PHYLOGEOGRAPHY AND MOLECULAR ECOLOGY OF SELECTED INVASIVE SPECIES IN THE HAWAIIAN ISLANDS.

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We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Tropical Plant and Soil Sciences.

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ABSTRACT

Most alien (non-native) species which become naturalized are not disruptive to natural ecosystems. However, the small fraction that do spread and become invasive can have severe environmental and economic impacts. These impacts are often irreversible as control efforts normally only start after species are widespread. Molecular ecology renders a new approach to better understand invasions and can help in their management by resolving taxonomic issues, elucidating geographical source(s), detecting hybridization and introgression, and tracking dispersal and spread. Using a molecular ecological approach, some of these phenomena were investigated in this dissertation research for three different plant invaders in Hawaii. Molecular markers were developed for *Pennisetum setaceum* (fountaingrass), *Miconia calvescens* (velvet tree) and *Senecio madagascariensis* (fireweed) to answer ecological and management related questions.

Molecular and quantitative genetic variation indicated that fountaingrass is monoclonal throughout Hawaii and furthermore, that this clone or "super-genotype" is shared globally among invasive and native ranges. This indicates phenotypic plasticity as the sole mechanism behind fountaingrass' invasive success. Fountaingrass is unlikely to evolve resistance against successful control mechanisms. Subsequent herbicide trails indicated that grass-selective herbicides are ineffective against fountaingrass.

Phylogenetic and population genetic structure showed that fireweed introduced to Hawaii originated from eastern South Africa. Effective and host-specific biological control agents against fireweed are most likely to be found in this region. The high genetic diversity found in fireweed is indicative of multiple introductions. Genetic spatial autocorrelation revealed a diffusive dispersal pattern in the Hawaiian Islands.

ABSTRACT (CONTD)

Genetic structure indicated that bottlenecked populations of velvet tree are highly inbred in Hawaii and southern Pacific Islands. The data further suggested that invasive populations throughout northern and southern Pacific islands are genetically similar despite differential invasive success. These results indicate that invasions in both hemispheres are potentially from similar geographic origin and/or that Hawaiian infestations are the result of a secondary introduction directly from Tahiti. The introduction of genotypes pre-adapted for various morphological, physiological and life history traits facilitate invasion success of velvet tree. Climatological similarities between the Society and the Hawaiian Islands indicate that Hawaiian infestations of velvet tree have not yet reached an optimum. Biological control would be the only effective control method against velvet tree with most productive control agents likely to be found in Mexico.

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SECTION 1 – LITERATURE REVIEW

CHAPTER 1: MOLECULAR SYSTEMATICS AND POPULATION GENETICS OF BIOLOGICAL INVASIONS: TOWARDS A BETTER UNDERSTANDING OF INVASIVE SPECIES MANAGEMENT

Abstract

The study of population genetics of introduced, invasive species offers opportunities to investigate rapid evolutionary responses at work. While the ecology of biological invasions has enjoyed extensive attention in the past, the recentness of molecular techniques makes their application in invasion ecology a fairly new approach. Despite this, molecular biology has already proved powerful in inferring aspects not only relevant to the evolutionary biologist but also to those concerned with invasive species management. Here, I review the different molecular markers routinely used in such studies and their application in addressing different questions in invasion ecology. I then review the current literature on molecular genetic studies aimed at improving management and the understanding of invaders by resolving of taxonomic issues, elucidating geographical sources of invaders, detecting hybridization and introgression, tracking dispersal and spread, and assessing the importance of genetic diversity in invasion success. Finally, I make some suggestions for future research efforts in molecular ecology of biological invasions.

Introduction

The recent extent of global trade and transport has led to an enormous displacement of biota across natural barriers into new environments, leading in some

cases to establishment and invasive spread. Species invasions are now considered a principle component in driving large-scale ecological changes given their ability to cause habitat degradation, lower native biodiversity (Welcome *et al.* 1998), contribute to ecosystem changes (D'Antonio and Vitousek 1992, Vitousek 1990) and change the evolutionary trajectories of species (Strauss *et al.* 2006a). While it is difficult or in most cases impossible to put monetary values on such ecological and ecosystem impacts (but see Morals *et al.* 2004) previous work has illustrated the devastating economic losses ascribed to invasive species in agricultural environments (Pimental *et al.* 2000). It is not surprising then that invasive species have been the research target in both natural and managed ecosystems to design appropriate management solutions, predict and prevent future invasions, and restore previously invaded areas. Despite most of these research efforts being purely ecological, the field of invasion ecology is still largely anecdotal with few reliable generalizations (Williamson, 1999) making the aforementioned research goals mostly of little use to land managers. This may reflect the tremendous amount of diversity involved in global biological invasions (taxonomic, environmental, reproductive, dispersal, genetic etc.), representing equally impressive variation in research findings aimed at identifying management, prevention and restoration solutions. A good example of this dilemma is found in the relative importance of a species' evolutionary ability or genetic variation in invasion success. Only recently have ecologists recognized that invasive species can evolve rapidly (for review see Lee, 2002) and that such rapid genetically based adaptation might be more important in the success of biological invasions than was previously thought (Mooney and Cleland 2001, Sakai *et al.* 2001). While supporting evidence for genetic variation and rapid post-introduction

evolutionary responses exists (e.g. see Lavergne and Molofsky, 2007, Maron *et al.* 2004, Bone and Farres 2001) other studies question the importance of such variation and indicate phenotypic plasticity as key to colonization success and to explain the robustness of some species in recipient communities (e.g. Parker *et al.* 2003, Poulin *et al.* 2005, Le Roux *et al.* 2007). This apparent paradox makes a generalization about the role of genetic diversity in invasion success unattainable but should direct future research efforts. Determining the species attributes (e.g. breeding system, dispersal mode, migration rates etc.) that are correlated with successful invaders showing high levels of phenotypic plasticity vs. high levels of genetic variation under certain environmental conditions might lead to novel insights or the emergence of more generalized patterns.

Molecular systematics and population genetics render new and exciting tools to better our understanding of population dynamics during biological invasions and have already shed light on management, prevention and restoration strategies. The use of molecular genetics in particular, gained substantial interest in invasion-evolution studies (Holland 2000, Sakai *et al.* 2001). Here I review the current literature on molecular systematics and population genetics of invasive species and their application to the management and control of these species. First, I briefly introduce molecular systematics and population genetics and the types of molecular markers routinely employed in studying invasive species. Then, I review the application and usefulness of such data to management and control strategies and make suggestions for future research efforts in this emerging and exciting field.

Population genetics and molecular markers

The ultimate fate of a particular species' genetic variants (genotypes) in time and space will be determined by the species' biology and the circumstances through which individuals pass, such as reproductive success, migration, population size, natural selection, historical events etc. Population genetics focuses on describing the amounts and distribution of genetic variation within and among populations and connecting the observed patterns to demographic features. At the DNA level, genetic variation can arise via base substitutions (single nucleotide substitutions), insertion or deletion of DNA sequences (indels), inversion of DNA segments, and the rearrangement of DNA segments. Over extensive evolutionary accumulation, many different instances of each type of mutation should be present in any given species, defining its genetic variation. The number of available molecular markers to study such variation has increased dramatically since the advent of DNA sequencing in the 1970's, including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) and microsatellites, for example.

As a result of differential actions of fundamental processes such as recombination, mutation and selective constraint, various markers differ in the amount of polymorphism that they display (Sunnucks, 2000). Marker choice is thus one of the most important aspects of any molecular genetic study (Brower and DeSalle 1994, Baverstock and Moritz 1996). In addition to polymorphism, main concerns include whether the characters examined exhibit variation appropriate to the questions posed, have a clear genetic basis, and whether the data are gathered and analyzed in such a way that one can compare and combine data derived from them (Moritz and Hillis 1996). For example, a

systematic study to detect cryptic species, would normally utilize faster evolving markers such as microsatellites that render high resolution on small temporal scales. However, if larger spatial scales are involved such as typically encountered in phylogeographical studies or those entailing deeper taxonomic phylogenetic reconstructions, slower evolving markers such as nuclear DNA gene sequences would be more appropriate markers. Using microsatellites for such large spatial scale studies will lead to too much information, where the entities are too different with nothing linking them (Sunnucks, 2000). I briefly outline the most frequently used markers and their respective uses below. Table 1 summarizes the general characteristics of these markers.

Allozymes – protein based markers

Allozymes are nuclear encoded enzymes visualized by starch gel electrophoresis (Murphy *et al.* 1996). DNA polymorphism may result in differences in the amino acid composition of polypeptide chains leading to different alleles that can be distinguished by their differential electrophoretic mobilities. These co-dominant Mendelian loci are routinely employed to determine standard estimates of genetic variation and gene flow (i.e. *F* statistics: Wright 1978) within and among populations (Hamrick and Godt 1990). Even though protein electrophoresis normally reveals only a small fraction of the genetic variation in a population and levels of polymorphism vary among different taxonomic groups (Parker *et al.* 1998) this approach can be useful for situations where budget is limited and high resolution data are not required. Limitations include a lack of polymorphism for non-coding regions of the genome, non-neutrality and a tendency

towards monomorphism despite underlying DNA sequence variation due to silent mutations.

DNA based markers

DNA can be classified in two broad categories: nuclear (nDNA) and organellar (chloroplast [cpDNA] and mitochondrial [mtDNA]), both of which can be utilized to address different questions in ecology. The former is much larger than organellar genomes, and depending on the ploidy level of the organism, normally represents multiple copies (genetic regions or loci) on homologous pairs of chromosomes. For example, a triploid organism would have three copies (alleles) of each region (locus). Nuclear DNA contains both unique single copy regions and repetitive (multiple copies) regions. The latter could consist of coding segments such as ribosomal RNA (rRNA) genes or non-coding tandemly repeated units. Tandemly repeated units represent some of the most variable regions in the genome and are frequently used to construct molecular markers such as microsatellites and minisatellites. Organellar DNA is found in cytoplasmic structures and is inherited in a non-mendelian fashion, often with uniparental (usually maternal) transmission.

Restriction fragment length polymorphism (RFLP) analysis

RFLPs are variations in restriction enzyme cleavage sites in DNA (Botstein *et al.* 1980). Genetic variation due to mutational changes in restriction endonuclease recognition sites (4-8 base pairs [bp]) can be assessed by digesting DNA with specific restriction enzymes and gel electrophoretic visualization. For nuclear material, gene-

specific probes are used in Southern blot hybridization to visualize polymorphisms. However, RFLP variation can be detected electrophoretically when dealing with smaller molecules such as mtDNA that yields fewer fragments (Tegelström 1992). Another approach is to make use of the polymerase chain reaction (PCR) by digesting amplified DNA fragments with restriction endonucleases (Karl *et al.* 1992). RFLP variation is generally low but in most cases still sufficient to investigate genetic questions both within and between populations. High costs of restriction endonucleases and intermediate to low reproducibility make RFLP analysis a less widely used approach.

Random amplified polymorphic DNA (RAPD) analysis

RAPD markers produce banding patterns resulting from genome wide PCR amplification of uncharacterized fragments with short randomly chosen oligonucleotide sequences as primers (Williams *et al.* 1990). RAPDs are presumed to be neutral and their variation detected as the absence or presence of PCR products as dictated by mutations at primer binding sites or indels in regions between primer binding sites. RAPD markers have some major limitations though, including dominant Mendelian inheritance, making differentiation between homozygous dominant and heterozygous individuals problematic. Adding to this problem is the inability to distinguish between bands that are the result of distinct separate loci vs. bands that are alleles from the same locus, leading to an erroneous assessment of the number of loci under investigation. For instance, two bands differing by substantial length due to an insertion or deletion event between primer binding sites will in most cases be scored as two separate loci. Or, alternatively, paralogous PCR products, i.e. different amplified sequences that are the same length, will

be interpreted as a single locus. Futhermore, reproducibility in RAPDs is normally low due to the low annealing temperatures used during PCR amplification. These markers have nevertheless successfully been used for species identification (e.g. Partis and Wells, 1996) and analysis of genetic variation within and among populations (e.g. Yue *et al.* 2002). However, given the problems mentioned above, RAPDs have become less preferable markers. Indeed, publication of data solely based on RAPD analysis has been denounced by many well-respected journals in population genetic and ecological fields.

Amplified fragment length polymorphism (AFLP) analysis

AFLP analysis is a multilocus PCR-based technique that combines the advantages and overcomes the limitations of both RFLP and RAPD analysis. As with RFLPs and RAPDs the variation observed for AFLPs is generated by mutations in restriction enzyme cleavage sites and primer binding sites. AFLP markers are constructed by digesting whole genomic DNA with restriction enzymes followed by the ligation of known adaptor sequences onto the ends of the resulting DNA fragments. These adaptors act as primer binding sites, enabling PCR amplification of fragments at high annealing temperatures. The tremendous number of fragments generated this way is reduced by the addition of known base pairs to the 3' end of the adaptor-specific primers. These additional base pairs will extend past the fragment-adaptor ligated site and into the DNA fragment, only allowing annealing of the primer if the correct DNA sequence is present in the DNA fragment. This greatly decreases the number of PCR products to more manageable levels. Genetic variation is analyzed in a similar fashion to RFLPs, but instead of analyzing one locus at a time, many different loci can be analyzed simultaneously. The dominant

inheritance of AFLPs reduces their informative ability, but the large numbers of polymorphisms revealed (often exceeding 100) and high reproducibility due to higher annealing temperatures make them efficient markers for inter- and intra-population studies.

Variable number of tandem repeats (VNTRs)

VNTRs are generally non-coding and thus selectively neutral (but see Li *et al.* 2002) genomic regions that consist of several to multiple copies of the same DNA sequence repeated tandemly. These repeat units can vary in length from 2-64 bp. When repeats are 2-9 bp in length the simple sequence repeats (SSRs) are referred to as microsatellites. Examples include dinucleotides such as $[CA]_6$ or [CACACACACACA], trinucleotides such as $[GTC]_5$ or [GTCGTCGTCGTCGTC], etc. Microsatellite sequences have been found in all organisms studied to date and tend to be evenly distributed in the genome on all chromosomes. These regions are hyper-variable and mutation rates of 10^{-2} per generation have been reported (Weber and Wong, 1993). Polymorphisms are generated by single strand slippage during DNA replication resulting in differences in the number of repeat units (Schlotterer and Tautz 1992). Given the high rate of mutation, it is not uncommon to find a large number of alleles per locus (up to 20-50 alleles). Microsatellite sequences are normally flanked by more conserved DNA regions enabling amplification via locus-specific primers. Primers can be labeled with fluorescent dyes, enabling high-throughput genotyping on automated sequencers. This, coupled with the high levels of polymorphism and co-dominant Mendelian inheritance, makes microsatellites some of the most informative markers currently available. This is clearly

reflected in the number of studies that employ them to determine genetic diversity at the population level, genome mapping, parentage, kinships etc.

Longer repeat units (>10 bp) are called minisatellites and as with SSRs are generally non-coding, neutral markers, distributed throughout the genome. Minisatellite polymorphisms can be detected using a variation of the RFLP method. Following restriction digestion of whole genomic DNA and fragment separation, Southern blot analysis is conducted with a VNTR-specific probe. Typically, one probe detects VNTRs at many different variable loci throughout the genome, giving rise to a multi-banded genotypic fingerprint. Due to their high variability minisatellites are frequently employed for individual identification, forensics and paternity exclusion (Bachmann, 1994).

Inter-simple sequence repeats (ISSR) analysis combines the advantages of high polymorphism levels exhibited by microsatellite sequences and the relative ease of applying RAPD- or AFLP-based PCR amplification. A multilocus fingerprint is generated by amplification of fragments flanked by oppositely oriented microsatellites, using microsatellite core sequences and a few selected nucleotides as a single primer. High rates of microsatellite mutation and the variability of these regions (primer binding and PCR fragment length) can reveal minute levels of genetic variation (Wolfe and Liston, 1998). Dominant ISSR loci generally have higher levels of polymorphism than RAPDs and higher annealing temperatures allow for greater reproducibility. ISSRs can be used to infer relationships among closely related taxa (Wolfe *et al.* 1998).

Gene sequencing

Point mutations are the most abundant polymorphisms and can reveal polymorphisms missed by other molecular methods, such that gene sequencing provides the ultimate level of resolution of genetic variation. Various universal and taxon-specific primers are available, enabling researchers to amplify precise gene sequences of known function for analysis. Primer binding sites usually entail highly conserved regions that flank variable gene sequences exhibiting polymorphisms. Since substitution rates of different loci vary widely, selecting appropriate markers can be guided according to the intended ecological application and the taxonomic level at which the questions are focused. For example, for phylogenetic reconstruction between genera, slower evolving nuclear genes such as elongation factor 1 alpha (EF1α) might more accurately reflect evolutionary relationships among taxa than a faster evolving gene such as the mitochondrial gene cytochrome oxidase *I* (CO*I*). Longer divergence times associated with taxa in deeper parts of the phylogeny will lead to homoplasy, where characters are identical by state but not due to decent, also termed saturation. When using gene sequences that evolve too fast, phylogentic signal is lost and erroneous phylogenetic relationships can be inferred.

Genealogies constructed from DNA sequence data and their relationship to demographics are a major area of expansion, revealing previously unimaginable advances in what can be deduced about population processes and history. For example, molecular approaches can help distinguish current demographic processes from the effects of historical events (Templeton, 1998).

The differential patterns of evolution and transmission in nDNA vs. organellar DNA causes genealogies derived from them to reflect different aspects of population biology and history (Rubinoff and Holland, 2005). Combining such data is useful in unraveling population processes that would otherwise be extremely challenging. Examples of these include detecting hybrid individuals, sex-biased dispersal, and asymmetrical mating preferences.

Application of molecular genetics to invasive species management

Invasive species are notoriously difficult and expensive to control and usually impossible to eradicate. Thus it is of utmost importance to identify the most efficient management strategies available (Byers *et al.* 2002). Prevention is clearly more cost-effective than post-establishment eradication or containment, but in most cases alien species are only identified after successful establishment. Here I will focus on the application of molecular markers to the management of invasive species by resolution of taxonomic issues such as the precise, repeatable identification of introduced species and cryptic species, elucidation of geographic source(s), detection of introgression, tracking dispersal and spread and the role of genetic diversity in invasive success.

Taxonomic identity

Identifying introduced species is often problematic for various reasons, including high species diversity in source regions, world regions that have been little studied using modern phylogenetic systematic methodology, introduction of multiple sympatric, cryptic species and multiple introductions (Stepien and Tumeo, 2006). Taxonomic

misidentification may obscure accurate invasion history and preclude appropriate management strategies, e.g. population regulation below economic threshold or containment to a particular area.

Although, integrated approaches to manage invasive species can be useful across multiple taxa, correct taxomonic identification can aid in determining the most effective management strategies. Indeed, some management practices, in particular biological control, may be more productive or only effective against certain species or even variants (genotypes or biotypes) within a species (e.g. Goolsby *et al.* 2006). Such inter- and intra-specific variants are often impossible to distinguish based on morphology alone, whereas molecular markers can often reliably and accurately identify variants and cryptic taxa.

Molecular systematics approaches are useful in biological invasions of aquatic environments where factors limiting dispersal and reproduction are not well understood (Palumbi, 1997). For example, Holland *et al.* (2004) showed that morphologically uniform introduced populations of upside-down jellyfishes, *Cassiopea* spp., in Hawaiian coastal waters represent two genetically distinct lineages. Furthermore, Holland *et al.* (2004) showed that these two cryptic species resulted from separate introductions from different geographic sources, and therefore likely arrived by separate vectors. Another study that involves discrimination between invasive and non-invasive species of oyster drills comes from Garcia-Meunier *et al.* (2002). Monomorphism between egg capsules and juveniles of introduced Japanese oyster drills, *Ocinebrellus inornatus,* and native European oyster drills, *Ocenebra erinacea*, compromised ongoing research efforts to assess predation risks on commercially cultivated oysters and mussels and environmental impacts of introduced drills (Garcia-Meunier *et al.* 2002). Characterization of primers to

amplify unique nuclear sequences from each species provided baseline information for taxonomic diagnostic markers for easy, fast, and reliable identification of any life stages for both oyster drills (Garcia-Meunier *et al.* 2002). These markers can be employed to determine ecological impacts (abundance and distribution) of *O. inornatus* and its influence on native *Ocenebra erinacea*. An interesting example of the consequences of taxonomic misidentification ironically involves some of the best-studied aquatic invaders in North America. Dreissenid mussels were first observed in the Laurentian Great Lakes in 1988 and up until 1992 it was assumed that all US dreissenid mussel invasions involved a single species of zebra mussel, *Dreissena polymorpha*. Genetic analysis using allozyme polymorphisms revealed the presence of a second species, *D. bugensis* (quagga mussel) (May and Marsden 1992) that is currently displacing zebra mussels in parts of the Great Lakes (Jarvis *et al.* 2000). This taxonomical error puts the validity of all life history experiments, specific invasion protocols and even genetic inferences about founder and heterosis effects prior to this discovery in serious doubt. This example illustrates accurate taxonomic identification as a fundamental first step to biological characterization of introduction events (Holland 2000). Native predators of dreissenid mussels, round gobies, *Neogobius melanostomus*, and tubenose gobies, *Proterorhinus marmoratus*, were also accidentally introduced into the Great Lakes and are now dominant benthic species (Jude 2001). These two species belong to the species-rich but poorly understood subfamily, Neogobiinae, and occur sympatric with numerous neogobiin species in their native ranges (Stepien and Tumeo, 2006). Neogobiine adaptability to heterogeneous habitats makes them good invaders and, coupled with the group's taxonomic uncertainty, prompted Stepien and Tumeo (2006) to develop DNA

markers to evaluate whether additional cryptic species are present in the Great Lakes. Part of the mtDNA cytochrome *b* gene proved diagnostic for all species at all life stages. Even though no additional cryptic species were detected in the Great Lakes, it is predicted that neogobiin congeners will invade in the future from the Ponto-Caspian region, making these markers valuable for rapid detection of future introduction events.

Among terrestrial organisms, plants are particularly notorious for having taxonomic uncertainties due to interbreeding in and among species complexes, introgression among closely related species and high levels of phenotypic plasticity. Le Roux *et al.* (2006) used internal transcribed spacer (ITS1 and ITS2) sequences to resolve species of invasive fireweed in the *Senecio madagacariensis* complex. This complex encompasses morphologically similar species such as *Senecio inaequidens, S. skirrhodon, S. burchellii* and *S. pellucidus*, frequently mistaken for fireweed. This analysis included populations from which potential biological control agents were collected. Phylogenetic reconstruction showed that some putative fireweed populations were in fact *S. inaequidens*, misidentified as fireweed (*S. madagascariensis*). These results will have important implications towards selecting host-specific biological control agents. Another good example of a study that used molecular markers to resolve species boundaries in a taxonomically difficult plant group focused on pigweeds, *Amaranthus* spp. (Wetzel *et al.* 1999). Pigweed species are aggressive competitors and produce allelopathic chemicals making them particularly noxious weeds in agricultural areas. In their study Wetzel *et al.* (1999) developed diagnostic markers for native and exotic *Amaranthus* species, allowing fast and reliable identification. Similarly Saltonstall (2002) used non-coding mtDNA markers to show that invasive populations of North American *Phragmites australis*

represent a unique invasive haplotypic variant that differs from native populations. Furthermore, Burdick and Konisky's (2003) correlation between landscape disturbance levels and enhanced aggressiveness of this invasive haplotype can be incorporated in management protocols: management prioritization of disturbed habitats and the reduction of human disturbances in vulnerable areas. Such decisions are based on a case-by-case basis and incorporate rapid discrimination between the invasive and native haplotypes using diagnostic RFLP markers (Saltonstall 2003).

The studies cited above illustrate both the importance of sound taxonomy, and the efficacy of molecular techniques towards this end when dealing with invasive organisms. In each example, taxonomic identification was the first step towards making effective management decisions.

Determining the native provenance of invasive species

Invasive species often have large native ranges (Lodge 1993) implying that it is not always easy to determine their geographical sources. It is of utmost importance to identify geographical origins when considering biological control as a management option. Biological control is the most economic and self sustained control strategy in natural ecosystems (Messing and Wright 2006) with about one in three attempts resulting in enemy establishment (Hall and Ehler 1979) and half of these leading to complete control of the target species (Hall *et al.* 1980). A survey of successful biological programs showed that most effective natural enemies are usually host-specific (Rosen, 1986). Failure to correctly identify invasive species' native origins and geographical sources could lead to unsuccessful enemy establishment, poor host-specificity and/or

incomplete control. This is especially true when dealing with biotypes of a single invasive species (e.g. Chaboudez 1994), invasive species complexes (e.g. *S. madagascariensis* [Le Roux *et al.* 2006]) or natural enemy host-races (e.g. skeleton weed rust, *Puccinia chondrillina* [Espiau *et al.* 1998]).

An example of determining the native provenance of an alien species and its direct application to biological control comes from invasive *Phragmites australis* discussed in the previous section (Saltonstall 2002). Determining the native origin of the invasive haplotype in Europe led to the discovery of specialized herbivore complexes and was a considerable advancement for the future control of this species (Häfliger *et al.* 2006). In a phylogeographical study, Le Roux *et al.* (2006) identified the geographical source of invasive fireweed (*Senecio madagascariensis*) in Hawaii as the east coast of South Africa. This information will direct the current biological control program of fireweed as to where the most productive agents are likely to be found (M. Ramadan, Hawaii Department of Agriculture, personal communication).

In a similar study Scott *et al.* (1998b) showed that invasive populations of *Chromolaena odorata* in Australia encompass early and late seasonal flowering types, representing two distinct ITS1 genotypes. Coincidently these two genotypes were also found sympatric only in Brazil, revealing Brazil as the native source of Australian infestations. Gwiazdowski *et al.* (2006) did a phylogeographical study in an attempt to identify regions to explore for co-evolved natural enemies of the North American invasive beech scale, *Cryptococcus fagisuga*. A mtDNA-based phylogeny suggested that the subspecies *Fagus sylvatica orientalis* is the native host of *C. fagisuga* and that natural enemies would be best sought on oriental beech in northeastern Greece, the Black Sea

drainage basin, the Caucasus Mountains, and northern Iran. In another example, Milne and Abbott (2004) used cpDNA RFLPs and RAPDs to elucidate the most likely geographical source(s) of Privet, *Ligustrum robustum*, an invader in the Mascarene Islands. Their analyses concluded that cpDNA haplotypes were identical for introduced and native Sri Lankan subspecies, *L. robustum* spp. *walkeri*, differing from other native range regions (North and South India).

Unfortunately, to date, very few studies have simultaneously investigated the native provenances of invasive species and the productivity of biological control agents from identified source regions. Typically, molecular biologists share phylogeographical data with authorities responsible for biological control programs of particular species. A recent study by Goolsby *et al.* (2006), however, gives an excellent example of such integration. Goolsby *et al.* (2006) analyzed cpDNA sequence data of the Old World climbing fern (*Lygodium microphyllum*), an invasive species in Florida, USA. The invasive haplotype was compared with those from the native range of *L. microphyllum* and subsequently led to an identical haplotypic match. *Floracarus perrepae,* the natural enemy of this fern throughout its native range, was also collected from all native range populations included in this study. Not surprisingly, the *F. perrepae* genotypes collected from the identified source population proved to be the most productive and damaging to *L. microphyllum* populations in Florida.

Determining the geographic source(s) of invaders is important not only to the success of biological control programs but also to understand basic processes in invasion ecology. Molecular techniques provide the only reliable way to determine source region(s) for species where introduction and invasion histories are not well documented.

Hybridization, introgression and invasiveness

Genetic recombination as a result of hybridization and introgression will almost certainly have ecological consequences and it has been suggested that this might be a strong determinant of fitness in invasive species (Ellstrand and Schierenbeck 2000). Hybridization, gene flow and admixture will infuse genetic diversity and novel genotypes, masking deleterious alleles and transferring favourable ones (Abbott 1992). In their review, Rieseberg and Brunsfeld (1992) showed that morphological evidence of introgression might be misleading or not evident at all despite underlying introgression. The contribution of molecular methods to detect and understand introgression and hybridization is already substantial.

Ellstrand and Schierenbeck's (2000) excellent review gives convincing evidence for numerous plant species where hybridization preceded the emergence of invasive populations. A treatment of this report here would be redundant and I only mention some of the examples cited in their review in order to focus on more recent reports.

A textbook example of hybridization leading to the evolution of an invasive species concerns cordgrass, *Spartina*. The highly aggressive hybrid *S. anglica* originated in England as a result of hybridization between native cordgrass *S. maritima* and introduced *S. alterniflora* (Ferris *et al.* 1997). *S. alterniflora* was also introduced on the west coast of the US and using RAPD markers Daehler and Strong (1997) showed that hybridization occurred with another native cordgrass species there, *S. foliosa.* These hybrids display vigorous growth as a result of greater pollen and seed output (Ayres *et al.* 2004) and are threatening to displace native the *S. foliosa* populations. Another salt marsh plant, *Sarcocornia perennis*, is an example where hybridization led to novel mechanisms

of successional invasion and species replacement (Figuerao *et al.* 2003). RAPD markers confirmed that the invasive genotype of *S. perennis* is a hybrid between *S. perennis* and *S. fruticosa*. This hybrid is now becoming the dominant species in salt marsh environments where it displaces its progenitors. Similarly, Bleeker (2003) used cpDNA markers and AFLP markers to show that hybridization between two Brassicaceae species, *Rorippa austriaca* (invasive) and *R. sylvestris* (native), led to the evolution of a new invasive taxon, *R.* x *armoracioides*, in Germany. Furthermore, the molecular data suggested that *R. austriaca* and *R. sylvestris* are likely to hybridize wherever they are sympatric and that current hybrid populations contain high genetic diversity due to high hybridization frequencies. These results have direct management applications. In addition to containing *R. austriaca* populations, potential contact zones between *R. austriaca* and *R. sylvestris* should be prioritized for eradication efforts of *R. austriaca*. In a similar example Abbot and Forbes (2002) resolved the origin of invasive *Senecio cambrensis* in the British Isles as a hybrid between the native *S. vulgaris* and an introduced species *S. squalidus*. The relative ease of hybridization between these two species was illustrated by identifying multiple origins of hybrids (Abbot and Forbes 2002). Milne and Abbot (2000) used a combination of chloroplast and nuclear RFLPs to confirm that *Rhododendron ponticum* reached England from the Iberia Peninsula and that hybridization occurred in its northern British range with yet another exotic *Rhododendron* species, *R. catawbiense*. Hybrid populations acquired increased tolerance to low temperatures leading to higher fitness and more aggressive invasive behaviour (Milne and Abbot, 2000). Similarly, in the US, it was found that highly invasive *Tamarix* species from Eurasia were hybridizing in their invasive range (Gaskin and Schaal, 2002). The most devastating and abundant

Tamarix genotype proved to be a hybrid between two previously introduced species. Pyšek *et al.* (2003) reported that introduced *Reynoutria* taxa and their associated hybrids show differential vegetative regeneration rates. Hybrids regenerated better than their closely related parents, a crucial advantage to a species that spread by water. Hybrid taxa reproduce mostly vegetatively that renders reproductive assurance and fixes new hybrid combinations, contributing to their abundance and persistence.

Increased invasiveness as a result of hybridization has also been documented for aquatic species. Moody and Les (2002) investigated the relationship between invasive and native watermilfoil (*Myriophyllum* spp.) in North America. DNA sequence data obtained from the nuclear (ITS) genome for four different *Myriophyllum* spp. (*M. heterophyllum, M. pinnatum, M. spicatum, M sibiricum*) indicated that extremely invasive populations (monospecific stands) were always characteristic of hybrid populations and that parental populations lacked aggressive growth. The aquatic weevil, *Euhrychiopsis lecontei,* is a host-specific biological control against exotic *M. spicatum* with no significant impact on native *M. sibiricum*. However, recently some *M. spicatum* populations showed resistance against *E. lecontei*. Moody and Les (2002) confirmed that these were hybrid populations that acquired resistance from the native *M. sibricum* parental lines. These findings will have serious implications for biological control of *Myriophyllum* spp. The marine alga *Caulerpa racemosa* was thought to have reached the Mediterranean Sea in the mid 1920's from the Red Sea (Durand *et al.* 2002). Stationary populations of *C. racemosa* were distributed throughout the Mediterranean Sea until the early 1990's after which some populations started spreading rapidly. Morphological characterization led to the recognition of three distinct taxa within the *C. racemosa*

complex, one of which represents the invasive taxon (Verlaque *et al.* 2000). Phylogenetic reconstruction based on ITS and 18S rDNA sequence data identified a recent hybridization event between two varieties of *C. racemosa* and as a result of heterosis led to invasive hybrid populations (Durand *et al.* 2002).

While the correlation between hybridization and invasive success has been extensively documented for plants, similar treatments for other organisms are lagging. Facon *et al.* (2005) showed that hybridization led to superior competitiveness of two morphs of the invasive freshwater snail, *Melanoides tuberculata,* over their parental lineages. They also showed that fecundity was lower in hybrid lines but that, as a result of heterosis, a shift in life-histories towards larger investment in juvenile biomass and growth has occurred. Invasion by rusty crayfish, *Orconectes rusticus*, in the US has led to the rapid displacement of native species. Perry *et al.* (2002) used diagnostic nDNA markers to detect hybridization between *O. rusticus* and *O. propinquus,* and inferred that hybrids are displacing native *O. propinquus* populations. Interestingly, displacement of *O. propinquus* was not due to hybrid vigor but was hypothesized to be the result of biased mating patterns (*O.rusticus* males outcompeting *O. propinquus* males). Unfit hybrid progeny decreases the reproductive output of *O. propinquus* or alternatively intermediate hybrid progeny outcompetes *O. propinquus* males for mating with *O. propinquus* females.

Hybridization preceding invasiveness is particularly relevant to genetically engineered crops (GM-crops) and their close wild relatives. Evidence for conventional gene flow (not involving transgenes) and hybridization between cultivated and wild populations are numerous (e.g. *Sorghum*, Arriola and Ellstrand 1996; *Brassica*, Rieger *et*

al. 2002). Genetic modification of crops is now commonplace and commonly involves the insertion of genetic material to express resistance against the effects of herbivores and parasites (e.g., *Bt* corn) or herbicides (e.g., Roundup-ready soybean). Introgression of transgenes into wild relatives may render the same resistance mechanisms to hybrids, leading to novel and enhanced fitness traits and increased persistence in agricultural and natural ecosystems.

Hybridization between GM-crops and their close relatives remains largely untested. Warwick *et al.* (2003) used AFLP markers and field data to assess whether gene flow between transgenic canola (*Brassica napus* L.) and closely related wild relatives occurred in Canada. This study revealed that *B. rapa* acquired glyphosate resistance via hybridization with commercial canola. Canada is currently commercially producing canola not only engineered for glyphosate- but also for glufosinate- and bromoxynil-resistance, implying that these traits also have potential to escape into wild populations of close relatives. Halfhill *et al.* (2004) showed that transgenic hybrids of *B. rapa* have the potential to produce viable transgenic seeds in backcrosses. Glyphosate transgene escape into native creeping bentgrass (*Agrostis stolonifera*) populations in the USA was recently reported by Reichman *et al.* (2006). Nuclear and cpDNA-based gene phylogenies revealed that both seed dispersal and pollen drift from GM-crops into wild populations were responsible for transgene escape. Transgenes would persist in wild *A. stolonifera* if constant selection pressure (herbicide application) ensures fitness advantages to hybrids, leading to weedy populations in agricultural ecosystems.

Molecular techniques remain the most reliable and conclusive approach to detect transgene escape via introgression. These studies will become more commonplace as

more evidence accumulates on hybridization between GM and non-GM plants and would help in resolving management strategies and regulations.

The positive correlation between hybridization and increased invasiveness illustrates how the detection of such events will improve management strategies, e.g. prioritization of contact zones for eradication, isolation distances between GM-crops and their wild relatives, removing or reducing pressures such herbicide application rates that select for introgressed transgenes.

Dispersal and spread of invasive species

Dispersal is a critical factor for the success and self-sustainability of introduced species (Shigesada and Kawasaki 1997). Quantifying dispersal, especially rare long-distance dispersal, is notoriously difficult and time consuming when measured directly. This problem is exacerbated in plant populations, especially for past dispersal events. Highly variable molecular markers now render the ability to quantify the movement and spatial distribution of alleles (gene flow), indirectly measuring dispersal as a function of individual, seed, and pollen movement (Ouborg *et al.* 1999). First, the distribution of alleles among populations is determined, a population genetic model applied, and the amount of gene flow that would result in a similar distribution inferred. Second, an indirect inference of dispersal pattern(s) is done by e.g. spatial regression of geographical distance and genetic distance or spatial auto correlation analysis. Alternatively individual-based assignment tests that assign individuals probabilistically to candidate populations by their genotypic makeup can be used.

The efficiency of this indirect approach was recently illustrated in a study by Berry *et al.* (2004) that demonstrated dispersal patterns of grand skink, *Oligosoma grande*, as inferred from microsatellite markers and individual-based assignment tests corroborated patterns previously inferred from a seven year long mark-and-recapture study of the same populations. This example clearly shows the reduction in time and costs when employing such alternative methods. A better understanding of invasive spread and dispersal patterns has important implications for management. For instance, eradication of small pioneering populations in front of a continuous invasion front can be the most effective means of slowing or even stopping spread (Moody and Mack, 1988). Alternatively, invaders exhibiting long-distance expansion may need biological control agents capable of dispersing over equally long distances in order to keep up with their targets (Fagan *et al.* 2002). Some species are capable of re-colonizing areas from which they were previously eradicated, emphasizing the need for simultaneous eradication efforts of connected populations (Hampton *et al.* 2004).

Recently, Hampton *et al.* (2004) used microsatellite markers to infer dispersal patterns of feral pigs (*Sus scrofa*) invading parts of Western Australia. Due to public health threats posed by feral pigs near water reservoirs control programs specifically targeted these areas, but re-invasion hampered eradication efforts. Genetic structure indicated that dispersal during re-invasion occurs almost exclusively from upper reaches within the same watercourse/river system and not from nearby neighboring systems. Furthermore, the study identified unidirectional "source" populations for subpopulations that were subjected to eradication efforts. More effective eradication will thus be achieved by simultaneously targeting "source" populations and populations within the

same catchments (Hampton *et al.* 2004). In another example, the dispersal patterns of the brown rat, *Rattus norvegicus*, were indirectly determined using individual-based assignment tests of microsatellite data, on South Georgia Island (Robertson and Gemmell 2004). The current extent of brown rat invasions makes eradication in a single effort economically unfeasible and potentially unsuccessful due to re-colonization by survivors. However, Robertson and Gemmell's (2004) study indicated that extensive glaciation subdivides the island's rat populations. Glaciers act as barriers to dispersal, leading to units of manageable size for eradication efforts without re-colonization risk from neighboring populations. Viard *et al.* (2006) investigated the dispersal capabilities of the introduced slipper limpet (*Crepidula fornicata*) using microsatellite markers. Spatial modeling using population genetic differentiation and a 2D-hydrodynamic model confirmed the same patterns of dispersal, namely, (1) high-density populations capable of releasing large numbers of larvae to neighboring populations and, (2) that larvae can disperse over very long distances within short time periods (21 days). Evidently, the management policy for *C. fornicata* invasion needs to start from the very beginning of its introduction, including larvae as part of the management scheme. Another example comes from Khudamrongsawat *et al.* (2004) who employed allozyme and RAPD markers to investigate genetic diversity in the asexual giant reed, *Arundo donax*, a serious invader in the USA. Spatial distribution of observed genetic variation indicated that this riparian species disperses vegetatively downstream and that, because of asexual reproduction and water dispersal, management strategies should focus on targeting upstream spread. A similar study on genetic variation in *Phragmites australis* showed that genetic variation grouped populations geographically with distance along the river, indicative of

downstream dispersal (Keller 2000). More recently, Walker *et al.* (2003) used microsatellite data and found the same general pattern for *Heracleum mategazzianum* introduced to England. However, for this species, several independent secondary introductions by human-mediated transport were also detected (Walker *et al.* 2003). Until recently, the invasive spread of *D. bugensis* (quagga mussel) in the Laurentian Great Lakes was thought to resemble a gradual diffusion from the initial introduction in Lake Erie (Mills *et al.* 1993). Microsatellite markers showed that leptokurtic dispersal (combination of rare long-distance dispersal with diffusive settlement) was the predominant pattern of dispersal (Wilson *et al.* 1999). Such data can be applied to illuminate the predicted ranges of quagga mussel invasions (Wilson *et al.* 1999). A similar approach was applied to endemic and invasive quagga mussels in Eurasia (Therriault *et al.* 2005). In contrast to Wilson *et al.* (1999), these Eurasian populations failed to support isolation-by-distance, indicating that long-distance dispersal via boats frequently occurs within these regions (Therriault *et al.* 2005). Recently, Williams *et al.* (2007) investigated the spatial genetic structure of the invasive Brazilian peppertree, *Schinus terebinthifolius*, in Florida, USA. Spatial structure of cpDNA haplotypes and nuclear microsatellite loci were applied to a geostatistical model to estimate dispersal. The results indicated a directional genetic cline and evidence for short-distance genetic spatial autocorrelation as well as occasional long-distance jumps exist (stratified dispersal). It was speculated that areas previously subjected to eradication efforts are vulnerable to rapid re-colonization, suggesting the need for concerted eradication efforts over large areas or the introduction of an effective biological control agent against *S. terebinthifolius*.

The indirect approach to infer dispersal from molecular data is a relatively novel approach and I anticipate that more studies will pursue this time- and cost-effective avenue. The application of Geographic Information Systems (GIS) data to such studies would prove particularly useful in correlating dispersal with environmental factors (Prather and Callihan 1993).

Genetic diversity

Population genetic theory predicts that high genetic diversity predisposes invasive populations to success at establishing, persisting, and dispersing into novel habitats. It is not surprising then that more and more population geneticists are turning their attention towards invasive species as model systems. The inference of the amount and distribution of genetic variation could also contribute towards better management practices.

Evolution in recipient communities has been documented for numerous introduced species (e.g. see Maron *et al.* 2004, Bone and Farres, 2001) resulting in increased fitness and invasive success. In most cases, especially intentional horticultural or agronomical introductions, elevated levels of genetic diversity resulted from multiple introduction events. The strong correlation between higher genetic diversities and multiple introductions has been extensively documented for many different invasive species, e.g. *Ambrosia artemisiifolia* introduced to France (Genton *et al.* 2005) and *Cirsium arvense* introduced to USA (Slotta *et al.* 2006). Consistent with this interpretation, Sexton *et al.* (2002) found evidence for genetic variation in root biomass and subsequent local adaptation in invasive populations of salt cedars (*Tamarix ramosissima*) in the US. These invasive populations arose from multiple introductions

from different native range regions and accumulated genetic diversity that facilitated local adaptation. Selection for adaptive genetic variation has been shown for a number of introduced species, e.g. *Heracleum* species (Umbelliferae) (Jahodová *et al.* 2007), *Conyza* species (Asteraceae) (Thebaut and Abbott 1995), and *Solidago* species (Asteraceae) (Weber and Schmid, 1998). More recently, Lavergne and Molofsky (2007) provided evidence that repeated introductions of reed canarygrass, *Phalaris arundinacea* L., to the United States resulted in exceptionally high levels of genetic diversity. Subsequent recombination of this continental scale genetic diversity led to the establishment of novel, highly invasive genotypes not found in native Europe.

Alternatively, genetically diverse source populations could harbor variants that are "pre-adapted" to conditions in the recipient environment. In these instances it is just a matter of introducing the right genotype(s) into the right environment(s). An example of a "pre-adapted" genotype leading to invasive spread comes from Neuffer and Hurka, (1999). Invasive populations of *Capsella bursa-pastoris* showed quantitative ecotypic variation similar to that observed in native ranges (Neuffer and Hurka, 1999). Here, multiple introductions led to the successful establishment and spread of "pre-adapted" genotypes.

From a management perspective the above-mentioned studies caution against repeated introductions of exotic species. This may be particularly applicable to agronomically or horticulturally important species that have been inbred for various traits and for species with mixed breeding systems, as sexual reproduction facilitates recombination and asexual clonal reproduction preserve successful genotype(s).

Intentional introductions should, as a precautionary mechanism, focus on importing founders containing low levels of genetic variation.

Additionally, genetic diversity may help predict the potential of invasive populations to evolve resistance to management practices such as herbicide or biological control. Invasive cordgrass, *Spartina alterniflora*, harbors genetic variation in both tolerance and resistance to its introduced biological control agent, *Prokelisia* hoppers (Garcia-Rossi *et al.* 2003). Similarly Hufbauer and Via (1999) found that the pea aphid, *Acyrthosiphon pisum*, shows genetic variation in resistance to parasitism by its parasitoid *Aphidius ervi*. Peever *et al.* (2000) found that the chestnut blight fungus, *Cryphonectria parasitica*, exhibits genetic variation in tolerance to strains of its hypovirus control agent. These examples illustrate that tolerance or resistance to control practices can be achieved in populations containing high levels of genetic diversity. Detection of extremely high levels of genetic diversity in invasive populations (potentially equal or higher than in native populations [e.g. Lavergne and Molofsky 2007]), calls for alternative control strategies to biological control, as the introduction of yet another alien species (control agent) might prove unsuccessful.

Food for thought

Phylogenetic relatedness is often cited as an important component of invasive potential or even the invasibility of communities (e.g. Kolar and Lodge 2001, Strauss *et al.* 2006b). The use of a "centrifugal phylogeny" approach (Wapshere 1974) is a common practice to assess potential non-target effects of biological control agents whereby organisms that are most closely related to the target species are tested first, expanding to

more distantly related taxa, until the full putative host range has been evaluated (Messing and Wright 2006). Centrifugal approaches might prove particularly useful to assess the invasive potential of different taxonomic groups, the assumption being that recentness of shared ancestry between closely related species will lead to similar responses when introduced into similar environments. For example, molecular phylogenies that describe relatedness among closely related taxa that are highly invasive, moderately invasive, minimally invasive and non-invasive should reveal whether a correlation exists between genetic relatedness and invasiveness. Environmental considerations would be important, as taxa will differ in their responses in different recipient environments. Invasive taxa with differential success under similar environmental conditions are numerous, e.g., the Melastomataceae is one of the most devastating invasive weed families in Hawaii, with noxious taxa (e.g. *Miconia calvescens, Clidemia hirta*), moderately invasive taxa (e.g. *Arthrostemma ciliatum*) and less invasive taxa (e.g. *Dissotis rotundifolia*) introduced to the archipelago. Altogether 15 melastome species are currently naturalized in the Hawaiian Islands and represent an ideal system to investigate the correlation between phylogenetic distances and degrees of invasiveness. To my knowledge such hypotheses remain untested. New and powerful risk assessment and management protocols could arise if molecular phylogenies support the current views on the phylogenetic importance in invasiveness. For example, species would be short listed as high risk if introduced to specific areas as a result of being within the identified "phylogenetic threshold" for invasiveness for that particular group.

The non-target impacts of introduced biological control agents are normally not easily determined (L. Kaufman, personal communication) and in many situations such

trophic interactions cannot be identified by conventional methods such as post-mortem gut content analyses or real-time observations. DNA-based approaches now offer new techniques to identify gut contents of predators, providing information on the dynamics of predator-pray interactions (Hoogendoorn and Heimpel 2001). Most research in this field focuses on the identification of one or two specific species in predator diet and has been successfully applied to most predator-pray/host systems (e.g. Jarman *et al.* 2002, Agusti *et al.* 2003, Juen and Traugott 2005)

After determining native geographical sources of invaders, biological control practitioners need to differentiate specialist, host-specific enemies from generalist, less host-specific enemies, a major constraint to the timely release of control agents (M.G. Wright, personal communication). Molecular characterization of putative biological control agent gut content in native ranges would be a fast and reliable method to distinguish generalists from specialists. Diagnostic gene amplification of species present in natural enemies' gut content would reveal diet diversity, a measure of host-specificity. Laboratory simulations to evaluate host-specificity are unable to recreate the vegetation structure and microclimates present in environments and could lead to changes in host preference or recognition (Symondson, 2002). Molecular gut content characterization would only be applicable to control agents such as Coleopteran species where the adult life stage is responsible for control and would be of little use for Lepidopteran species where the larval stage is the primary controlling stage. The recent advent and extent of "DNA bar coding" would make species identification from molecular data relatively easy (Herbert *et al.* 2003). Additionally, identification of gut content can also be applied to assess environmental impacts of invasive species on native biodiversity.

Border control and quarantine inspectors are not trained taxonomists and consequently many unwanted species are introduced unnoticed. Many of the examples cited throughout this review used molecular markers diagnostic for specific taxa. Training authorities in a relatively simple laboratory procedure would enhance the accuracy and rapidity of species identification and, therefore, reduce unwanted species introductions. In Hawaii, for example, all possible measures are taken to prevent the introduction of additional Melastomataceae species. Our laboratory, in collaboration with the Hawaii Department of Agriculture, is currently developing diagnostic molecular markers for particularly unwanted species that are morphologically difficult to identify, providing a basis for fast and reliable molecular identification of intercepted materials (Wieczorek *et al.* unpublished data).

Conclusions

Invasion ecology and its application to management, prediction and restoration of introduced species remains largely anecdotal. Here, I underscored some of the recent advancements made towards effective management, control and restoration of invasive species when incorporating molecular approaches. Invasion ecologists are faced with numerous challenges, which prior to molecular techniques, proved difficult and cumbersome to overcome. Ironically, these obstacles remain some of the most important aspects when management is the ultimate goal of investigation. Molecular techniques now offer new approaches to better understand the complexity of invasive organisms below and above the species level, the importance of past (e.g. dispersal), recent (e.g. hybridization), and future (e.g. predictive) events in biological invasions and their

impacts on environments. I do not intend to advocate molecular systematics to replace the tremendous amount of ongoing ecological research on invasive species, but rather want to demonstrate its usefulness as a supplemental approach. Indeed, many of the inferences derived from the molecular ecology of biological invasions rely on the findings of previous ecological research. Molecular ecology as a science is still in its cradle rendering major advances and powerful inferences for future research efforts in this field.

Table 1.1. Summary and characteristics of molecular markers routinely applied in molecular ecological studies of invasive species

Marker type	Acronym	Variability[d]	Reproducibility	Precision[a]	PCR assay	Single locus	Inheritance	Allele genealogy feasible	Integrating data among studies	Major applications
Organellar										
Mitochondrial	mtDNA	Low (multiple)	High	High	Yes	Yes	Maternal, codominant[b]	Yes	Direct	Maternal lineage, phylogeography, population genetic
Chloroplast DNA	cpDNA	Low (multiple)					Maternal, but sometimes paternal, codominant[b]	Yes	Direct	Parental lineage, phylogeography, population genetic
Multilocus nuclear										
Restriction fragment length polymorphism	RFLP	Low (2)	Intermediate	Intermediate	Few	Yes	Mendelian, codominant	No	Limited	Linkage mapping, genetic diversity
Randomly amplified polymorphic DNA	RAPD	Intermediate (2)	Low	Intermediate	Yes	No	Mendelian, dominant	No	Limited	DNA fingerprinting, population genetic, hybrid detection
Amplified garment length polymorphism	AFLP	High (2)	High	Intermediate	Yes	No	Mendelian, dominant	No	Limited	Linkage mapping, population genetic
Inter-simple sequence repeat	ISSR	High (multiple)	Intermediate	Intermediate	Yes	No	Mendelian, dominant	No	Limited	DNA fingerprinting, population genetic, hybrid detection
Single-locus nuclear										
Allozyme		Low (2-6)	Intermediate	Intermediate	No	Yes	Mendelian, codominant	Rarely	Direct	Linkage mapping, population genetic
Minisatellites		High (multiple)	High	Intermediate	Yes	Yes	Mendelian, codominant	Rarely	Indirect[c]	Linkage mapping, population genetic, paternity analysis
Microsatellites	SSR	High (multiple)	High	Intermediate	Yes	Yes	Mendelian, codominant	Yes	Indirect[c]	Linkage mapping, population genetic, paternity analysis
DNA fragment sequence		Low/moderate	High	High	Yes	Yes	Mendelian, codominant	Yes	Direct	Phylogeographic, population genetic

[a] Differs from "reproducibility" in that a reproducible results may not accurately reflect the underlying variation, e.g. paralogous PCR products during RAPD analysis, or the presence of null alleles in microsatellite analysis.
[b] mtDNA and cpDNA are haploid but represent one of numerous possible states, in contrast dominant markers the state is either present or absent.
[c] Data from these markers are indirectly, but meaningfully, integrated given adequate models of molecular evolution.
[d] Number of alleles typically observed per locus are given in parenthesis for each marker type.

SECTION 2 – *PENNISETUM SETACEUM* (FORSSK.) CHIOV. (FOUNTAINGRASS)

CHAPTER 2: SUPER-GENOTYPE: GLOBAL MONOCLONALITY DEFIES THE ODDS OF NATURE

Abstract

The ability to respond to natural selection under novel conditions seems critical for the establishment and persistence of introduced alien species and their ability to become invasive. Here, neutral and quantitative genetic diversity of the weed *Pennisetum setaceum* Forsk. (Poaceae) were correlated with differing global (North American and African) patterns of invasiveness and this diversity compared to native range populations. Numerous molecular markers indicate complete monoclonality within and among all of these areas (F_{ST} = 0.0), and this is supported by extreme low quantitative trait variance (Q_{ST} = 0.00065 - 0.00952). The results support the general-purpose-genotype hypothesis that can tolerate all environmental variation. However, a single global genotype and widespread invasiveness under numerous environmental conditions suggests a super-genotype. The super-genotype described here likely evolved high levels of plasticity in response to fluctuating environmental conditions during the Early to Mid Holocene. During the Late Holocene, when environmental conditions were predominantly constant but extremely inclement, strong selection may have resulted in only a few surviving genotypes.

Introduction

Biological invasions offer real-life situations that demonstrate the roles of basic evolutionary processes, such as the essentiality of genetic diversity, the release from key

limiting factors in native ranges and phenotypic plasticity in self-sustained population growth (Sakai *et al.* 2001, Cox 2004). While general trends and characteristics of successful invasions remain for the most part elusive, many studies suggest that the ability to respond to natural selection might contribute more to invasion success than broad physiological tolerance or plasticity (reviewed in Lee 2002). Introduced ranges typically represent sub-adapted environments akin to a "valley" on the adaptive topography; species without pre-adapted histories will only persist if plasticity allows genetic assimilation or if post drift allele frequencies allow for the adaptation towards an alternative fitness peak.

Founding plant populations often have depauperate genetic diversity and following range expansion, will generate low intra-populational genetic variation. This situation is exacerbated in self-pollinating and apomictic species with limited or no gene flow. As isolated selection and mutations are the only mechanisms for creating new alleles and variation, apomixes may represent an evolutionary dead-end (Asker and Jerling 1992), leading to genotypic diversity decay over time. Despite this, apomicts are frequently found as highly tolerant and successful invaders (Rambuda and Johnson 2004) supporting two of Baker's hypotheses (Baker 1965; 1967). First, apomictic species have the opportunity for a single propagule to colonize and spread into new environments, satisfying Baker's rule (Baker 1967). Secondly, many apomicts show developmental and phenotypic plasticity with broad environmental tolerances, conforming to a "general-purpose-genotype" as coined by Baker (Baker 1965).

The allopolyploid apomictic grass weed, *Pennisetum setaceum* Forsk. (fountaingrass) was used to study whether global invasion gradients are shaped by

genetic differentiation between areas that are differentially impacted by this plant. This species' native range spans parts of the Middle East and North Africa and as a popular ornamental plant it has escaped cultivation and invaded areas of Australia, Democratic Republic of Congo, Fiji, Hawaii, continental USA, Namibia, South Africa, Swaziland, Zambia and Zimbabwe (Joubert and Cunningham 2002). Neutral and quantitative genetic diversity of globally invasive and native fountaingrass populations was quantified to determine the importance of such variation in invasion success.

Materials and Methods

Population sampling

Leaf material and where possible seeds were randomly collected from 20-30 individuals per population of *P. setaceum* in South Africa, Namibia, Hawaii, California and Egypt (Table 2.2).

DNA extraction and sequencing of Internal Transcribed Spacer Regions (ITS1 and ITS2)

Genomic DNA was extracted with the DNeasy Plant mini kit (Qiagen, Germantown, USA). The entire ITS region was PCR amplified using conditions and primers described by Martel *et al.* (2004). Each reaction contained 5 ng of total genomic DNA, 0.4 (v/v) HotMasterMix (HotMaster Taq DNA polymerase, 0.3 U; 2.5 X HotMaster Taq Buffer pH 8.5, 45 mM KCl and 2.5 mM $MgCl_2$; 200 μM of each dNTP [Brinkman Instruments Inc., Westbury, USA]) and 25 pmol of both primers. Purified PCR products were sequenced in both directions and were run on an ABI377 automated

sequencer (Applied Biosystems, Foster City, USA.) using standard dye-terminator chemistry. DNA sequences were manually aligned and submitted to GenBank (AY944426-AY944463).

Microsatellite libraries and marker development

Microsatellite-enriched libraries were developed as described by Hamilton *et al.* (1999) with slight modifications. Fifty micrograms of extracted genomic DNA were digested with *Rsa*I, *Hae*III, *Alu*I and *Hinc*II and the resulting fragments simultaneously ligated onto SNX oligonucleotide linkers. Following enrichment with biotin-labeled microsatellite oligoprobes (AAC_8 and GC_{12}) and streptavidin-coated magnetic beads, PCR amplification and purification, putative microsatellite-containing fragments were digested with *Nhe*I, and ligated into the *Xba*I site of pUC19 prior to transformation. Transformed bacterial colonies were transferred onto nylon membranes and screened using digoxigenin labeled microsatellite probes to identify microsatellite-containing clones. Following DNA extraction from clones, vector insert fragments were PCR amplified, purified and sequenced. DNA sequences were visually inspected to identify perfect and compound microsatellite sequences and PCR primers specific to each locus designed using the program FASTPCR (Kalendar 2004).

Screening for polymorphism and genotyping

Initial screening for polymorphisms was done by analyzing alleles for 6 different individuals from each global region (total of 24 individuals). PCR reactions were carried out in 15 μL total reaction volumes. Each reaction contained 0.4 (v/v) HotMasterMix,

7.5 pmol of each primer and approximately 5 ng total genomic DNA. A thermocycle of an initial denaturation of 94 °C for 2 min followed by 35 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperature (Table 2.2) and 1 min at 72 °C, followed by a final 12 min extension at 72 °C. Purified PCR products were run on an Agilent 2100 Bioanalyzer analysis LabChip (Agilent Technologies, Inc.) to detect fragment size differences at each locus.

Loci were also genotyped by fluorescently labeling PCR products using PCR cycle conditions described above and fluorophore-labeled primers. Separation of specific alleles was carried out on 5% polyacrylamide gels using an ABI377 sequencer. Analysis of the gels and the fragment lengths were carried out using the software GeneMarker (Softgenetics LLC^{TM}, PA). Fragments of lengths 50-700 bp were manually scored.

Inter-simple sequence repeat marker (ISSR) diversity

ISSR diversity was assessed using primers described by Poulin *et al.* (2005). Each PCR reaction contained approximately 2 ng of total genomic DNA, 0.4 (v/v) HotMasterMix and 50 pmol of ISSR primer. A thermocycle of an initial denaturation of 94 °C for 2 min followed by 35 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperature (Table 2.3) and 1 min at 72 °C, followed by a final extension of 12 min at 72 °C. PCR products were run on an Agilent 2100 Bioanalyzer analysis LabChip (Agilent Technologies, Inc.) to visualize and compare banding patterns for each ISSR marker.

Developmental traits

Twenty families from South Africa, Hawaii and Egypt were included for analysis of developmental variation under various conditions. Each family consisted of seedlings produced from seeds harvested from one of 20 randomly chosen individuals from each geographic location. Prior to all treatments, seeds were allowed to germinate and grow for two weeks in a 1:1 vermiculite and potting soil mixture before being transplanted into 800 ml plastic pots. A total of 10 replicates from each geographical location, i.e. 10 randomly chosen families, were used in each treatment. Seedlings were grown under full sunlight at ambient temperatures in a greenhouse. Individual pots were randomly repositioned weekly in the greenhouse for the duration of all experiments. Responses to all different treatments were measured as the amount of above-ground biomass produced after a certain growth period. Egyptian populations were only included for the nitrogen gradient due to limited seed stock available and as all experimental procedures were terminated prior to plant flowering (seeding stage).

Water stress

Seedlings were maintained at five different levels of water availability representing well-watered (obtained by watering the potting soil and vermiculite mixture (1:1) and allowing percolation to finish) to severe water stress (50, 40, 30, 20 % of the maximum water retention capacity i.e., well-watered) conditions. Plants were watered with the appropriate amount of water each day. Above-ground biomass was harvested and weighed after 43 days.

Nitrogen Availability

After transplanting seedlings in vermiculite a nitrogen gradient was simulated by applying complete micronutrient solutions with nitrate concentrations fixed at 0, 0.5, 1, 2 and 4 mM NO_3^-, all other elements applied at concentrations as described by Broughton and Dilworth (1971). Fifty milliliters of the appropriate nutrient solution were applied twice a week throughout the duration of the experiment to each pot. Plants were watered daily to keep vermiculate at saturated moisture content. Above-ground biomass was harvested and weighed after 60 days.

Nutrient availability

A nutrient gradient was created by applying slow-releasing fertilizer (Osmocote® [total nitrogen, 9.0 %; available phosphate, 6.0 %; soluble potassium, 6.0 %; total sulphur, 18.7 %; iron, 2.0 %]) at different rates (24.4 g/m², 73.2 g/m², 244 g/m², 610 g/m² and 1.83 kg/m²) to seedlings planted in a 1:1 vemiculite:potting soil mixture. Plants were watered daily to simulate well-watered conditions. Above-ground biomass was harvested and weighed after 45 days. The slow-releasing fertilizer promoted acidic soil conditions, simultaneously creating a pH gradient. Soil pH was measured at the time of harvest after continuous stirring of 50 g of soil in distilled water for 20 min.

Analytical methods

A nested ANOVA (PROC NESTED [SAS Institute, 2001]) was used to partition and estimate variance components of developmental responses, and to estimate Q_{ST} values as $Q_{ST} = V_o/(V_o + 2V_t)$, where V_o is variance among families by geographical

origin (country), and V_t is variance among families by treatment (nested within origin) (Stenøien *et al.* 2005). Q_{ST} values were calculated for the quantitative traits measured as accumulated biomass in response to water, nutrients and nitrogen availability. Responses to treatments (water, nutrient and nitrogen availability) of plants from various origins were compared using linear regression (PROC REG [SAS Institute, 2001]). To compare the effect of the qualitative variable "origin", indicator variables were generated and full model multiple regressions (including indicator variables as independent variables) were compared to reduced models (with origins pooled) to determine if origin had any significant effect on the slope and intercept of the response curves. This analysis was conducted for each of the treatments separately.

Results

Molecular genetic diversity

DNA sequencing of the internal transcribed spacer (ITS) regions 1 and 2 showed that all populations from all geographical regions share identical haplotypes for both genes. ITS2 was heterozygous in all individuals with the two alleles differing by 1 bp transitions at positions number 38 (T and C) and 101 (A and G), fixed for all populations. The 19 species-specific and diverse (length and motive class; Table 2.1) microsatellite markers developed during this study furthermore showed a complete absence of allelic diversity. Genotyping of 320 individuals from all sampled locations (Table 2.2) revealed that all microsatellite loci were fixed across all individuals (Fig. 2), indicating a single genotype shared globally and a panmixic F_{ST} value of 0.0. Microsatellite sequences used as primer binding sites (inter simple sequence repeats [ISSR]) supported all other

molecular data in resolving a single genotypic fingerprint for all populations investigated (Fig. 2).

Quantitative genetic diversity

Consistent with the molecular data, less than 1 % of the variation in seedling above ground biomass accumulation was explained among differentially invaded (South Africa and Hawaii) and native range (Egypt) countries (Nitrogen gradient Q_{ST} = 0.00952; Nutrient and soil pH gradient, Q_{ST} = 0.00065 and Water gradient, Q_{ST} = 0.00562 [see Fig. 3 for genotypic reaction norms]) for all fitness correlates. Phenotypic plasticity was apparent for all populations investigated and a lack of additive genetic variation in plasticity was reflected in the similarity of the slopes and intercepts of the genotypic reaction norms (Indicator Variable Analysis; nutrient and soil pH gradient [F = 0.84; P = 0.362], water gradient [F = 0.16; P = 0.687] and nitrogen gradient [F = 0.42; P = 0.661], Fig. 3).

Discussion

Understanding the underlying processes and variables that affect a population's ability to adapt and survive in changing and/or novel environments is a critical issue in evolutionary biology, conservation biology, and ecology (Sakai *et al.* 2001, Reed *et al.* 2003). Biological invasions pose ideal study systems to investigate such processes. For example, with invasive species often having to respond rapidly to novel abiotic and biotic forces of selection one might expect local adaptation and thus genetic diversity to play a major role in population fitness and survival.

The initial lack of phylogeographical differentiation within and among extremely invasive *Pennisetum setaceum* populations characterized by nearly monotypic stands and aggressive competitiveness (Hawaiian archipelago), moderately invasive populations confined to disturbed habitats (South Africa), introduced but non-invasive populations limited to roadsides (Namibia) and native range populations (Egypt) (see Fig. 1) are indicative of an apomictic breeding system. I therefore turned my attention to molecular markers rendering higher intra-specific resolution. In general, genetic polymorphism is anomalously high for plant species with no known reproductive alternative(s) to apomixes and could be the result of mutations and/or multiple origins of clones (for e.g. see Ellstrand and Roose 1987; Chaboudez, 1994; Novak and Mack 2000). Despite this generalization, the subsequent usage of "high resolution" markers (microsatellites and ISSR's) failed to resolve any further differentiation. Previous work (Poulin *et al.* 2005) showed a similar lack of genetic differentiation for a similar invasive gradient of North American fountaingrass populations from Hawaii (extremely invasive), Arizona (moderately invasive) and California (less invasive) using these same ISSR markers. Most plant introductions to Hawaii originate from the continental US and considering the relative geographical scale of Poulin *et al.*'s (2005) study, the monoclonality observed could be the result of a single initial introduction event of fountaingrass to the USA. However, based on the ISSR markers findings, it appears that *P. setaceum* from South Africa, Namibia, Egypt, Hawaii, Arizona and California share a single genotype, supporting monoclonality on a much larger, inter-continental scale. Not surprisingly, additional genotyping of individuals from two Californian populations also proved that all fixed microsatellite alleles were shared with all other global populations investigated.

More convincing of global monoclonality is that this single genotype also conforms to native Egyptian populations. Coupled with the broad native range of this species the Egyptian populations included in this study are unlikely to be the direct sources of the current globally introduced fountaingrass populations and do not explain the sharing of a single genotype.

The widespread global distribution of fountaingrass exposes it to unique and divergent selection pressures and that prompted investigation of ecologically meaningful fitness correlates (biomass accumulation) to different environmental gradients (drought, nitrogen, total nutrients and soil pH) for Hawaiian, South African and where possible, Egyptian populations. The choice of treatments was based on the marked differences or similarities for these abiotic components between the study regions. For example, in South Africa moderately invasive populations were found in both summer (monsoonal) and winter (polar front) rainfall areas that pose unique nitrogen fluxes, whilst highly aggressive Hawaiian populations inhabit volcanic ash soils that are typically poor in nitrogen. Furthermore, in South Africa, invading populations can be found in different biomes including temperate coastal Fynbos, sub-tropical Savannah, arid Succulent Karoo and Nama Karoo regions (Milton *et al.* 1998), representing diverse and distinctive environmental conditions. Measures of accumulated above-ground biomass were useful in two ways: 1) to assess the degree of phenotypic plasticity and its adaptive variation and 2) to partition quantitative genetic differentiation among these regions, thereby allowing comparison between neutral genetic variation (F_{ST}) to non-neutral adaptive genetic variation (Q_{ST}). Quantitative traits vary continuously owing to their polygenic nature and environmental influences, and coupled with selection strength, determine

evolutionary potential. A strong correlation exists between quantitative trait variation (Q_{ST}) and that observed for neutral markers (F_{ST}) with Q_{ST} typically exceeding F_{ST} in natural populations (Merilä and Crnokrak 2001). This is consistent with the interpretation that polygenic traits are under directional selection varying in magnitude and direction as a function of differential selection pressures among different populations in different environments.

As with the adaptive evolution of any trait, polyphenism requires genetic variation in, and selection on that variation. Considering the geographical scale of this study, the lack of variation in ecological meaningful fitness correlates and adaptive plasticity is surprising. Most studies do reveal genetic variation in plasticity, even over very small spatial scales (van Kleunen and Fischer 2005) and coupled with the known complexity of developmental plasticity, underscores the uniqueness of this study's findings. These data were furthermore predisposed to maternal effects as treatments were started from seed directly collected in the field from all locations and were not subjected to a common environment prior to experimental manipulation making these results even more intriguing. *Pennisetum setaceum*'s native range populations and those introduced globally (in most instances more than a century ago) seemingly have undergone no local adaptation for developmental traits related to water, nitrogen or nutrient stresses, but adequate levels of plasticity guaranteed broad ecological range tolerance and fitness.

Selective evolution of plasticity for any given population will in part depend on whether the plastic response has high energetic, functional and/or genetic costs (De Witt *et al.* 1998). Due to the lack of phenotypically more or less plastic individuals estimates of such costs were impossible to obtain during this study. Although plasticity costs are

likely to constrain the evolution of plasticity for a given trait, the ubiquity of plasticity in many species suggests that the benefits outweigh the costs under a wide variety of conditions. Costs related to plasticity do, however, prevent the continued evolution of plasticity to the point where a species could be successful in most environments (Agrawal 2001). Opposing this general perception, the single genotype described here supports the hypothesis that plasticity is the sole mechanism driving this genotype's success in heterogeneous and novel environments. Similarly, Williams and co-workers (Williams and Black 1993; Williams *et al.* 1995) found that high levels of plasticity exist in ecophysiological traits in reciprocally transplanted fountaingrass populations conforming to an altitudinal gradient spanning sea level to sub-alpine habitats in Hawaii. Without sufficient plasticity, rapid changes in conditions and environments such as those to which *P. setaceum* may never have been exposed, will pose a particular risk of local extinction. Costs associated with maintaining such high levels of polyphenism are expected to trigger the evolution of reaction norms that facilitate adaptation to more frequently encountered environments (Pigliucci 2005). Therefore, I postulate that the single genotype described here was historically extremely cost-effective even when multiple plastic hybrid genotypes likely existed. For the evolution of plastic genotypes such as the one reported here, single genotypes or genotypes over a few generations must be subjected to heterogeneous environmental conditions. However, this situation still does not explain why apparently only one genotype exists. To explain this, I furthermore speculate that at some point, native genotypes of *P. setaceum* were exposed to constant environmental conditions that were extremely hostile and caused hard purifying

selection, depriving these populations of genetic variation to very low or possibly non-existing levels, allowing only for the most plastic genotype(s) to persist.

Numerous paleontological records attest that the Late Pleistocene epoch (10, 000 BP) was characterized by arid conditions accompanied by aeolian activity in Egypt and Sudan. Aridity abated during the Early to Middle Holocene as a transition towards episodic humid and wetter conditions punctuated by intervening arid phases occurred with primarily steppe vegetation covering aeolian sands (Gasse 2000; Prentice and Jolly 2000; Nicoll 2004). These fluctuations, corresponding to Indian Ocean monsoon (IOM) intensity oscillations resulting from glacial boundary forcing (e.g. sea surface temperatures), occurred on timescales as short as decadal to multidecadal phases (Fleitmann *et al.* 2003). *Pennisetum setaceum*'s agamospermous reproductive system likely assured single genotypes' preservation and thus exposure to such fluctuations over the course of multiple generations. These variable conditions in the ancestral environment would have favored the survival of highly plastic genotypes(s). Review of botanical evidence from the Eastern Sahara furthermore suggests that the grasslands in Egypt and Sudan were diminishing around 7000 BP when these fluctuating climate conditions waned and constant aridity began to set in, dramatically reducing the desert flora to a similar composition to the present day flora (Neumann 1989). Aridification led to the establishment of arid-to-hyperarid conditions across the region by ~ 4500 BP with the extant flora disappearing from most habitats, and becoming restricted to only the hardiest desert-adapted plants (Bornkamm and Kehl 1989). Such inclement environmental conditions would have exerted extremely strong selection on ancestral

populations of *P. setaceum* and might have allowed only a few of the most plastic genotypes to explore the new adaptive landscape and to survive.

In addition, an agamospermous reproductive system would have stabilized hybridity and thus conserve this seemingly well-adapted genotype(s) of *P. setaceum*. Two possible attributes could have further contributed to the success of this single allotriploid genotype since its prehistoric origin. First, stabilized hybridity also leads to fixed heterotic genotypes and boosts fitness as afforded by fixed heterozygosity. Secondly, this fixed heterozygosity leads to a dumping of genetic load by masking detrimental mutations accumulated by the pre-hybridization parental lineages.

Plasticity is essential for populations to persist in novel environments and following establishment, heritable differences can be accumulated by natural selection to the extent where the adaptive phenotype achieved via plasticity becomes genetically fixed (Pigliucci *et al.* 2006). However, traits that are highly plastic are unlikely to be subject to divergent selection and may not become genetically divergent from the source (Price *et al.* 2003). Extremely high levels of plasticity enabled *P. setaceum* to cross adaptive valleys (analogous to maladapted conditions) on a changing adaptive topography that resulted from environmental change(s) (Fear and Price 1998) and to reach alternative optimal fitness peaks without the need for local adaptation and thus the chance to differentiate from source populations (Fig. 2.4). *Pennisetum setaceum* is thus a classic, but extreme example of a general-purpose genotype (Baker 1965). Generality is fundamental for apomictic survival as clones surviving for many generations could only have done so by virtue of being able to tolerate all environmental variation exposed to since their origin, while more specialized genotypes would have rapidly gone extinct.

Here, evidence is provided for the existence of a single, successful apomictic genotype on a global scale showing wide environmental tolerance and I propose the term super-genotype to define this unique phenomenon. A super-genotype for *P. setaceum* is justified by the apparent lack of neutral and adaptive genetic differentiation within the region of origin and among globally introduced populations and the grass' capability to survive under a wide array of environmental conditions. High levels of phenotypic plasticity ensure self-sustainability in disturbed habitats (Parker *et al.* 2003) but do not seem to allow for fountaingrass to overcome the biological resistance-buffer posed by undisturbed habitats and intact ecosystems. The gradient of invasiveness observed here is indeed correlated to some degree to a similar disturbance-level gradient between differentially invaded areas. Namibian fountaingrass populations are only found in human-derived disturbance areas such as roadsides with no spread or establishment in undisturbed native vegetation (Joubert and Cunningham 2002). In South Africa, however, where limited spread and persistence in native vegetation does occur (Milton *et al.* 1998), ecosystems are characterized by intermediate disturbance levels. In these Mediterranean-climate shrubland (Fynbos) areas fire disturbance often yields spatial heterogeneity and intermediate environmental disturbances (Schwilk *et al.* 1997). The islands of the Hawaiian archipelago's, and in particular the island of Hawaii's, geological recentness (0 – 0.5 Ma) encompasses habitats that are overall in a constant state of extreme disturbance (Price and Clague 2002). Even though this genotype is well-adapted to disturbed habitats, evolutionary potential is essentially impossible and any fitness valley resulting from environmental changes such as novel biotic interactions (e.g. phytopathogens, herbivores), will result in rapid extinction. Despite this contrast to the

hypothesis that typical Darwinian evolution contributes significantly to invasive species survival, the single super-genotype described here persists and survives exposure under many different conditions. Further examination of other species may reveal further super-genotypes, and it may be found that this is a more common, significant but hitherto overlooked mechanism driving survival and local fitness of plant populations.

Table 2.1. Characteristics of the 19 microsatellite markers developed for *Pennisetum setaceum*.

Locus name	Forward/reverse primer sequences (5'→ 3')	Repeat unit & length	Annealing temperature	Genbank accession number
Penset4	TATGGTTCGCCACTTGGTGC/ACCCTCTCACACCCTGGGAG	$(GA)_{18}$	48.0 °C	DQ899151
Penset6	CATATTTCAGACCGGGAACACC/AGGTCAGGGTCTCGGGTCG	$(TC)_{23}$	60.0 °C	DQ899152
Penset14	TGTCACCAATGGAGTTGCTC/GCGTATGTGGGTGTGTTGC	$(AAC)_6$	58.5 °C	DQ899153
Penset18	TCACTTTTGTGCCAGACTGC/TCAGCAGCTTGTGGCCCAC	$(CT)_{24}$	50.0 °C	DQ899154
Penset21	TTGGGATGGTGTGGACACC/ACCAAAGGATAAATCTCGCTGC	$(TA)_7(AC)_6$	48.5 °C	DQ899155
Penset24	TCCTCACTCTTGCTCTCACG/CCCACATAGTTTGCGGTAGG	$(CT)_{15}$	49.0 °C	DQ899156
Penset28	GTGGTCTAACCGCCGATTAG/ACTAGCCAAACTTGGTTGATCG	$(GA)_{13}$	50.0 °C	DQ899157
Penset35	GCGAGCCTAACAGCGTTTC/CTCGTGTGGGCAGCAATGC	$(GA)_{33}$	56.2 °C	DQ899158
Penset37	TTGACGGGAAGAGCAAAGC/TGAATCGAGCCCAGGCTGC	$(CT)_{12}$	52.1 °C	DQ899159
Penset95	GGAGTGCTTGGAGACTTGC/CCAAATGGTACATACTAGCGGTTC	$(GTT)_{17}$	53.0 °C	DQ899160
Penset99	GCAATCAACGTGCCTGAACC/ATCCAGTGCCAGAGGCTCC	$(GTT)_7$	48.5 °C	DQ899163
Penset104	TGTTTCAGTCATGGGCTGAC/GCTTGCGATTGGGTCCTGAG	$(CAA)_{18}$	60.5 °C	DQ899165
Penset105	AGCAATTAGTGTGCCTGTAACC/TTTGCCACCAGCCGAGAGTC	$(GTT)_7$	45.5 °C	DQ899166
Penset110	CAATGTGTCTGAACCATGACCTC/AGCCTTTTGTCCCAAGCAAG	$(GT)_8(GGGG)(GT)_{12}$	60.0 °C	DQ899168
Penset111	TGGGGTTGTCCTGGGGTGG/TGAGGAAGACAAAGCAATCACC	$(GTT)_{27}$	50.5 °C	DQ899169
Penset114	ACCCCAACTTGCTTGGGAC/TCTACGAGGACGCCTGTGG	$(GTT)_6$	48.0 °C	DQ899170
Penset117	CGCCATGCAACACAAGCAC/TCAAAGTGGTTGAGGGTTGC	$(AAC)_{21}$	60.0 °C	DQ89917

Table 2.1. (Continued) Characteristics of the 19 microsatellite markers developed for *Pennisetum setaceum*.

Locus name	Forward/reverse primer sequences (5'→ 3')	Repeat unit & length	Annealing temperature	Genbank accession number
Penset119	TCACGTCGTAACAATGCACC/TGCTCAGGTGACTGCTCTG	$(CAA)_8$	60.0 °C	DQ899172
Penset120	ACAATCCCTGTGCCCAAAC/AGCTATCAACGTGCTTGAACC	$(AAC)_6$	49.0 °C	DQ899173

Table 2.2. Globally invasive and native populations of *Pennisetum setaceum* used in this study.

Origin (country)	Region*	Habitat/Vegetation	Latitude, Longitude
Native range			
Egypt	Medan Gohainah*	Desert sand dune/Sparse steppe	N 30.00825°, E 30.98019°
Egypt	Al-Geiza*	Desert sand dune/Sparse steppe	N 29.99233°, E 30.98936°
Introduced and non-invasive			
Namibia	Windhoek	Highland Savannah/Trees and grassland	S 22.58327°, E 016.97473°
Namibia	Windhoek	Highland Savannah/Trees and grassland	S 22.58243°, E 016.97683°
Namibia	Windhoek	Highland Savannah/Trees and grassland	S 22.58243°, E 016.97682°
Moderately invasive			
South Africa	Northern Cape*	Semi-desert/Succulent, shrub and grassland	S 30.47577°, E 017.95216°
South Africa	Northern Cape	Semi-desert/Succulent, shrub and grassland	S 30.47893°, E 017.94686°
South Africa	Western Cape*	Mountain Fynbos/Woody tree and shrub fynbos	S 31.94066°, E 018.69687°
USA	California	Mountain grassland	N 33.97183°, W 117.72305°
USA	California	Shrubland	N 34.10388°, W 118.6025°
Highly invasive			
USA	Kona, Hawaii*	Semi-arid/Dry forest, shrub and grassland	N 19.81157°, W 155.97464°
USA	Kona, Hawaii	Semi-arid/Dry forest, shrub and grassland	N 19.73449°, W 155.53534°
USA	Kona, Hawaii	Semi-arid/Dry forest, shrub and grassland	N 19.73663°, W 156.03279°
USA	Lanikai, Oahu*	Semi-arid/Shrub and grassland	N 21.46888°, W 157.74333°
USA	Lanikai, Oahu	Semi-arid/Shrub and grassland	N 21.47111°, W 157.735°

* Populations that were used for quantitative trait variance analysis.

Table 2.3. ISSR primers used in this study.

Marker name	Sequence	Annealing Temperature
Primer 7	CACACACACACAGA	43.0 °C
Primer 8	CTCTCTCTCTCTCTCTRG	43.0 °C
Primer 10	GAGAGAGAGAGAGAGA	44.5 °C
Primer 14	GTGTGTGTGTGTGTYG	50.0 °C
Primer 16	GACGACGACGACRC	50.0 °C
Primer 17	GTCGTCGTCGTCRC	48.0 °C
Primer 18	GTGGTGGTGGTGRC	50.0 °C

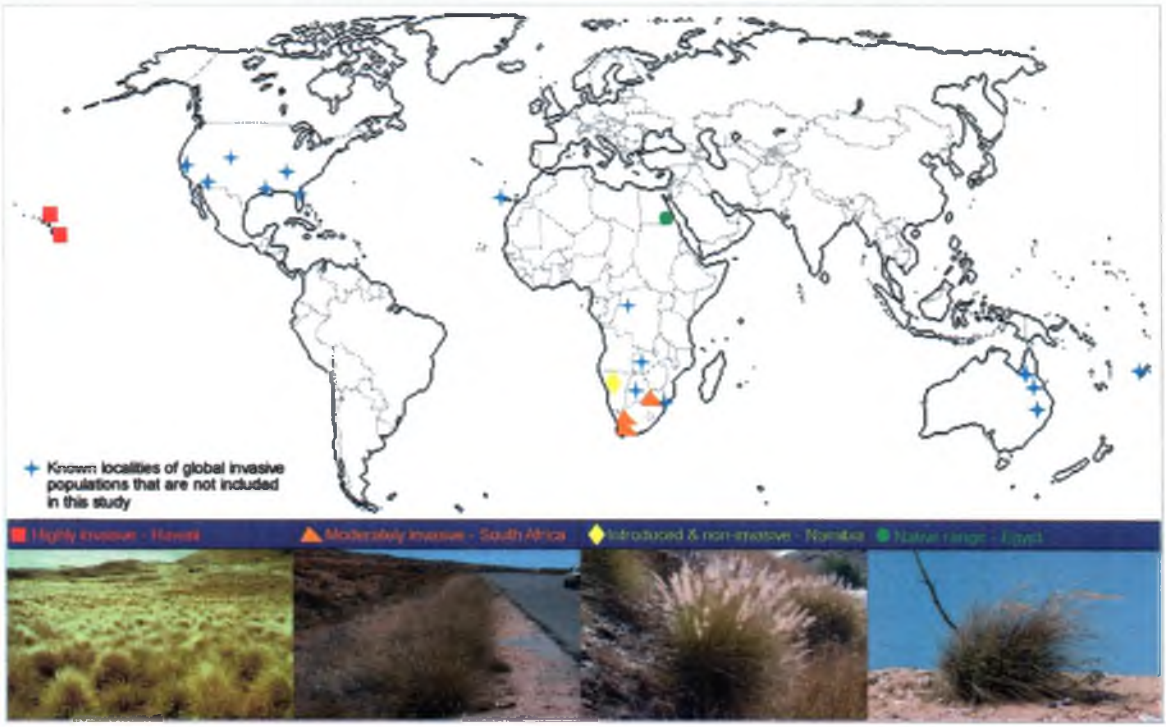


Figure 2.1. Global locations where differentially invasive and native range populations of *Pennisetum setaceum* were collected for this study. Additional world locations where fountaingrass has been introduced and are considered invasive are also indicated.

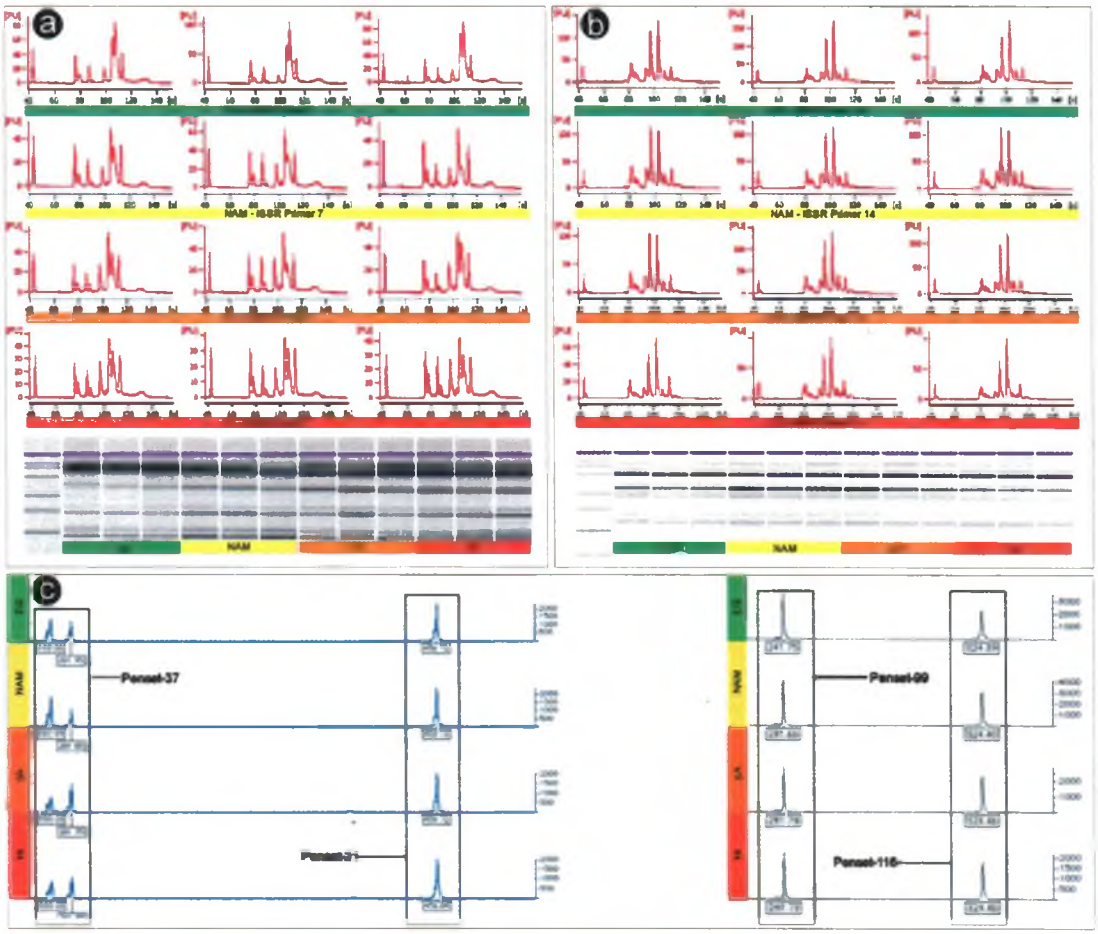


Figure 2.2. Results for selected molecular markers. ISSR banding pattern data for primer 7 (a) and primer 14 (b) indicating no variation between Egypt (EG, green), Namibia (NAM, yellow), South Africa (SA, orange) and Hawaii (HI, red). Electropherograms and their associated gel images are shown for 3 individuals from each of these locations. An illustration of 4 selected microsatellite loci (c) shows complete fixation for all alleles between all locations.

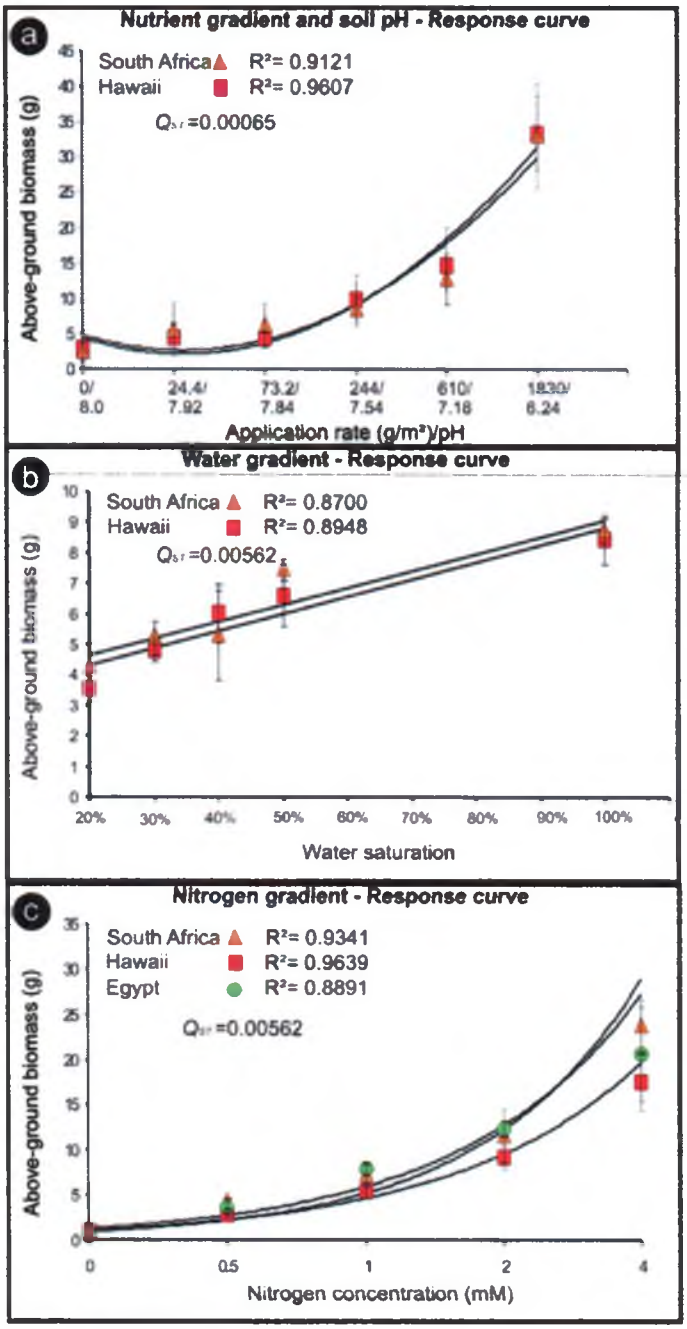


Figure 2.3. Genotypic reaction norms for (a) nutrient and soil pH gradient, (b) water gradient and (c) nitrogen gradient showing mean (± s.e.m.) biomass accumulated in response to each treatment level. Egyptian populations were only included for the nitrogen gradient. The corresponding Q_{ST} values for fitness correlates are indicated on each graph.

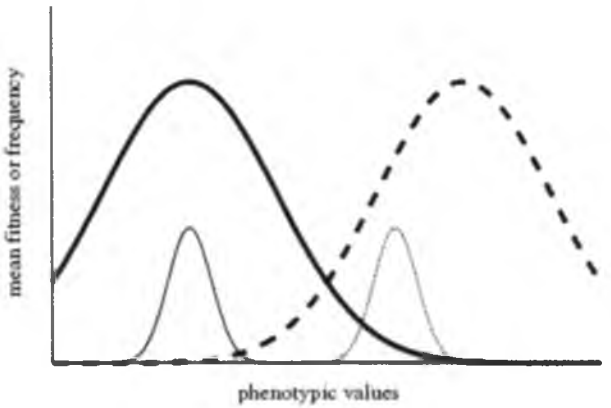


Figure 2.4. The contribution of phenotypic plasticity to a peak shift on the adaptive topography in a changing environment (after Fear and Price 1998). Bold lines are mean fitness; the dashed line represents fitness in the new environment. The thin solid line represents the population distribution in the old environment (e.g. native range) and the thin dotted line represents the population distribution after a plastic response to the new environment (e.g. introduced range). Note that without a plastic response no peak shift will occur and the population will go extinct.

CHAPTER 3: THE EFFECTS OF HERBICIDES ON INVASIVE AND NATIVE POPULATIONS OF *PENNISETUM SETACEUM* (FORSSK.) CHIOV. (FOUNTAINGRASS)

Abstract

The effects of two herbicides, fluazifop-p-butyl and glyphosate were assessed on the growth performances of fountaingrass (*Pennisetum setaceum*) representing invasive (Hawaiian and South African) and native (Egyptian) geographic populations. Based on the global monoclonality of these fountaingrass populations all populations were predicted to respond in a similar fashion to all treatments. Fountaingrass ramets were grown for three months in a greenhouse before being transferred to an open air facility. Plants were cut to uniform habit and herbicides applied using a diaphragm pump calibrated to deliver 374 liters per hectare. The effects of herbicides were monitored over an eight week period as visual injury and amount of accumulated regrowth. Even though the two herbicides differed in their overall effects on fountaingrass from all these regions, all regional populations reacted similarly to treatments, supporting very low or possibly no genetic variation. In addition, different surfactant combinations did not influence herbicide performance significantly. Non-selective glyphosate caused 100 % visible injury and mortality (average biomass ratio percentage: 15.12 %) in all treatments while treatments with fluazifop-p-butyl resulted in less visible injury (average visual injury: 42.43 %) and growth retardation (average biomass ratio percentage: (24.22 %).

Introduction

It is a fundamental tenet of evolutionary biology that the rate of change in response to natural selection is proportional to the amount of additive genetic variation present (Fisher 1930). Such heritable variation may thus help to predict the potential and rate of populations of invasive species to evolve resistance in response to management practices such as chemical or biological control. For example, empirical evidence suggested that invasive cordgrass, *Spartina alterniflora*, harbors genetic variation in both tolerance and resistance to its biological control agents (Garcia-Rossi *et al.* 2003). Similarly Hufbauer and Via (1999) found that the pea aphid, *Acyrthosiphon pisum*, shows genetic variation in resistance to parasitism by its parasitoid. Mengistu *et al.* (2005) showed that wild oat, *Avena fatua,* evolved resistance to multiple herbicides as a result of highly genetically diverse founding populations.

The single genotype and apparent lack of genetic structure observed for *Pennisetum setaceum* (Chapter 2, Le Roux *et al.* 2007) would thus predict that this grass is unlikely to evolve resistance to selection pressures exerted by effective control mechanisms, even over extensively long periods of exposure. Once potential control strategies have been identified this would be an important attribute towards the successful management of this species. The genetic structure of populations has been shown to affect the efficacy of control of invasive species. Burdon and Marshal (1981) noted that, as a result of genetic homogeneity, asexually reproducing weeds are more effectively controlled by biological control than sexually reproducing weeds. Not only is it easier to match biological control agents to asexual host genotypes, but lower genetic diversity in asexual species also implicates a lower likelihood to evolve resistance against control

agents. Here, the effects of two herbicides, glyphosate and fluazifop-p-butyl, was tested on the performance of fountaingrass from different invasive ranges (Hawaii and South Africa) and its native range (Egypt). Under the assumption of the single "super-genotype" (Chapter 2, Le Roux *et al.* 2007) it is expected that all regional populations should respond similarly to different herbicide treatments.

Materials and Methods

Growth conditions

Pennisetum setaceum ramets from different geographical sources (Egypt, Hawaii and South Africa; location data can be found in Chapter 2) were grown in 1:1 vermiculate and potting soil (Miracle-Grow ®) mixture in 800 mL plastic pots and slow-releasing fertilizer (total nitrogen, 9.0 %; available phosphate, 6.0 %; soluble potassium, 6.0 %; total sulphur, 18.7 %; iron, 2.0 %) applied at 24.2 $g.m^{-2}$. Pots were randomly repositioned twice monthly in the greenhouse and kept under well-watered conditions. Plants were kept in the greenhouse for three months after which they were transferred to an open air facility. Following three weeks of exposure to outside conditions, plants were cut to uniform habits, fertilized with turf fertilizer (total nitrogen, 22%; available phosphate, 2%; soluble potassium, 9%; total sulphur, 12.1%; iron, 5%; chlorine, 2%) at 9.8 $g.m^{-2}$ and allowed to grow for an additional two weeks prior to herbicide treatments.

Herbicide treatments and response measurements

Two herbicides, fluazifop-p-butyl (Fusilade DX® , Syngenta) and glyphosate (Roundup Pro®, Monsanto Technology, Inc.) were used in herbicide trials. Glyphosate is

a non-selective but generally very effective broad-range herbicide while fluazifop-p-butyl is frequently applied as a grass-selective herbicide. Each herbicide was applied at labeled recommended rates (0.84 kg/ha, fluazifop-p-butyl; 2.3 kg/ha, glyphosate). To examine the effects of different surfactants, application mixtures contained either 0.1 % (v/v) methylated seed oil (MSO®, Loveland products, Inc.) or a combination of 0.1 % (v/v) methylated seed oil and 0.1 % (v/v) silicone (Sylgard 309®, Norac concepts, Inc.). Treatments consisting of surfactant-only combinations and no herbicides were applied to control plants. Applications were done with an electric powered diaphragm pump calibrated to deliver 374 liters per hectare. Each treatment was replicated four times.

Visual ratings of injury to different herbicides treatments were obtained by averaging observations of two persons for each of the four replicates (0-100%). A 100% rating corresponded to treatments where leaf coloration and necrosis affected 100% of the treated foliage, whereas 0% corresponded to treatments where foliage was completely unaffected. Visual injuries were scored in 5 % increments (resolution). Quantitative responses to different herbicide treatments were measured by collecting all visible re-growth accumulated 6 weeks after herbicide application (measurement *r*). Due to trimming, plants had uniform habits prior to herbicide application (45 cm above soil surface) and this, coupled with distinct trimming lines, allowed us to estimate re-growth for control treatments and lesser effected plants. Also, at this time the remaining above-ground biomass for all treatments was cut down to 15 cm above the base (measurement *m*) (Fig. 3.1). A secondary response measurement (measurement *z*) was taken as re-growth accumulated after an additional 14 days of growth after first harvesting. All biomass collections (*r*, *m* and *z*) were allowed to dry at 70 °C in a forced air oven for 14

days prior to weighing. Overall responses were calculated as a biomass ratio percentage (BRP) between all dried re-growth accumulated after herbicide application and the amount of "basal" biomass prior to herbicide treatment, where percentage biomass ratio = $[^{(r+z)}/_{m}]$ x 100.

Data were treated as a 3-way factorial design with factors designated as herbicide type, surfactant and geographical source and analyzed using ANOVA. Values for visual injury were arcsine square root transformed in order to stabilize variance. To determine the effects of individual herbicides and different surfactant combinations, both visible injury and BRP data were analyzed separately for each herbicide in a two way factorial analysis. Following significant ANOVA, mean separation was completed using Tukey's studentized range test with ($P \leq 0.05$) using SAS (SAS Institute, 2001).

Results

Average BRP measurements and visual ratings (Fig. 3.2) for all geographical regions for all treatments are given in Table 3.1. Glyphosate and fluazifop-p-butyl differed significantly in their effects irrespective of type of surfactant added ($P < 0.05$). Surfactant type (methylated seed oil or combination of methylated seed oil and silicone) did not influence herbicide performance ($P > 0.05$). Separate two-way factorial analysis for glyphosate and fluazifop-p-butyl indicated that there was no significant difference in responses to treatments among Egyptian, South African and Hawaiian populations for both herbicides ($P > 0.05$).

The results for fluazifop-p-butyl for BRP measurements (Fig. 3.2a) showed that treatments containing methylated seed oil had BRP values ranging from 25.9 % to 30.1 %

and those containing a combination of methylated seed oil and silicone from 21.0 % to 22.7 %, while those for glyphosate ranged from 14.1 % to 16.5 % and 11.6 % and 16.8 % respectively. Treatments involving surfactant-only applications (controls) had an average BRP estimate of 42.1 %. Visual injury (Fig. 3.2b) for fluazifop-p-butyl ranged from 36.3 % to 71.25 % for treatments containing methylated seed oil (average: 40.0 %) and from 53.3 % to 71.7 % (average: 44.86 %) for treatments containing methylated seed oil and silicone. All treatments that involved glyphosate resulted in complete necrosis (100 % injury) of treated foliage (Fig. 3.2b).

Discussion

Tolerance or resistance against control practices can be achieved in invasive species populations that contain high levels of genetic diversity and have been documented for numerous species (e.g. Garcia-Rossi *et al.* 2003; Hufbauer and Via, 1999; Peever *et al.* 2000). Determining the amount and distribution of such genetic variation (genetic structure) in invasive populations could thus aid in predicting the chances and rapidity at which evolution for resistance to control practices may be acquired (Sakai *et al.* 2001). Extremely low genetic diversity and genetic homogeneity would predispose populations to lower fitness and ultimately extinction in the event of unfavorable, detrimental conditions, whereas high genetic variation renders the opportunity for local adaptation to overcome the consequences of such conditions.

The single global genotype of *P. setaceum* (Chapter 2, Le Roux *et al.* 2007) precludes or minimizes the ability of these invasive populations to adapt in response to the selection pressures resulting from control strategies (chemical or biological). A

confirmatory test for this hypothesis would be to assess the relative responses of all putative monoclonal populations to different herbicide treatments. Indeed, while glyphosate and fluazifop-p-butyl differed in their respective effects on monoclonal fountaingrass populations, populations from South Africa, Hawaii and Egypt did not differ significantly in their responses to treatments, thus supporting the previously observed genetic homogeneity. Fluazifop-p-butyl only had moderate effect on growth performance of all populations while glyphosate lead to complete necrosis and mortality in all treatments (Fig. 3.1). Most treatments with glyphosate yielded positive BRP estimates despite all treatments resulting in 100 % necrosis and mortality. As with many herbicides, glyphosate may allow plant survival for a few days after application before complete physiological shutdown of the plant (e.g. Madsen *et al.* 1995). In fountaingrass' case, this would have resulted in some initial re-growth (measurement *r*) and thus positive BRP values. Indeed, all secondary response measurements (measurement *z*) for glyphosate treatments were zero, i.e no re-growth. Glyphosate could thus be a potentially useful herbicide against monotypic fountaingrass populations. Chemical control, however, like biological control, may have non-target effects when desirable species occur sympatric with weedy species. In Hawaii fountaingrass is often found sympatric with the native piligrass (*Heteropogon contortus*) (Williams and Black 1993) and glyphosate would also severely impact this native species. Future research involving herbicide trials should thus focus on grass-selective herbicides that retard fountaingrass growth while simultaneously allowing favorable growth of native species such as piligrass. In Chapter 4 the effects of two grass-selective herbicides were assessed against

three different *Pennisetum* species, including fountaingrass, and the native Hawaiian piligrass, *Heteropogon contortus*.

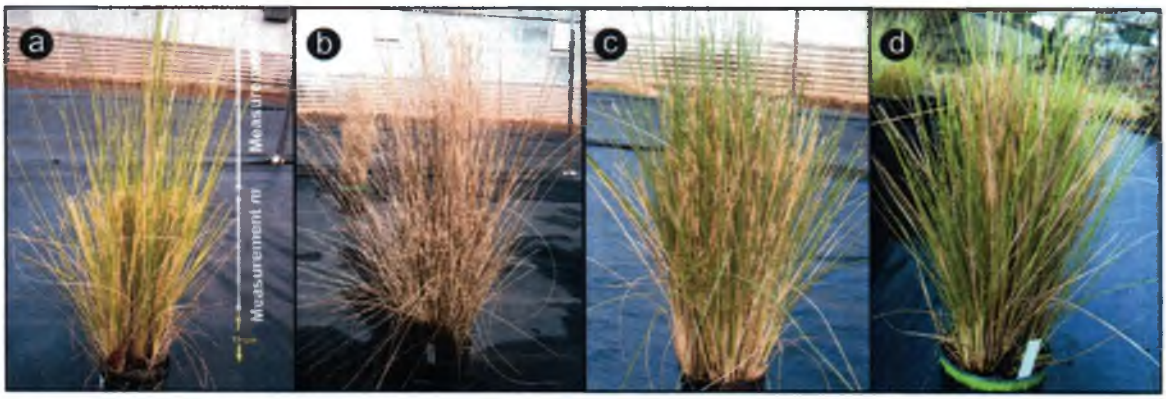
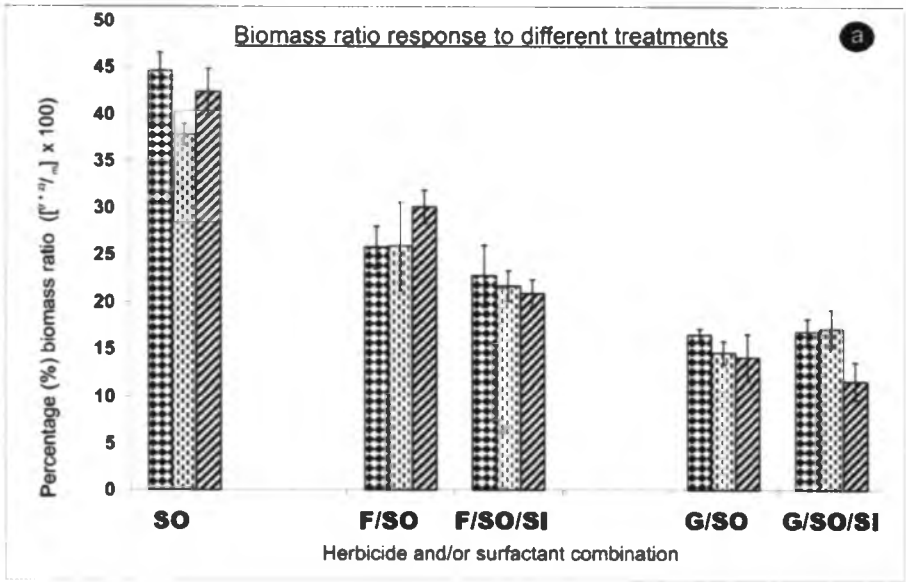
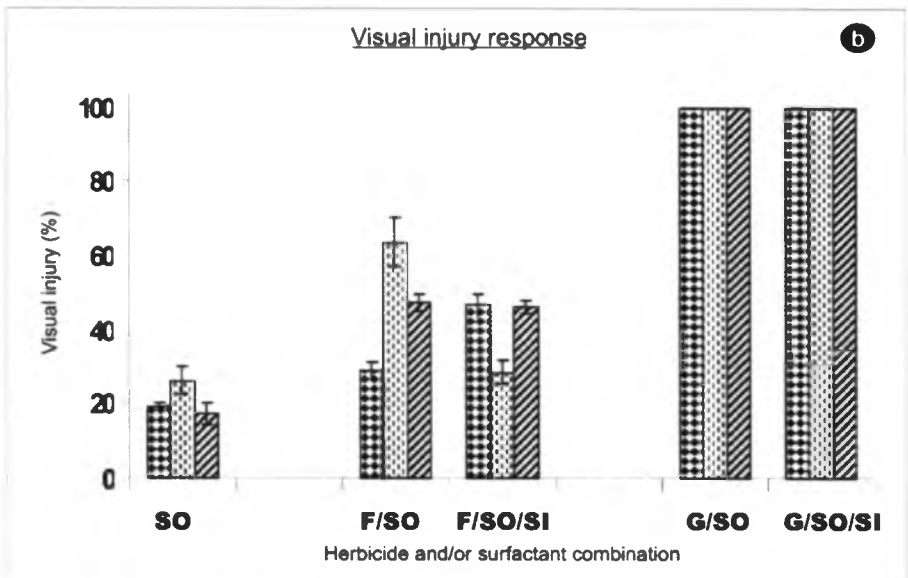


Figure 3.1. Illustration of measurements taken six (measurement *m*) and eight (measurement *z*) weeks after herbicide application to infer BRP (a), and visual injury for glyphosate (b), showing 100 % necrosis, and fluazifop-p-butyl (c), showing intermediate (40 %) necrosis, and a control treatment (no herbicide) (d).





South Africa
Egypt
Hawaii

Figure 3.2. Average biomass ratio percentage (BRP) measurements (a) and visual injury response observations (b) (± SEM) for all regional populations included in this study. SO = surfactant-only controls, F/SO = fluazifop-p-butyl with seed oil, F/SO/SI = fluazifop-p-butyl with seed oil and silicone, G/SO = glyphosate with seed oil, G/SO/SI = glyphosate with seed oil and silicone.

CHAPTER 4: EFFECTS OF TWO SELECTIVE POST-EMERGENCE HERBICIDES ON THREE ALIEN AFRICAN GRASSES THREATENING *HETEROPOGON CONTORTUS* IN THE HAWAIIAN ISLANDS.

Abstract

African C_4 grasses encompass a substantial proportion of invasive alien species that threatens ecosystem processes and biodiversity in the Hawaiian archipelago. Here, growth responses to two different selective post-emergence herbicides (fluazifop-p-butyl and imazapic ammonium salt) applied at different rates was assessed against three introduced African grass species from the genus *Pennisetum* (fountaingrass [*Pennisetum setaceum* (Forssk.) Chiov.], kikuyugrass [*Pennisetum clandestinum* Hochst. ex Chiov.], buffelgrass [*Pennisetum ciliare* L. (Link.) syn. *Cenchrus ciliaris* L.]) that threaten the native Hawaiian piligrass (*Heteropogon contortus* (L.) P. Beauv. ex Roem. & Schult.). Ramets of each species were allowed to grow under fertilized, well-watered, conditions in a greenhouse for four months before being transferred to an open air facility. Herbicides were applied in concentration gradients at 0.5X, 1X, 2X and 4X the manufacture's recommended rates with an electric powered diaphragm pump. The effects of herbicides were monitored over an eight week period as the amount of accumulated regrowth (biomass).

Selectivity was obtained for imazapic ammonium salt at various application rates with severe growth retardation and suppression of kikuyugrass and buffelgrass. Imazapic ammonium salt, applied at 0.2 kg/ha, proved the most promising, showing no visible damage or reduced performance of piligrass. Fountaingrass was relatively unaffected by

both herbicides, not responding to any of the treatment rates applied, even at 4X the recommended rates (15.8 % and 34.3 % reduction in biomass accumulation for fluazifop-p-butyl and imazapic ammonium salt respectively). Imazapic ammonium salt herbicides could be a potential useful tool in projects aimed at the restoration of piligrass populations in areas invaded by buffelgrass and kikuyugrass in Hawaii.

Introduction

Non-native species introduced to new environments may establish, spread and become invasive, in many instances causing substantial environmental and economic damage (Mack *et al.* 2000, Pimental 2000, Sakai *et al.* 2001). Like most insular habitats, native ecosystems of the Hawaiian archipelago have proven to be notoriously susceptible to the devastating effects of alien species invasions (Lonsdale 1999). However, prioritizing the control of weeds in Hawaii remains problematic with the estimated number of plant species introduced to the archipelago now nearing 5000 (St. John 1973). Of these, C_4 African grasses contribute significantly to the threats posed to Hawaii's biodiversity and environments with, at one time, almost 50% of the islands' land converted to grasslands (Gange and Cuddihy 1990). Most of these African grasses were introduced to Hawaii as pasture grasses for grazing purposes and have extended their geographical ranges over the last century to the extent where they are now present in almost all dry and mesic environments (Elmore *et al.* 2005). These grasses are often fire-promoting and pose a unique challenge to the native Hawaiian flora and fauna to which fire was historically not a major evolutionary force. In most instances these grasses catalyze positive feedback loops in which the fire regime increases in intensity and

frequency, leading to further reduction of poorly adapted native vegetation and a corresponding increase in fire-adapted biomass (Cabin *et al.* 2002). Such major alterations to disturbance regimes could, in the worse case scenario, lead to irreversible whole-ecosystem modifications (D'Antonio and Vitousek 1992).

Heteropogon contortus (L.) P. Beauv. ex Roem. & Schult. or piligrass is one of the many native Hawaiian species that are directly threatened and being displaced by African grasses and as a result has rapidly declined, especially over the past 35 years (Daehler and Carino 1998). Piligrass is a valuable forage grass for cattle and was also valued by ancient Hawaiians as preferred high-quality thatching material (Degener and Degener 1968). Historical records attest of ancient Hawaiians intentionally modifying the environment to promote the growth of piligrass (Cuddihy and Stone 1990). Although piligrasslands were once common in the seasonally drier leeward or rain shadow habitats of the Hawaiian archipelago, these grasslands have steadily been replaced by African grasses. For example, 30% of some native piligrasslands on the island of Oahu was replaced by these African grasses within 30 years (Daehler and Carino 1998). The urgent need to find effective methods to control these invasive grasses and relieve remaining native piligrass populations from being out-competed and extirpated is thus evident.

Pennisetum setaceum (Forssk.) Chiov. (fountaingrass) was first observed in Hawaii in 1914 and is a highly aggressive, fire-adapted colonizer that readily out-competes native plants leading to the formation of nearly-monotypic stands. This species is also capable of prolific regeneration following burning (Tunison 1992). Furthermore, in contrast to native piligrass, fountaingrass is unpalatable and is avoided by grazers. Fountaingrass continues to colonize and spread across an impressive diversity of habitats

(Wagner *et al.* 1999) ranging from coastal, sub-alpine to alpine habitats (Williams and Black 1993). It also colonizes bare lava flows (Tunison 1992), and in many instances displaces and dominates former piligrasslands (Williams and Black 1993).

The congeneric species, *Pennisetum clandestinum* Hochst. ex Chiov. (kikuyugrass), was introduced to Hawaii as a pasture grass. Currently weedy kikuyugrass populations can be found on all major Hawaiian Islands from 500-2,000 meters above sea level in dry and mesic habitats. Kikuyugrass is one of the most destructive invasive species threatening Hawaii's native vegetation. Its smothering, thick dense growth can prevent new seedling establishment (Wagner *et al.* 1999), including, to some extent, that of *Heteropogon contortus*. Piligrass rarely occurs above 500 meters elevation, thus restricting sympatric growth with kikuyugrass to relatively small ranges in Hawaii (C. Daehler, University of Hawaii, personal communication). Yet another grass species intentionally introduced for pastures to the islands, *Pennisetum ciliare* L. (Link.) syn. *Cenchrus ciliaris* L. (buffelgrass), has spread quickly to dominate major areas of the leeward Hawaiian Islands, often replacing native piligrass populations by forming nearly-monotypic stands (Daehler and Carino 1998).

In this study, the responses of piligrass and these three African C_4 grasses to two different selective post-emergence grass herbicides was evaluated. The aim was to determine under which application rates and what herbicide treatments selectivity can be obtained so that minimum damage is caused to piligrass while simultaneously providing significant growth retardation and suppression of the introduced *Pennisetum* species.

Materials and Methods

Growth conditions

In September 2005, four to eight mature plants from each of the study species were separated into 50-60 ramets, each comprising 5-8 tillers. Ramets were transplanted in 1:1 vermiculate and potting soil (Miracle-Grow ®) mixture into 800 mL plastic pots and slow-releasing fertilizer (total nitrogen, 9.0 %; available phosphate, 6.0 %; soluble potassium, 6.0 %; total sulphur, 18.7 %; iron, 2.0 %) applied at 24.2 $g.m^{-2}$. Pots were randomly repositioned twice monthly in the greenhouse and kept under well-watered conditions. Plants were kept in the greenhouse until February 2006 after which they were transferred to an open air facility. Following three weeks of exposure to outside conditions, plants were cut to uniform habits (~ 45 cm above ground), fertilized with turf fertilizer (total nitrogen, 22 %; available phosphate, 2 %; soluble potassium, 9 %; total sulphur, 12.1 %; iron, 5 %; chlorine, 2 %) at 9.8 $g.m^{-2}$ and allowed to grow for an additional 2 weeks prior to herbicide treatments.

Herbicide treatments and response measurements

Two herbicides, fluazifop-p-butyl (Fusilade DX®, Syngenta) and imazapic ammonium salt (Plateau®, BASF) were chosen due to their grass-selective properties. During March 2006, each herbicide was applied in a concentration gradient at 0.5X, 1X, 2X and 4X the manufacture's recommended rates, where X equals the recommended rate. Recommended rates (X) of 0.84 kg/ha and 0.2 kg/ha was used for fluazifop-p-butyl and imazapic ammonium salt respectively. Each application mixture contained 0.25% (v/v) of LI700 surfactant (Loveland Industries). Applications were done with an electric powered

diaphragm pump calibrated to deliver 374 liters per hectare. Each treatment was replicated four times.

Quantitative responses to different herbicide treatments were measured by collecting all visible re-growth accumulated 6 weeks after herbicide application (measurement *r*). At this time the remaining above-ground biomass for all treatments were cut down to 15 cm above the base (measurement *m*). A secondary response measurement (measurement *z*) was taken as re-growth accumulated after an additional 14 days of growth after first harvesting. All biomass collections (*r*, *m* and *z*) were allowed to dry at 70 °C in a forced air oven for 14 days prior to weighing. Since the general habit varied substantially among different species, overall responses were calculated as a ratio percentage between all dried re-growth accumulated after herbicide application and the amount of "basal" biomass prior to herbicide treatment, where percentage biomass ratio (BRP) = $[^{(r+z)}/_{m}]$ x 100.

Data were treated as a 3-way factorial design with factors designated as herbicide type, herbicide concentration and grass species and analyzed using ANOVA. Mean separation was completed using Tukey's studentized range test with ($P \leq 0.05$) using SAS (SAS Institute, 2001), following significant ANOVA. Response curves were determined by linear regression (PROC GLM) for all BRP measurements against recommended rates for each herbicide. For control treatments BRP values associated with untreated controls were used.

Results

Average ratio percentage response measurements (Fig. 4.1) for all four grass species for all treatments are given in Table 4.1. Overall, the data showed a significant interaction between grass species and herbicide concentration ($F = 2.03$, df = 9,107, $P < 0.05$ and $F = 2.22$, df = 9,107, $P < 0.05$, respectively) but not for all three factors combined (grass species, herbicide and herbicide application rate) ($F = 1.10$, df = 12,107, $P > 0.05$). Mean separations indicated that, overall, only kikuyugrass and buffelgrass responded similarly to all treatments ($P > 0.05$) and that all other species differed in their responses to all treatments ($P < 0.05$). Application rates at 1X, 2X and 4X of the recommended labeled rates did not differ significantly from each other in their overall, pooled effects for both herbicides ($P > 0.05$).

Imazapic ammonium applied at 0.1 kg/ha or 0.5X the recommended rate did not affect BRP for any of the species when compared to untreated controls ($P > 0.05$). Application at 0.2 kg/ha resulted in a significant effect on BRP for only kikuyu and buffelgrass when compared to untreated controls ($P < 0.05$). Higher application rates ($\geq$ 2X the recommended rate) had the same effect when compared to 1X the recommended application rate. Fountaingrass appeared to be unresponsive to any of the application rates used ($P > 0.05$).

The results for fluazifop-p-butyl (Fig. 4.1a) showed that kikuyu and fountaingrass were unaffected by any of the treatments applied. Piligrass and buffelgrass responded to all treatments but with no significant response differences between different application rates ($P > 0.05$).

For imazapic ammonium salt, piligrass showed no growth retardation at 0.1 kg/ha or 0.5X the recommended rate (BRP, 228.4 %) and slight growth retardation at 0.8 kg/ha or 4X the recommended rate (BRP, 94.8 %), the latter corresponding to a relative BRP decrease of 50.3 % when compared to untreated controls.

Once again, fountaingrass seemed overall relatively unaffected by all treatments. The relative decrease in BRP, when compared to untreated controls, was only 7.3 % at 0.5X the application rate and 34.3 % at 4X the recommended rate. Selectivity against buffelgrass and kikuyugrass was obtained at the recommended rate application of 0.2 kg/ha with no impact on piligrass growth. At this application rate, buffelgrass and kikuyugrass showed relative biomass accumulation (BRP) decreases of 90.4 and 72.5 % respectively. Mortality (no visible secondary re-growth [measurement *z*]) at this rate was found in 25 and 50 % of treatments involving buffelgrass and kikuyugrass respectively.

Discussion

Introduced African grasses are aggressively invading extensive dryland habitats on the leeward sides of the Hawaiian Islands and pose a unique threat to native biota if left unabated. These exotic species alter plant community composition, interfere with natural succession, and change natural fire regime by carrying fires more frequently and intensely than they would otherwise occur. Following fire, grasses generally out-compete natives and increase in cover and fuel loading (D'Antonio *et al.* 2000).

The native lowland Hawaiian piligrass, *Heteropogon contortus* (L.) P. Beauv. ex Roem. & Schult., is currently one of many native species being threatened by such grass invasions. Despite being a fire-stimulated species it has been displaced and out-competed

by various C_4 African grasses (Daehler and Carino 1998) and could be a result of its inferior competitive ability and/or its preference and selective grazing by ungulates over alien grasses (Goergen and Daehler 2001).

The large distribution ranges of some introduced species in Hawaii make biological control in many instances the only sustainable method for control over the long run. However, the African grasses in this study have been purposefully introduced as pasture grasses and/or have closely related species that are of high agricultural value, making biological control problematic. It is thus evident that chemical control is the most cost- and time-effective method against these species in non-agricultural areas. Chemical control, however, like biological control, may have non-target effects when desirable species occur sympatric with weedy species. In this study, both herbicides tested (fluazifop-p-butyl and imazapic ammonium salt) proved promising candidates as chemical control agents against some of the species threatening piligrass-dominated landscapes in Hawaii. Satisfactory growth retardation of both kikuyugrass and buffelgrass was obtained using both these herbicides following a single application. Imazapic ammonium salt caused no visible signs of growth stunting on piligrass at the recommended rate of 0.2 kg/ha. Under the same conditions substantial growth suppression and in most cases, complete necrosis, was evident for buffelgrass and kikuyugrass. Fluazifop-p-butyl on the other hand, did show some growth stunting at application rates above the recommended rates for piligrass. Surprisingly, fountaingrass showed very little to no growth suppression to either of the herbicides used in this study even when applied at 4X the recommended rates. From our preliminary findings imazapic ammonium salt is a potentially good selective herbicide against buffelgrass and

kikuyugrass and should warrant further testing and investigation. Even at high application rates this herbicide showed little to moderate growth suppression in piligrass but severe growth retardation for invasive buffelgrass and kikuyugrass. Many threatened piligrass populations are dominated by buffelgrass (Daehler and Goergen 2005), clearly illustrating the value of selective herbicides such as imazapic ammonium salt. Indeed, Daehler and Goergen (2005) showed that pili-dominated grasslands can be restored through one-time removal of buffelgrass followed by the addition of piligrass seeds. Imazapic ammonium salt applied at the recommended rate resulted in mortality in 25 % of buffelgrass and 50 % of kikuyugrass treatments that increased to 75 % and 100 % at 4X the recommended rate. The results from this preliminary herbicide trial indicate that imazapic ammonium salt to be potentially effective against both these species. Future trials should assess the effect of multiple applications at the recommended rate on mortality of these unwanted species and the performance of piligrass. With many of the current pili populations being small and fragmented, selective herbicide treatment using imazapic ammonium salt could potentially be a feasible tool to use in restoration efforts.

Table 4.1. Average biomass ratio percentage (BRP) responses to fluazifop-p-butyl and imazapic ammonium salt for all species included in this study.

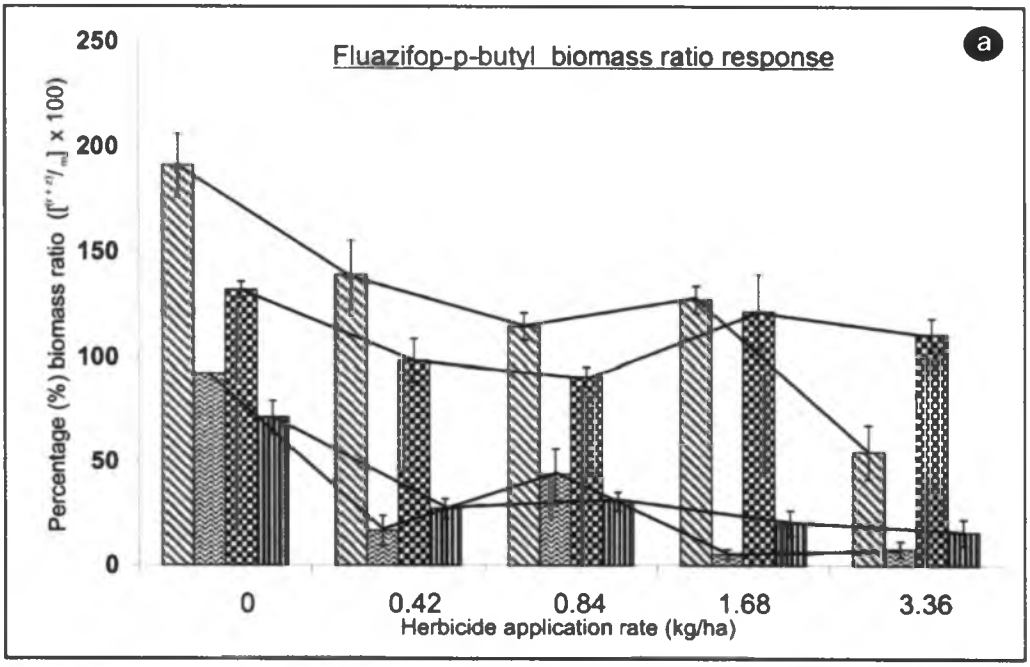
Species	Control Treatments Untreated	Herbicide application rate (X recommended rate) 0.5X	1X	2X	4X
Fluazifop-p-butyl					
H. contortus (piligrass)	223.3a	138.9b	114.6b	127.2b	54.7b
P. setaceum (fountaingrass)	106.8a	98.5a	90.7a	121.7a	111.0a
P. clandestinum (kikuyugrass)	54.55a	27.3 a	30.7a	20.3a	15.9a
P. ciliare (buffelgrass)	80.9a	16.7b	43.9b	5.4b	7.8b
Imazapic ammonium salt					
H. contortus (piligrass)	223.3a	228.4a	178.1ab	134.2ab	94.8b
P. setaceum (fountaingrass)	106.8a	122.2a	105.2a	91.2a	86.6a
P. clandestinum (kikuyugrass)	54.55a	32.9ab	19.7b	38.1ab	7.6b
P. ciliare (buffelgrass)	80.9a	61.2a	8.9b	19.0b	2.7b

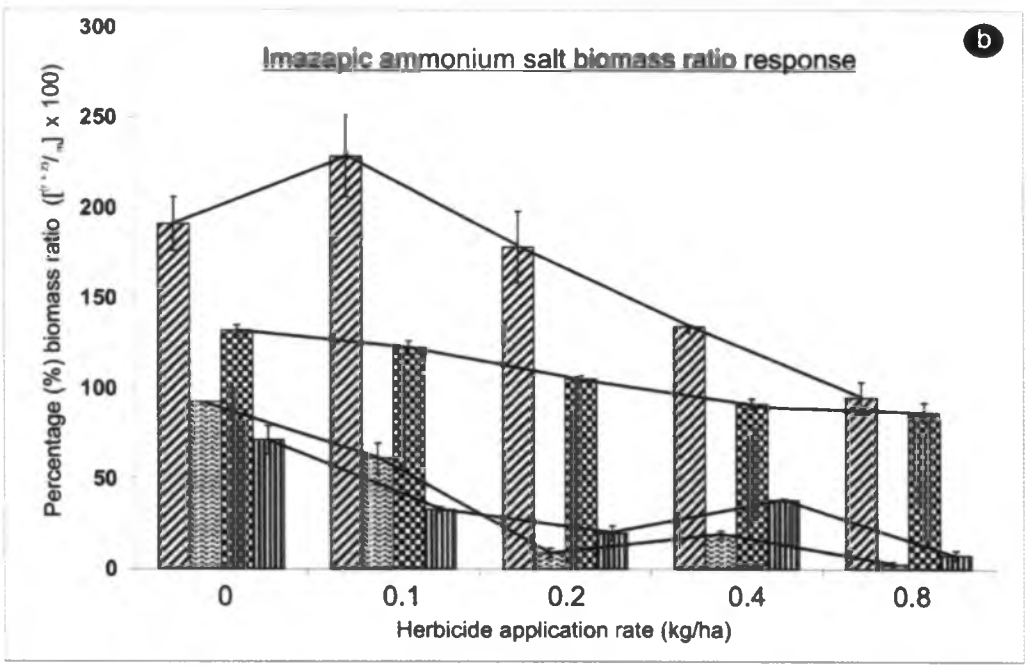
Means in rows followed by the same letter are not significantly different ($P > 0.05$, Tukey pairwise comparisons)

Table 4.2. Response curves for different grass species to different herbicides used in this study.

Grass species	Response curve	R^2-value	*P*
Fluazifop-p-butyl			
H. contortus (piligrass)	y = -28.425x + 210.56	0.84	0.002
P. setaceum (fountaingrass)*	y = -1.8434x + 116.25	0.03	0.96
P. clandestinum (kikuyugrass)	y = -11.795x + 68.494	0.71	0.028
P. ciliare (buffelgrass)*	y = -18.056x + 87.385	0.61	0.06
Imazapic ammonium salt			
H. contortus (piligrass)	y = -28.653x + 251.26	0.76	0.005
P. setaceum (fountaingrass)	y = -12.129x + 143.82	0.97	0.0003
P. clandestinum (kikuyugrass)	y = -12.223x + 70.612	0.65	0.004
P. ciliare (buffelgrass)	y = -22.165x + 103.33	0.83	0.003

* No statistically significant association between biomass ratio percentage and herbicide concentration.





piligrass (*Heteropogon contortus*)
buffelgrass (*Pennisetum ciliare*)
fountaingrass (*Pennisetum setaceum*)
kikuyugrass (*Pennisetum clandestinum*)

Figure 4.1. Average percentage biomass ratio (± SEM) measurements for all species included in this study for (a) fluazifop-p-butyl and (b) imazapic ammonium salt treatments at different concentrations.

SECTION 3 – *SENECIO MADAGASCARIENSIS* POIR.

(FIREWEED)

CHAPTER 5: RESOLVING THE NATIVE PROVENANCE OF INVASIVE FIREWEED (*SENECIO MADAGASCARIENSIS* POIR.) IN THE HAWAIIAN ISLANDS AS INFERRED FROM PHYLOGENETIC ANALYSIS.

Abstract

Accurate identification of weedy species is critical to the success of biological control programs seeking host-specific control agents. Phylogenetic relationships based upon internal transcribed spacer region (ITS1, ITS2) DNA sequence data were used to elucidate the most likely origin and taxonomic placement of *Senecio madagascariensis* Poir. (fireweed; Asteraceae) in the Hawaiian archipelago. Putative *S. madagascariensis* populations from Madagascar, South Africa, Swaziland and Hawaii were included in the analysis. Different phylogenetic models (Maximum parsimony and Maximum likelihood) were congruent in suggesting that Hawaiian fireweed is most closely related to populations from the KwaZulu Natal region in South Africa. Phylogenetic divergence and morphological data (achene characteristics) suggest that the *S. madagascariensis* complex is in need of revised alpha-level taxonomy. Taxonomic identity of invasive fireweed in Hawaii is important for finding effective biological control agents as native range populations constitute different biotypic variants across a wide geographical area. Based on these phylogenetic results research directed at biological control of Hawaiian infestations should focus on areas in the KwaZulu Natal region in South Africa where host-specific natural enemies are most likely to be found. This study's results show that phylogeographical analysis is a potential powerful and efficient tool to address questions relevant to invasion biology of plants.

Introduction

Senecio madagascariensis Poir. (fireweed) was accidentally introduced to Hawaii in the early 1980's and became naturalized over a wide range of different habitats within 20 years. On the island of Hawaii, it has infested pastureland in the north eastern and western sides of the island and from there expanded its range towards the southern areas (Motooka *et al.* 1999). On Maui, fireweed infests roadsides and pastures on eastern parts of the island with naturalized populations found from sea level up to 1600 meters above sea level. *Senecio madagascariensis* competes strongly with existing pasture flora for light, moisture and soil nutrients (notably phosphorus and nitrogen), leading to the ultimate deterioration of pastures (Watson *et al.* 1994). In addition, like many other *Senecio* species, fireweed produces pyrrolizidine alkaloids that reduce growth and in severe cases cause mortality when ingested by livestock (McBarron 1976). In Australia annual losses amounting up to US$ 2 million directly linked to fireweed infestations have been reported (Motooka *et al.* 1999).

Prioritising the control of weeds in Hawaii remains problematic with the estimated number of introduced plant species to the archipelago now nearing 5000 (St. John 1973). Coupled with the large distribution ranges of some of these species, biological control is in many instances the only sustainable method for control over the long run. *Senecio madagascariensis*' extensive spread and the lack of closely related relatives in the Hawaiian Islands would make biological control a feasible option. Even though several herbicides proved successful against fireweed (Motooka *et al.* 1999), the extent of Hawaiian infestations would make such control uneconomical. A biological control program aimed at *S. madagascariensis*, like any other biological pest control

initiative, would require sound systematics. It would be of utmost importance to identify the most likely native origin of invasive Hawaiian fireweed, the possible existence of cryptic species and/or intraspecific variants if the introduction of natural enemies is being considered.

The generalization that invasive species often have large native ranges is well documented (Lodge 1993). This phenomenon implies that it is not always easy to determine the native origin of such species. *Senecio madagascariensis*, an Afro-Madagascan native, is widely distributed from southern Madagascar and the Mascarene islands through coastal southern Natal and Transkei and inland in the Swaziland and Cape regions of South Africa. This geographical range represents an equally impressive array of climatological habitats, e.g. in these areas precipitation varies from approximately 500 to 3000 mm annually and mean annual temperatures from 18.0 °C to 27.0 °C. Considerable morphological variation has also been reported for specimens identified as *S. madagascariensis* between Madagascar and regions in southern Africa (Radford *et al.* 2000) and coupled with the large native range distribution, implies that determining the native origin of Hawaiian *S. madagascariensis* would be problematic. The native origin of Hawaiian *S. madagascariensis* remains elusive though it supposedly reached the islands from Australia, where it is also considered a serious invasive pasture weed, through the importation of contaminated fodder.

The taxonomic identity of fireweed in Australia has been the subject of considerable attention after it became apparent that it was not part of the native *Senecio lautus* complex as was traditionally thought (Sindel, 1986). Numerous studies attempting to differentiate Australian fireweed from the *S. lautus* complex suggest that fireweed in

Australia is more closely related to South African *S. madagascariensis* and *S. inaequidens* than to *S. madagascariensis* from Madagascar (Radford *et al.* 1995; Radford 1997; Scott *et al.* 1998a). *Senecio inaequidens* is one of several closely related and widespread species in southern Africa (others include *S. skirrhodon, S. burchellii* and *S. pellucidus*) sharing overlapping morphological characteristics and frequently mistaken for *S. madagascariensis* (Hilliard 1977). Genetic analysis by Scott *et al.* (1998a) did not differentiate between fireweed in Australia, *S. madagascariensis* and *S. inaequidens* from South Africa leading them to conclude that these encompass a single species that differed from putative *S. madagascariensis* specimens from Madagascar. A more recent study by Radford *et al.* (2000) differentiated *S. inaequidens* and *S. madagascariensis* on the basis of achene morphology and chromosome counts. Genetic distances as inferred from isozyme polymorphisms and morphological data suggested that a considerable amount of variation exits within the current *S. madagascariensis* species complex and its close relatives, and concluded that the taxonomy of the complex needs to be revised (Radford *et al.* 2000).

Fireweed populations in Hawaii show a high degree of morphological variation in plant size and leaf shape (pers. observation). Phenotypic plasticity could explain these variations, but with the known variation that exists within the fireweed species complex, Hawaiian infestations could potentially represent more than one *S. madagascariensis* variant or even different *Senecio* species. Determining the taxonomic position of fireweed in Hawaii is important as the Hawaii Department of Agriculture is currently assessing the possibility of biological control for fireweed in Hawaii. Correct taxonomic placement and phylogeographical analysis of Hawaiian *S. madagascariensis* would

contribute significantly to identify area/s where foreign exploration for parasites and/or pathogens is likely to be most productive. Biological control agents against fireweed have been tested in Australia but proved to be unacceptable there, due to their low host-specificity, potential non-target effects on the closely related *S. lautus* complex and the temporal and spatial coincidence of acceptable host plants (Radford, 1997). Many of these problems can be circumvented when seeking possible biological control agents for Hawaiian infestations since no closely related *Senecio* species are native to the archipelago, and thus non-target impacts are unlikely.

To this end, a phylogenetic study was undertaken in an attempt to elucidate the taxonomic placement and geographical origin of invasive fireweed in Hawaii. The Internal Transcribed Spacer Regions (ITS1 and ITS2) DNA sequences were used to construct a phylogeny to infer relationships between putative *S. madagascariensis* populations from Hawaii, South Africa, Swaziland and Madagascar. ITS demonstrates a unique pattern of evolution, featuring interspersion of relatively rapidly evolving sequences with some of the most highly conserved sequences known (Hillis *et al.* 1996) and has previously been used to infer relationships within other *Senecio* species complexes (Bain and Jansen, 1995; Scott *et al.* 1998a). In addition, morphological features of achenes were used as supplemental data to infer taxonomic relationships as these characteristics were previously reported as useful characters for inter species and intraspecific discrimination for the *S. madagascariensis* complex and closely related species (Radford *et al.* 2000).

Materials and Methods

Population sampling

Leaf and seed (achene) material were collected during March 2005 in the known geographical ranges of *S. madagascariensis* in South Africa, Swaziland and Madagascar. Field collections were made during a survey and collection of potential natural enemies for biological control by M. Ramadan (Hawaii Department of Agriculture). Flower material was placed in containers containing 95% ethanol in the field and subsequently stored at – 80 °C. Leaf material was placed and dehydrated in plastic containers containing Drierite™. Locality name, region, latitude, longitude, and elevation were recorded for all populations sampled (Table 5.1). Specimens were classified according to Hilliard (1977) and current herbarium practices in South Africa.

Morphological characteristics

Achenes of putative *S. madagascariensis* populations were characterized by recording the surface hairiness and lengths of at least ten achenes per population. In addition, the number of involucral bracts was identified and leaf morphology recorded for each population. Achene lengths were measured for each population (n = 10) with an electronic micrometer and a phase contrast microscope.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted according to the manufacturer's protocol with the Wizard® genomic DNA purification kit (Promega, Madison, USA) from 40 to 50 mg dried leaf material that was frozen in liquid nitrogen, ground by hand, and stored at – 80

°C. In total, three nuclear regions (ITS1, 5.8S, and ITS2) were sequenced. These genes were PCR amplified and sequenced using primers described elsewhere (White *et al.* 1990). A thermocycle of 35 cycles: denaturation for 1 min at 94 °C, annealing for 1 min at 50 °C, and extension for 1 min at 72 °C was used for PCR amplification. Purified PCR products were sequenced in both directions and were run on an ABI 377 automated sequencer (Applied Biosystems, Foster City, USA.) using standard dye-terminator chemistry following the manufacturer's protocol.

DNA sequence alignment and phylogenetic analysis

DNA sequences were aligned using the Clustal X program (Thompson *et al.* 1997) followed by manual editing of the alignment. In addition to DNA sequences generated in this study DNA sequences for other relevant *Senecio* species were obtained from GenBank; *S. inaequidens* (accession numbers AF459943 and AF097537), *S. lautus* (accession numbers AY554113 and AF097540). Species from the more distantly related sister group (Senecioneae, section *Rowleyani*), *Senecio serpens* and *S. rowleyanus* (accession numbers AF459930 and AF459933 respectively), were chosen as outgroup taxa.

Phylogenetic analyses were conducted on the ITS1 and ITS2 regions (excluding the non-variable 5.8S rDNA region) using PAUP* 4.0b10 (Swofford, 2001). Maximum-parsimony (MP) analysis was performed with the option "Heuristic search" with TBR, MULTREES and COLLAPSE (max) options in effect. Deletion and insertion events (gaps [indels]) were treated as missing data. Confidence in tree topologies was assessed using bootstrap analysis (Felsenstein, 1985) of 1000 replicates. The partition

homogeneity test, as implemented in PAUP*, was used to test for incongruence between the phylogenetic signals of the separate ITS1 and ITS2 datasets. In addition to MP a maximum likelihood (ML) analysis was performed using parameter estimates for ML obtained by a hierarchical likelihood ratio testing approach using the program MODELTEST version 3.06 (Posada and Crandall, 1998). Heuristic searches were carried out with TBR, MULTREES, and COLLAPSE options in effect. Confidence in tree topology was assessed using bootstrap analysis of 100 replicates with the same heuristic settings used for tree construction.

Results

Morphology

Achene surface morphology appeared to be of three distinct types (Fig. 5.1). Two of these types showed definite grooves on the surfaces of achenes differing in the pattern of papillose (mixogenic) hair coverage. One of these types was sparsely covered with hairs on separate grooves with only a few hairs on each groove (Fig. 5.1 A) while the second type had intermediate hair coverage on all grooves (Fig. 5.1 B). A third type showed complete hair coverage and the apparent absence of grooves or very slightly grooved (Fig 5.1 C). The papillose hairs of this type also appeared to be longer and thinner. For these three achene types the average lengths and associated standard deviations (sd) were: Sparsely covered (1.35 mm, sd 0.6); intermediately covered (1.66 mm, sd 0.19); totally covered (1.75 mm, sd 0.37), representing significantly different length classes ($P = 0.001498$, One-way ANOVA).

Leaf morphology varied between and in some instances within populations from slender oblanceolate, broad oblanceolate, to pinnately lobed (Fig. 5.2). The number of involucral bracts ranged consistently from 19 to 22 for all the populations collected. Table 5.2 summarizes morphological data for all populations included in this study.

Sequence variation

The amplified ITS1 and ITS2 regions were between 189 -236 base pairs (bp) and 229 -230 bp, respectively. All DNA sequences were submitted to Genbank (accession numbers DQ322598 – DQ322621).

The alignment matrices for ITS1 and ITS2 gene sequences constructed using data generated in this study and additional sequence data obtained from GenBank (http://www.ncbi.nlm.nih.gov) required 53 and 7 gaps, respectively, ranging from 1 to 44 bp in size. Individual gaps for all ingroup taxa never exceeded 3 bp. The combined datasets comprised of 474 characters, which included 384 (81.0%) constant characters. Sequence divergence (measured as uncorrected *P* distance) ranged from 0.0% (e.g. putative *S. madagascariensis* individuals SM182 - SM232) to 5.8% (*S. madagascariensis* - *S. lautus*) between ingroup taxa and from 11.59 % (*S. rowleyanus* – SM255) to 13.18% (*S. serpens* - *S. inaequidens*) between ingroup and outgroup taxa.

Phylogenetic analysis of ITS sequences

Partition homogeneity analyses showed that the phylogenetic signal for the two separate internal transcribed spacer regions were congruent ($P = 1.0$). Parsimony analysis of the combined ITS1 and ITS2 regions yielded 572 most parsimonious trees

with a length of 101 steps, a consistency index (CI) of 0.9307, a retention index (RI) of 0.9598, and a rescaled consistency index (RC) of 0.8933 (Fig. 5.3). For ML the TrNef + G substitution model was selected. This uses a general time-reversible model and gamma-distributed (G) among-site rate variation. The ML tree (-ln likelihood = 1209.30) was similar in topology to the MP tree. Overall, both trees were well supported by bootstrap. Both ML and MP analysis placed *S. inaequidens* and two of the putative *S. madagascariensis* populations (Blythedale and Park Ryne Beach populations [100% and 99% bootstrap support respectively]) as a sister group to the otherwise monophyletic *S. madagascariensis*-only clade (Fig. 5.3). Both ML and MP analysis indicated that a strong geographical relationship exists among putative *S. madagascariensis* populations. Populations from Swaziland (86% and 90% bootstrap support respectively) and Madagascar (99% and 100% bootstrap support respectively) formed individual monophyletic clades. With the exception of the Blythedale and Park Ryne Beach populations all the South African east coast populations were unresolved sharing 100% DNA similarity within the monophyletic *S. madagascariensis*-only clade. Both ML and MP analyses placed *S. lautus* as basal to *S. madagascariensis* and *S. inaequidens*. The phylogram generated by ML indicated that the closest phylogenetic relationship between Hawaiian *S. madagascariensis* populations was shared with the South African east coast populations (Durban city, Umhlanga and Camperdown [1bp substitution, 0.21 % divergence]) (Fig. 5.4). More distant relationships were between Swaziland (0.86% divergence) and Madagascar (1.29 % divergence). Within the Madagascan-only clade sequence divergence varied between 0.22 % (between Tulear and Fort Dauphin Airport populations) and 0.65 % (between Tulear and Saint Luce populations). The two South

African populations (Park Ryne Beach and Blythedale Beach) within the monophyletic *S. inaequidens* clade formed a sister group to the monophyletic *S. madagascariensis* clade (1.50 % sequence divergence).

Discussion

Successful invasive species often have large native ranges (Lodge, 1993), complicating efforts aimed at determining their origin. The identification of the region/s of origin, cryptic species and/or intraspecific variants is especially important when seeking host-specific natural enemies for biological control purposes. Where morphological or physiological traits fail to reveal taxonomic relationships, phylogenetic studies of plant and/or herbivore taxa can give clues to the evolution of host-choice in control agents and/or the most likely region of origin.

Senecio madagascariensis is currently considered a serious pasture weed in Hawaii. Its supposed introduction from Australia via contaminated fodder remains to be verified. *Senecio madagascariensis*' native range spans Madagascar, its surrounding islands and a great part of southern Africa. Not surprising, as mentioned, this species shows a vast amount of biotypic variation such as plant habit and leaf morphology and is considered a species complex (Radford *et al.* 2000). The occurrence of several closely related, very similar, and widespread *Senecio* species further complicates this group's taxonomy. For example, *S. inaequidens* is frequently mistaken for *S. madagascariensis* in the field. In addition Scott *et al.* (1998a) have showed that no genetic differences existed between *S. inaequidens* and *S. madagascariensis* based on ITS 1 DNA sequence data. Indeed, closely related species such as *S. burchellii*, *S. skirrhodon*, *S. pellucidus* are

frequently considered to be part of the *S. madagascariensis* complex when observed in the field (P.A. Muller, personal communication).

Understanding the identity and number of species involved in biological invasions and their native origin(s) are attributes that will contribute valuable insights in predicting potential range expansions, impacts, invasiveness etc. as well as suggesting prevention, control and management strategies (Courtenay and Stauffer 1984). Such information is especially vital to the success of biological control programs. Failure to correctly identify invasive species and/or their native origin could lead to unsuccessful natural enemy establishment, poor host-specificity and/or incomplete control, especially when dealing with biotypes of a single weed species (e.g. Chaboudez 1994), weed species complexes (e.g. *S. madagascariensis* [Scott *et al.* 1998a]) or natural enemy host-races (e.g. skeleton weed rust, *Puccinia chondrillina* [Espiau *et al.* 1998]). A survey of successful biological programs showed that most effective natural enemies are usually host-specific (Rosen, 1986). Therefore, understanding the phylogenetic relationships of target species and their close relatives will greatly contribute to improving biological control, particularly given the current concern for non-target impacts of biological control agents (Louda *et al.* 1997).

In an ongoing biological control program targeting fireweed in Hawaii it is necessary to elucidate the taxonomic placement of Hawaiian infestations. Given the known variation within the *S. madagascariensis* complex and the amount of morphological variation observed in Hawaiian populations, it might seem reasonable to suspect that more than one species/biotype are responsible for these infestations. The phylogenetic results, however, showed otherwise, indicating that all Hawaiian

populations included in this study share 100 % DNA sequence similarity regardless of morphological and ecological differences. Phylogenetic relationships furthermore suggest that the most likely origin of Hawaii's fireweed populations is the KwaZulu-Natal region on the east coast of South Africa, excluding populations sampled in the Blythedale and Park Ryne beach areas. The latter two populations formed a monophyletic group with *S. inaequidens*. Even though achene and flower morphology overlapped among and within different native range regions, Hawaiian fireweed furthermore also had morphological characteristics that were observed within the South African east coast populations. Achene hairiness and leaf and flower morphology appear to be uninformative for taxonomical inferences within the *S. madagascariensis* complex and there seem to be no distinct regional or species-specific patterns evident for these traits. Hawaiian *S. madagascariensis* was constantly invariable for involucral bract number (21) and achene hairiness (intermediate) across all populations whilst the South African east coast populations showed variation in these traits. In addition to the specimens included in this study, the invariability in involucral bract number for Hawaiian fireweed was consistently observed for numerous individuals in the field ($n >$ 100). These differences could be indicative of a single introduction event and/or a founder effect coupled with reduction in genetic diversity in these populations.

Precise elucidation of the invasion route(s) of *S. madagascariensis* to Hawaii cannot be determined with 100 % certainty from this study's results, however, phylogenetic results support the notion that fireweed might have reached Hawaii secondarily from Australia rather than directly from any of the native range regions. DNA sequence data for fireweed from Australia were not included here as only partial

ITS sequences were available for Australian accessions (Scott *et al.* 1998a). Even though Australian fireweed populations were not include in the analysis, the fact that Australian fireweed is more closely related (1 bp substitution) to KwaZulu-Natal region populations whilst only distantly related to Madagascan populations (Radford *et al.* 2000; Scott *et al.* 1998a) is very similar to the relationships between Hawaiian fireweed and native range populations observed in this study. Whilst the existing trade market for agricultural products, especially animal fodder, between Hawaii and Australia would create an opportunity for fireweed to easily hitch a ride to the Hawaiian Islands, no such pathways exist between Hawaii and any of the native range countries.

Populations sampled in Swaziland and Madagascar clearly had a more distant relationship to Hawaiian populations. Populations from the Western Cape Province in South Africa were excluded since putative fireweed populations from there has previously been shown to be genetically only distantly related to other South African and Madagascan populations (Radford *et al.* 2000). Populations identified as fireweed from this region are most likely *S. burchellii* since they normally possess 13 involucral bracts instead of the 19-22 characteristic of *S. madagascariensis* (Muller, P.A., unpublished report). *Senecio madagascariensis* populations are more common and localized within the KwaZulu-Natal region than other parts of the native range regions and are thus more likely to be associated with highly adapted and co-evolved predators and/or pathogens. Prati and Bossdorf (2004) recently reported that invasive populations of *S. inaequidens* showed a reduced incidence of parasitizing insects, suggesting that escape from natural enemies may play a role in the invasion success of this species (now considered a part of the *S. madagascariensis* species complex [Radford *et al.* 2000]). Madagascan control

agents failed in Australian fireweed populations due to low host-specificity (Marohasy 1989, Holtkamp and Hosking 1993, McFadyen and Sparks, 1996, Sindel *et al.* 1998). These failures could be explained by the phylogenetic evidence of this study and other inferences (Radford *et al.* 1995, Radford 1997; Scott *et al.* 1998a) that proved Madagascan populations to be only distantly related to Hawaiian/Australian fireweed populations.

Individuals from the two South African coastal populations collected at Blythedale and Park Ryne Beaches formed a monophyletic group with *S. inaequidens,* and a sister group to all other *S. madagascariensis* populations. The identification key of the *S. madagascariensis* complex described by Radford and co-workers (2000) suggests *S. madagascariensis* and *S. inaequidens* are differentiated based on chromosome counts ($n = 20$ and $n = 40$ respectively), and achene hairiness, with only *S. inaequidens* having complete mixogenic hair coverage. On the contrary, even though this study did not include any chromosome counts, it confirms that populations identified as *S. inaequidens* based on ITS1 and ITS2 DNA sequence data had achenes of the sparsely hairy type (Fig. 5.1A). Mapping of achene morphology types onto the phylogeny (Fig. 5.4) illustrates that no relationship exists between geographical regions or even between species and the observed variances for this trait. The only observation worth mentioning is that complete achene hairiness, that is markedly different from the other two achene hairiness types, seems to have evolved only in eastern Madagascan populations that are most likely reproductively isolated from the western (Tulear) population having intermediately covered achenes (Fig. 5.1B & C). South African east coast populations also varied having achenes of the sparse and intermediate types. These observations reiterate the

futility of using this trait for taxonomic inferences. Also, the amount of DNA sequence divergence between different taxa within the monophyletic *S. madagascariensis* clade is surprisingly high considering the relatively short length of DNA sequence (474 bp) used in the phylogeny construction. Coupled with the variation observed for achene morphology and length this study strongly recommends a revised alpha-level taxonomy for the *Senecio madagascariensis* species complex and a need for a more "total evidence" approach, making use of all available data, to overcome confusion resulting from the numerous morphological overlaps.

In this study, a DNA-based phylogeny proved to be a powerful tool to better understand the system in which biological control of *S. madagascariensis* is being considered. Future biological control research for *S. madagascariensis* in Hawaii should focus on those natural enemies collected from the South African east coast populations (excluding Blythedale and Park Ryne Beach areas) rather than those collected from Madagascar and Swaziland.

Until recently most plant genetic studies typically have turned to genome-wide markers such as random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), but these data cannot be ordered in a historic sequence, which precludes the construction of gene trees and subsequent phylogeographical inferences (Schaal *et al.* 2003). This lag in molecular markers suitable for plant phylogenetics led to phylogeographical studies primarily being dominated by animal taxa, far outnumbering plant taxa (Avise, 2000). However, recent developments in this field have seen an increase in the identification of molecular markers in plants that present variation at the intraspecific level, in the nuclear genome, and in the chloroplast

and mitochondrial genomes. This will undoubtedly warrant phylogeography to become a popular practice when closely related plants are being studied in the range between geographic races and cryptic species, as is often the case in research efforts involving biological control of invasive plants. Indeed, recent studies using this approach for plant invasions revealed aspects concerning cryptic species, hybridization events and source populations that would otherwise have been difficult or even impossible to detect (see McIvor *et al.* 2001, Gaskin and Schaal, 2002, Saltonstall 2002, Gaskin *et al.* 2005). The natural enemies collected during this study are currently being tested in quarantine and, once behavioural data are available, pose an excellent opportunity to infer correlations between phylogenetic relationships of plant taxa and the corresponding effectiveness of their associated herbivores on Hawaiian fireweed populations.

Table 5.1. Location data for putative *S. madagascariensis* populations used in this study.

Specimen number/s (meters)	Region	Locality	Latitude/longitude	Elevation
	North America			
SM149/SM150	Maui	Haleakela HWY	N20° 51.449'/W156°21.570'	303
SM323	Hawaii	Parker ranch	N19° 59.121'/W155° 33.04'	1140
SM462	Hawaii	Parker ranch	N19° 54.436'/W155° 20.577'	1766
SM553	Hawaii	Mamalahoa HWY	N19° 50.502'/W155° 45.496	227
	South Africa			
SM153/SM155	KwaZulu-Natal	Hilton, Pietermaritzburg	S29° 34.015'/W30° 19.059'	980
SM165/SM170	KwaZulu-Natal	Murray Road	S29° 39.409'/E30° 23.751'	15
SM172*	Kwazulu-Natal	Park Ryne beach	S30 °18.839'/W30° 44.576'	14.5
SM193*	KwaZulu-Natal	Blythedale beach	S29° 22.596'/W31° 20.807'	26
SM182/SM191	KwaZulu-Natal	Umhlanga	S29° 42.812'/E31° 04.729'	135
SM222	KwaZulu-Natal	Camperdow	S29° 43.867'/E30° 33.748'	745
SM231/SM232	KwaZulu-Natal	Durban city	S29° 51.570'/E31° 2.326'	14
	Madagascar			
SM241/SM242	Saint Luce	Azafady	S24° 57.992'/E47° 5.437'	16
SM261/SM263/SM264/SM267	Fort Dauphin	Fort Dauphin airport	S25° 3.05'/E46° 56.73'	33
SM275/SM276	Tulear	Belembika village	S23° 19.706'/E43° 41.288'	10
	Swaziland			
SM255/SM259	Motshane	Motshane	S26° 14.508'/E31° 3.179'	1334

* Putative *Senecio inaequidens* specimens.

Table 5.2. Morphological data (achene, leaf and involucral bract) recorded for all populations used in this study.

Specimen number/s number/s	Region	Achene type*	Leaf morphology	Involucral bract
	North America			
SM149/SM150	Maui	Intermediate	Pinnately lobed	21
SM323	Hawaii	Intermediate	Slender oblanceolate	21
SM462	Hawaii	Intermediate	Broad oblanceolate	21
SM553	Hawaii	Intermediate	Pinnately lobed	21
	South Africa			
SM153/SM155	KwaZulu-Natal	Intermediate	Slender oblanceolate	19-22
SM165/SM170	KwaZulu-Natal	Intermediate	Slender oblanceolate	19-22
SM172§	KwaZulu-Natal	Sparsely	Broad ablanceolate	19-22
SM193§	KwaZulu-Natal	Sparsely	Broad oblanceolate	19-22
SM182/SM191	KwaZulu-Natal	Sparsely	Slender oblanceolate	19-22
SM222	KwaZulu-Natal	Intermediate	Pinnately lobed	19-22
SM231/SM232	KwaZulu-Natal	Intermediate	Slender oblanceolate	19-22
	Madagascar			
SM241/SM242	Saint Luce	Fully	Broad oblanceolate	19-22
SM261/SM263/SM264/SM267	Fort Dauphin	Fully	Slender oblanceolate	19-22
SM275/SM276	Tulear	Intermediate	Slender oblanceolate	19-22
	Swaziland			
SM255/SM259	Motshane	Intermediate	Slender oblanceolate	19-22

§ Putative *Senecio inaequidens* specimens
* Refers to hair coverage of achenes as discussed in the text

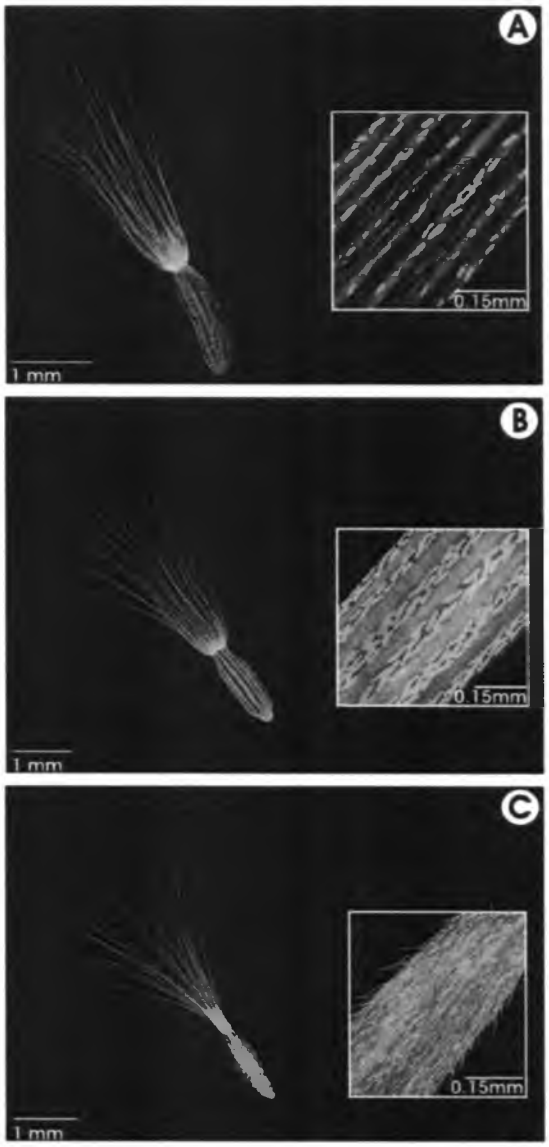


Figure 5.1. Illustrations of the three achene types identified for taxa used in this study. (A) Sparsely covered achene surfaces with few mixogenic hairs on individual grooves (collected from Umhlanga, South Africa), (B) Intermediately covered achene surfaces with mixogenic hairs concentrated on individual grooves (collected from Pietermaritzburg, South Africa) and (C) Fully covered achenes having total surface coverage by mixogenic hairs (Collected from Azafady, Madagascar). For each morphological type a higher resolution illustration of the surface hairiness is included.

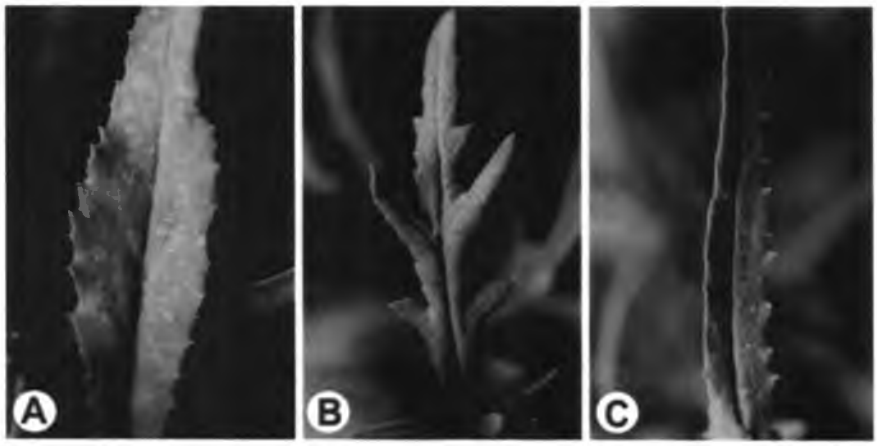


Figure 5.2. Photographs illustrating the variation observed in leaf morphology for putative *Senecio madagascariensis* populations in the Hawaiian Islands. (A) Broad oblanceolate, (B) Pinnately lobed, and (C) Slender oblanceolate.

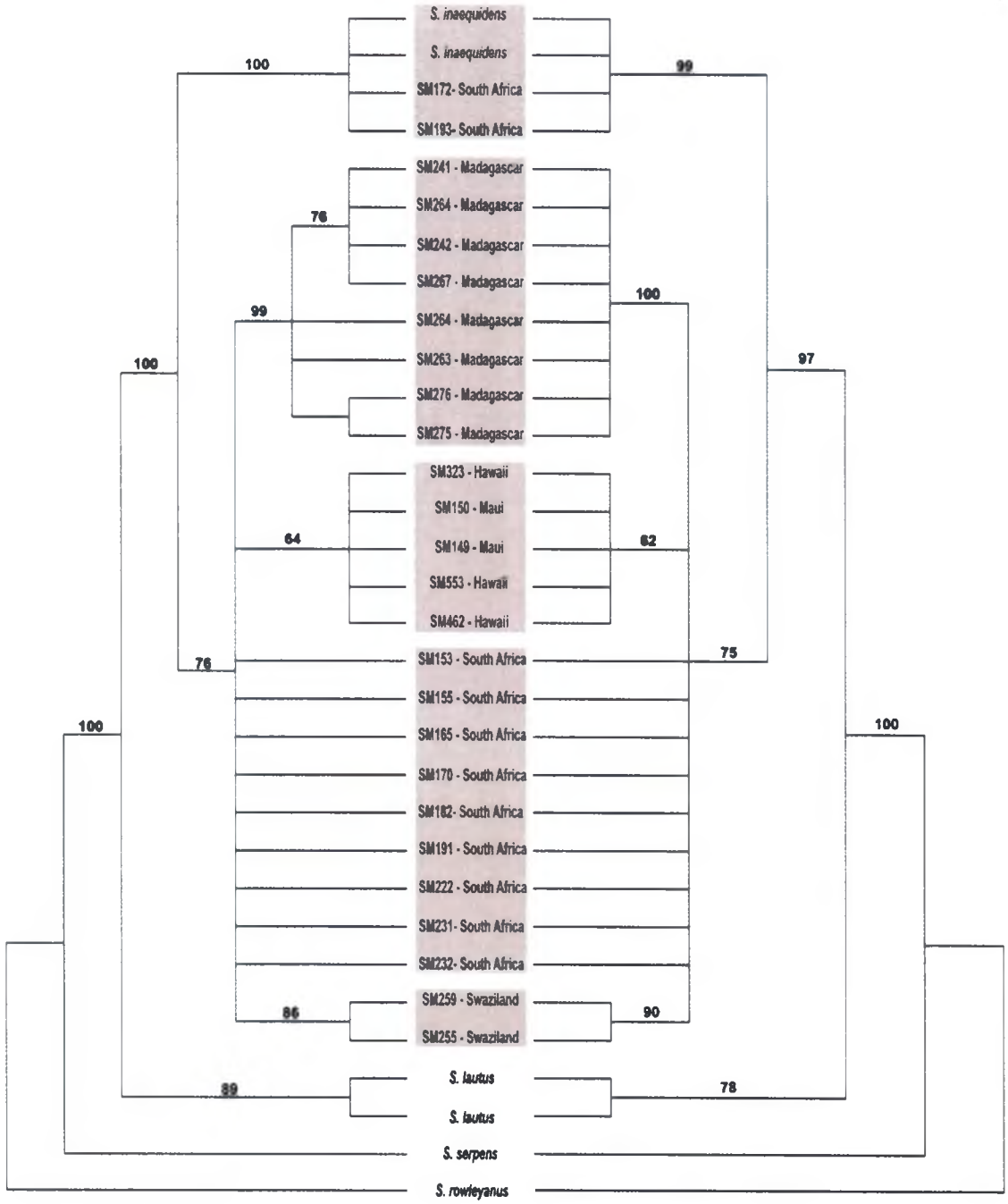


Figure 5.3. The ML tree (-ln likelihood = 1209.30) on the left hand side and a strict consensus MP tree (CI = 0.9307, RC = 0.8933) on the right hand side constructed by the combined ITS1 and ITS2 datasets. Confidence in tree topologies is indicated as bootstrap values on branches (100 replicates for ML and 1000 replicates for MP).

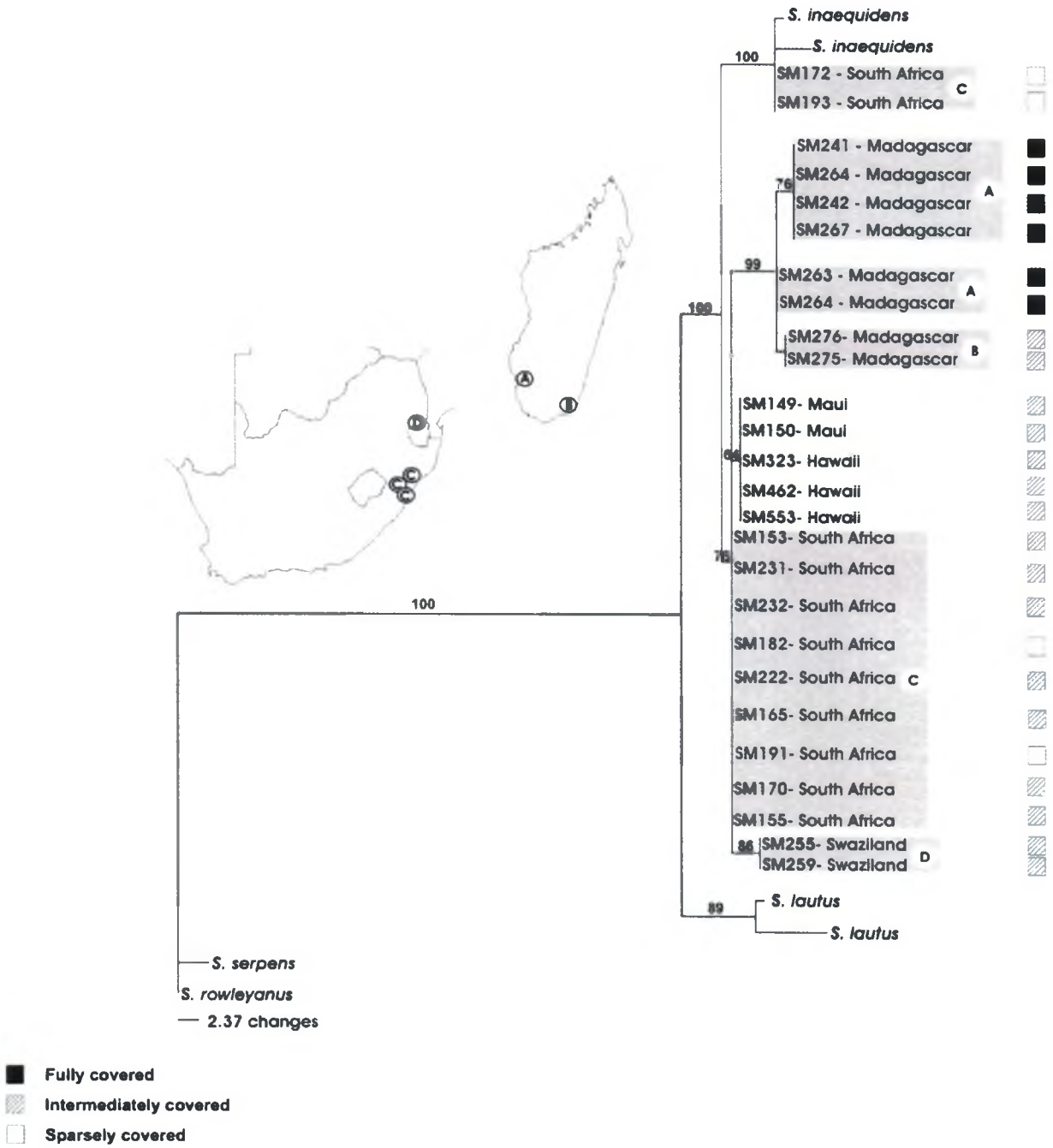


Figure 5.4. The ML phylogram (-ln likelihood = 1209.30) indicating phylogenetic distances between taxa. The approximate regions for all putative native range *Senecio madagascariensis* populations are shown on the inserted maps and indicated accordingly on the phylogram. Achene morphology (full, intermediate and sparse hair coverage) is indicated for all studied taxa on the right-hand side of the phylogram. Tree topology confidence is indicated as bootstrap values at nodes.

CHAPTER 6: ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE MARKERS FROM FIREWEED, *SENECIO MADAGASCARIENSIS* POIR. (ASTERACEAE)

Abstract

Ten polymorphic microsatellite loci were isolated and characterized from invasive fireweed (*Senecio madagascariensis*) populations in the Hawaiian archipelago. These loci provided markers with polymorphism of six to 24 alleles per locus within 96 individuals collected from two populations from the island of Maui. The expected and observed heterozygosities ranged from 0.31 to 0.91 and 0.056 to 1, respectively. These markers should be useful to study the importance of genetic diversity in invasion success of this species.

Introduction

Senecio madagascariensis Poir. (Asteraceae), an Afro-Madagascan native, has been introduced outside its native range and invades regions in Kenya, Argentina, Colombia, Australia and Hawaii (Le Roux *et al.* 2006; Sindel *et al.* 1998). *Senecio madagascariensis* forms part of a species complex and its taxonomy is problematic and in need of revision (Le Roux *et al.* 2006). The population biology and genetics of this species have not been studied extensively and coupled with its invasive success and large geographical native range, *S. madagascariensis* poses a good system to investigate the role of genetic diversity, multiple introductions and dispersal in invasion success. Here,

10 microsatellite markers were developed from *S. madagascariensis* to address some of these issues.

Materials and Methods

Total DNA was extracted with the DNeasy Plant mini kit (Qiagen) from 40 to 50 mg of fresh leaf material. Extracted genomic DNA was digested with *Rsa*I, *Hae*III, *Alu*I and *Hinc*II and the resulting fragments simultaneously ligated onto SNX oligonucleotide linkers (Hamilton *et al.* 1999). Linker-ligated digests were hybridized to biotinylated oligoprobes $(AAC)_8$, $(AAT)_8$, $(GC)_{12}$ and $(GT)_{12}$ and subsequent probe-bound DNA extracted using streptavidin-coated magnetic beads. Following PCR with the SNX forward primer only, PCR products of microsatellite-enriched DNA were purified with the QIAquick PCR purification kit (Qiagen). These purified fragments were digested with *Nhe*I, and ligated into the *Xba*I site of pUC19 followed by transformation into competent *Escherichia coli* DH5α (Invitrogen).

Colonies were transferred onto nylon membranes after transformation and screened using digoxigenin labeled $(AAC)_8$, $(AAT)_8$, $(GC)_{12}$ and $(GT)_{12}$ probes to identify positive clones. The inserts of 162 putative microsatellite-containing colonies were amplified with universal M13 plasmid primers, purified and subjected to sequencing using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and an automated sequencer (ABI PRISM 377XL DNA sequencer, PE Applied Biosystems). One hundred and thirteen of the 162 sequences had limited flanking region(s) for primer design or no or imperfect microsatellite sequence repeats.

PCR primers were designed from the remaining 49 sequences using the program FASTPCR (Kalendar 2004).

I tested and optimized amplification with a gradient PCR at 48 – 60 °C range of annealing temperatures. Each reaction contained 6 μL HotMasterMix (HotMaster Taq DNA polymerase, 0.3 U; 2.5 X HotMaster Taq Buffer pH 8.5, 45 mM KCl and 2.5 mM $MgCl_2$; 200 μM of each dNTP; [Brinkman Instruments, Inc.]), 7.5 pmol of each primer and approximately 5 ng total genomic DNA. Thirty-six primer sets were successfully amplified. To detect polymorphism at each locus PCR products from 12 different fireweed individuals were run on a GeneChip™ microarray analysis chip (Affymetrix, Inc.) (Banerjea *et al.* 2003). One of the primers of loci that showed polymorphism were fluorescently labeled with either HEX, NED or 6-FAM (Integrated DNA Technologies, Inc.) and re-optimized as described above. PCR was conducted on an MJ Research PTC 100 cycler with a thermocycle of: initial denaturation of 94 °C for 2 min; 35 cycles at 94 °C for 60 s, locus-specific annealing temperature (see Table 1) for 60 s, elongation at 72 °C for 90 s; and final extension at 72 °C for 12 min. Polymorphisms were screened using an ABI PRISM 377XL DNA sequencer (PE Applied Biosystems) and PCR products sized relative to a molecular size marker (LIZ500, PE Applied Biosystems). DNA fragments were analyzed the GeneMarker version 1.4 program (SoftGenetics, LLC). For each locus expected and observed heterozygosities were inferred, significant deviation from Hardy-Weinberg equilibrium (HWE) and the existence of genotypic linkage disequilibrium (LD) using the GENEPOP version 3.4 program (Raymond and Rousset 1995).

Results and Discussion

Table 6.1 summarizes the characteristics of the 10 primer pairs developed from fireweed. Allelic variation at the 10 microsatellite loci was assessed in 96 individuals of *S. madagascariensis* from two different populations (approximately 800 m apart) on Maui. The number alleles detected at the 10 loci ranged from six to 24 and expected and observed heterozygosity ranged from 0.31 to 0.91 and 0.056 to 1. For both populations only three loci (Se-116, Se-176(a) and Se-220) had heterozygosities conforming to those expected under HWE. All other loci had heterozygosities that deviated from HWE expectations ($P < 0.01$) and could be the result of null alleles and/or inbreeding effects often experienced by introduced species originating from a single introduction event. No significant LD was found for all loci.

These ten polymorphic loci will be utilized to assess the genetic diversity, genetic structure and dispersal patterns of invasive fireweed populations in and among the different Hawaiian Islands.

Table 6.1. Characteristics of ten microsatellite markers isolated from *Senecio madagascariensis*. Results are given for two different populations collected from the Kula region in Maui.

Locus	Repeat motif	Primer sequence (5'-3')	T_a (°C)	No. of alleles	Allele size range (bp)	Pop 1 $H_O H_E$	Pop 2 $H_O H_E$	GenBank Accession no.
Se-46*	$(GT)_{32}$	F: HEX-GGGTTAAAAGTGTAATTATGGC R: TGAAACCCGAATCGCCGTC	52	6	183-197	1/0.748	1/0.704	DQ886396
Se-76*	$(GTT)_{13}$	F: NED-GGAGGTCCAAATACGTTTGCAC R: TCGTCAAATGAAACTCACGGAC	58	8	537-615	0.47/0.841	0.3/0.82	DQ886397
Se-116	$(AAC)(AC)(AAC)_{14}$	F: NED-CCTTCTGGTTGATTTGGCTAAGC R: AGAACTGCACATTTGAAGCCTG	48	12	418-466	0.667/0.642	0.71/0.77	DQ886398
Se-136*	$(GA)_{25}$	F: HEX-CAAAGGTAGGATGATGTGAAGCTC R: TCTTGTTGGGTCAATGCTCG	51	20	315-395	0.056/0.859	0.292/0.904	DQ886399
Se-138*	$(TC)_{17}$	F: HEX-ACTTCGTGGGCCATTCCAG R: CTTCCTGCATAACATCCACCAC	58	19	180-232	0.482/0.847	0.419/0.902	DQ886400
Se-176(a)	$(GT)_{12}$	F: FAM-AGCATAGTGCAAGCATGTTCG R: CTTTGATGTTGGCTGCAATGC	60	21	232-288	0.778/0.862	0.839/0.91	DQ886401
Se-194*	$(GT)_{11}$	F: FAM-GTCGCAGTCACCGTCACTG R: GAGCAGCAGACAACGACAC	58	8	316-392	0.111/0.389	0.069/0.31	DQ886402
Se-206*	$(CT)_{43}$	F: HEX-ACGGGCGTTAAACTGCTCC R: TCCCCACCACCATCACCTC	58	18	352-404	0.333/0.82	0.581/0.616	DQ886403
Se-208*	$(TC)_{24}$	F: NED-TTTTGGGCAGGCCATATCC R: AGTGTCTCCACGGTTGTCG	55	24	301-379	0.593/0.907	0.6/0.796	DQ886404
Se-220	$(GT)_{10}$	F: NED-AACTCGACCAGTCCTCAGC	58	11	156-208	0.815/0.846	0.677/0.662	DQ886405

* Significant deficit of heterozygotes from expected under HWE ($P < 0.01$).

CHAPTER 7: GENETIC INSIGHTS INTO NATIVE AND INVASIVE FIREWEED, A MEMBER OF THE *SENECIO MADAGASCARIENSIS* POIR. SPECIES COMPLEX

Abstract

Genetic variation is essential for local adaptation to new biotic and abiotic environments and may play a key role in the successful establishment of introduced species. Studies investigating the importance of genetic variation in invasive species' success render the opportunity to better our understanding of biological invasions as manifested by the elucidation of geographic source(s), taxonomic identity, dispersal patterns, number of introductions and adaptive potential. However, to date, such studies rarely include comparisons between invasive and non-invasive, native range, populations.

In this study, microsatellite variation in eight polymorphic loci was studied among and within 26 invasive (Hawaiian Islands) and 11 native (Southern Africa and Madagascar) populations of fireweed (*Senecio madagascariensis* Poir.). This Afro-Madagascan native is a serious pasture weed in the Hawaiian Islands, posing a particular threat to the archipelago's livestock industry due to the production of toxic pyrrolizidine alkaloids.

Microsatellite analysis revealed the majority of native range populations to be tetraploid while all invasive populations in Hawaii were diploid. Fireweed was traditionally thought to be diploid ($2n = 20$), but here, only two South African east coast and all Hawaii populations were diploid. These findings, coupled with the substantially higher proportion of shared alleles between Hawaii and eastern South African

populations support eastern South Africa as the likely native geographic source for Hawaii infestations. The differential ploidy levels in fireweed could also pose interesting considerations for its biological control, as elevated alkaloid gene expression in tetraploid populations are expected to deter generalist enemies and attract more host-specific, specialist enemies.

Bayesian and frequency-based analyses revealed fine-scale genetic structure of invasive Hawaii populations with two genetically defined demes corresponding to the two islands of Maui and Hawaii. Furthermore, spatial genetic autocorrelation analyses revealed high correlation between genetic similarity and geographical proximity for populations up to 2 km apart, followed by a gradual decline until correlation is lost at ~ 9 km scale. Fireweed appears to spread via a diffusive strategy, although, a single population (SM1) was identified that likely resulted from a human- or animal-mediated long distance dispersal event from Maui to Hawaii. Despite relatively low overall genetic structure, two populations from Maui (SM 63 and SM 941), showed high differentiation from all other populations. This, coupled with the absence of inbreeding or a genetic bottleneck in Hawaii populations, the observed differentiation between Hawaii and Maui and relatively high allelic diversity, suggests that fireweed was introduced repeatedly to the Hawaiian Islands.

Introduction

Plant biological invasions are key factors leading to changes in the functioning of ecosystems (D'Antonio and Vitousek 1992, Vitousek 1990), native species' biodiversity (Wilcove *et al.* 1998) and changes in evolutionary trajectories of species (Strauss *et al.*

2006a). The attributes of some successful plant invaders have been determined, e.g. the release from natural enemies and increased competitiveness in introduced ranges (Keane and Crawley 2002), but a general consensus as to which characteristics are associated with invasiveness has not yet been reached. Past research efforts to unravel these attributes focused mainly on the ecological aspects of biological invasions, but recent studies recognize the importance of evolutionary processes resulting from drift, inbreeding, inter- and intraspecific hybridization, and selection as essential or equally important to the success of some introduced species (for reviews see Lee 2002, Mooney and Cleland 2001, Sakai *et al.* 2001). Reduced genetic diversity and novel conditions in new environments that often accompany species introductions will lead to rapid evolutionary responses. For any introduction the amount and distribution of genetic variation (genetic structure) is determined by that of founder population(s), number and source of founders, and life history characteristics of the species (Lee *et al.* 2002). Genetic diversity will form the foundation for post-introduction evolutionary responses and the lack thereof is often cited for being partially responsible for the inability of a species to persist in new environments. This causes a so-called lag phase, which could represent the time lapse between colonization and initial spread during which genetic variation is acquired for sufficient local adaptation to occur (Sakai *et al.* 2001).

Although numerous studies have investigated population genetic structure within and among populations of introduced species (e.g. May *et al.* 2006, Walker *et al.* 2003) surprisingly few studies have compared the amount and distribution of genetic variation among native and introduced populations (for review see Bossdorf *et al.* 2005). This might be the consequence of the logistical difficulties of collecting material over large

spatial scales that such work usually entails. These studies could provide valuable insights into the population dynamics of biological invasions and, in addition, will aid in identifying geographical source(s), dispersal route(s), and the roles of adaptation and 'pre-adaptation' in invasion success. These large spatial scale population genetic studies may also shed light on population processes not evident at local scales, e.g. variation in mating patterns or breeding system evolution (Eckert *et al.* 1996).

Here, a large scale population genetic study was done on fireweed (*Senecio madagascariensis* Poir. [Asteraceae]), invasive in the Hawaiian Islands and native in Southern Africa and Madagascar.

Introduced to numerous world locations outside of its native ranges, this pasture weed strongly competes with existing flora for light, moisture and soil nutrients (notably phosphorus and nitrogen), ultimately leading to pasture deterioration (Watson *et al.* 1994). Fireweed, furthermore, produces numerous pyrrolizidine alkaloids that are toxic to livestock (Gardner *et al.* 2006). Fireweed was accidentally introduced to the Hawaiian Islands in the early 1980's and is now found over a wide range of different habitats, mainly on the islands of Hawaii and Maui (Motooka *et al.* 1999). Fireweed forms part of a species complex and, using a phylogeographical approach, Le Roux *et al.* (2006) (Chapter 5) provided support for the hypothesis that Hawaiian infestations originated secondarily from Australia (Gardner *et al.* 2006), where it is also considered a serious pasture weed. Furthermore, Le Roux *et al.* (2006) (Chapter 5) showed that Hawaiian populations are most closely related to fireweed from its native range in eastern South Africa.

The taxonomy of the *S. madagascariensis* species complex is problematic. Genetic distances from isozyme polymorphisms and morphological data revealed a considerable amount of variation within the complex and its close relatives, and suggested that the complex is in need of taxonomic revision (Radford *et al.* 2000). For example, studies attempting to differentiate Australian fireweed from the closely the related native *S. lautus* complex suggested that fireweed in Australia was more closely related to South African *S. madagascariensis* and a sister species, *S. inaequidens* than to *S. madagascariensis* from Madagascar (Radford *et al.* 1995; Radford 1997; Scott *et al.* 1998a).

Senecio inaequidens is one of several morphologically similar and widespread species in the southern African *S. madagascariensis* complex (including *S. skirrhodon, S. burchellii, S. harveianus* and *S. pellucidus*) and these species are frequently mistaken for fireweed (Le Roux *et al.* 2006, Hilliard 1977). A phylogenetic analysis by Scott *et al.* (1998a) did not differentiate between Australian fireweed, *S. madagascariensis* and *S. inaequidens* from South Africa leading the authors to conclude that these encompass a single species that differed from putative *S. madagascariensis* specimens from Madagascar. A more recent study by Radford *et al.* (2000) differentiated *S. inaequidens* and *S. madagascariensis* on the basis of achene morphology and ploidy level. However, Le Roux *et al.* (2006) refuted the usefulness of achene morphology (mixogenic hair coverage) as a taxonomic informative character when no correlation was found between phylogenetic signal and achene hairiness. In the current study, genetic diversity of South African, Swazi, Madagascan and Hawaiian *S. madagascariensis*-complex populations

was compared using recently developed microsatellite markers (Chapter 6, Le Roux and Wieczorek 2006).

The following key questions were adressed: (i) How is allelic diversity partitioned between individuals, populations and native and introduced ranges of *S. madagascariensis*? (ii) How does genetic diversity between introduced populations and previously identified geographical sources for Hawaiian populations correspond? (iii) Do *S. madagascariensis* infestations in Hawaii represent one or multiple introduction events? (iv) Is dispersal in introduced ranges primarily diffusive, long-distance, or a combination of both (stratified dispersal)?

Materials and Methods

Study species

Fireweed, *Senecio madagascariensis* Poir., is assumed to be a diploid ($2n = 20$) member of the Asteraceae family native to Madagascar, the Mascarene islands and Southern Africa. Fireweed is a short-lived perennial herb but behaves most commonly as an annual, growing strongly from autumn to spring (Sindel *et al.* 1998). *Senecio madagascariensis* is an out-breeder and is mainly insect-pollinated (Sindel *et al.* 1998). Dispersal is mostly by wind dispersed seeds, but animals and animal feed (human-mediated dispersal) may occasionally act as dispersal vectors (Sindel *et al.* 1998).

Like many other *Senecio* species, fireweed produces pyrrolizidine alkaloids that reduce growth of and in severe cases, cause mortality when ingested by livestock (Gardner *et al.* 2006). In the native Afro-Madagascan range this species is widely distributed and common in disturbed habitats such as contour banks and road verges and

can occur up to 1500 m above sea level. Outside of its native range, fireweed has established and is spreading in cool moist equatorial highland areas in Kenya and Colombia and is a serious pasture weed in agricultural areas in Argentina, Hawaii and the south-eastern coastal areas of Australia. In Australia annual losses amounting up to US$ 2 million directly linked to fireweed infestations, have been reported (Motooka *et al.* 1999). Fireweed is currently the target of a biological control program in Hawaii (M. Ramadan, Hawaii Department of Agriculture, personal communication).

Population sampling and DNA extraction

Eleven *S. madagascariensis* populations were sampled from within the native range (South Africa, Madagascar and Swaziland) and 26 populations from introduced areas in the Hawaiian archipelago (Hawaii and Maui) (Fig. 7.1). For each population, material from 10-30 individual plants was collected. A population was arbitrarily defined as any patch or community of fireweed plants (between 20 – 150 individuals) that was separated by at least two kilometres from any other population. Leaf material was collected during March 2005 in the known geographical ranges of *S. madagascariensis* in South Africa, Swaziland and Madagascar. Field collections were made during a survey and collection of natural enemies to be assessed as potential biological control agents against fireweed in Hawaii by M. Ramadan (Hawaii Department of Agriculture). Plant material was placed and dehydrated in plastic containers containing Drierite^{TM}. Hawaii populations were collected throughout 2005 and leaf material was kept on ice in the field for no longer than 24 hours before being transferred to a – 80 °C freezer. Locality name, region, latitude, and longitude were recorded for all populations sampled (Table 7.1). All

specimens were classified according to Hilliard (1977) and current herbarium practices in South Africa. In total 804 individuals were sampled (110 from Southern Africa and Madagascar, 694 from Hawaiian Islands) representing 37 putative populations.

Total genomic DNA was extracted according to the manufacturer's protocol with the Wizard® genomic DNA purification kit (Promega) from 40 to 50 mg leaf material that was frozen in liquid nitrogen and ground by hand. All extractions were stored at – 80 °C.

Microsatellite analysis

Details concerning the isolation, characterization, and internal repeat structure of the *S. madagascariensis* microsatellite loci used in this study can be found in Chapter 6 (Le Roux and Wieczorek 2006). Polymerase chain reaction (PCR) amplification of loci was done in 10 µL volumes with the Multiplex PCR Kit (Qiagen) by combining primer pairs into two different multiplex reactions. Each PCR reaction contained 5 µL 2X Qiagen Multiplex PCR Mastermix [HotStarTaq DNA Polymerase; Qiagen Multiplex PCR Buffer (6 mM $MgCl_2$, pH 8.7); dNTP mix], 1 µL 10X primer mix (see Table 7.2), 1 µL Q-solution [PCR additive(Qiagen)] and approximately 5 ng total genomic DNA. Table 7.2 gives the concentrations of individual primers used to make up 10X primer mix solutions that resulted in optimum multiplex amplification. All reactions followed a thermal cycle consisting of an initial denaturation at 95 °C for 15 min, followed by 35 cycles at 94 °C for 60 s, annealing at 55 °C (multiplex 1) or 60 °C (multiplex 2) for 60 s, elongation at 72 °C for 90 s, and final extension at 72 ° for 30 min. Polymorphisms were screened using an ABI PRISM 377XL DNA sequencer (PE Applied Biosystems) and PCR products sized relative to the LIZ500 molecular size marker (PE Applied

Biosystems). Gel analysis was done using the GeneMarker version 1.4 program (SoftGenetics, LLC).

Data analyses for diploid, invasive (Hawaii) populations

Statistical analysis of allele frequency data

The number of alleles was calculated for each population using GenAlEx6 (Peakall and Smouse 2006), and observed (H_O) and expected (H_E) heterozygosities using Arlequin version 3.01 (Schneider *et al.* 2000). Statistically significant deficits of heterozygotes from that expected under Hardy-Weinberg equilibrium (HWE), linkage disequilibrium among all pairwise sets of loci, pairwise F_{ST}, and inbreeding coefficient (F_{IS}) values were estimated using Arlequin version 3.01 (Schneider *et al.* 2000). For HWE, a Monte Carlo approximation of the Fisher's exact test was used (Guo and Thompson 1992) and a standard Bonferroni correction for multiple comparisons where the Markov chain algorithm was run for 100 000 steps following 10 000 dememorization steps.

Transient excess of heterozygosity relative to that expected under mutation-drift equilibrium is a signature of recent population bottleneck (Cornuet and Luikart 1997). Populations that have experienced a recent reduction of their effective population size (N_e) exhibit a correlative reduction in numbers of alleles and gene diversity (H_E, or Hardy-Weinberg heterozygosity) at polymorphic loci. However, allele numbers decline more rapidly than gene diversity so that in recently bottlenecked populations, the observed gene diversity is higher than the expected equilibrium gene diversity (Luikart *et al.* 1998). An excess of heterozygosity was tested for microsatellite data with the

Bottleneck program (Cornuet and Luikart 1997) under a 100% stepwise mutation model (SMM) and a two-phase mutation model (TPM with 70% SMM). Significance was tested by the the Wilcoxon test (Luikart and Cornuet 1998). These tests were applied globally, to the entire Hawaiian sample size (n = 694).

To test for isolation by distance, Mantel tests with 1000 permutations as implemented in Arlequin version 3.01 (Schneider *et al.* 2000) were used. A matrix of pairwise F_{ST} values was tested against a matrix of geographical distances between populations that was calculated from geographical coordinates using GIS-Arcview.

An analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was used to examine the distribution of genetic variation at three hierarchical levels: within populations, among populations within islands, and among islands. This test, implemented in Arlequin version 3.01 (Scneider *et al.* 2000), partitions total genetic variance into covariance components and calculates fixation indices (Wright 1965) for which statistical significance is determined by comparison with a null distribution derived from permuting haplotypes, individuals or populations at the appropriate hierarchical level (Excoffier *et al.* 1992).

Bayesian estimates of population structure

Bayesian assignment techniques were used to test for population structure among populations throughout Hawaii and to assess the geographical scale of population differentiation, using STRUCTURE version 2.2 (Falush *et al.* 2007). This method identifies clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities. The model assumes K genetic clusters,

each having a characteristic set of allele frequencies at each locus; the admixture model then probabilistically estimates the proportion of individuals with ancestry in each cluster. A series of pilot runs were used to estimate $Pr(X \mid K)$, where X represents the data, for K between 1 (the expected value if all populations represent a single panmixic unit) and 26 (the maximum number of populations). Using the options to ignore population affiliation when defining genetic clusters, assuming independence among loci, and allowing admixture, four independent runs of 300 000 iterations were done, following a burn-in period of 100 000, for each value of K (Pritchard *et al.* 2000). From these initial runs, the true value of K (the highest posterior probability) was shown to fall between 2 and 14. Pritchard and Wen (2003) warned that $Pr(X \mid K)$ is, in reality, only an indication of the number of clusters and an ad hoc guide, and potentially difficult to interpret biologically. This is especially true in cases were LnProb values increase with stepwise values of K and can lead to the overestimation of K. To minimize such overestimation, ΔK was calculated (Evanno *et al.* 2005) by taking into account the shape of the log-likelihood curve with increasing K and variance among estimates among multiple runs. Once the number of genetic clusters was established, each individual was assigned to a cluster and the overall membership of each sampled individual in the clusters was estimated.

Global spatial autocorrelation analysis

Spatial autocorrelation analysis was used to identify spatial structure by testing the relationship between genetic similarity and spatial proximity, following the method of Smouse and Peakall (1999) as implemented in GenAlEx6 (Peakall and Smouse 2006).

This method uses a multilocus genetic distance, thus strengthening the spatial signal by reducing stochastic (locus-to-locus and allele-to-allele) noise. These methods have been applied to animal and plant taxa with different dispersal strategies and have contributed to the better understanding of dispersal behavior and dynamics (Peakall *et al.* 2003; Double *et al.* 2005; Zamudio and Wieczorek 2007; Williams *et al.* 2007). Under restricted gene flow, no selection, and a sampling scheme that includes the geographical scale of positive genetic structure, populations should show significant positive spatial autocorrelation at short distances and these will decline to zero followed by stochastic oscillations of positive and negative values (Sokal and Wartenberg 1983; Smouse and Peakall; 1999). The autocorrelation coefficient, *r*, is a measure of genetic similarity between individuals that fall within a defined distance class and is closely related to Moran's-*I*, a weighted correlation coefficient used to detect departures from spatial randomness. In cases of positive genetic structure, the first x-intercept in the autocorrelogram (*r* plotted as a function of distance, where $r = 0$) provides an index of the spatial extent of nonrandom (positive) genetic structure (Peakall *et al.* 2003). Individual pairwise genetic and geographical distance matrices derived from the data were used to calculate *r*. The statistical significance of *r* was determined by random permutation of all individuals among distance classes and computing the null distribution for *r* in cases of no genetic structure. One thousand permutations were used to set the lower and upper 95% confidence limits for the populations in the dataset. Ninety-five percent confidence intervals were also calculated around each *r* value by bootstrapping *r* values within each distance class 1000 times. Following Peakall *et al.* (2003), the null hypothesis of no spatial autocorrelation was rejected only when *r* exceeded the 95 % CI derived from the

among-population permutation test, and when the 95 % CI about *r* (derived from bootstrapping) did not intercept the axis of $r = 0$. In order to more accurately determine the distance where *r* approximates zero, i.e. loss of spatial structure, the spatial autocorrelation analysis was run independently making use of different distances (50 km, 30 km, 10 km and 1 km) starting with the largest, proceeding to the smallest intervals.

Genetic analysis of native and invasive fireweed populations

The proportion of shared and unique alleles among different native range and introduced regions was calculated for all populations. Due to differential ploidy levels (see results) an analysis of microsatellite data was done based on the presence/absence of bands, similar to random amplified polymorphic DNA bands (e.g. see Lian *et al.* 2003), rather than allele frequencies. Nei's (1978) unbiased genetic distances of the nuclear genome between populations was calculated and a dendrogram constructed based on these genetic distances using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis as implemented in Popgene, version 1.31 (Yeh *et al.* 1997).

Due to the lack of fresh plant tissue from native ranges, ploidy levels could not be determined with 100% certainty. To confirm polyploidy for suspected tetraploid populations, a comparison of the relative intensity of allelic electrophoretic peaks was done with the GeneMarker version 1.4 program (SoftGenetics, LLC). For instance, a triallelic locus in a tetraploid will harbor two single-copy alleles and one double-copy allele, with the latter, given optimal amplification conditions, exhibiting relative peak intensity similar to that of the two single-copy alleles combined. The ratio of these intensities should thus approximate one. These proportions were determined and

compared for all possible allele combination scenarios for a subset of 40 suspected polyploid individuals for all loci. These ratios were compared among individuals using ANOVA (SAS Institute, 2001) to determine the efficacy of this approach in confirming polyploids. Using ratios eliminated any bias between individuals resulting from PCR amplification artifacts within individual reactions (e.g. due to template quality).

Results

Two loci, Se-46 and Se-206, gave consistent amplification problems (null alleles and excessive stutter bands) during multiplex PCR amplification and were subsequently omitted from all analyses.

Scoring of microsatellite alleles revealed that almost all native-range fireweed populations harbored more than two sets (diploid) of chromosomes. Polyploid populations were assumed to be tetraploid based on previous reports on the ploidy levels of members of the *S. madagascariensis* complex (Lafuma *et al.* 2003) and the fact that the maximum number of alleles observed at any locus (i.e. complete heterozygosity) never exceeded four. As outlined in the materials and methods section, the relative intensity of allelic electrophoretic peaks corresponded to those expected for tetraploid genomes. Figure 7.2 illustrates all possible allelic combinations for locus Se-176(a) for different individuals. Intensity ratios for the different allelic combinations did not differ significantly (One way ANOVA: three alleles, $P = 0.394$; two alleles [both with two copies], $P = 0.097$; two alleles [one with one and the other with three copies], $P = 0.247$) among individuals. Two South African populations (SM151 and SM161) and all Hawaii populations appeared to be diploid, i.e. never harboring more than two alleles per locus.

Therefore, standard estimates of genetic indices dependant on allele frequency data were only determined for introduced, Hawaii fireweed populations, unless otherwise stated.

Species-level allelic diversity and relatedness

Two hundred and sixty-three alleles were identified in the eight microsatellite loci analyzed. Twenty-three alleles (9% of the total) were shared among all regions (Hawaii, South Africa, Madagascar and Swaziland) (Fig. 7.1). Hawaii and South African populations shared the highest number of "unique" alleles, 65 or 25% of the total, not present in any other regional (Madagascar and Swaziland) populations. This was followed by South African and Madagascan populations sharing eight (3% of total) unique alleles and Swazi and South African populations also sharing 3 % of the total number of alleles. Table 7.3 summarizes the proportions of shared alleles among all regions.

Nei's unbiased nuclear genetic distances, based on the absence/presence matrix of microsatellite allele data, between native and introduced populations ranged from 0.0005 (between Hawaiian populations SM281 and SM311) to 0.0115 (between SM231 [South Africa] and SM261 [Madagascar]) (Table 7.4). Not surprising, consistent with the high number of common alleles shared only between Hawaii and South Africa, hierarchical cluster analysis (UPGMA) nested Hawaiian populations within South African populations (Cluster 2) and formed a sister cluster to other South African and Swazi populations (Cluster 1) (Fig. 7.3). South African population SM221 was basal to Cluster one and two, with Madagascan populations forming a more distantly related sister cluster to all of these (Cluster 3). Confidence in tree topology, i.e. bootstrap analysis, was not

determined as the dendrogram was constructed from entities that did not evolve independently (different microsatellite alleles from the same locus) (Felsenstein 2004).

Population genetic analysis of introduced Hawaii S. madagascariensis

Within- and among-population patterns of genetic diversity

The microsatellite loci used in this study were highly polymorphic across populations sampled. The number of alleles per locus ranged from 19 (locus Se-194) to 46 (locus Se-136); within populations the mean number of alleles ranged from 4.750 to 16.875. Expected heterozygosity ranged from 0.55 to 0.93, with a mean of 0.75 across all loci. Observed heterozygosity ranged from 0.14 to 1.00, with a mean of 0.56 across all loci. Overall, most Hawaii populations showed a deficit of heterozygotes from that expected under HWE, with some loci (Se-220, Se-176(a) and Se-116) conforming to HWE proportions, but not for all populations. No linkage disequilibrium was detected among any of the eight loci across all populations.

The computer program Bottleneck (Cornuet and Luitkart 1997) was used to test whether introduced *S. madgascariensis* populations showed a transient excess in heterozygosity, as expected under a bottleneck event. No significant excess of heterozygosity was obtained under both the full SMM and TPM model of mutation with 70% SMM ($P = 1.00$ and $P = 0.98$ respectively for Wilcoxon test), indicative that *S. madagascariensis* is not currently going through a population bottleneck or has potentially recovered from such an event.

Regressions of genetic distances (pairwise F_{ST} values) over geographical distances were significant (Mantel test, $r^2 = 0.43$, $P < 0.0001$). Thus, fireweed showed isolation by distance at the spatial scales investigated.

Overall, population genetic structure was moderate ($0.05 \leq F_{ST} \geq 0.15$) ranging from 0.0002 to 0.18 (Table 7.5). There was no significant genetic differentiation for 4.3 % or 28 of all population pairwise comparisons. However, two populations from Maui, SM63 and SM941, showed high genetic differentiation from each other and most other island populations (Table 7.5).

The hierarchical AMOVA based on the putative field populations revealed that the majority of genetic variation (93.68 %) resided within populations, 4.14 % was distributed among populations within islands, and 2.18 % of the variation can be explained by differentiation between Maui and Hawaii (Table 7.6). Although the proportion of genetic variation accountable at higher levels is small (2.18 %), all fixation indices were statistically significant. Consistent with the pairwise F_{ST} values, very little genetic variation was attributed to differences among populations within clusters (islands), suggesting that populations from the same island show very low genetic structure. Most genetic variation at this geographical scale was found at the population level.

Bayesian estimates of population structure

The model-based clustering method implemented in STRUCTURE showed that the model with $K = 2$ (where K is the number of population genetic clusters) was substantially better than alternative models. The highest posterior probabilities for K

varied among multiple runs with the their associated K ranging from 1 to 14, demonstrating that posterior probability alone is not a good measure of the true K. Values of LnProb(data) showed a pattern of incremental increase with increasing K; leading to potential overestimates of the number of genetic clusters. To overcome this problem Evanno *et al.* (2005) suggested the use of ΔK, which takes into account the shape of the log likelihood curve. For this study's data, $\Delta K = 2$ was 762, the highest value, whereas estimates for all other possible runs were less than 500.

The genetic identity of individuals (the average per-individual proportion of ancestry) from each of the 26 Hawaii populations in the two STRUCTURE-defined clusters, corresponded to the two islands included in the sample area (Fig. 7.4). Indeed, with the exception of population SM1 (Southern Hawaii), all other populations were probabilistically correctly assigned to the genetic deme that corresponded to the island from where they were collected (Hawaii or Maui). Population SM1 was collected in southern part of Hawaii, where fireweed was historically not known to occur. This population, with 80 % of its individuals assigned to the Maui deme, was indeed very sparse. Given the improbability for admixture, this observation potentially reflects an independent long-distance introduction from Maui to Hawaii.

Spatial autocorrelation analysis

Spatial autocorrelation analyses helped to resolve the scale of spatial connectivity among invasive populations of fireweed in the Hawaiian Islands. Across all populations included, the autocorrelogram showed significant positive genetic correlations among geographically close populations and an overall clinal pattern of genetic structure (Fig.

7.5). The decrease in *r* associated with increased distance is indicative of restricted gene flow among subsets of geographical samples in this study. The geographical distance among populations where genetic correlations are expected to be random, i.e. the x-intercept for *r*, was ~ 9 km. In other words, on average, populations separated by 9 km or less shared a higher proportion of genes, while those separated by greater distances are on average genetically independent. At smaller spatial scales (1 km intervals) the genetic patch associated with high positive spatial correlation (within 9 km) revealed that genetic correlation increased between 1 km ($r = 0.59$) to 2 km ($r = 0.067$) and then droped drastically at 3 km ($r = 0.021$). At distances greater than 3 km spatial correlation fluctuated but gradually declined until the 9 km distance class is reached.

Discussion

Differential ploidy levels within a species

Further complicating a species complex, which already is beset by extremely difficult taxonomic problems, this study provides the first evidence for the existence of different ploidy levels among regional *S. madagascariensis* populations. Previously, Lafuma *et al.* (2003) reported the same phenomenon for *S. inaequidens*, an invasive weed throughout Europe and also a member of the *S. madagascariensis* complex. *Senecio inaequidens* appeared to be tetraploid throughout the introduced ranges while both diploid and tetraploid populations were found in native South Africa. *Senecio madagascariensis* populations included in their study appeared diploid. Based on inferences derived from intraspecific DNA variants found among diploid populations, Lafuma *et al.* (2003) argued that tetraploid populations of *S. inaequidens* likely arose as a

result of allopolyploidization (hybridization between different DNA variants). In contrast, the combination of two facts supports autopolyploidization rather then allopolyploidization as more parsimonious in explaining the origin of tetraploid *S. madagascariensis*. Firstly, tetraploid homozygous loci, i.e. having four copies of a single allele, frequently involved alleles also found in diploid native range (SM151 [Pietermaritzburg] and SM161 [Murray road]) and Hawaii populations. Hybridization among closely related species would lead to alleles that are only found in hybrids when compared to either one of the parental lineages. Secondly, Le Roux *et al.*'s (2006) phylogenetic analysis resolved all *S. madagascariensis* populations included here as a monophyletic clade with *S. inaequidens* paraphyletic and basal to this clade (Chapter 5). Once again, in the event of hybridization, different nuclear gene copies would exist, a problem that was not encountered in their study. Indeed, diploid and putative tetraploid native range populations shared 100% internal transcribed spacer regions sequence similarity.

The identification key of the *S. madagascariensis* complex described by Radford *et al.* (2000) suggested that *S. madagascariensis* and *S. inaequidens* should be differentiated based on chromosome counts ($n = 20$ and $n = 40$ respectively), and achene hairiness, with only *S. inaequidens* having complete mixogenic hair coverage. The validity of this achene morphological trait as a taxonomic informative character has been refuted by Le Roux *et al.* (2006) (Chapter 5). Additionally, based on this study's findings and those of Lafuma *et al.* (2003), I now argue that cytotype (diploid or tetraploid) is also inadequate to differentiate between *S. madagascariensis* and *S. inaequidens*.

Different cytotypes within *S. madagascariensis* could furthermore have implications for future exploration for biological control agents and the current biological control program aimed at controlling Hawaii infestations. Host-specificity is normally sought in potential sap-sucking, root- and stem-boring, larval, or seed-damaging biological control agents, and different levels of alkaloid gene expression due to different ploidy levels pose two important considerations. Firstly, higher concentrations of alkaloids due to gene duplication (polyploidization) might deter generalists while attracting specialists enemies (Mark Wright, University of Hawaii, personal communication). For example, iridoid glycosides are toxic to many generalist herbivores, but Niemenen *et al.* (2003) found that, under field conditions, individual plants of *Plantago lanceolata* with high iridoid glycoside concentrations were sigificantly more used for oviposition by the specialist lepidopteran, *Melithaea cinxia*, than plants with low concentrations. Similarly, Honda *et al.* (1997) found that pyrrolizidine alkaloids exerted significant stimulatory activities on the specialist herbivore of *Parsonsia laevigata,* a lepidopteran species, *Idea leuconoe*. Oviposition by *I. leuconoe* females relied on alkaloids as principle cues in recognizing *P. laevigata* host plants. In *S. madagascariensis*,, tetraploid-specific enemies might be particularly host-specific and thus effective against diploid populations. The effects of alkaloids are also expected to be lower due to lower levels of alkaloid gene expression, leading to lower defence against host-specific herbivores. On the other hand, these tetraploid-specific enemies may have evolved to respond to elevated levels of alkaloids as a cue to locate or to accept host plants for oviposition and/or feeding (phago-stimuli), in which case lower expression levels in diploids may lead to reduced host recognition and thus ineffective control. These

hypotheses remain largely untested but is worthy to persue and could contribute towards biological control of *S. madagascariensis* and biological control in general.

Genetic diversity among and within native and introduced populations

The polyploid nature of most native range populations precluded a direct comparison to introduced populations based on inferences requiring allele frequency data. The mean number of alleles was on average higher for native range populations than for introduced populations (12.95 and 9.62 respectively) despite smaller native range sample sizes. This observation is consistent with a reduction in gene diversity that often accompanies species introductions, but could also be a consequence of polyploidy, i.e. the opportunity to have more than two alleles per locus. In fact, overall Hawaii populations harbored 134 or 51 % of the total number alleles followed by South African populations with only 107 or 40 % of the total number of alleles found within and among populations. In addition, no transient excess in heterozygosity was observed in Hawaii populations, suggesting that these populations are not currently experiencing a genetic bottleneck. These results were supported by low population-level inbreeding coefficients (F_{IS}: -0.00216 - 0.06897, mean: 0.0064). Given the relative short time lapse since fireweed was first introduced to Hawaii (± 50 generations or less than 30 years), two scenarios can explain these results. First, the founding population(s) consisted of many individuals with sufficient genetic variation to overcome the effects of a severe bottleneck or alternatively, these invasive populations represent multiple independent introductions. Based on quantitative and genetic analysis it is thought that *Senecio madagascariensis* represents a secondary introduction from Australia (Chapter 5, Le

Roux *et al.* 2006, Gardner *et al.* 2006) that reached the Hawaiian Islands as contaminated animal feed. The diploid nature of Hawaii populations further supports this hypothesis as Australian fireweed is also diploid (Radford *et al.* 1995). Given the high genetic diversity observed in Hawaii populations of fireweed, the apparent lack of inbreeding and the differentiation among islands, this study indicates that multiple introductions to the Hawaiian Islands have most likely occurred. The extensive agricultural trade in place between Hawaii and Australia around the suspected time of initial fireweed introductions to the islands (early 1980's) would have furthermore favored multiple introductions. Trade figures indicate Australia as the third largest overall international exporter to the Hawaiian Islands during the late 1970's and early 1980's (State of Hawaii, Department of Planning and Economic Development) and that agricultural products, including animal fodder, encompassed ~ 30 % of these commodities (Stegmaier, 1980). No trade agreements were in place between Hawaii and any of the native range countries during this period. As a result of differences in the intensity of livestock farming among the Hawaiian Islands, animal fodder potentially contaminated with fireweed seeds was distributed disproportionally. Indeed, the heavily fireweed-infested Maui and Hawaii harbor 80 % of the total livestock farms in the islands (National Agricultural Statistics Service, USDA), and likely explain the restriction of fireweed to these two islands given the higher likelihood of imported animal fodder reaching them.

Several other lines of argumentation support the notion of multiple introductions. Fireweed was supposedly first introduced to the island of Hawaii from where it spread to Maui (Motooka *et al.* 1999, Gardner *et al.* 2006). However, mean observed heterozygosity was 5 % higher in Maui populations compared to Hawaii populations. A

secondary introduction to Maui from Hawaii would have been accompanied with a further decrease in heterozygosity, an observation not supported by this study's results. If anything, one Hawaii population was identified (SM1) that seemed to have been introduced in the opposite direction. The overall clustering of island populations into two genetic demes is maybe not surprising with the obvious dispersal boundary between them. However, separate introductions are the most parsimonious explanation for these genetic entities, as the ~ 30 years since introduction is unlikely to be an adequately long timeframe to for the observed genetic structure to accrue. In addition, fireweed was introduced to Hawaii in the early 1980's (Chaper 5, Le Roux *et al.* 2006) but was only observed for the first time in 1997 on Maui (Mohsen Ramadan, Hawaii Department of Agriculture, personal communication).

Populations SM63 (Kula, Maui) and SM941 (Waiohiwi falls, Maui) consistently showed higher genetic differentiation to all other populations and among each other, despite the relatively low overall differentiation among populations, and this may also be the result of additional introductions. And lastly, the recent discovery of fireweed on the windward side of Oahu near the Castle junction area is thought to represent a separate independent introduction. This population probably arose from roadside plantings of ground cover grass seeds from Australia contaminated with fireweed seeds (M. Ramadan, Hawaii Department of Agriculture, personal communication).

Genetic distances obtained from microsatellite data, in congruence with phylogenetic analysis (Chapter 5, Le Roux *et al.* 2006), also indicated South African east coast populations as the most likely geographic source(s) of Hawaii, and thus Australian, populations. Interpretation of genetic clustering from these data should be done with

caution as microsatellite data on this geographical scale are prone to exhibit characters identical by state but not descent (homoplasy) (Sunnucks 2000). However, the high number of different alleles included, higher allelic diversity in Hawaii, and the presence of a number of regional private alleles, provide confidence in the observed genetic clustering. South African east coast regions were also the only native range areas included in this study that, similar to Hawaii and Australia, harbored diploid populations (SM151 [Pietermaritzburg] and SM161 [Murray road]).

Spatial autocorrelation

A significant pattern of isolation by distance suggests that dispersal is restricted at the spatial scales investigated here and that the current spatial genetic structure of fireweed in Hawaii is consistent with a diffusive pattern of dispersal. Fireweed populations included here showed a nonrandom, clinal pattern of genetic structure at a scale of approximately 9 km. A dramatic decrease in spatial genetic correlation at scales between 2 and 3 km is most likely the result of a frequent dispersal and or pollen movement at this scale.

Despite the relative recentness of fireweed's introduction to Hawaii this species has established and is spreading over extensive areas, showing that dispersal, even though mainly diffusive, occurs rapidly. Similarly, Sindel and Michael (1988) reported that farmlands in New South Wales infested by fireweed increased exponentially since initial population establishment. Empirical studies have related basic plant traits such as seed mass to species distributions and changes in distributions (Kahmne and Poschlod, 2004, Ozinga *et al.* 2005). Preliminary studies indicate that the hairy pappus of fireweed may be

caught up in wind more easily than other anemochorous *Senecio* species (Sindel *et al.* 1998). This, coupled with seed production of up to 18,000 seeds per individual and high frequency of strong trade winds in the Hawaiian Islands, contributes to the rapid expansion of fireweed populations in the islands. Rare events such as human-, animal- and vehicle-aided dispersal could lead to the establishments of outlying populations (long-distance jumps) (Sindel *et al.* 1998) that can act as new pioneering sources for diffusive spread. The southern Hawaii population, SM1, appeared to be the result of such a rare long-distance dispersal event that originated from Maui. Such accidental long-distance jumps coupled with the primary diffusive dispersal mode of fireweed have implications relevant to its management. Eradication of small pioneering populations in front of a continuous invasion front is reasonably achievable, and can be the most effective means of slowing or even stopping spread (Moody and Mack, 1988).

In conclusion, despite the added complication of mixed ploidy levels to an already-problematic taxonomic group, this study adds supporting evidence to a previous report (Chapter5, Le Roux *et al.* 2006) that the native origin of Hawaiian *S. madagascariensis* is eastern South Africa. This first report of different ploidy levels within fireweed further supports a re-evaluation of the whole complex's alpha taxonomy (Radford *et al.* 2000). Hawaiian infestations most likely involved multiple introductions as contaminated animal feed from Australia. Comparative studies at the University of Adelaide, Australia, are currently underway to investigate population genetic similarities between Hawaii and Australian fireweed and should contribute towards a more comprehensive understanding of the invasion ecology of fireweed (Peter Prentis, University of Adelaide, personal communication).

Table 7.1. Location and genetic diversity data for *S. madagascariensis* populations included in this study.

Location information					Genetic diversity				
ID	Region	Locality	Latitude/Longitude	Ploidy level*	*N*	*A*	H_O	H_E	F_{IS}
Hawaii									
SM1	Hawaii	Kahuku ranch	N 19°11.117'/ W 155°67.596'	DIP	31	10.875	0.48	0.79	-0.00108
SM32	Maui	Haleakela	N20° 84.374'/W156°' 33.806'	DIP	31	11.000	0.48	0.81	-0.00216
SM63	Maui	Kula	N20° 84.435'/W156°' 33.890'	DIP	29	7.250	0.41	0.74	0.000
SM92	Maui	Lower Kula	N20° 47.961'/W156°19.624'	DIP	30	10.875	0.53	0.83	0.000
SM122	Maui	Haleakela	N20° 51.449'/W156° 21.570'	DIP	29	12.875	0.52	0.83	0.000
SM881	Maui	Makawao	N20° 51.173'/ W156° 18.777'	DIP	7	4.750	0.47	0.81	0.000
SM911	Maui	Kokomo Rd.	N20° 52.492'/W156°18.894'	DIP	24	7.750	0.52	0.76	0.000
SM941	Maui	Waiohiwi falls	N20° 50.233' /W156° 16.802'	DIP	12	5.250	0.40	0.79	0.000
SM1001	Maui	Baldwin & Halimaile	N20° 52.453'/ W156° 19.809'	DIP	17	6.500	0.40	0.81	0.000
SM281	Hawaii	Makahalau	N20° 00.366'/W155° 35.632'	DIP	28	9.750	0.41	0.79	0.000
SM311	Hawaii	Mana Rd	N19° 59.121'/W155° 33.04'	DIP	30	10.000	0.42	0.77	-0.00058
SM341	Hawaii	Hanaipoe	N19° 57.602'/ W155° 30.857'	DIP	29	9.750	0.40	0.79	0.000
SM371	Hawaii	Keanakolu Rd	N19° 56.758'/ W155° 29.304'	DIP	30	10.250	0.46	0.80	0.000
SM401	Hawaii	Koholalele gulch	N19° 56.787'/ W155° 24.987'	DIP	30	11.125	0.46	0.79	0.000
SM431	Hawaii	Parker ranch	N19° 55.389'/ W155° 20.678'	DIP	30	7.375	0.34	0.72	0.000
SM461	Hawaii	Pu'u Lahohinu	N19° 54.436'/W155° 20.577'	DIP	29	8.875	0.37	0.74	0.06897
SM491	Hawaii	Laupahoehoe forest reserve	N19° 56.109'/ W155° 23.598'	DIP	30	8.625	0.41	0.79	0.000
SM521	Hawaii	Pu'u Alau'awa	N19° 46.665'/ W155° 54.885'	DIP	28	9.250	0.40	0.77	0.03571
SM551	Hawaii	Mamalahoa Hwy	N19° 50.502'/W155° 45.496'	DIP	29	10.250	0.46	0.80	0.000
SM582	Hawaii	Saddle Road Junction	N19° 57.468' /W155° 40.845'	DIP	30	10.000	0.43	0.81	0.000

Table 7.1. (Continued) Location and genetic diversity data for *S. madagascariensis* populations included in this study.

Location information					Genetic diversity				
ID	Region	Locality	Latitude/Longitude	Ploidy level*	*N*	*A*	H_O	H_E	F_{IS}
SM611	Hawaii	Waimea	N20° 02.257' W155° 42.716'	DIP	30	10.000	0.41	0.81	0.000
SM641	Hawaii	Pu'u Hue ranch	N20° 09.254' /W155° 48.722'	DIP	30	10.625	0.45	0.84	0.000
SM671	Hawaii	Waiki'I ranch	N19° 52.387'/ W155° 39.572'	DIP	25	9.250	0.4	0.79	0.000
SM701	Hawaii	Pu'u La'au	N19° 47.649'/ W155° 37.588'	DIP	30	9.125	0.42	0.78	0.06667
SM731	Hawaii	Bradshaw Airfield	N19° 45.339'/ W155° 32.829'	DIP	30	9.625	0.41	0.76	0.000
SM761	Hawaii	Humuula Trail	N19° 47.729'/ W155° 27.390'	DIP	16	6.250	0.38	0.76	0.000
South Africa									
SM151	KwaZulu-Natal	Pietermaritzburg	S29° 34.015'/W30° 19.059'	DIP	10	13.25	0.57	0.85	-----
SM161	KwaZulu-Natal	Murray Road	S29° 39.409'/E30° 23.751'	DIP	10	13.625	0.68	0.82	-----
SM182	KwaZulu-Natal	Umhlanga	S29° 42.812'/E31° 04.729'	TET	10	15.000	-----	-----	-----
SM202	KwaZulu-Natal	Mtunzini	S28° 58.246'/E31° 45.369'	TET	10	8.250	-----	-----	-----
SM211	KwaZulu-Natal	Tinely Manor	S29° 27.129'/E31° 17.161'	TET	10	8.125	-----	-----	-----
SM221	KwaZulu-Natal	Camperdown	S29° 43.867'/E30° 33.748'	TET	10	17.75	-----	-----	-----
SM231	KwaZulu-Natal	Durban city	S29° 51.570'/E31° 2.326'	TET	10	16.875	-----	-----	-----
Madagascar									
SM241	Saint Luce	Azafady	S24° 57.992'/E47° 5.437'	TET	10	11.500	-----	-----	-----
SM261	Fort Dauphin	Fort Dauphin airport	S25° 3.05'/E46° 56.73'	TET	10	12.000	-----	-----	-----
SM271	Tulear	Belembika village	S23° 19.706'/E43° 41.288'	TET	10	12.125	-----	-----	-----
Swaziland									
SM251	Motshane	Motshane	S26° 14.508'/E31° 3.179'	TET	10	14.000	-----	-----	-----

* DIP = Diploid, TET = Tetraploid

Table 7.2. Concentrations of forward and reverse primers in the two 10 X primer mixes used in multiplex PCR reactions.

Multiplex 1		Multiplex 2	
Primers[1]	cons.[2]	Primers[1]	cons.[2]
Se-208-NED	2.3	Se-136-HEX	0.6
Se-208-R	2.3	Se-136-R	1.8
Se-116-NED	1.2	Se-138-HEX	0.6
Se-116-R	1.2	Se-138-R	1.5
Se-194-FAM	2.4	Se-76-NED	0.6
Se-194-R	2.4	Se-76-R	0.2
Se-176(a)-FAM	1.2	Se-220-NED	1.2
Se-176-R	1.2	Se-220-R	0.8
Se-206-HEX*	1.1		
Se-206-R*	2.4		
Se-46-HEX*	2.4		
Se-46-R*	2.3		

[1] Fluorescent labels are included in forward primer names, R = reverse primers. [2] Concentration in μM. * Loci excluded from this study due to amplification problems.

Table 7.3. Pairwise distribution of 263 alleles among native and introduced populations of *Senecio madagascariensis*. Diagonal represents number of regional private alleles (green).

	Hawaii	South Africa	Swaziland	Madagascar
Hawaii	57			
South Africa	65	27		
Swaziland	5	7	1	
Madagascar	7	8	1	14

Table 7.4. Nei's unbiased nuclear genetic distances between native and a subset of the introduced populations of *Senecio madagascariensis* included in this study.

	SM151	SM161	SM182	SM202	SM211	SM221	SM231	SM241	SM251	SM261	SM271	SM281	SM311	SM1	M32
SM151	0.0000														
SM161	0.0034	0.0000													
SM182	0.0035	0.0028	0.0000												
SM202	0.0049	0.0040	0.0039	0.0000											
SM211	0.0045	0.0071	0.0056	0.0040	0.0000										
SM221	0.0062	0.0070	0.0066	0.0073	0.0076	0.0000									
SM231	0.0042	0.0054	0.0043	0.0049	0.0051	0.0054	0.0000								
SM241	0.0065	0.0075	0.0064	0.0056	0.0059	0.0085	0.0065	0.0000							
SM251	0.0034	0.0038	0.0047	0.0057	0.0056	0.0068	0.0051	0.0079	0.0000						
SM261	0.0100	0.0097	0.0095	0.0094	0.0110	0.0109	0.0115	0.0051	0.0109	0.0000					
SM271	0.0090	0.0089	0.0091	0.0083	0.0098	0.0097	0.0097	0.0049	0.0103	0.0042	0.0000				
SM281	0.0047	0.0037	0.0030	0.0035	0.0052	0.0063	0.0052	0.0054	0.0058	0.0064	0.0060	0.0000			
SM311	0.0050	0.0040	0.0032	0.0034	0.0053	0.0060	0.0051	0.0052	0.0058	0.0061	0.0055	0.0005	0.0000		
SM1	0.0056	0.0035	0.0034	0.0038	0.0068	0.0077	0.0059	0.0073	0.0066	0.0090	0.0086	0.0018	0.0018	0.0000	
SM32	0.0067	0.0052	0.0042	0.0039	0.0065	0.0062	0.0065	0.0058	0.0077	0.0066	0.0061	0.0017	0.0015	0.0020	0.0000

Table 7.5. Genetic structure in *Senecio madagascariensis* in Hawaii given as pairwise F_{ST} values between populations.

	SM1	SM32	SM63	SM92	SM122	SM881	SM911	SM941	SM1001	SM281	SM311	SM341	SM371
SM32	0.037	0											
SM63	0.082	0.089	0										
SM92	0.027	0.022	0.075	0									
SM122	0.025	0.01*	0.086	0.013*	0								
SM881	0.058	0.032*	0.113	0.05	0.043	0							
SM911	0.086	0.074	0.18	0.073	0.063	0.107	0						
SM941	0.089	0.082	0.156	0.087	0.074	0.077*	0.092	0					
SM1001	0.09	0.066	0.145	0.07	0.053	0.065	0.018	0.068	0				
SM281	0.047	0.039	0.136	0.047	0.028	0.058	0.067	0.104	0.065	0			
SM311	0.061	0.044	0.136	0.056	0.031	0.05	0.058	0.099	0.045	0.013*	0		
SM341	0.035	0.041	0.119	0.036	0.02	0.072	0.073	0.107	0.077	0.01*	0.021	0	
SM371	0.044	0.03	0.115	0.034	0.017	0.064	0.069	0.093	0.069	0.013*	0.016*	0.004*	0
SM401	0.037	0.033	0.116	0.032	0.019	0.054	0.073	0.09	0.065	0.014*	0.019	0.004*	0.006*
SM431	0.095	0.081	0.174	0.074	0.072	0.092	0.114	0.138	0.098	0.048	0.046	0.04	0.043
SM461	0.106	0.063	0.171	0.079	0.063	0.066	0.101	0.137	0.067	0.046	0.034	0.057	0.062
SM582	0.031	0.03	0.107	0.035	0.015	0.035	0.065	0.093	0.057	0.009*	0.015	0.009*	0.009*
SM611	0.056	0.038	0.13	0.037	0.025	0.064	0.069	0.101	0.064	0.019	0.022	0.019	0.019
SM641	0.047	0.033	0.115	0.026	0.026	0.053	0.076	0.106	0.069	0.026	0.035	0.019	0.016
SM671	0.059	0.06	0.152	0.052	0.044	0.095	0.076	0.101	0.079	0.049	0.046	0.035	0.029
SM761	0.066	0.061	0.17	0.063	0.045	0.074	0.108	0.107	0.01	0.045	0.05	0.048	0.047
SM701	0.053	0.052	0.149	0.051	0.041	0.076	0.081	0.104	0.082	0.042	0.039	0.025	0.019
SM491	0.07	0.056	0.154	0.058	0.041	0.07	0.085	0.11	0.074	0.033	0.03	0.031	0.031
SM521	0.06	0.042	0.132	0.047	0.035	0.052	0.073	0.101	0.064	0.017*	0.006*	0.017*	0.011
SM551	0.04	0.045	0.122	0.038	0.025	0.064	0.071	0.108	0.07	0.021	0.018	0.014*	0.009*
SM731	0.05	0.052	0.144	0.046	0.037	0.086	0.07	0.113	0.081	0.039	0.042	0.027	0.024

Table 7.5. (Continued) Genetic structure in *Senecio madagascariensis* in Hawaii given as pairwise F_{ST} values between populations.

	SM401	SM431	SM461	SM582	SM611	SM641	SM671	SM761	SM701	SM491	SM521	SM551	SM731
SM431	0.037	0											
SM461	0.049	0.067	0										
SM582	0.005	0.044	0.041	0									
SM611	0.01	0.055	0.045	0.022	0								
SM641	0.015	0.052	0.053	0.014*	0.017*	0							
SM671	0.032	0.065	0.094	0.038	0.044	0.044	0						
SM761	0.04	0.078	0.082	0.037	0.056	0.052	0.036	0					
SM701	0.023	0.055	0.079	0.028	0.038	0.033	0.0002*	0.035	0				
SM491	0.021	0.044	0.056	0.025	0.031	0.029	0.04	0.052	0.039	0			
SM521	0.011	0.027	0.039	0.009*	0.022	0.028	0.03	0.045	0.022	0.022	0		
SM551	0.016	0.049	0.069	0.001*	0.022	0.017*	0.029	0.048	0.024	0.027	0.016	0	
SM731	0.021	0.064	0.088	0.029	0.035	0.04	0.008*	0.052	0.01*	0.043	0.028	0.02	0

*No significant genetic differentiation ($P > 0.05$)

Table 7.6. Results of hierarchical AMOVA comparing genetic variation within populations, among populations, and among islands of invasive fireweed. Significance was tested against a null distribution of 10 000 random permutations.

Source of variation	d.f.	Sum of squares	Fixation index	Percent variation	*P*-value
Among Islands	1	45.131	$\Phi_{ST} = 0.06276$	1.99	< 0.001
Among populations within islands	24	253.654	$\Phi_{SC} = 0.04378$	4.29	< 0.001
Within populations	1362	4185.770	$\Phi_{CT} = 0.01985$	93.72	< 0.001

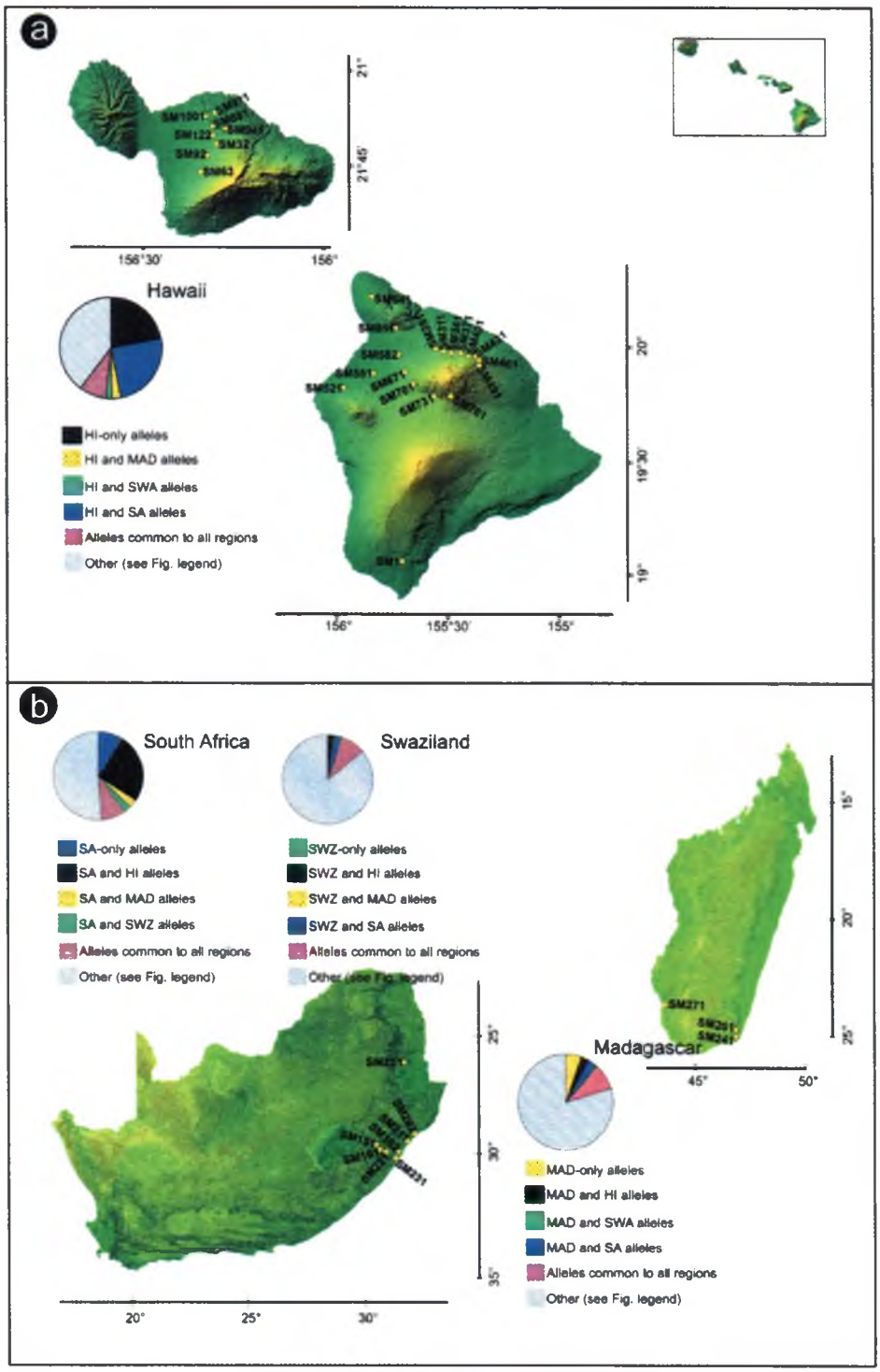


Figure 7.1. Maps of regional areas included in this study showing the location of all populations. For each region (Hawaii, South Africa, Swaziland, Madagascar) the proportion of common alleles and those shared with individual regions are shown. The proportion referred to as 'Other' represents all alleles that are absent from the specific region under consideration.

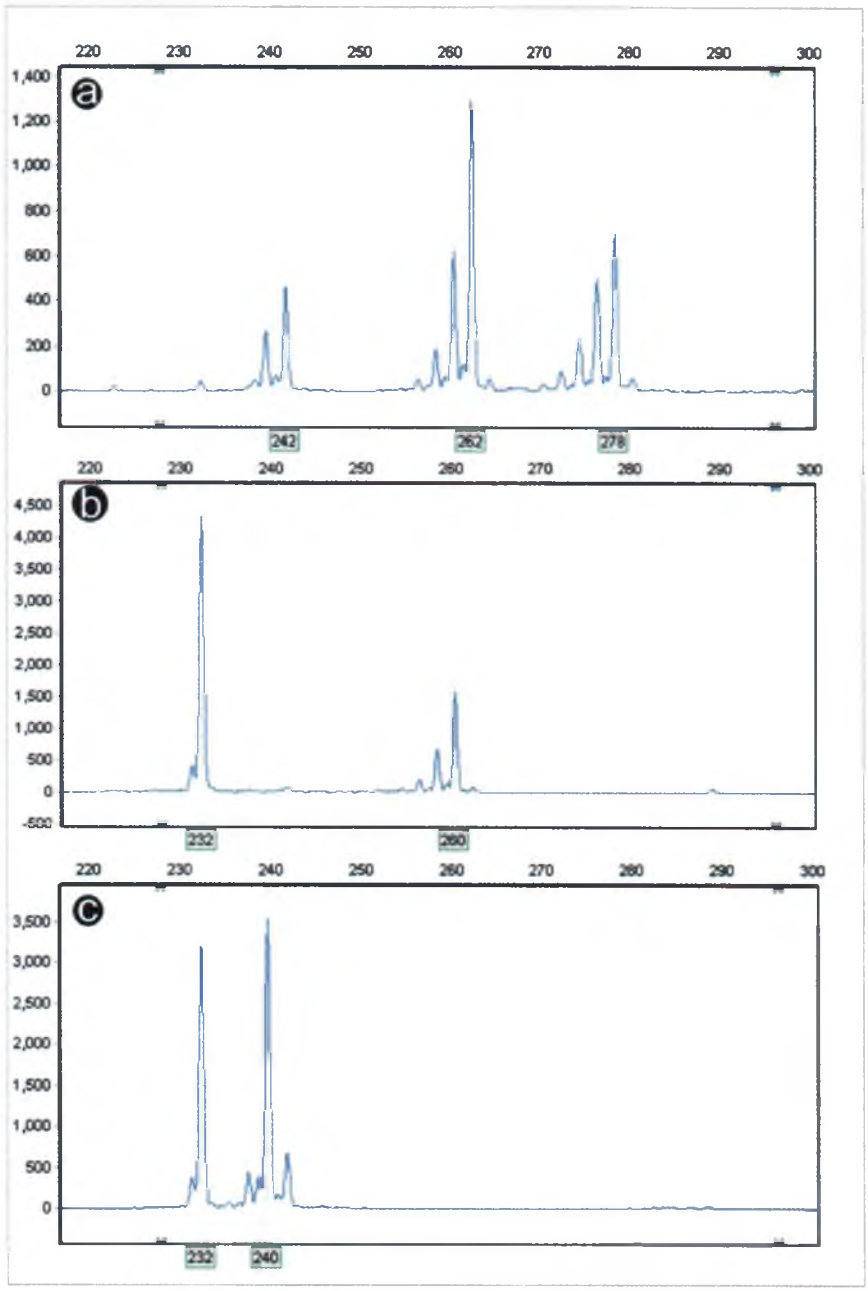


Figure 7.2. Electropherograms depicting different peak intensities for different allele scenarios for tetraploid fireweed individuals. Here, locus Se-176(a) is shown for individuals having (a) three alleles [one double copy (262 bp) and two single copy (242 bp and 278 bp)], (b) two alleles [one single copy (260 bp) and one tri-allelic (232 bp)] and (c) two alleles both with two copies. The relative intensity is given on the Y-axis.

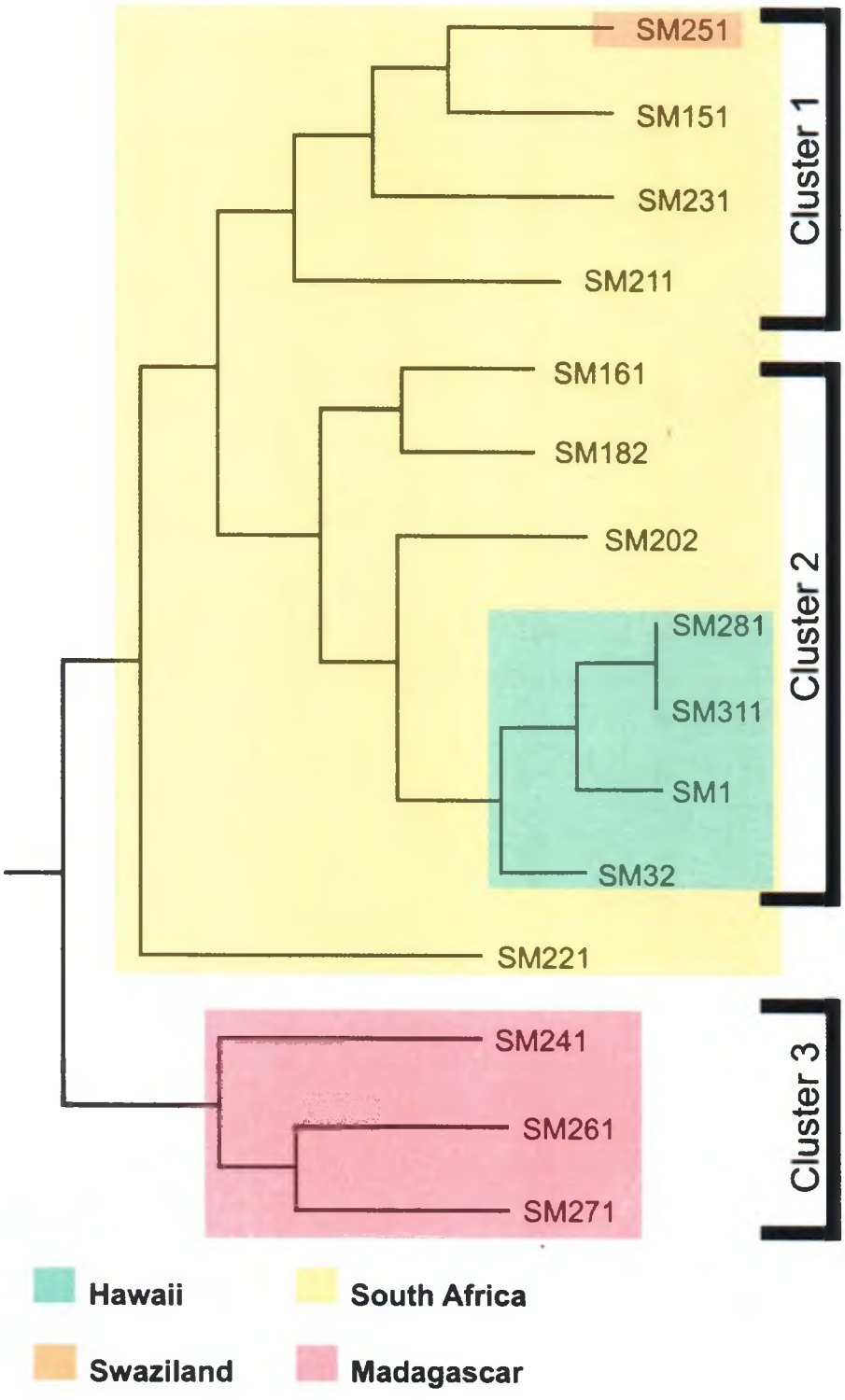


Figure 7.3. A dendrogram based on Nei's (1978) unbiased genetic distances constructed by the using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis.

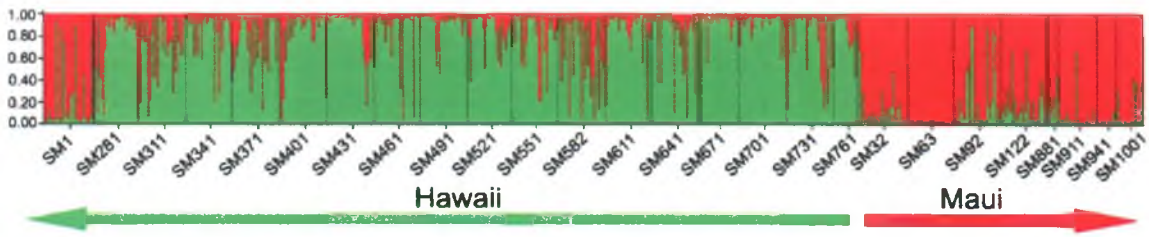


Figure 7.4. Population structure inferred by Bayesian assignment of 694 Hawaii individuals of *Senecio madagascariensis* shown as individual membership coefficients in the STRUCTURE-identified genetic demes. Fireweed populations in the archipelago can be assigned to two geographical genetic demes that corresponded to two islands (Maui and Hawaii), each represented by a cluster of populations. Population SM1 was the only population not clustering within the 'right' deme.

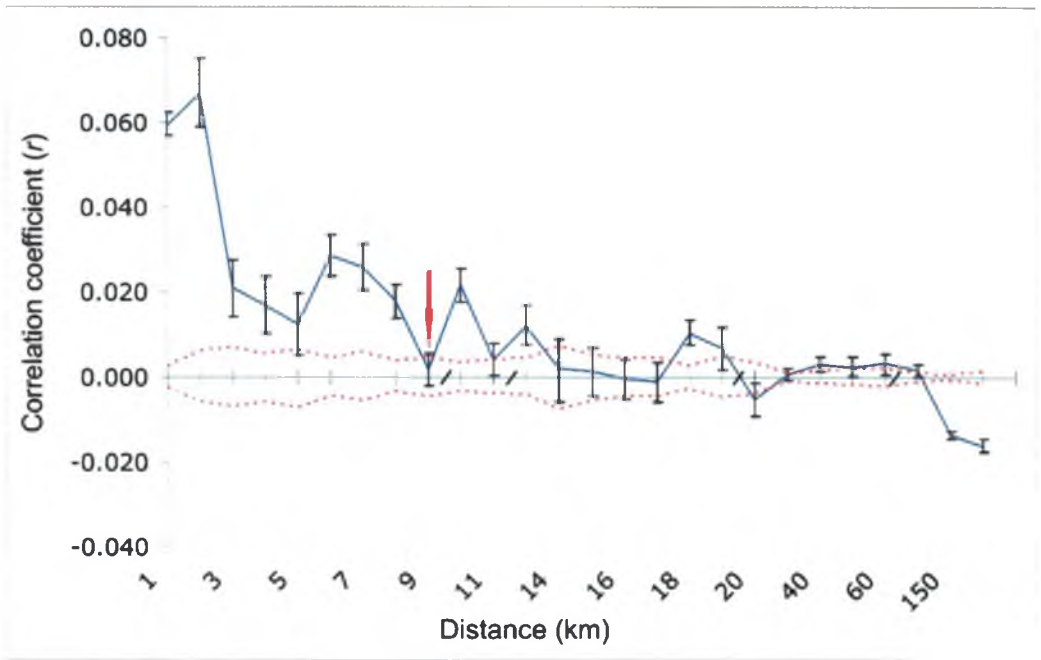


Figure 7.5. Autocorrelogram of the spatial coefficient, *r*, as a function of distance. The null hypothesis of no spatial genetic structure is bounded by the 95% confidence intervals (dashed lines) derived from randomly permuting individual genotypes over geographical locations. Error bars for mean *r* at each distance class were estimated with bootstrapping. Significant spatial genetic autocorrelation can be assumed when mean *r* exceeds the 95% CI and the error bars for each distance class do not intercept the *X*-axis of $r = 0$. The distance scale where positive spatial autocorrelation ceases is indicated by the arrow.

SECTION 4 – *MICONIA CALVESCENS* DC. (VELVET TREE)

CHAPTER 8: ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE MARKERS FROM VELVET TREE, *MICONIA CALVESCENS* DC. (MELASTOMATACEAE)

Abstract

Nine polymorphic microsatellite loci were isolated and characterized from invasive velvet tree (*Miconia calvescens* DC.) a serious forest invader in tropical oceanic islands in the North and South Pacific. These loci provided markers with polymorphism of three to ten alleles per locus within 95 individuals collected from the island of Hawaii. The expected and observed heterozygosities ranged from 0.0367 to 0.5053 and 0.0370 to 0.2473 respectively. These markers should be useful to study dispersal and the importance of genetic diversity in invasion success of this species.

Introduction

Miconia calvescens DC. (velvet tree; miconia) is a small tree belonging to the Melastomataceae family and is native to forests in Neotropical Central and South America. As a popular ornamental it has been introduced outside its native range and is now considered one of the worst invaders of especially tropical insular habitats such as Hawaii, the Society and the Marquesas Islands, and New Caledonia (Gagné *et al.* 1992, Meyer and Florence 1996, Meyer 1998). In Tahiti velvet tree has spread over two thirds of the island, forming dense monotypic stands over 25 % of the island (± 260 km^2) (Thomas 1997), that significantly endanger native species (Meyer and Florence, 1996). Even though velvet tree is considered one of the worst noxious weeds in Hawaii, the

extent of spread and damage is less than in Tahiti. In Tahiti *Miconia calvescens* infestations can be found at wider elevational ranges (10–1300 m) than Hawaiian infestations (10-760m) (Meyer 1998a). Furthermore, in the Hawaiian Islands, monotypic stands similar in density to those in Tahiti, are found in substantially smaller areas, even on the most severely infested islands (Maui, ± 5 km^2 [Medeiros and Loope 1997] and Hawaii, ± 12.14 km^2 [Kaiser 2006]). Ecological and climatological similarities between Tahiti and the Hawaiian Islands raised the question whether Hawaii could potentially experience invasions to the extent that Tahiti already has.

The attributes contributing to the differential success of *Miconia calvescens* in these North and South Pacific islands remain unknown. Whether this is the result of differences in introduction times (the velvet tree was first introduced in Tahiti in 1937 and in Hawaii in the early 60's [Meyer 1998]), in soil or climate conditions, management practices, or in limited adaptive potential due to severe bottlenecks, remains undetermined. Here, nine microsatellite markers were developed from *M. calvescens* to test for differences in allelic diversity within and among these differentially invaded areas.

Materials and Methods

Total DNA was extracted with the DNeasy Plant mini kit (Qiagen) from 40 to 50 mg of fresh leaf material. Sequences for 81 di- and trinucleotide repeat microsatellite loci were identified by Genetic Identification Services (GIS, Chatsworth, CA), using an enrichment protocol similar to Edwards *et al.* (1996). Extracted genomic DNA was digested with a cocktail of seven blunt-end cutting enzymes (*Rsa*I, *Hae*III, *Bsr*B 1, *Pvu*II,

*Stu*I, *Sca*I, *Eco*R V) and the resulting fragments ligated onto oligonucleotide linkers. Linker-ligated digests were hybridized to biotinylated oligoprobes $(AAC)_8$, $(ATG)_8$, $(GA)_{15}$ and $(CA)_{15}$ and subsequent probe-bound DNA extracted using streptavidin-coated magnetic beads. Following PCR amplification, PCR products of microsatellite-enriched DNA were purified and digested with *Hind*III. Digested fragments were ligated into pUC19 followed by transformation into competent *Escherichia coli* DH5α (Invitrogen). Recombinant clones were randomly chosen for sequencing. The inserts of putative microsatellite-containing colonies were amplified with universal M13 plasmid primers, purified and subjected to sequencing using the ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and an automated sequencer (ABI PRISM 377XL DNA sequencer, PE Applied Biosystems).

PCR primers were designed for 59 sequences based on microsatellite purity (uninterrupted repeats) and available flanking regions, using the programs PRIMER3 (Rozen and Skaletsky 1996) or FASTPCR (Kalendar 2004). Amplification was tested and optimized with a gradient PCR at 50 – 62 °C range of annealing temperatures. Numerous PCR conditions and enzyme systems failed to produce any PCR products. Indeed, using 11 different DNA extraction protocols and various enzyme systems failed to produce PCR amplified fragments from developed primers (M. Todd, Genetic Identification Services Inc., personal communication). However, good amplification of individual loci was obtained using the Qiagen multiplex PCR kit (Qiagen). This system utilizes a PCR additive (Q-Solution) that changes the melting behavior of DNA to help overcome amplification problems associated with extensive secondary DNA structure and/or GC-richness (≥ 65 % GC content) of template DNA. Using the Qiagen multiplex PCR kit,

each 10 μL reaction contained 5 μL 2X Qiagen Multiplex PCR Mastermix [HotStarTaq DNA Polymerase; Qiagen Multiplex PCR Buffer (6 mM $MgCl_2$, pH 8.7); dNTP mix], 1 μL Q-solution [PCR additive(Qiagen)], 12 pmol of each primer and approximately 5 ng total genomic DNA. Thirty-one loci were successfully amplified. To detect polymorphism at each locus PCR products from 12 different velvet tree individuals from Honokua Bay, Hawaii, were run on an Agilent 2100 Bioanalyser analysis LabChip (Agilent Technologies, Inc.) (Banerjea *et al.* 2003). One of the primers of loci that showed polymorphism was fluorescently labeled with either HEX, NED or 6-FAM (Integrated DNA Technologies, Inc.) and re-optimized as described above. PCR was conducted on an MJ Research PTC 100 cycler with a thermocycle of: initial denaturation of 95 °C for 15 min; 35 cycles at 94 °C for 60 s, locus-specific annealing temperature (see Table 1 and Appendix) for 60 s, elongation at 72 °C for 90 s; and final extension at 72 °C for 12 min. Polymorphisms were screened using an ABI PRISM 377XL DNA sequencer (PE Applied Biosystems) and PCR products sized relative to a molecular size marker (LIZ500, PE Applied Biosystems). DNA fragments were analyzed the GeneMarker version 1.4 program (SoftGenetics, LLC). For each locus expected and observed heterozygosities were inferred, significant deviation from Hardy-Weinberg equilibrium (HWE) and the existence of genotypic linkage disequilibrium (LD) using the GENEPOP version 3.4 program (Raymond and Rousset 1995).

Results and Discussion

Table 8.1 summarizes the characteristics of the nine primer pairs of polymorphic loci developed from velvet tree. Allelic variation at the nine microsatellite loci was

assessed in 95 individuals of *M.calvescens* collected from the Honokua Bay area in Hawaii. The number alleles detected at the nine loci was generally low and ranged from three to ten. For most loci one allele was fixed in almost all individuals while alternative alleles at the same locus were less common and restricted to very few individuals. The expected and observed heterozygosities ranged from 0.0367 to 0.5053 and 0.0370 to 0.2473. Except for locus D118, all other loci had heterozygosities conforming to those expected under HWE. The presence of null alleles is most likely responsible for this deficit in heterozygosity as amplification of D118 alleles failed for many individuals that successfully amplified alleles at all other loci. For those loci that were genotyped and scored no significant LD was found.

The polymorphic loci identified in this study will be utilized to assess the genetic diversity and structure within and among northern and southern Pacific islands invaded by *M. calvescens*.

Table 8.1. Characteristics of nine polymorphic microsatellite markers isolated from *Miconia calvescens*.

Locus	Repeat motif	Primer sequence (5'-3')	T_a (°C)	No. of alleles	Allele size range (bp)	H_O	H_E	GenBank Accession no.
B2	$(GA)_{12}$	F: NED-GTCGCGTTTCCAGAATACTG R: CTACCACCGTGCAAAGAATC	56	7	226-264	0.2473	0.2532	EF595210
B9	$(CT)_{16}$	F: FAM-TTCCCTTATGCGACGAGTAC R: GGTCCATTCACACCACAAAC	60	10	226-304	0.1647	0.1685	EF595213
B102(a)	$(CT)_{14}$	F: NED-GGTGCCGAAGCATTGCCTG R: AGCCACGATGAGTCTGCTG	60	4	363-379	0.1034	0.1003	EF595216
B109	$(CT)_{14}$	F: HEX-GCCACCTATCCGAACATCG R: AGGCTGTGCCGCCAACGAC	62	5	315-363	0.0423	0.0693	EF595222
B117	$(GA)_{17}$	F: HEX-TTGGTGTCCCTCTTGCTCC R: ACAAGCTCTTTACTGTGGTTTCC	55	5	322-410	0.0435	0.5053	EF595225
C103	$(GTT)_6$	F: HEX-AGAAGAACGAGGCAAACTGC R: TCCTCCCGCACAAGCCCAG	56	5	200-251	0.0568	0.0561	EF595226
D101	$(GAT)_6$	F: FAM-TGCTATCGCCGAACCACTG R: GTTGGATTCGGACACCATCG	64	3	432-444	0.0370	0.0367	EF595233
D114	$(CAT)_5$	F: NED-TGGCTTTGATTACGATTATCTG R: GTTAGGTTAGCAGGTGATTTG	60	4	201-248	0.2308	0.2076	EF595234
D118*	$(CT)_5$	F: HEX-GGATCTGGCAATTTATTTC R: TGAAAGAGGGAGAAGTAGC	55	7	204-244	0.1053	0.1029	EF595235

* Significant deficit of heterozygotes from expected under HWE ($P < 0.01$).

CHAPTER 9: GENETIC DIVERSITY AND STRUCTURE OF INVASIVE VELVET TREE (*MICONIA CALVESCENS* DC.) ON ISLANDS THROUGHOUT THE PACIFIC OCEAN

Abstract

Conventional theory suggests that high genetic diversity is a determinant of success in invasive species, as the potential to adapt to new environments is increased and inbreeding is reduced. *Miconia calvescens* DC. (velvet tree) is one of the most destructive invaders in insular habitats throughout the Pacific. Northern and southern Pacific islands (Hawaiian, Society, Marquesas Islands and New Caledonia) are currently experiencing differential impacts from velvet tree invasions. Climatological similarities between some of these archipelagos (Hawaiian and Society Islands) suggest that these differences could reflect differences in introduction times (lag phases), differences in genetic diversity (adaptive ability) or introductions of different pre-adapted genotypes. This study, using microsatellite and highly variable inter-simple sequence repeat markers(ISSRs), characterized genetic diversity among and within differentially invaded areas to determine the role of genetic diversity in invasiveness. All markers showed low genetic differentiation among velvet tree populations from northern and southern Pacific hemispheres (3 %, microsatellite; 0 %, ISSR), indicative of similar geographic sources for both hemispheres and small founding populations. Bayesian and frequency-based analysis also failed to support geographic structure, confirming considerable low genetic differentiation throughout the Pacific. Both dominant and co-dominant marker data showed that velvet tree populations throughout the Pacific are currently undergoing

severe bottlenecks and high levels of inbreeding ($f = 0.91$ and $F_{IS} = 0.27$ respectively). Pre-adapted traits such as physiological responses to light and water, life history (reproductive), dispersal capability, and morphological adaptations that have evolved under dense, shaded, native range conditions appear to give velvet tree a competitive advantage in the less saturated forest environments characteristic of Pacific islands. The differential impact and severity of infestations among Pacific Islands are most likely the result of different control strategies applied to infestations and differences in introduction times, i.e. a lag phase. The implications of these findings towards management of velvet tree are discussed.

Introduction

Introduced, alien species that persist and spread in their new environment(s) offer ideal situations to investigate such basic evolutionary processes as drift, gene flow, migration and local adaptation (Sakai *et al.* 2001). New environment(s) present unique conditions to which introduced species normally may have not been exposed historically, in many cases representing sub-adapted environments that will exert strong selection on founders. Founding populations typically introduce only a small fraction of the available genetic variation from native gene pools, undergoing, in most cases, severe bottlenecks, likely resulting in reduced fitness. Genetically depauperate populations without pre-adapted histories can only persist if ecological flexibility (plasticity) allows genetic assimilation or if post-drift allele frequencies allow for local adaptation to occur. While many introductions fail to persist, others experience a so-called lag phase; when species are persisting but not spreading rapidly in the new environment (Sakai *et al.* 2001). This

lag phase is thought to partially represent the time lapse that results from the accumulation of sufficient genetic variation and/or from recombination of existing genetic variation to allow local adaptation to occur.

Population genetics theory predicts that high genetic diversity predisposes invasive populations to success at establishing and persisting in novel habitats, reducing such lag phases. A correlation between higher genetic diversities and invasion success has been documented for many species, e.g. *Ambrosia artemisiifolia* introduced to France (Genton *et al.* 2005), *Cirsium arvense* (Slotta *et al.* 2006) and *Tamarix ramosissima* (Sexton *et al.* 2002) introduced to the US. Furthermore, other studies have shown how post-introduction adaptation for phenotypic traits among invasive populations contributed towards their invasiveness (e.g. Huey *et al.* 2000, Maron *et al.* 2004).

In contrast to the to the population genetics dogma, many successful invaders represent bottlenecked populations that typically have low genetic diversity, low evolutionary potential and perhaps low reproductive fitness (Frankham 2005). Such species' success in new environments has been attributed to wide environmental tolerance (Parker *et al.* 2003), escape from natural enemies (Wolfe 2002), reproductive assurance (Rambuda and Johnson 2004) and/or the presence of pre-adapted genotype(s) (Hurka *et al.* 2003). For example, high levels of phenotypic plasticity can allow species to maintain high fitness levels in new environments without the need of local adaptation and thus genetic diversity (Price *et al.* 2003). Poulin *et al.* (2005) showed that highly invasive fountaingrass (*Pennisetum setaceum*) appeared monoclonal throughout its North American invasive range. This was also true on an intercontinental scale, and phenotypic plasticity is very likely the sole mechanism driving this species' invasive success under

different environmental conditions (Chapter 2, Le Roux *et al.* 2007). Furthermore, a strict apomictic breeding system preserves the successful fountaingrass genotype and renders breeding assurance to this species. Indeed, apomicts are frequently found to be highly tolerant and successful invaders (Rambuda and Johnson 2004) due to the potential for a single propagule to colonize and spread into new environments (Baker, 1967) and for frequently exhibiting broad environmental tolerances, conforming to "general-purpose-genotypes" (Baker, 1965).

Even native species may only reach local levels of fitness and never reach optima due to the constraints of limited genetic variation on the outcomes of local adaptation. On the other hand, it is also possible for some organisms to posses a superior, pre-adapted, combination of attributes for their introduced environment(s) by sheer chance alone.

Given the arguments above, a generalization about the importance of genetic diversity during biological invasions is clearly lacking. Adding to the lack of data is the difficulty to interpret molecular genetic variation in the context of adaptive potential (McKay and Latta 2002). Despite a strong correlation between adaptive quantitative trait variation and variation observed for neutral molecular markers (Merilä and Crnokrak 2001), the relationship is not always clear and interpretation should be done with caution.

The purpose of this study was to investigated whether a correlation exists between neutral genetic variation and the invasion success of a plant invader throughout island habitats in the Pacific. *Miconia calvescens* DC. (velvet tree [Melastomataceae]) has been introduced and is an aggressive invader in climatologically similar insular habitats throughout the Pacific. Velvet tree was introduced as a popular ornamental for its

attractive foliage, and is now considered one of the most destructive invaders in insular tropical forest habitats (Gagné *et al.* 1992, Meyer 1996).

In the North Pacific velvet tree invasions have severely impacted lowland rainforests in the Hawaiian Islands (Medeiros *et al.* 1997). Velvet tree was introduced to Hawaii between the late 1960s and early 1980s and since has established and is spreading mainly on the two islands of Maui and Hawaii. Hawaiian infestations are considered "moderate" in comparison to invasions in the South Pacific. Among the Society Islands, Tahiti is the worst hit by this invader, where velvet tree introductions preceded Hawaiian introductions by about 23 years (1937) (Meyer, 1996, Medeiros *et al.* 1997). Currently velvet tree has spread over more than two thirds of Tahiti, forming dense nearly-monotypic stands over 25 % (± 260 km^2) of the island (Thomas 1997), significantly impacting native biodiversity (Meyer and Florence, 1996). In the Hawaiian Islands, similar monotypic stands are found in substantially smaller areas, even on the most severely infested islands (Maui, ± 5 km^2 [Medeiros *et al.* 1997] and Hawaii, ± 12.14 km^2 [Kaiser 2006]). Furthermore, Tahitian *Miconia calvescens* infestations can be found at wider elevational ranges (10–1300 m) when compared to Hawaiian infestations (10-760m) (Meyer 1998b).

Velvet tree was also secondarily introduced from Tahiti to other Society Islands such as Moorea, Raiatea and Tahaa. Furthermore, *Miconia calvescens* also naturalized and is spreading in the Marquesas Islands and southwards in New Caledonia.

Even though velvet tree is considered one of the biggest threats to Hawaiian forest ecosystems, the extent of invasions is less than in Tahiti. This might be a reflection of the rigorous containment and control efforts against velvet tree in Hawaii. Nevertheless,

velvet tree infestations in steep mountainous areas that are inaccessible for control efforts, remain relatively moderate compared to southern Pacific infestations (Julie Leialoha, Big Island Invasive Species Committee, personal communication). Ecological and climate similarities between these northern and southern Pacific islands raised the question whether Hawaii could potentially experience and expect invasions to the extent that Tahiti has (Medeiros *et al.* 1997). Other than control and containment efforts, the attributes potentially contributing to differential success of *Miconia calvescens* in northern and southern Pacific islands have not been investigated. Whether differences in introduction times, i.e. lag phases, or differential adaptive potential due to the presence of severe bottleneck(s) play a role, remain unknown.

Using microsatellite and hyper-variable inter-simple sequence repeat (ISSR) markers the following questions were addressed: (1) Do Hawaiian velvet tree populations differ from southern Pacific island populations (Moorea, Raiatea, Tahiti, Nuku Hiva and New Caledonia) in the amount of genetic diversity they harbor, and if so, (2) How is neutral genetic diversity correlated to invasiveness among northern and southern Pacific islands, and, (3) What are the implications with regard to the management of velvet tree?

Materials and Methods

Study species

Velvet tree, *Miconia calvescens* DC, is a diploid ($2n = 32$) member of the Melastomataceae with a neotropic native range extending 40 ° of latitude from southern Mexico to northern Argentina and southern Brazil (Meyer, 1996). Velvet tree is a fast-growing small tree (up to 15 m tall) that readily outcompetes other species for available

light owing to its large (up to 1m in length) leaves. Pollen to ovule ratios indicate that *Miconia calvescens* is facultative xenogamous, having a mixed breeding system (Meyer, 1998a). Dispersal of seeds is accomplished either passively through wind and water, or actively over long distances by frugivorous birds feeding on fruits. In its introduced ranges, velvet tree occurs in both disturbed vegetation and in native lowland and montane rain forests.

Population sampling and DNA extraction

Eight *M. calvescens* populations were sampled from northern Pacific Hawaiian Islands and 11 populations from southern Pacific Society Islands, Marquesas Islands and New Caledonia (Fig. 9.1). For each population leaf material from 10-30 individual plants was collected. Leaf material was collected from July till November of 2006 throughout all southern Pacific Islands by J-Y Meyer (Délégation à la Recherche, Papeete, Tahiti). Plant material from New Caledonia was collected and donated by Jérôme Munzinger, (Institut de Recherche pour le Développement, Nouméa Cedex, New Caledonia). Plant material was placed and dehydrated in sealed plastic pouches containing Drierite™. Hawaiian populations were collected throughout 2005 by J.J. Le Roux and leaf material kept on ice in the field for no longer than 24 hours before being transferred to a –80 °C freezer. Locality name, region, and where possible, latitude and longitude were recorded for populations sampled (Table 9.1). In total 500 individuals representing 19 putative populations were collected.

Total genomic DNA was extracted according to the manufacturer's protocol with the DNeasy Plant mini kit (Qiagen) from 40 to 50 mg leaf material that was frozen in liquid nitrogen and ground by hand. All extractions were stored at – 80 °C.

Microsatellite analysis

Details concerning the isolation, characterization, and internal repeat structure of the *M. calvescens* microsatellite loci used in this study can be found in chapter 8. Polymerase chain reaction (PCR) amplification of loci was done in 10 μL volumes with the Multiplex PCR Kit (Qiagen) by combining primer pairs into two different multiplex reactions. Each PCR reaction contained 5 μL 2X Qiagen Multiplex PCR Mastermix [HotStarTaq DNA Polymerase; Qiagen Multiplex PCR Buffer (6 mM $MgCl_2$, pH 8.7); dNTP mix], 1 μL 10X primer mix, 1 μL Q-solution [PCR additive (Qiagen)] and approximately 5 ng total genomic DNA. For multiplex 1 the 10X primer mix consisted of all primers (amplifying loci B9, B102a, C103, D101, B109 and D114) mixed in equal amounts (1.4 μM final concentration each). To make up the 10X primer mix for multiplex 2, primers amplifying locus D118 had a final concentration of 2.0 μM each and those amplifying loci B2 and B117, 3.0 μM. All reactions followed a thermal cycle consisting of an initial denaturation at 95 °C for 15 min, followed by 35 cycles at 94 °C for 60 s, annealing at 59 °C (multiplex 1) or 58 °C (multiplex 2) for 60 s, elongation at 72 °C for 90 s, and final extension at 72 ° for 30 min. Polymorphisms were screened using an ABI PRISM 377XL DNA sequencer (PE Applied Biosystems) and PCR products sized relative to a molecular size marker (LIZ500, PE Applied Biosystems). DNA fragments were analyzed using the GeneMarker version 1.4 program (SoftGenetics, LLC).

ISSR amplification and scoring

A subset of the ISSR PCR primers described in Poulin *et al.* (2005) was selected for this study. Three primers were chosen based on high number of bands and the robustness and reproducibility of banding profiles. A subset of populations used in the microsatellite data analysis and the Maui population SM17 was used for ISSR analysis (Table 9.1). For each population ISSR diversity was analyzed in 24 individuals. Each 10 µL ISSR PCR reaction contained 5 µL 2X Qiagen Multiplex PCR Mastermix [HotStarTaq DNA Polymerase; Qiagen Multiplex PCR Buffer (6 mM $MgCl_2$, pH 8.7); dNTP mix], 1 µL Q-solution [PCR additive (Qiagen)], 12.5 pmol of individual ISSR primer and approximately 5 ng total genomic DNA. PCR was conducted on an MJ Research PTC 100 cycler with a thermocycle of: initial denaturation of 95 °C for 15 min; 35 cycles at 94 °C for 60 s, primer-specific annealing temperature: Primer1 ($[AC]_7RG$), 52 °C; Primer3 ($[AG]_8TG$), 48 °C; Primer5 ($[CA]_6RY$), 50 °C for 60 s, elongation at 72 °C for 90 s; and final extension at 72 °C for 12 min.

Agarose gel electrophoresis with ethidium bromide detection or polyacrylamide gel electrophoresis (PAGE) combined with either silver staining or isotopic detection is frequently used to detect ISSR polymorphisms. Instead of these conventional methods, the Agilent 2100 Bioanalyser analysis LabChip (Agilent Technologies, Inc.) was used for high resolution separation of fragments (Banerjea *et al.* 2003) (Fig. 9.2). This analysis allows for detecting smaller differences (sizes) between fragments than allowed by conventional methods and also detects minute quantities of DNA (as low as 0.01 ng/µL) that would otherwise appear absent with conventional methods. Data were analyzed with

the 2100 expert program (Agilent Technologies, Inc.) and to ensure that poorly amplified bands were not missed during the scoring of profiles, the global height threshold setting was set to 0.02 in all analysis. Adjusting the contrast of gel images to the appropriate levels furthermore helped to detect potential "null" (poorly amplified) loci. For each individual, each locus (size fragment) was scored as present or absent ('1' = locus present, '0' = locus absent).

Statistical analysis of microsatellite data

Genetic diversity

The number of alleles and private alleles were calculated for each population using GenAlEx6 (Peakall and Smouse 2006), pairwise F_{ST} and observed (H_O) and expected (H_E) heterozygosities using Arlequin version 3.01 (Schneider *et al.* 2000). Statistically significant deficits of heterozygotes from that expected under Hardy-Weinberg equilibrium (HWE), linkage disequilibrium among all pairwise sets of loci and inbreeding coefficient (F_{IS}) values were estimated using Arlequin version 3.01 (Schneider *et al.* 2000). For HWE a Monte Carlo approximation of the Fisher's exact test (Guo and Thompson 1992) and a standard Bonferroni correction for multiple comparisons where the Markov chain algorithm was run for 100 000 steps following 10 000 dememorization steps were used.

Transient excess of heterozygosity relative to that expected under mutation-drift equilibrium is a signature of a recent population bottleneck (Cornuet and Luikart 1997). Populations that have experienced a recent reduction of their effective population size (N_e) exhibit a correlative reduction in numbers of alleles and gene diversity (H_E, or

Hardy-Weinberg heterozygosity) at polymorphic loci. However, allele numbers decline more rapidly than gene diversity, so that in recently bottlenecked populations, the observed gene diversity is higher than the expected equilibrium gene diversity (Luikart *et al.* 1998). An excess of heterozygosity was tested for microsatellite data with the Bottleneck program (Cornuet and Luikart 1997) under a 100% stepwise mutation model (SMM) and a two-phase mutation model (TPM with 70% SMM). Significance was tested by the sign and Wilcoxon tests (Luikart and Cornuet 1998). These tests were separately applied to Hawaii (n = 192) and southern Pacific islands (n = 281) samples.

To test for isolation by distance, Mantel tests with 1000 permutations as implemented in Arlequin version 3.01 (Schneider *et al.* 2000) were used. A matrix of pairwise F_{ST} values was regressed against a matrix of geographical distances between populations.

All populations were used in an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) to examine the distribution of genetic variation at three hierarchical levels: within populations, among populations within southern and northern Pacific regions, and among southern and northern Pacific regions. This test, implemented in GenAlEx6 (Peakall and Smouse, 2006), partitions total genetic variance and calculates *Φpt*, an analog of F_{ST} (Wright 1965). The significance is determined by comparison with a null distribution derived from permuting haplotypes, individuals or populations at the appropriate hierarchical level (Excoffier *et al.* 1992). The use of *Φpt* enabled me to compare patterns of molecular variance at the same levels between co-dominant microsatellite and dominant ISSR data (e.g. see Maguire *et al.* 2002). Finally, pairwise *Φpt* values were used in a Principle Components Analysis (PCA) to examine the genetic

clustering of populations from throughout the Pacific Ocean using the GenAlEx6 program (Peakall and Smouse 2006).

Bayesian estimates of population structure

Bayesian assignment techniques were used to test for population structure among populations throughout the Pacific and to assess the geographical scale of population differentiation, using STRUCTURE version 2.2 (Falush *et al.* 2007). This method identifies clusters of genetically similar individuals from multilocus genotypes without prior knowledge of their population affinities. The model assumes K genetic clusters, with each having a characteristic set of allele frequencies at each locus; the admixture model then probabilistically estimates the proportion of individuals with ancestry in each cluster. A series of pilot runs were used to estimate $\Pr(X \mid K)$, where X represents the data, for K between 1 (the expected value if all populations represent a single panmixic unit) and 18 (the maximum number of populations). Using the options to ignore population affiliation when defining genetic clusters, assuming independence among loci, and allowing admixture, four independent runs of 300 000 iterations were run, following a burn-in period of 100 000, for each value of K (Pritchard *et al.* 2000). From these initial runs, it was determined that the true value of K (the highest posterior probability) fell between 2 and 10. Pritchard and Wen (2003) warned that $\Pr(X \mid K)$ is in reality only an indication of the number of clusters and an ad hoc guide and potentially difficult to interpret biologically. This is especially true in cases were LnProb values increase with stepwise values of K and can lead to the overestimation of K. To minimize such overestimation, ΔK was calculated (Evanno *et al.* 2005) by taking into account the shape

of the log-likelihood curve with increasing K and variance among estimates among multiple runs. Once the number of genetic clusters was established, each individual was assigned to a cluster and the overall membership of each sampled individual in the clusters estimated.

ISSR genetic diversity

Within population genetic diversity was estimated as heterozygosity using a Bayesian approach as implemented in the program Hickory 1.0.4. (Holsinger *et al.* 2002). Similar to microsatellite data, all populations were used in an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) to examine the distribution of genetic variation (Φpt) within populations, among populations within southern and northern Pacific regions, and among southern and northern Pacific regions. Overall, population differentiation was calculated as G_{ST}-B, a Bayesian analog of the coefficient of gene differentiation among populations, G_{ST} (Nei 1973). This was done separately for northern and southern Pacific regions using the Hickory 1.0.4. program (Holsinger and Lewis 2003). Pairwise genetic distances between populations based ISSR profiles were calculated according to Huff *et al.* (1993) using the program GenAlEx6 (Peakall and Smouse 2006). The subsequent genetic distance matrix was used in cluster analysis and a dendrogram generated for the 11 populations using the unweighted pair-group method with arithmetic averages (UPGMA). A phenetic rather than parsimony-based method was used as this study did not verify that all co-migrating loci were homologous or that they sorted independently. Population genetic distances were also used in a Principle

Components Analysis (PCA), to examine genetic clustering of populations from throughout the Pacific.

Results

Microsatellite genetic diversity

Within-population genetic diversity

In the nine microsatellite loci analyzed a total of 69 alleles was identified, an average of 7.67 alleles per locus. The number of alleles ranged from 5 (locus D101) to 11 (locus D118); within populations the mean number of alleles ranged from 1.2 to 3.0 (Fig. 9.3). The effective number of alleles (corrected for expected heterozygosity) allows for more meaningful comparisons of allelic diversity across populations with different allele distributions. The effective number of alleles did not differ among sampled populations (Fig. 9.3), and could be a consequence of two scenarios. First, both southern and northern Pacific populations represent invasions founded by the same or similar source(s) or secondly, Hawaii infestations resulted from a secondary introduction that originated from southern Pacific islands. Indeed, a larger number of alleles, twenty-eight alleles (40.6 % of the total), were shared among southern Pacific islands and the Hawaiian Islands (Fig. 9.1) while only twenty-six alleles (37.7 % of total) were restricted to southern Pacific islands and 15 alleles (21.7 % of total) were restricted to the Hawaiian Islands. Expected heterozygosity ranged from 2 % to 25 %, with a mean of 11 % across all loci. Observed heterozygosity ranged from 2 % to 44 %, with a mean of 8 % across all loci. On average the majority of loci displayed a significant deficit of heterozygotes from that expected

under HWE, with some loci conforming to HWE for some populations. No linkage disequilibrium was detected between any of the 9 loci across all populations.

The computer program Bottleneck (Cornuet and Luitkart 1997) was used to test whether introduced *M. calvescens* populations showed a transient excess in heterozygosity, as expected under a bottleneck event. A significant excess of heterozygosity was detected under all models tested (full SMM model, TPM model of mutation with 70% SMM) for both northern and southern Pacific regions for the sign and Wilcoxon tests ($P < 0.001$), indicating that *M. calvescens* is currently going through a severe bottleneck. The average population inbreeding coefficient was 0.27, giving further support to severely bottlenecked populations in both regions. However, four Society Island populations (T31, T210, T234 and T263), had negative inbreeding coefficients.

Among-population genetic diversity

Pairwise F_{ST} values ranged from 0.009 between population T263 (Raiatea) and populations T31 (Moorea) and T234 (Raiatea) to 0.197 between population T234 (Raiatea) and T298 (Tahaa) (Table 9.2). Overall, except for one Raiatea population (T180) and the Tahaa population, T298, these values represented very low population differentiation. Genetic distances among pairs of populations were not significantly correlated to geographical distance between localities (Mantel test, $P > 0.05$).

Genetic distances (Φpt) were used in a PCA to investigate the relative position of populations in multidimensional space. The first two principle components (PC) axes explain 73.04 and 14.52 % of the genetic variation among populations respectively, for a total of 87.52 %. A Scattergram of these two axes show little geographic correlation (Fig.

9.4). While the southern Society Islands of Tahiti and Raiatea were separated from all other populations, primarily due to displacement along PC Axis 1, the rest of the southern and northern Pacific islands grouped at random with no apparent geographic correlation.

The hierarchical AMOVA based on sampled populations revealed that the majority of genetic variation (81.00 %) resided within populations, 17.00 % was distributed among populations within islands, and 3.00 % of the variation can be explained by differentiation between northern and southern Pacific regions (Table 9.3). Although the proportion of genetic variation accountable at higher levels is small (3.00 %), all fixation indices were statistically significant. In agreement with pairwise F_{ST} values and PCA, very little genetic variation was attributed to differences among populations between hemispheres, suggesting genetic very little genetic differentiation on this large geographical scale. In addition, most genetic variation at this geographical scale is found at the population level.

Bayesian estimates of population structure

The model-based clustering method implemented in STRUCTURE suggested that the model with $K = 8$ (where K is the number of population genetic clusters) was substantially better than alternative models. The highest posterior probabilities for K varied among multiple runs with the their associated K ranging from 4 to 10, demonstrating that posterior probability alone is not a good measure of the true K. Values of LnProb(data) showed a pattern of incremental increase with increasing K; leading to potential overestimates of the number of genetic clusters. Evanno *et al.* (2005) suggested the use of ΔK, which takes into account the shape of the log likelihood curve, to overcome this problem. For this study's data, $\Delta K = 8$ was 234.09, the highest value,

whereas estimates for all other possible runs were less than 71.08. The genetic clusters identified by STRUCTURE, in congruence with all other estimates, supported low genetic differentiation and high similarity by probabilistically assigning individuals from different hemispheres to the same genetic clusters (Fig. 9.3).

ISSR genetic diversity

Within- and among-population genetic diversity

The three ISSR primers generated a total of 77 scorable bands (average ca 26 bands/primer and range of 13-36) of which 73 (95 %) were polymorphic in the 251 genotypes examined. The sizes of bands ranged from 260 bp to 3400 bp.

A Bayesian approach (Holsinger *et al.* 2002), was used to calculate heterozygosity estimates. The average heterozygosity across populations was slightly higher for Hawaiian Islands populations (0.280) than for southern Pacific islands populations (0.243). The Hickory 1.0.4. program was used to calculate, *f,* an estimate of F_{IS} (Holsinger *et al.* 2002). On average values were high (0.91) ranging from 0.850 to 0.927 (Table 9.1). Holsinger and Lewis (2003) emphasized the problems associated with estimating F_{IS} from dominant data and warned that programs such as Hickory can lead to an overestimate of inbreeding, especially when sample sizes are small. However, given large sample sizes, the bottlenecks and high levels of inbreeding detected for microsatellite markers, the high inbreeding coefficients estimated for ISSR data are a further indication of severe inbreeding in these populations.

The Bayesian analog of Nei's G_{ST}, G_{ST}-B, showed very low differentiation between Maui and Hawaii populations (0.063) compared to all southern Pacific islands

(0.1071). Nei's unbiased nuclear genetic distances, based on the absence/presence matrix of ISSR fragments, between northern and southern populations of velvet tree ranged from 0.030 (between the New Caledonia population, T150, and Tahiti population T61) to 0.106 (between Moorea population T31 and the Nuku Hiva population, T89) (Table 9.4). In congruence with the co-dominant data, UPGMA cluster analysis based on Nei's unbiased nuclear genetic distances based on ISSR data failed to support geographic clustering. For example, Hawaiian populations M4 and M17 fell within a southern Pacific cluster (Cluster 1, Fig. 9.6) joined by a more distantly related sister cluster of Society Island populations (Cluster 2). These two clusters were basally joined by the Marquesas population, T89, followed by Moorea population T31.

Similar to microsatellite data, genetic distances (*Φpt*) inferred from the ISSR presence/absence matrix were used in a PCA to investigate the relative positions of populations in multidimensional space. The first three principle component (PC) axes explained 27.83, 18.83 and 18.48 % of the genetic variation among populations respectively, for a total of 65.14 %. Scattergrams of these three axes, similar to that obtained for microsatellite data, showed little geographic correlation (Fig. 9.4). These results provide further support to the pattern of very little genetic differentiation among southern and northern Pacific populations and regions.

Furthermore, hierarchical AMOVA revealed that the majority of genetic variation (88.00 %) resided within populations, 12.00 % was distributed among populations within islands, while 0.00 % of the variation can be explained by differentiation between northern and southern Pacific regions (Table 9.3). Once again, these results were similar to those obtained for the co-dominant marker dataset with the exception that no genetic

exception that no genetic variation existed among hemispheres. Although the proportion of genetic variation accountable at higher levels was zero, all fixation indices were statistically significant. Consistent with pairwise values of genetic distances and the PCA, little genetic variation was attributed to differences among populations among hemispheres, also suggesting very little genetic differentiation on this large geographical scale.

Discussion

Large geographical scales, inbreeding and low genetic differentiation

Throughout the Pacific, highly invasive populations of *M. calvescens* appear to be highly inbred and genetically depauperate. The obvious explanation for this observation would be a single or small founding source for all Pacific Island populations. Convincing evidence exists in support of this hypothesis for at least the southern Pacific regions. Introduced from Sri Lanka where it is also considered invasive in 1937, dense stands of velvet tree were first observed by the early 1970's in Tahiti (Meyer 1996). During this timeframe velvet tree was deliberately and accidentally introduced to the neighboring island of Raiatea (Meyer 1998b). Similarly, infestations on neighboring Tahaa and more distant Nuku Hiva and New Caledonia are thought to be of Tahitian origin (Meyer, 1998b). The origin of Hawaiian velvet tree is less clear. In Central and South America native velvet tree has two distinct leaf morphological types, bi- and mono-colored, with the former restricted to the northern areas of the native range (Mexico, Gautemala and Costa Rica) and the latter to the southern parts of the native range (Argentina and southern Brazil). Similar to southern Pacific Islands, Hawaii is invaded by velvet tree

with bi-colored leafs and it is speculated that both regions' invasions resulted from a similar source. (Medeiros *et al.* 1997). The lack of genetic differentiation (i.e. similarity) based on numerous molecular markers found in this study supports a similar geographic source for infestations in both Pacific hemispheres. Alternatively, Hawaiian populations of velvet tree might be the result of a secondary introduction from Tahiti (Tracy Johnson, USDA Forest Service, personal communication).

Sri Lankan populations of *M. calvescens* originated from Mexico, suggesting that Hawaiian and southern Pacific populations, are also of Mexican origin. To date the identification of potential natural enemies to be used in biological control programs aimed at Pacific velvet tree infestations focused on the southern native regions in Costa Rica and Brazil and led to the identification of numerous fungal pathogens, witches' broom-causing phytoplasmas, and foliar nematodes (Killgore *et al.* 1999, Seixas *et al.* 2002; 2004). One fungus, *Colletotrichum gloeosporioides* f. sp. *miconiae,* was introduced into Hawaii and the Society Islands and became established. Post-establishment impacts are evident but not nearly sufficient to control or contain the current infestations (J-Y Meyer, Délégation à la Recherche, Tahiti, personal communication). A survey of successful biological control programs showed that the majority of effective natural enemies used in biocontrol programs are host-specific (Rosen, 1986). Failure to correctly identify invasive species and/or their native origin could thus lead to unsuccessful natural enemy establishment, and no or incomplete control, especially when dealing with biotypes of a single weed species (e.g. Chaboudez 1994), weed species complexes (e.g. *S. madagascariensis* [Chapter 5, Le Roux *et al.* 2006]) or natural enemy host-races (e.g. skeleton weed rust, *Puccinia chondrillina* [Espiau *et al.* 1998]). Given the magnitude of

velvet tree's native range (spanning 40 ° of latitude) it is reasonable to speculate that regional genotypic variants or biotypes exist and these might have different specialized and co-evolved natural enemies (e.g. Goolsby *et al.* 2006). More productive and damaging control agents targetting Pacific island infestations are thus more likely to be found in velvet tree's native ranges in Mexico.

The founders for both northern and southern Pacific regions consisted of only a few individuals and resulted in severely bottlenecked invasive populations. Low genetic structure among different populations is further exacerbated by two reproductive characteristics of *M. calvescens*. First, and the most obvious, is the ability of velvet tree to self pollinate. Selfing may keep genetic diversity low, especially within populations, and coupled with active seed dispersal by frugivorous birds, suggests that outlying foci may frequently result from a single propagule. Secondly, and maybe less obvious, is seed bank formation and dormancy of seeds. Throughout the Pacific, velvet tree reproduces trimodally each year with individual mature trees capable of producing up to eight million seeds during each cycle (Meyer 1997). Seed banks are dense (>50,000 seeds/m^2) and dormant under shaded conditions, stimulated by light when canopy gaps open (Medeiros *et al.* 1997). Dormant seeds can maintain viability for up to 7 years (J-Y Meyer, Délégation à la Recherche, Tahiti, personal communication) resulting in the overlap of generations as trees can reach a reproductive age after only 4-5 years. This can result in an increase in the effective generation time, which prevents genetic decay, the formation of spatial genetic structure between geographically distinct populations, and the ability of genetic drift to drive unique alleles to fixation (Loveless and Hamrick, 1984). Similarly, Bahulikar *et al.* (2004) showed that a combination of self pollination

and seed dormancy was partially responsible for a lack of genetic structure in wild tobacco, *Nicotiana attenuata*, over large spatial scales.

Genetically depauperate populations have particularly low adaptive potential and are vulnerable to the effects of sub-adapted conditions. This suggests that velvet tree is unlikely to rapidly evolve resistance against effective control mechanisms, including biological control. The presence of high genetic diversity and the subsequent rapid evolution of resistance against control mechanisms have been demonstrated for numerous invasive species. For example, cordgrass, *Spartina alterniflora*, harbors genetic variation in both tolerance and resistance to its introduced biological control agent (Garcia-Rossi *et al.* 2003) while the pea aphid, *Acyrthosiphon pisum*, shows genetic variation in resistance to parasitism by its parasitoid (Hufbauer and Via 1999).

The role of pre-adapted genotypes in invasion success

Given high levels of inbreeding and low genetic diversity, velvet tree's success throughout the Pacific can be ascribed either to the introduction of pre-adapted genotypes or wide environmental tolerances (phenotypic plasticity) or a combination of both. Although these two phenomena are not necessarily mutually exclusive, several lines of argument are in favor of pre-adaptation as a more parsimonious explanation.

Reproductive strategies

The mixed breeding system of velvet tree conforms to Baker's rule (1967) that would allow for a single propagule to disperse, establish foci and spread into the new environment. Synchronous flowering and strongly sweet-scented flowers are consisted

with adaptation for biotic pollination and thus outcrossing. In Hawaii non-indigenous syrphid species have been observed visiting velvet tree flowers (Medeiros and Loope, 1997). Thus, while selfing ensures reproductive assurance, occasional outcrossing events may relieve the effects of inbreeding due to selfing. The enormous seed output of single mature trees is dispersed both actively and passively. Passive dispersal occurs by gravity (most frequent mode), wind carrying the light and small seeds, or water. Meyer (1996) showed that velvet tree seeds remained viable after being immersed in water for up to three months. Active dispersal in velvet tree over long distances is most commonly by various non-indigenous frugivorous birds in Pacific regions and also by small rodents such as rats. Meyer (1994) found that velvet tree seeds can survive a transit through the digestive tract of Polynesian rats, *Rattus exulans* Peale. The correlation between reproductive traits such as small seed size and high dispersion capability and invasion success has previously been demonstrated (Rejmanek and Richardson 1996) and would be enhanced by velvet tree's mixed breeding system.

Physiological adaptations and the importance of light availability

Light and water are the most limiting resources to plant growth and are responsible for a wide array of adaptations to their availability in plants. For example, shade tolerant plants benefit from adaptations that maximize exposure to light and minimize respiratory costs in low light environments (Pearcy and Sims 1994). Velvet tree evolved shade tolerance in dense neotropical canopy rain forests, and evolved pioneer-like behavior to compete with other canopy-forest species, growing rapidly to fill available forest gaps. Indeed, light requirements for phenology and fruiting may be one

of the main limiting factors to the persistence of velvet tree populations in neotropical forests (Meyer, 1998a). Meyer and Malet (1997) speculated that the sudden and extensive range expansion of velvet tree on the island of Tahaa (Society Islands) was likely the result of canopy damage (openings) caused by a hurricane. Velvet tree forms sapling banks (with slow growth rates [less than 30 cm per year]) in dense shaded understory habitats that will act as sources for pioneering plants with rigorous growth (up to 1.5 m/yr) in the event of forest gap formation (Meyer and Malet 1997). Compared to neotropical forests, the native forests of the regions included in this study are relatively unsaturated, with high solar radiation levels and less canopy tree species to intercept available sunlight before it reaches the forest floor (Medeiros *et al.* 1997). As velvet tree evolved to respond positively to light stimuli, introduced environments, given favorable growth conditions such as rainfall, temperature etc., are especially vulnerable to invasion. Once light gaps are filled, the dense foliage of velvet tree creates light conditions to which native plants are not adapted, displacing them and thus securing available resources. This positive feedback cycle is typically observed in Tahiti, where velvet tree is present in over 65 % of the vegetation, forming nearly-monotypic stands over 25 % of the island (Thomas 1997).

Superior competitiveness of velvet tree over native Hawaiian trees and shrubs has been demonstrated for various physiological fitness correlates. A comparison between the findings of Pattison *et al.* (1998) and Baruch *et al.* (2000) indicates that, under similar conditions, the relative growth rate of velvet tree is 74 % greater than that for a group of native Hawaiian trees and herbs (*M. calvescens,* 0.0224 g $g^{-1}d^{-1}$; natives, 0.0129 g $g^{-1}d^{-1}$). Similarly, a comparison between Baruch and Goldstein (1999) and Baruch *et al.* (2000)

shows that velvet tree has higher concentrations of leave nitrogen and phosphorous when compared to native Hawaiian species under similar environmental conditions (*M. calvescens,* 1.90 %; natives, 1.36 % N, and *M. calvescens,* 0.17 %; natives, 0.08 P). Higher foliar nitrogen content promotes higher carbon assimilation and is correlated with enhanced plant growth. Also, under similar light conditions, the amount of fixed carbon per unit leaf, an index of the efficiency of carbon use, was lower for velvet tree than for native Hawaiian scrubs and trees (*M. calvescens,* 1.24 g glucose g^{-1}; natives, 1.39 g glucose g^{-1}) (Baruch and Goldstein 1999, Baruch *et al.* 2000). Lower levels of carbon fixation indicate that velvet tree use carbon more efficiently than native species by investing less energy per unit biomass produced (Baruch *et al.* 2000). *Miconia calvescens'* large leaves ($\leq$ 1 m in length) and the absence of its natural foliar herbivores would furthermore give it a photosynthetic advantage over Hawaiian natives. Indeed, Baruch and Goldstein (1999) found specific leaf area (SLA; leaf area per unit leaf mass) to be significantly higher for velvet tree than for 46 different Hawaiian forest herbs, shrubs and trees (*M. calvescens,* 138.4 cm^2g^{-1}; natives 122.06 cm^2g^{-1}). By having leaves with high SLA, *M. calvescens* can produce larger assimilatory surfaces for a given amount of carbon fixed, and this, coupled with higher levels of leave nitrogen (see above), will result in higher growth rates compared to Hawaiian natives.

These physiological traits evolved in velvet tree in dense native range forest habitats where velvet tree competes with many shade tolerant species, facing challenges not present in introduced Pacific regions. A lack of many of these selection pressures in introduced ranges may result in pre-adaptive traits rendering higher fitness under novel conditions throughout the Pacific.

Baruch *et al.*'s (2002) investigation also showed that phenotypic plasticity exists in velvet tree saplings under different light and water stress conditions. This is maybe not surprising given the differences in growth responses of saplings in response to light availability under natural conditions. Plasticity might further contribute to velvet tree's success in Pacific islands.

Given, a species that evolved strategies to cope with competition under low light environments, low genetic diversity throughout its introduced range, and favorable environmental conditions in all the introduced ranges, it appears that pre-adapted physiological, morphological and life history traits are the main factors driving *M. calvescens*' invasion success. The success of plant biological invasions afforded by the introduction of such competitively superior pre-adapted genotypes is well documented (e.g. see Woitke and Dietz 2002, Hurka *et al.* 2003). In addition, the release of velvet tree from its natural enemies most certainly contributed to its invasive success (see above). Various hemopteran, lepidopteran and coleopteran enemies have been found to cause substantial damage to plants in native ranges (Picanço *et al.* 2005). The highly inbred Pacific populations are unlikely to rapidly evolve increased competitive ability (Lee 2002) by reallocating resources used for defence against natural enemies to growth and reproduction. *Miconia calvescens'* success as an invasive species appears to be consistent with a previous meta-analysis showing that invaders are generally better competitors than natives for available resources (Vilà and Weiner 2004).

Conclusions

Miconia calvescens in the Pacific represents an invader with a suit of pre-adapted traits that virtually guarantee high fitness and competitive success in tropical insular forest habitats. The high output of small seeds and various means of effective dispersal coupled with a mixed breeding system contribute to this species' success throughout the Pacific. An evolutionary history under shade and dense forest conditions favored traits that are superior to those in natives from less saturated forests characteristic of the Hawaiian and Society Islands. Coupled with genetic similarity of velvet tree populations from the Hawaiian and Society Islands, the highly comparable climate, topography, relative geographic location and biota should caution Hawaiian authorities against the potential magnitude of invasion if current control methods are relaxed or outlying foci left unchecked. Recently Kaiser (2006) illustrated the enormous economic repercussions if the spread of velvet tree is left unabated in Hawaii. Finally, the current differential impact in northern and southern Pacific regions seems to be the result of two considerations. First, rigorous containment and control efforts have been in place for over a decade against velvet tree infestations in the Hawaiian Islands compared to the relatively less rigorous attempts in the southern Pacific regions. Secondly, lag phases (±30 y) associated with velvet tree infestations in the Pacific are not due to the accumulation of genetic diversity or of natural selection on such diversity but rather appear to reflect the consequences of absolute growth rates that accompany small founding populations. Assuming initial establishment of a single propagule, it would take approximately 30 years for a dense population of between 100-1000 trees to establish. If one assumes that 5-10 years as the average time to form a reproductive tree from one

will be necessary to build the second generation of approximately 10-100 reproductive trees (Meyer, 1998b). After a period of time between 15-30 years, the third generation will be formed by a dense cover of about 100-1000 trees. This is consistent with observations on the plateau of Taravao in Tahiti, where, in the early 1970's, nearly-monotypic stands were first observed about 30 years after its introduction (Meyer, 1998b).

Differences in introduction times (± 25 y) have likely played an important role in the differences observed in *Miconia calvescens* infestations in northern and southern Pacific islands

Table 9.1. Geographic position of sampling sites with measurements of genetic diversity at nine microsatellite and 77 ISSR loci: *A*, mean number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; *hs*, panmictic heterozygosity; *f*, inbreeding coefficient.

				Co-dominant marker genetic diversity				Dominant marker genetic diversity	
ID	Island	Locality	Lat/Long[§]	*A*	H_O	H_E	F_{IS}	*hs*	*f*
Northern Pacific									
M1	Hawaii	South Hilo	19.830°/-155.108°	2.00	0.443	0.166	0.649	------	------
M2	Hawaii	North Hilo	19.869°/-155.271°	2.556	0.081	0.176	0.407	------	------
M3	Hawaii	South Hilo	19.811°/-155.115°	1.556	0.016	0.031	0.185	------	------
M4	Hawaii[†]	Honokua Bay	19.980°/-155.253°	2.111	0.039	0.090	0.260	0.261	0.871
M7	Kauai	UH Research	22.079°/-159.408°	2.222	0.086	0.161	0.190	------	------
M8	Kauai	UH Research	22.068°/-159.396°	1.222	0.034	0.104	0.410	------	------
M9	Hawaii	Leilani Estates	19.676°/-154.989°	2.222	0.068	0.124	0.253	------	------
M17	Maui	Huelo Honokala	20.855°/-156.135°	------	------	------	------	0.2989	0.911
Southern Pacific									
T1	Moorea[†]	Maatea valley	-17.579°/-149.813°	1.778	0.038	0.077	0.403	0.259	0.895
T31	Moorea[†]	Vaianae valley	-17.573°/-149.834°	1.556	0.020	0.020	-0.062	0.237	0.878

Table 9.1. (Continued) Geographic position of sampling sites with measurements of genetic diversity at nine microsatellite and 77 ISSR loci: A, mean number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; hs, panmictic heterozygosity; f, inbreeding coefficient.

				Co-dominant marker genetic diversity				Dominant marker genetic diversity	
ID	Island	Locality	Lat/Long[§]	A	H_O	H_E	F_{IS}	hs	f^*
T61	Tahiti[†]	Papenoo valley, Vainavenave	-17.521°/-149.441°	2.111	0.077	0.084	0.032	0.254	0.912
T89	Nuku Hiva[†]	Taipivai-Hatiheu	-140.053°/-8.814°	3.000	0.031	0.249	0.873	0.231	0.922
T120	Tahiti[†]	Taravao plateau	-17.743°/-149.298°	1.333	0.029	0.042	0.286	0.253	0.907
T150	New Caledonia[†]	Mount Dore	-22.299°/166.792°	2.556	0.075	0.128	0.298	0.247	0.916
T180	Raiatea[†]	Mount Tohiea	-16.864°/-151.448°	3.333	0.102	0.240	0.532	0.211	0.916
T210	Raiatea	Uturaerae valley	-16.747°/151.474°	1.444	0.050	0.047	-0.060	-----	-----
T234	Raiatea	Tetooroa valley	-16.822°/-151.466°	1.333	0.016	0.016	-0.031	-----	-----
T263	Raiatea[†]	Anatorea	-16.750°/-151.442°	1.444	0.017	0.017	-0.024	0.233	0.850
T289	Tahaa[†]	----------	-16.598°/-151.501°	1.889	0.193	0.180	0.252	0.260	0.927

[†] Populations that were included in both dominant and co-dominant marker datasets.

[*] f is an estimate of F_{IS} for dominant marker data after Holsinger *et al.* (2002).

[§] Latitude and longitude coordinates were not determined in the field for southern Pacific populations and values given in table are approximate locality waypoints estimated from geographical information systems (GIS) maps as GPS waypoints are unavailable for these locations.

Table 9.2. Genetic structure in *Miconia calvescens* throughout the Pacific based on microsatellite data, given as pairwise F_{ST} values between populations.

	M1	M2	M3	M4	M7	M8	M9	T1	T31	T61	T89	T120	T150	T180	T210	T234	T263
M2	0.023																
M3	0.065	0.049															
M4	0.062	0.050	0.018														
M7	0.057	0.050	0.046	0.032													
M8	0.127	0.091	0.066	0.046	0.045												
M9	0.064	0.056	0.030	0.027	0.027	0.027											
T1	0.071	0.052	0.017	0.017	0.035	0.052	0.023										
T31	0.067	0.049	0.012	0.024	0.048	0.087	0.039	0.023									
T61	0.058	0.040	0.015	0.016	0.033	0.048	0.024	0.014	0.021								
T89	0.042	0.038	0.052	0.045	0.041	0.078	0.045	0.044	0.059	0.042							
T120	0.076	0.051	0.022	0.031	0.051	0.104	0.041	0.029	0.019	0.021	0.059						
T150	0.050	0.037	0.022	0.023	0.028	0.039	0.018	0.017	0.027	0.017	0.034	0.024					
T180	0.156	0.148	0.143	0.125	0.115	0.134	0.120	0.134	0.152	0.140	0.118	0.163	0.120				
T210	0.064	0.045	0.016	0.026	0.048	0.085	0.042	0.018	0.019	0.017	0.046	0.023	0.022	0.159			
T234	0.078	0.053	0.015	0.028	0.054	0.112	0.042	0.025	0.011	0.021	0.061	0.021	0.030	0.169	0.018		
T263	0.066	0.048	0.013	0.026	0.048	0.090	0.040	0.024	0.009	0.021	0.059	0.020	0.028	0.166	0.018	0.009	
T298	0.188	0.162	0.164	0.144	0.120	0.144	0.123	0.158	0.193	0.130	0.133	0.184	0.133	0.138	0.166	0.197	0.173

Table 9.3. Results of hierarchical AMOVA comparing genetic variation within populations, among populations within regions, and among southern and northern Pacific regions invaded by velvet tree for both dominant and co-dominant data. Significance was tested against a null distribution of 10 000 random permutations.

Source of variation	d.f.	Sum of squares	Fixation index	Percent variation	*P*-value
Co-dominant microsatellite data					
Among regions	1	77.494	$\Phi_{RT} = 0.028$	3.00	< 0.01
Among populations within regions	16	537.153	$\Phi_{PR} = 0.170$	17.0	< 0.01
Within populations	455	2405.129	$\Phi_{PT} = 0.193$	80.0	< 0.01
Dominant ISSR data					
Among regions	1	32.422	$\Phi_{RT} = 0.000$	0.00	< 0.01
Among populations within regions	5	253.654	$\Phi_{PR} = 0.122$	12.0	< 0.01
Within populations	240	4185.770	$\Phi_{PT} = 0.114$	88.0	< 0.01

Table 9.4. Nei's pairwise unbiased nuclear genetic distances based on ISSR diversity between northern and southern Pacific populations of *Miconia calvescens*.

	M17	M4	T120	T150	T180	T1	T163	T289	T31	T61	T89
M17	0.000										
M4	0.065	0.000									
T120	0.052	0.048	0.000								
T150	0.043	0.039	0.047	0.000							
T180	0.053	0.063	0.049	0.032	0.000						
T1	0.050	0.058	0.044	0.040	0.038	0.000					
T163	0.067	0.062	0.035	0.046	0.050	0.045	0.000				
T289	0.055	0.070	0.062	0.052	0.079	0.056	0.076	0.000			
T31	0.091	0.075	0.093	0.078	0.059	0.058	0.085	0.101	0.000		
T61	0.051	0.052	0.045	0.030	0.034	0.039	0.048	0.053	0.068	0.000	
T89	0.058	0.048	0.055	0.059	0.074	0.071	0.075	0.076	0.106	0.050	0.000

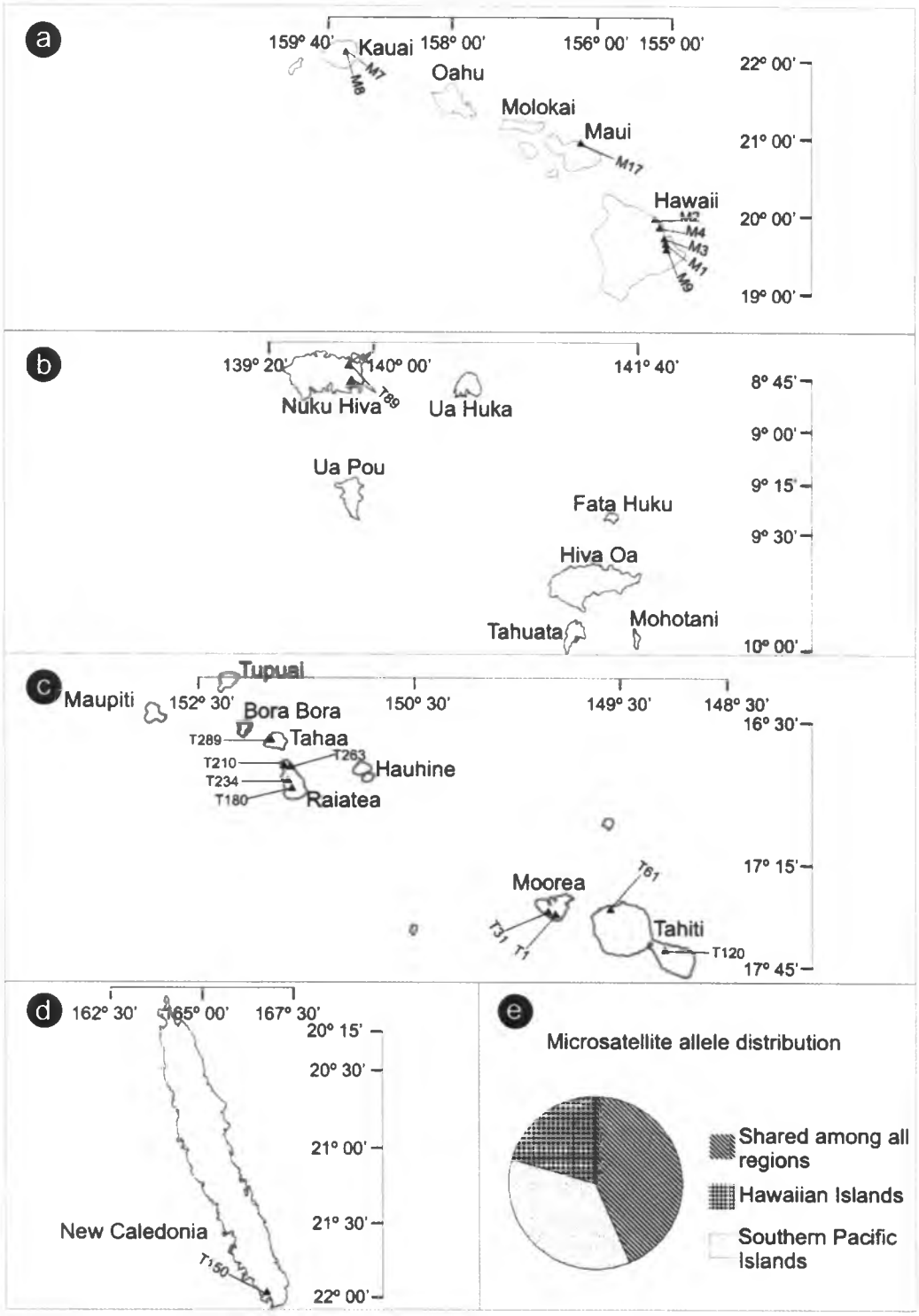


Figure 9.1. Maps indicating the collection sites in the (a) Hawaiian Islands, (b) Marquesas Islands, (c) Society Islands and (d) New Caledonia. Inset e illustrates the distribution of 69 microsatellite alleles between and among northern and southern Pacific populations of *Miconia calvescens*.

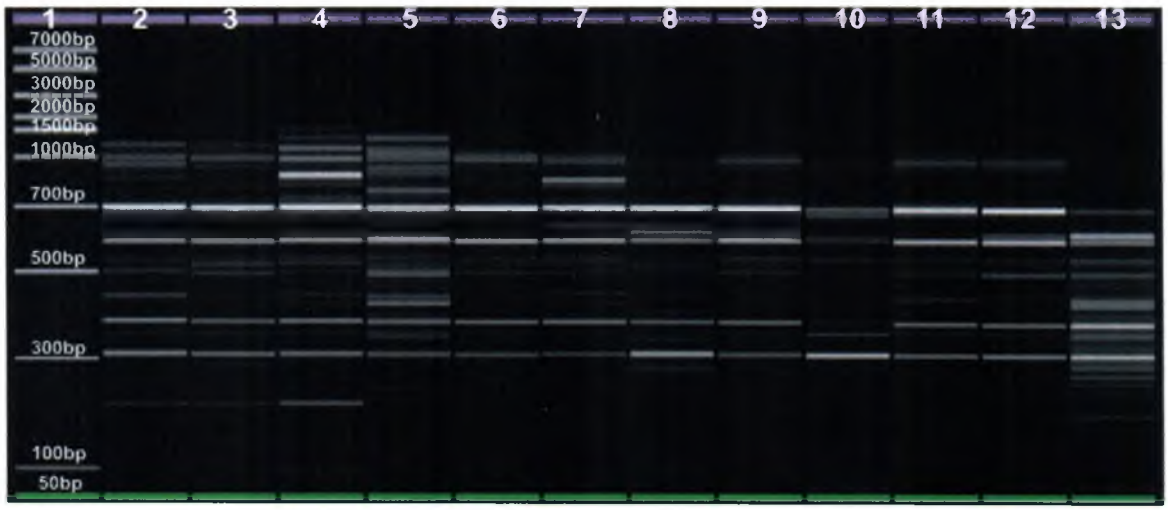


Figure 9.2. An ISSR gel picture generated with the 2100 expert software. Lane 1 shows the standard marker used and lanes 2-13 the banding patterns generated for 12 different velvet tree individuals for ISSR Primer3.

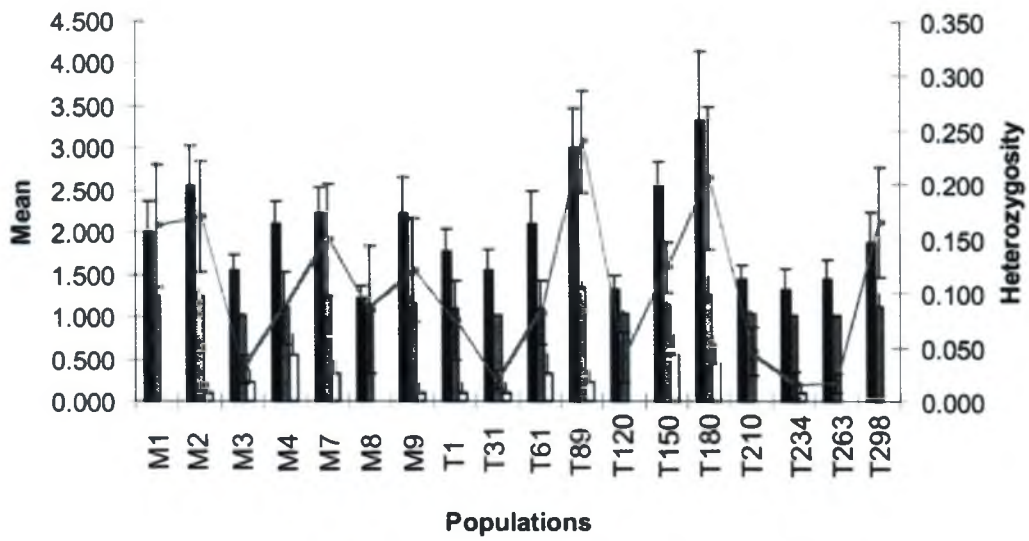


Figure 9.3. Patterns of microsatellite allelic richness and heterozygosity in 18 populations of *Miconia calvescens* genotyped at nine microsatellite loci. Bars represent mean ± SD number of alleles (black bars), mean ± SD number of effective alleles (grey bars), and mean ± SD number of private alleles (white bars). Mean ± SD heterozygosities for each population (across all loci) are represented by the grey line.

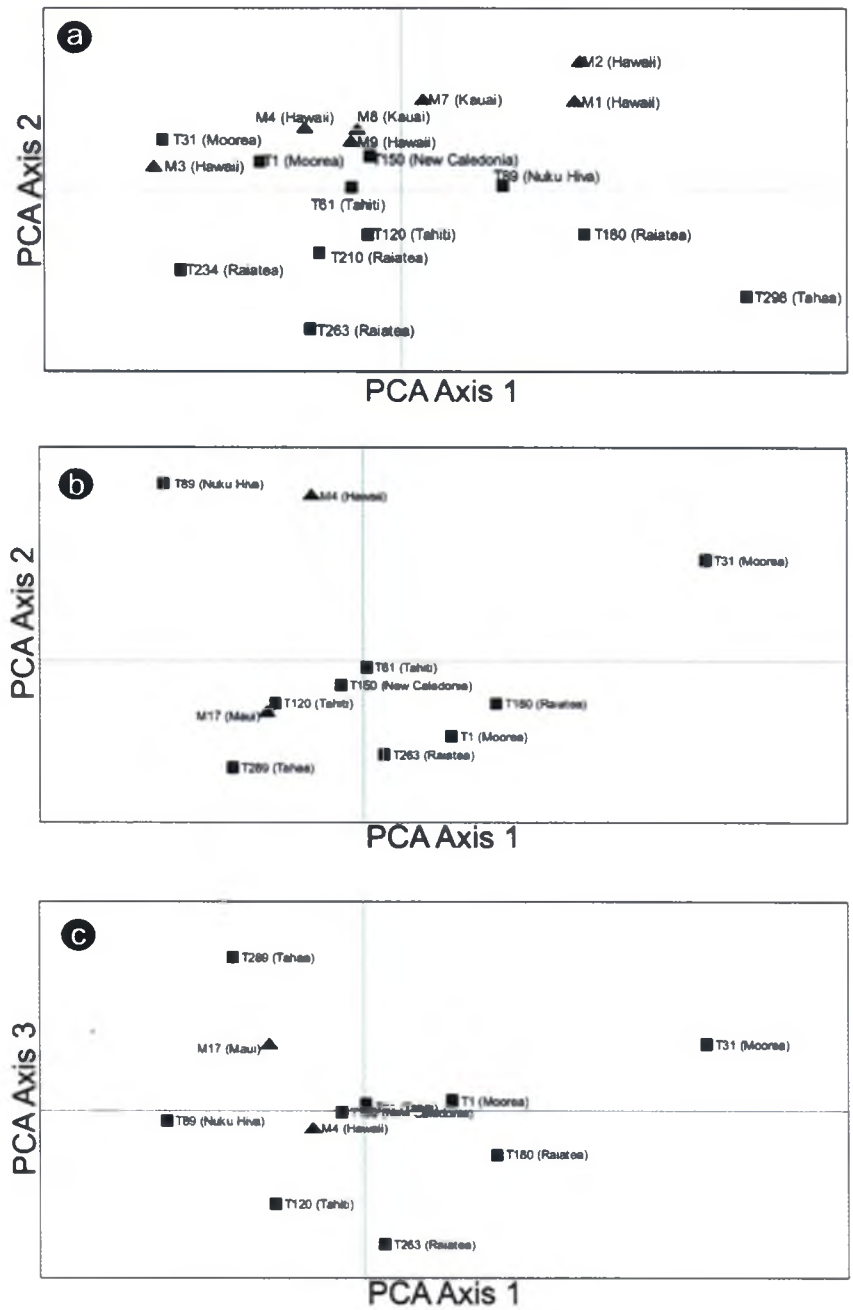


Figure 9.4. Scattergrams of the first two axes of a PCA of genetic variation for nine microsatellite loci (a) and the first three axes for ISSR data (b and c) based on pairwise *Φpt* values in *Miconia calvescens.* Symbols represent northern (black triangles) and southern (black squares) Pacific populations. Clustering of populations is concordant with Bayesian assignment based on microsatellite genotypes (see Fig. 5) and UPGMA analysis of ISSR data (see Fig. 6) in showing no apparent geographic structuring.

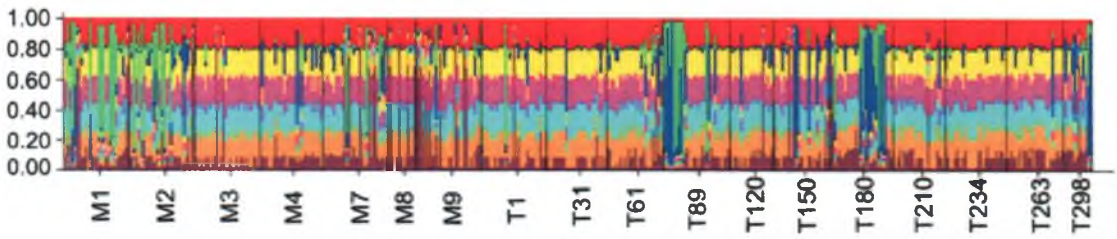


Figure 9.5. Population structure inferred by Bayesian assignment of northern and southern Pacific individuals of *Miconia calvescens* shown as individual membership coefficients in the STRUCTURE-identified genetic demes. Velvet tree populations can be assigned to eight geographical genetic demes that did not corresponded to geographic location.

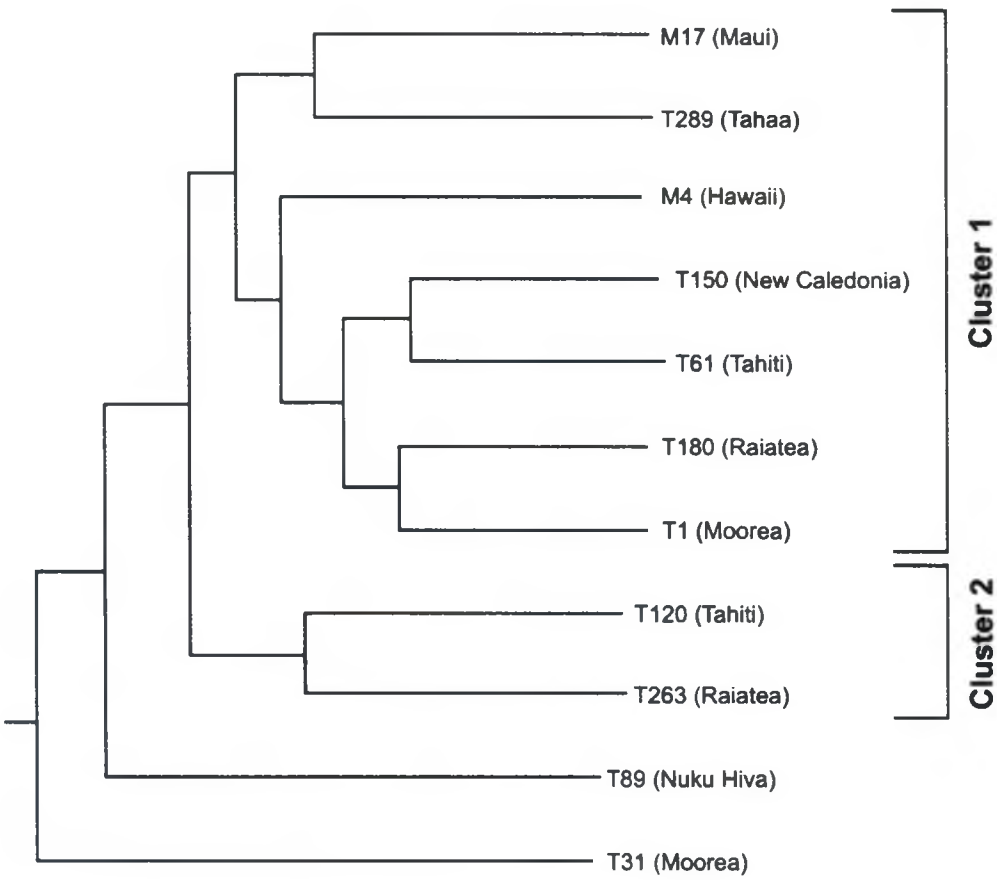


Figure 9.6. A dendrogram based on Nei's (1978) unbiased genetic distances (ISSR data) constructed by the using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis.

CONCLUDING REMARKS

Environmental and economic consequences of the establishment and spread of non-indigenous species that become invasive have been emphasized throughout this dissertation. Like most insular habitats, native ecosystems of the Hawaiian Islands have proven particularly susceptible to these devastating effects (Courchamp *et al.* 2003). The geographic isolation of the Hawaiian Archipelago has provided for the evolution of a flora so unique that 95 % of native Hawaiian plants are found nowhere else on the planet (Fosberg 1948). Many of these species have been replaced by or are facing unique challenges as a result of over 5,000 species of introduced plant species. The necessity to better understand the processes and traits involved in successful invasions and their ecological and evolutionary effects is evident, and may have considerable implications for control measures. This dissertation research mainly focused on the molecular ecology of biological invasions in the Hawaiian archipelago. A starting point in such research efforts is often to determine the species' phylogenetic origin, to unravel its colonizing history, and to estimate its genetic diversity. These inferences are important to determine the role of local adaptation in invasive success, whether invaders resulted from single or multiple introduction(s), and whether invasiveness is acquired after colonization or resulted from the introduction of pre-adapted genotype(s). In a broader sense such studies will help in determining whether adaptive strategies are associated with several life history traits.

This dissertation research shed light on numerous aspects of biological invasions that will not only be valuable for future control efforts of the species studied here but also will give insights into biological invasions in general. This research effort identified a

identified a plant species, *Pennisetum setaceum* (fountaingrass), which appears monoclonal throughout its invasive and native ranges (Le Roux *et al.* 2007). This was the first report on such a phenomenum and the implications of these findings reach far beyond the better understanding of this plant's invasion biology. The common usage of clones (polyploids) in the ornamental industry targets two biological traits to prevent ornamentals from becoming invasive: 1) the construction of clones that lack genetic diversity that could otherwise facilitate local adaptation to new environments and 2) the inability to produce seeds by such clones. This research's findings on fountaingrass' super-genotype caution against the first consideration, as a single clone can clearly have highly invasive potential and success. These findings should thus appeal to the general public involved in nursery and ornamental industries and their relation to environmental management in a general sense. The hypothesis that Hawaiian populations of *Senecio madagascariensis* (fireweed) originated secondarily from Australia was supported here. The results furthermore indicated that eastern South African regions are the most likely source for these infestations and thus for effective biological control agents. Multiple introductions of fireweed to Hawaii suggest that contaminated materials (in this case animal feed and potentially landscape (cover) plants) arrive to the islands on several separate occasions. This is alarming and should caution border officials against more unintentional introductions as current screening and quarantine measures appear to be inadequate.

Dispersal patterns identified for fireweed (diffusive) could help in the management of outlying foci as eradication of small pioneering populations in front of a continuous invasion front is often plausible. Such management strategies can be applied

leading to the successful eradication. Coupled with climatological similarities, genetic similarity between *Miconia calvescens* (velvet tree) in Hawaii and southern Pacific islands, suggest that Hawaiian infestations have not yet reached full invasive potential. Brazilian biological control agents may not be suitable for control of the current infestations of velvet tree throughout the Pacific and more effective agents are likely to be found closer to geographic source(s) of Pacific infestations (Mexico). These findings also caution against the introduction of early successional tree species that originated from dense canopied rainforests for ornamental purposes into areas with less saturated forest habitats, as pre-adaptation could lead to invasiveness when they escape cultivation.

Exotic plant introductions represent a grand, if unfortunate, experiment in evolutionary ecology, and as illustrated in this dissertation research, the understanding of these evolutionary processes is fundamental to our basic understanding of biological invasions and ecological management.

GLOSSARY

Achene: A small, dry, indehiscent one-seeded fruit with a thin wall, as in the sunflower.

Adaptive topography: A multi-dimensional graph that shows the frequencies of genes and their combinations plotted against average fitness for a given set of environmental conditions.

Additive genetic (variation, effects): A mechanism of quantitative inheritance such that the combined effects of genetic alleles at two or more gene loci are equal to the sum of their individual effects.

Admixture: Refers to reproductive output from different groups (populations) of individuals having different allele frequencies

Agamospermous: The asexual formation of embryos and seeds without the occurrence of fertilization.

Allele: A variant segment of the genetic material.

Allopolyploidization: Changes in **ploidy levels** due to hybridization between different species and thus combination of chromosome sets from different parents.

Apomixes: Any of several kinds of reproduction without fertilization.

Assignment (test): A method of assigning individuals to the populations from which they were most likely to have originated (regardless of where they dispersed to or were sampled).

Autopolyploidization: Changes in **ploidy levels** due to within-individual processes such as chromosome doubling.

Biotype A subspecies of organism morphologically similar to but physiologically different from other members of the species.

Bottleneck: Reduction in population size that can have major influence on genetic variation because of the relationship between genetic drift and population size.

Codominant: expression of heterozygote phenotypes that differ from either homozygote phenotype.

DNA bar coding: A taxonomic method which uses a short genetic marker in an organism's mitochondrial DNA to identify it as belonging to a particular species.

Effective population size: The size of a hypothetical stable, randomly-mating population that would have the same rate of gene loss or increase in inbreeding as the real population (size N).

Fitness: The capability of an individual of certain genotype to reproduce, and usually is equal to the proportion of the individual's genes in all the genes of the next generation.

***F*ST:** The proportion of the total genetic variance contained in a subpopulation (the s subscript) relative to the total genetic variance (the $_T$ subscript). Values can range from 0 to 1. High F_{ST} implies a considerable degree of differentiation among populations.

Gene flow: A mechanism for evolutionary change resulting from the movement of genes from one population to another. Gene flow introduces new genes into a population and also acts to make populations more similar genetically to one another.

Genetic assimilation: The process that occurs when selection acts upon heritable variation in **phenotypic plasticity** to turn a phenotype directly stimulated by an altered environment into a fixed phenotypic response no longer sensitive to the ancestral environmental triggers.

Genetic distance: A measure of the dissimilarity of genetic material between different species or individuals of the same species.

Genetic drift: a force that reduces **heterozygosity** by the random loss of alleles. Drift is inversely related to **population size**. Infinitely large populations (an assumption of the **Hardy-Weinberg equilibirum**) will not experience drift, whereas small populations will experience major effects of drift.

Genetic engineering: The process whereby any organism's genes are directly altered through adding DNA from a completely different type of organism rather than through cross-breeding or cross-pollination of closely related organisms, as in hybrids.

General-purpose-genotype: Denotes a strategy that weeds utilize extensively in having **genotypes** that allow a wide degree of **phenotypic plasticity** and an adequate and sustained level of **heterozygosity**.

Genotype: The entire genetic identity of an individual, including alleles, or gene forms, that do not show as morphological characteristics.

Genotypic reaction norm: Describes the pattern of phenotypic expression of a single genotype across a range of environments.

Haplotype: The genetic constitution of an individual chromosome. May also refer to one locus or to an entire genome.

Hardy–Weinberg equilibrium (HWE): Under certain conditions, after one generation of random mating, the genotype frequencies at a single gene locus will become fixed at a particular equilibrium value.

Heterozygosity: An individual or population-level parameter. The proportion of loci that are heterozygous in an individual or population (ranging from 0 to 1.0).

Holocene: Refers to a period of time between the present and 10,000 years before present.

Hybridization: Crossing of individuals from genetically different strains, populations, or species.

Homoplasy: Any characteristic found in two taxa whose similarity is not due to common descent.

Hybrid vigor: Increased size or fitness of a hybrid, generally a result of its increased heterozygosity.

Inbreeding: Reproduction between closely related individuals; includes self-fertilization.

Introgression: The movement of a gene from one species into the gene pool of another by backcrossing an interspecific hybrid with one of its parents.

Isolation-by-distance: Refers to the idea that individuals may be spatially distributed across some region, with slow dispersal with allele frequencies varying gradually across the region.

Linkage disequilibrium (LD): Greater co-occurrence of two genetic markers (on the same chromosome, as a **haplotype**) in a population than would be expected for independent markers. Usually, LD is generated when the markers are located close together on the same chromosome.

Maternal effects: The phenomenon where the genotype of a mother is expressed in the phenotype of its offspring, unaltered by paternal genetic influence.

Mixogenic (hair): see **Papillose (hair)**

Molecular genealogy: The application of the techniques for characterizing an individual's genotypic make-up to the task of testing a hypothesis that two or more

individuals share a common ancestor and of estimating how far back in time that ancestor lived.

Molecular systematics: A product of the traditional field of systematics and the growing field of bioinformatics. It is the process of using data on the molecular constitution of biological organisms' DNA, RNA, or both, in order to resolve questions in systematics, i.e. about their correct scientific classification or taxonomy from the point of view of evolutionary biology.

Monoclonality (genetic): Refers to genotypes that are genetically indistinguishable and thus identical clones.

Monophyletic (group or clade): Evolutionary assemblage of taxa that includes a common ancestor and all of its descendants.

Naturalized: Introduced from another region and persisting without cultivation.

Neutral genetic diversity: Genetic diversity that is inferred from molecular markers in non-coding regions of the genome and is thus not influenced by natural selection.

Oblanceolate: A shape (usually referring to leaves) which is tapered to a point at both ends but broadens towards the apex.

Panmixic: Referring to unstructured (random-mating) populations.

Papillose (hair): Small, round or cone-shaped protuberances (hair) on the top of the surface (e.g. **achene**).

Phenotypic plasticity: The ability of an organism with a given **genotype** to change its phenotype in response to changes in the environment.

Phylogeography: The study of biogeography as revealed by a comparison of estimated phylogenies of populations or species with their geographic distributions.

Pinnately lobed: Variable divisions of leaflets arranged along the long axis of the leaf.

Pleistocene: The geologic timescale referring to the period from 1,808,000 to 11,550 years before present.

Ploidy level: Refers to the number of complete sets of chromosomes in each cell (except reproductive and some other specialised cells).

Polygenic: A trait whose expression is influenced by more than one gene.

Polyphenism: The occurrence of several phenotypes in a population which are not due to different genetic types. Also see **phenotypic plasticity**.

Polyploid: having more than two sets of homologous chromosomes.

Pre-adaptation: A characteristic evolved by an ancestral species or population that serves an adaptive though different function in a descendant species or population.

Private alleles: Alleles unique to a given deme.

Purifying selection: A type of natural selection in which genetic diversity decreases as the population stabilizes on a particular trait value.

***Q*ST:** The proportion of the total quantitative genetic variance contained in a subpopulation (the s subscript) relative to the total genetic variance (the *T* subscript). Values can range from 0 to 1. High Q_{ST} implies a considerable degree of differentiation among populations.

Quantitative genetic diversity: Genetic diversity that is inferred from the variation in phenotypes that are quantitative in nature and continuous in distribution. These traits are normally under selection.

Recombination: The formation of new combinations of genes.

Restriction endonucleases: Enzymes that cut double-stranded DNA. The enzymes make two incisions, one through each of the phosphate backbones of the double helix without damaging the bases.

Spatial Autocorrelation: Property of random variables which take values, at pairs of sites a given distance apart, that are more similar (positive autocorrelation) or less similar (negative autocorrelation) than expected for randomly associated pairs of observations.

Species complex: A group of species that satisfy the biological definition of species, that is, they are reproductively isolated from each other, but which are not morphologically distinguishable.

Sympatric: Populations of organisms that inhabit the same or overlapping geographic area.

Transgenic: Containing genes from another species.

Transition: A point mutation in the DNA in which replacement is by a similar nucleotide. i.e., a purine (A and G) by a purine or a pyrimidine (C or T) by a pyrimidine.

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