see also Doskotch *et al.* 1980), and the present study shows that *O. vitiosa* populations should perform well on these plants. Conversely, *M. quinquenervia* populations along the Atlantic Coast are characterized by plants in which viridiflorol and/or 1,8-cineole are more abundant (Chapter IV, see also Doskotch *et al.* 1980), and the present study predicts that *O. vitiosa* populations will establish less readily and increase more

slowly on such plants. Center *et al.* (2000) argued that failed establishments likely resulted from an absence of suitable pupation sites in *M. quinquenervia* populations growing under long-hydroperiod conditions. If these sites were, as I suspect, dominated by type II plants then it is likely that few of the progeny produced on-site survived to pupation. Failure to establish at long-hydroperiod sites might then be understood as a convergence of limited larval survivorship with the scarcity of suitable pupation sites. If so, then greater number of larvae seeking pupation sites might have assured that the few suitable sites would have been discovered. This conjecture is supported by recent observations (personal communication: Drs. Thai Van and Min Rayamahji, USDA-ARS Invasive Plant Research Lab, Fort Lauderdale, Florida, 2003) that sites where *O. vitiosa* originally failed to establish now harbor viable populations.

The association between plants and insects is ancient (Wooten 1981), and often the relationship has been adversarial (Price 1975). Plants have had to develop effective defensive strategies to be able to resist an onslaught of herbivory. Variability in chemical defenses: phenological, ontological, and compositional, is an adaptation that has proven effective in this evolutionary arms race. Monocultures of plants are more susceptible to insect pests than mixed crops (Ricklefs 1990). Similarly, populations of a plant species containing only a single chemical defensive compound should be more susceptible to specialist insects than are populations containing individuals presenting a variety of chemical defenses. This assertion is affirmed by the present study wherein *M. quinquenervia* trees of the (E)-nerolidol (type I) chemotype were apparently less

resistant to the biological control agent *O. vitiosa* than were trees of the viridiflorol/1,8-cineole (type II) chemotype.

This development of multiple defensive chemotypes suggests that the selective pressures brought against *M. quinquenervia* by an abundance of herbivores (over 450 insect species, including specialists such as *Oxyops vitiosa*; Balciunas *et al.* 1994a) has been sufficiently strong to promote a radiation of chemical defenses. The high levels of survivorship of snout beetles reared on the type I plants could, under this scenario, be construed to represent the more ancient association of this herbivore and its host. Under this premise, populations of *O. vitiosa* would have evolved mechanisms to detoxify the (E)-nerolidol that was the principle constituent among the secondary metabolites conferring resistance to early *M. quinquenervia*. In a classical co-evolutionary response, individuals presenting novel chemotypes containing almost no (E)-nerolidol would therefore have had a selective advantage over their type I neighbors. Thus, plants whose principle chemical constituents are viridiflorol and 1,8-cineole should enjoy increased fitness and become prominent in the landscape. In fact, this is what we see in *M. quinquenervia*'s native range. Plant populations sympatric with *O. vitiosa*'s geographic range (Queensland and northern New South Wales; Purcell & Balciunas 1994, Madeira *et al.* 2001, Ireland *et al.* 2002) are largely composed of type II trees resistant to this herbivore. In contrast, type I trees susceptible to the weevil are more abundant where *O. vitiosa* is scarce or unknown (Purcell & Balciunas 1994, Madeira *et al.* 2001, Ireland *et al.* 2002). While this certainly is not the only possible

interpretation for these data, it is a plausible explanation that can serve as the foundation for future research.

Conclusion

Wapshere (1981) argued that the most virulent weed biological control agents will be those deriving from regions of the plant's native range wherein the genotypes found in its adventive range originated. Other biocontrol researchers (e.g., van den Bosch & Messenger 1973, Crawley 1983, Harris 1989, Harley & Forno 1992), echoing that sentiment, have called for biocontrol researchers to explicitly test the hypothesis that target plant genotype may have a strong influence on success of weed biocontrol projects. This study was, in part, a response to that challenge. In it, I empirically tested the hypothesis that performance of the melaleuca snout beetle, *Oxyops vitiosa,* would differ according to which genotype (as expressed by chemical phenotype) of its host, the paperbark *Melaleuca quinquenervia*, served as the weevil's food source.

The results of this study confirmed the basic premise that plant genotype affects the population dynamics of biological control insects. Population parameters such as larval mortality, rate of growth, and adult biomass were directly impacted by the *M. quinquenervia* chemotype upon which *O. vitiosa* fed. However, the weevils imported into Florida as biological controls of paperbark derive from an Australian population in an area where the viridiflorol/1,8-cineole (type II) chemotype of *M. quinquenervia* is exclusive or at least predominates (Ireland 1999, Ireland *et al.* 2002). Thus, if Wapshere's (1981) contention were

true for the *M. quinquenervia/O. vitiosa* system, one would expect that type II plants would be most susceptible to the weevils. Yet results from the present study show that *O. vitiosa* performance is substantially depressed on this chemotype in Florida. Instead, the greatest virulence is shown against a plant chemotype (the (E)-nerolidol type) that occurs largely outside of the weevil's known range in Australia (Purcell & Balciunas 1994, Madeira *et al.* 2001, Ireland *et al.* 2002).

The outcome of the present study thus failed to support the contention that matching target weed genotypes in native and adventive ranges will necessarily produce the most potent weed biological control agents. Instead, the data suggest that the most effective natural enemies may be found on genotypes other than those being targeted. This is mildly reminiscent of the "new associations" hypothesis in which Hokkanen and Pimentel (1984; see also Goeden & Kok 1986, Aldrich 1995) argue that prey species will have adapted defenses against closely associated predators (including herbivores) that will render the latter incapable of achieving outbreak densities. They thus conclude that the most effective biological control agents will derive from close relatives to the targeted pest. While not exactly a "new association" (*O. vitiosa* is, after all, a specialist on *M. quinquenervia*) the fact that the weevils are appear to be more virulent on a chemotype which they don't generally encounter in their native range seems to lend credence, in a limited sort of way, to this hypothesis. In any event, data from the present study suggest that weed bioagent efficacy might be enhanced by assaying the performance of proposed agents derived from a

variety of weed genotypes occurring throughout a target's native range (c.f. Hoffmann *et al.* 1999, Sobhian *et al.* 2003).

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Chapter VI. General Summary

Differential response of herbivorous insects to variation among host-plant genotypes is commonly reported in the general insect-plant interaction literature. For example, genotype differences among cottonwood hybrids (*Populus angustifolia* x *P. fremontii*) have a strong effect on bud-gall mite (*Aceria parapopuli* Kiefer: Eriophyidae) population growth rates (McIntyre & Whitham 2003). Such differential responses to plant variation may be particularly prominent among insects selected for use as biological control agents because they are so closely adapted to their host plants. Relatively few biocontrol projects have investigated this possibility, however, despite repeated calls for such studies (van den Bosch & Messenger 1973, Crawley 1983, Harris 1989, Harley & Forno 1992).

Australian populations of *Melaleuca quinquenervia* differ in the principle terpenoid constituents in their leaves (Ireland *et al.* 2002), and terpenoids often serve a defensive function in myrtaceous plants (Morrow and Fox 1980, Edwards *et al.* 1993, Schmidt *et al.* 2000). Further, the terpenoid compounds that characterize essential oils in *M. quinquenervia* are known to be genetically controlled (Shen & Dooner 2000, Trapp & Croteau 2001). Thus, it is likely that selective pressures caused by differences in these defensive compounds might have casued the hervirous insects that feed on this tree to specialize on subpopulations of *M. quinquenervia* that are characterized by different terpenoid compounds. Thus, importation of the Australian snout beetle *Oxyops vitiosa* as a

biological control of the Everglades invader *Melaleuca quinquenervia* afforded an opportunity to test the hypothesis that biocontrol agent performance can be influenced by the genetics of the target weed in its adventive range.

Formally stated, the purpose of this study was to (1) determine the number and origins of *M. quinquenervia* introductions into Florida; (2) determine whether multiple introduction events resulted in the partitioning of Florida's *M. quinquenervia* populations into discrete biotypes; and (3) determine whether *Oxyops vitiosa*, an Australia weevil imported into Florida as a biocontrol agent, discriminates among *M. quinquenervia* populations or biotypes.

Examination of the invasion history of this plant (Chapter II) showed that *M. quinquenervia* populations in Florida derive from at least a dozen introduction events, involving six source populations. The earliest introduction occurred during 1886 in Oneco, Florida, and preceeded by twenty years the best known introductions prior to this study. The principle reason for *M. quinquenervia*'s importation was as a landscape tree, but some proponents also sought a timber crop. Some founder populations escaped cultivation to become naturalized within two decades, but the principle means of dispersal was through human transport.

Allozyme analyses (Chapter III) indicated that this invasion history resulted in high levels of genetic heterogeneity in Florida. Measures of allelic richness and diversity were consistent with values for other tropical woody plants. Further, the pattern of the introductions, and the subsequent redistribution of progeny, resulted in geographic structuring among the populations. This structuring follows the primary distributional pattern for *M.*

quinquenervia in Florida: populations along the Gulf Coast are genetically distinct from those along the Atlantic Coast. Rates of gene flow were quite low,

Herbivorous insects are unable to directly measure genetic variation, however. So it was important to assay phenotypic differences that might be recognized by biological control agents. Some of the principle essential oil constituents in *M. quinquenervia* have been found to improve plants' resistence to herbivores (Morrow and Fox 1980, Edwards *et al.* 1993, Schmidt *et al.* 2000), so Florida populations were surveyed for differences in chemotypes (i.e., chemical phenotypes; Chapter IV). Foliar oil composition followed similar trends to the allozyme analysis, in that Gulf Coast populations differed from Atlantic Coast populations. For instance, Gulf Coast trees yielded nearly twice as much oil as Atlantic Coast trees when both were grown in a common garden. These differences were partially explained by the predominance of a chemotype very rich in the sesquiterpene (E)-nerolidol in *M. quinquenervia* trees from the Gulf Coast, but rich in a mixture of the monoterpene 1,8-cineole and the sesquiterpene viridiflorol in trees from the Atlantic Coast.

This biochemical variation among *M. quinquenervia* populations in Florida provided the foundation for investigating the principle question of this dissertation: whether biocontrol agents might be influenced by host-plant genotype differences. Bioassays showed that *O. vitiosa* performance differed dramatically depending on the chemotype of the foliage they were fed (Chapter V). Larval survivorship was four-fold greater on the (E)-nerolidol chemotype. Growth was also greater, with adult *O. vitiosa* gaining nearly 50% more biomass

on the (E)-nerolidol plants than on the second chemotype. These results offered some insight as to why a few of the initial *O. vitiosa* releases in Florida failed to establish persistent populations. They also helped confirm a hypothesis postulated by Wheeler (2003) to explain unexpected results in trials investigating the effects of differential foliar nitrogen concentrations on *O. vitiosa* performance.

Finally, the results from this study confirmed the premise that plant genotype can affect the population dynamics of insects released as weed biocontrols. Implications of this research can be summarized as follows:

- Invasion history offers important insights into the genetics and biology of weedy species in their adventive ranges.
- Population genetics can help differentiate among genotypes (biotypes, ecotypes, demes) present in a weed's adventive range.
- Plant defensive compounds are also useful indicators of the numbers and kinds of biotypes present.
- Specialist insects are, by definition, highly adapted to their hosts and are likely to be sensitive to differences among biotypes of their weed targets.
- Efficacy of biological control programs can be enhanced by investigating these parameters early in the process.

Biological control of weeds is a scientific discipline founded on the evolutionary premise that through geologic time many herbivorous insects have become specialized to breed, feed, and develop on one or a very few plant species (Huffaker 1971, DeBach 1974, Harley & Forno 1992, Center *et al.* 1997). Results from the present study argue that weed biocontrol researchers must be aware that

such close host fidelity may cause some biocontrol agents to be adapted to a restricted subset of genotypes within the target weed species. Such extreme specialization may explain some of the "failures" (i.e., no establishment, poor performance) that have occurred in various previous weed biocontrol programs. Remaining vigilant for potential genotype effects could help improve success rates in the future.

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