

POPULATION GENETICS OF *PHANAEUS VINDEUS* AND *P. DIFFORMIS*
AND CONGRUENCE WITH MORPHOLOGY ACROSS A
GEOGRAPHIC ZONE OF SPECIES OVERLAP

by

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ABSTRACT

POPULATION GENETICS OF *PHANAEUS VINDEUS* AND *P. DIFFORMIS* AND CONGRUENCE WITH MORPHOLOGY ACROSS A GEOGRAPHIC ZONE OF SPECIES OVERLAP

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Baseline studies are the cornerstone of any new program of research. Long held assumptions about the taxonomic status of an organism and its phylogenetic relationships to other organisms may be called into question and reassessed with different tools. Taxonomy as a field finds itself in somewhat of a crisis, dealing with several major questions that get to the core of species concepts and recognition while still fitting the ever-widening data stream into existing concepts and analysis methods.

Contact zones lie at an important crossroads between taxonomy and evolution. Taxonomy, as the study of individuals and populations in the confines of a specific

spatiotemporal frame of reference; and evolution, as the study of the on-going process that continues to refine, reinforce, and at times disrupt existing taxonomy. Contact zones are affected by both extrinsic and intrinsic factors and teasing apart the myriad of factors affecting a contact zone takes time and approaches from several angles.

Amplified Fragment Length Polymorphisms (AFLPs) reveal that the rainbow scarabs *Phanaeus vindex* (MacLeay) and *Phanaeus difformis* (LeConte) are distinct evolutionary units which occur in sympatry at the Fort Worth Nature Center. The genetic structure at this location is distinctly bimodal with strong segregation of taxa based on soil type. Elsewhere in the zone of geographic overlap, *P. vindex* is an edaphic generalist, being found on several different soil types. In addition, certain allele frequencies within *P. vindex* locations exhibited a significant correlation with a precipitation gradient and other allele frequencies within *P. vindex* locations were significantly different on loam soil than on both sand and clay soils. These results provide a testable hypothesis of selection acting on certain AFLP alleles within *P. vindex* as they relate to soil type and annual precipitation.

Phanaeus vindex and *P. difformis* exhibit continuous morphological character states between two extremes, with character states of one species periodically occurring in the other species, particularly within the zone of geographic overlap. Individual specimens may therefore be misidentified based on any given character within a morphological character suite. A very high degree of congruence between principal component 1 of morphological variance and principal component 2 of molecular variance was obtained using a taxonomic congruence approach. Presence/absence of

mid-longitudinal costae on elytral interstriae was 99% accurate in predicting an individual's genotype within my data set.

The results of these studies confirm the current taxonomic classification of these beetles and provide strong evidence that past laboratory crosses were dominated by heterospecific pairings.

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CHAPTER 1

CONTACT ZONES, ECOLOGY, AND A NEW SYSTEM: PHANAEUS DUNG BEETLES

1.1 Bimodality: a Central Question in Evolutionary Biology

A central question of interest in evolutionary biology is, “what maintains bimodality in contact zones?” A bimodal zone is a contact zone dominated by parental genotypes with hybrids being rare or absent (e.g. Bridle & Butlin 2002). At the other end of the continuum, a unimodal zone is one in which hybrids dominate and parental genotypes are rare (e.g. Childs *et al.* 1996). Bimodality can be maintained by many prezygotic and postzygotic isolation mechanisms alone or in concert (Jiggins & Mallet 2000).

Postzygotic isolation can maintain bimodality either via endogenous or exogenous selection against hybrids (Bronson *et al.* 2003). First, divergent populations under endogenous selection will have few surviving hybrids because hybrid genotypes are less fit than parental genotypes (Carney *et al.* 1994, Shaw *et al.* 1982). Such hybrid genotypes produce individuals which are unfit due solely to unfavorable interactions between alleles, genes, and linkage groups within the hybrid genotype regardless of the environment to which the phenotype is exposed. Alternatively, bimodality maintained by populations undergoing exogenous selection will have few hybrids because hybrid phenotypes are less fit with respect to their environment (Grant & Grant 1993, 1996).

Bimodality can also be the result of prezygotic isolation or the failure of divergent populations to reproduce successfully. Some known prezygotic mechanisms include conspecific sperm precedence (Chang 2004, Geyer & Palumbi 2005, Howard 1999), mechanical (Peretti 2003) and cytoplasmic incompatibilities (Engelstadter *et al.* 2006), ecological/ spatial separation (Via & Hawthorne 2002), geographical isolation and conspecific recognition (Bridle & Butlin 2002, Smith *et al.* 2003).

Conspecific sperm precedence and reproductive incompatibilities will produce positive assortative fertilization regardless of whether mating is positively or negatively assortative. The last four mechanisms will produce positive assortative mating in nature even when they do not produce positive assortative fertilization under laboratory conditions. Jiggins and Mallet (2000) argue that bimodality is associated most strongly with prezygotic rather than postzygotic factors, but see Coyne and Orr (2004) for a counter argument.

1.2 Contact Zones

Contact zones occur when individuals from two closely related but genetically distinct populations occur sympatrically (Harrison 1990, 1993). The most commonly observed phenomenon in contact zones is bimodality with any admixing of genetic information producing hybrids (Howard *et al.* 2003, Jiggins & Mallet 2000). Hybrid outcomes in nature were once thought to be limited to complete sterility, hybrid swarm formation, or the reinforcement of reproductive isolation mechanisms when two taxa come into secondary contact (Dobzhansky 1940, Marshall *et al.* 2002). In fact, outcomes occur along a broad continuum ranging from bimodality, where parental

genotypes dominate (Bridle & Butlin 2002, Peterson *et al.* 2000, Szymura & Barton 1986), to rapid and complete introgression leading to a hybrid swarm (Childs *et al.* 1996, Taylor *et al.* 2006). Understanding contact zone dynamics is important to our understanding of speciation (Seehausen 2004, Turelli *et al.* 2001), as well as the formation, maintenance, and disruption of species boundaries (Cicero 2004, Cruz *et al.* 2004, Vines *et al.* 2003).

1.2.1 Hybrids and hybridization

A hybrid can be defined as any individual of mixed ancestry between two populations which show fixed differences at a minimum of one genetic locus (Harrison 1993). Generally hybrids as recognized in most studies are recent hybrids, individuals whose ancestry traces back to “pure species” in three or fewer generations. Natural hybridization is a fairly common phenomenon in both plants and animals (Arnold 1997, Mallet 2005). With modern phylogenetic techniques it is also possible to detect less recent hybridization. The so-called “Ghost of hybridization past” is evidenced by reticulate evolution of one genome into another over the course of evolutionary history or even in the course of a research career (Besansky *et al.* 2003, Carney *et al.* 2000, Herben *et al.* 2005). Such historical gene flow is often uncovered by using genetic data or a combination of data sets in hybrid zone studies where genetic maps are available for parental taxa.

Hybridization can also lead to the formation of new sympatric or parapatric species (Arnold 1997, Jiggins 2006, Richmond & Reeder 2002, Schilthuizen *et al.* 2004, Schneider 2000). This occurs through chromosome arrangements, polyploidy,

novel character development, or intermediate characters in a novel habitat. Evidence of species with hybrid origins is not uncommon in the literature (see review in Arnold [1997]).

1.2.2 Contact Zone Models

Tension - Several models can be invoked to explain spatial variation of hybrid genotypes in contact zones. The most widely used model is the Tension Zone model (Barton & Hewitt 1985, Key 1968) where there is no barrier to mating between species coming into secondary contact, usually within a narrow geographic area (Bronson *et al.* 2003, Phillips *et al.* 2004, Virdee & Hewitt 1994). The tension zone model assumes that hybrids have reduced fitness compared to parentals and that hybrid genotypes are swamped by continuous dispersal of parental genotypes into the zone. A tension zone is often cited as a way to speed up reproductive isolation between two taxa that evolved in allopatry, where they had no need for such isolation (Dobzhansky 1940, Peterson *et al.* 2004).

Bounded Hybrid Superiority - The Bounded Hybrid Superiority model (Moore 1977) invokes an assumption that while hybrids are less fit than parentals in parental habitats, they may actually be equally fit or more fit in a transitional ecotone, disturbed habitat, or unrealized niche (Anderson 1948, Moore 1977, Remington 1968). The notion that hybrids are not always less fit than parentals is very important to the increasing number of studies pointing to sympatric and parapatric hybrid origins of many groups (Rieseberg 1995, Arnold 1997, Schilthuizen 2000).

Mosaic - The Mosaic model invokes a patchily distributed habitat mosaic over which differentially adapted species are found (Bierne *et al.* 2003, Bridle & Butlin 2002, Harrison & Rand 1989, Rand & Harrison, Ross & Harrison 2002). This model in its original form (Howard 1986) assumed less fit hybrids in both habitats but acknowledged the existing habitat gradients of the bounded hybrid superiority model. Recent work by Vines *et al.* (2003) showed that hybrids between the toads *Bombina bombina* and *Bombina variegata* in a mosaic were indeed more numerous in intermediate habitat types than either parental. They also showed that dispersal into the zone by parentals was not swamping hybrid genotypes. This may be a case of more gradual species boundary collapse as opposed to examples of rapid collapse creating a hybrid swarm following an exotic species introduction.

Evolutionary Novelty - A fourth model by Arnold (1997), the Evolutionary Novelty model, assumes that interspecific matings can give rise to novel traits making a hybrid equally fit or more fit than parentals in parental habitats, unexploited niches and transitional ecotones (Arnold & Emms 1998). It also acknowledges that maladaptive hybridization does produce new lineages in both plants and animals (Reviewed in Arnold and Emms [1998]). While the literature is admittedly dominated by tension zones, it remains vital to recognize the importance of evolutionary novelty, landscape structure, and chance in producing new lineages (Carney *et al.* 1994, DeMarais *et al.* 1992, Rieseberg 1991).

1.3 Effects of Habitat on Contact Zones

Habitat and ecology can have profound effects on contact zone structure and the maintenance of bimodality (species boundaries) under the first three hybrid zone models, but the Mosaic and Bounded Hybrid Superiority models in particular. Mosaic and Bounded Hybrid Superiority hybrid zone structure are both characterized specifically as they relate to habitat patches or ecotonal clines. On a local scale, this may simply mean soil type segregation or host plant segregation over a broad landscape patchwork or across a distinct ecotone. On a continental scale this leads to the idea of suture zones, specific areas of a continent where an abrupt ecological transition promotes contact zone formation between multiple differently adapted species pairs (Remington 1968, Swenson & Howard 2004, Swenson & Howard 2005). The tension zone model often evokes an ecological transition zone but generally assumes secondary contact between divergent previously allopatric populations.

Habitat can have an effect on contact zone structure and bimodality via both prezygotic and postzygotic mechanisms. Its effect on prezygotic boundaries takes the form of habitat associations or preferences (MacCallum *et al.* 1998, Nosil *et al.* 2006, Pereyra *et al.* 2004, Prowell *et al.* 2004, Sandoval & Nosil 2005). The effect habitat has on postzygotic boundaries lies in the form of exogenous selection acting on the hybrid genotype (Schneider *et al.* 2005).

1.3.1 Prezygotic habitat effects

In a two species, two habitat scenario, prezygotic habitat segregation alone promotes positive assortative mating which in turn preserves bimodality and species

boundaries (Via & Hawthorne 2002). An additional component, selection against immigrants from one habitat to the other, may also operate in concert to preserve bimodality and species boundaries (Nosil *et al.* 2005). Contact zones are likely to occur where strikingly different habitats either abut or are found in relative close proximity. Local proximity increases the odds of two species with different habitat preferences hybridizing under natural conditions.

One can imagine habitat segregation itself occurring along a continuum from weak to strong ecological association. In the absence of other prezygotic boundaries, positive assortative mating should be weakened by weak habitat associations and conversely strengthened by strong habitat associations. If both species in a contact zone do not show equal habitat preference, perhaps one species is a habitat generalist and its congener is a habitat specialist, this could impose an asymmetry on positive assortative mating. Hybrid crosses would occur more frequently in the specialist's habitat than the generalists. If natal habitat association was strong, hybrid offspring of such encounters would more likely backcross to the specialist making gene flow unidirectional in the direction of the specialist. While the possibility exists for such an asymmetry explained by habitat association, asymmetry in assortative mating and unidirectional gene flow are most often attributed to postzygotic isolating mechanisms.

When the transition between two habitat types is gradual rather than abrupt, creating an intermediate habitat, assortative mating can break down in the intermediate habitat (Virdee & Hewitt 1994). In this case prezygotic bimodality can still be maintained though continuous dispersal of parental genotypes into the zone. This is the

classic tension zone model long thought to be the first stage in the formation of postzygotic boundaries (Barton & Hewitt 1985, Dobzhansky 1940).

1.3.2 Postzygotic habitat effects

Habitat associations can also be invoked in the postzygotic maintenance of bimodality and species boundaries via exogenous selection acting on hybrid fitness (Schneider *et al.* 2005). Three of the four contact zone models view all hybrids as unfit in parental habitats. Several models however leave open the option of increased hybrid fitness in intermediate habitat types leading to either speciation via lineage founding or reticulate evolution. Under purely exogenous selection, hybrids with increased fitness in parental habitats will produce more offspring and bimodality will collapse over time. If hybrids under exogenous selection are less fit than parents in parental habitat types, bimodality can be maintained with some gene flow through semi-permeable species boundaries.

If hybrids have higher fitness than parents only in an intermediate habitat type, bimodality in the intermediate habitat will break down under purely exogenous selection. Bimodality would however be maintained between each parent in their respective habitat with some gene flow through semi-permeable species boundaries. While bimodality can theoretically be maintained exclusively by other habitat effects as listed above, the main way it is thought to operate is through promoting positive assortative mating between conspecifics (Via & Hawthorne 2002).

1.4 Amplified Fragment Length Polymorphisms

1.4.1 Definition of AFLPs

AFLPs or Amplified Fragment Length Polymorphisms are selectively amplified fragments of DNA which serve as dominant genetic markers (Vos *et al.* 1995). These fragments are created in a restriction ligation reaction by digesting genomic DNA with two restriction enzymes, and then ligating adaptors to the end of each fragment. The most common restriction enzymes used are MseI and EcoRI. Once the DNA is chopped into fragments and adapters attached to the ends of each fragment, two successive amplification reactions are generally employed to bring the number of markers down to a set more reasonably visualized and measured on an electrophoretic gel run or a DNA sequencer, usually about 100 markers. The first amplification step amplifies only those fragments that have a certain base pair sequence 1 selective base from each original cut end. The second amplification step amplifies only those fragments remaining from the first amplification step that have a certain base pair sequence 3 or 4 selective bases from each cut end.

Amplification occurs by creating multiple copies of the selected fragments mediated by the enzyme Taq DNA polymerase. This is known as a polymerase chain reaction (PCR). One of the selective oligonucleotide DNA primers used in the second amplification step is labeled with either radioactive or fluorescent dye to allow visualization of the fragments. Generally the labeled oligonucleotide is the EcoRI sequence. AFLPs are visualized either with electrophoresis through a polyacrylamide gel or with a capillary DNA sequencer. Both methods achieve separation of fragments

based on their size in base pairs. The fragments will show up as visible bands on a gel and as fluorescent peaks on an electropherogram generated by a capillary sequencer.

When visualizing markers with a sequencer, it is recommended to mix the twice-amplified samples with a size standard labeled with a different color dye. The size standard is a series of DNA fragments of known base pair (bp) sizes (i.e. 50bp, 100bp, 150bp, 200bp.....400bp). This allows a computer program to compare amplified fragments of unknown size to a series of standard fragments of known size. Using the size curve created by the standard, a software package can accurately measure the base pair sizes of the unknown AFLP markers. A peak or band is scored as allele presence at a specific locus (i.e. 256 bp). If this band or peak is absent in an individual it is scored as allele absence at that locus.

1.4.2 General utility of AFLPs

AFLPs are a very valuable tool in population genetics studies because they allow a researcher to generate hundreds of polymorphic markers genome wide with relatively little time and low cost compared with other methods. One drawback to AFLPs is that they are dominant markers so there is no way to know if band presence is a product of a homozygous dominant allele pair (AA) or a heterozygous dominant pair (Aa). Thus heterozygote deficiencies are difficult to estimate without additional assumptions but see Lynch and Milligan (1994). The ability to generate lots of markers can be highly valuable in species phylogenies (Albertson *et al.* 1999). AFLPs are also useful for locating QTLs or Quantitative Trait Loci responsible for specific traits and in genetic linkage maps (van Haeringen *et al.* 2002, Rogers *et al.* 2001, Howard *et al.*

2002). In addition, AFLPs have been shown very effective at discriminating hybrid individuals (Congiu *et al.* 2001, Haig *et al.* 2004) and have the potential to discriminate F1 and F2 hybrids from early backcrosses and parentals (Miller 2000). For a detailed review see Bensch and Akesson (2005).

1.4.3 Utility of AFLPs in contact zones and species boundaries

AFLPs are very effective at detecting hybrid individuals in contact zones even when other methods fail to do so (Bensch *et al.* 2002). A single fixed allele present in all individuals of each population means hybrids will have the diagnostic allele from both populations. Since a hundred polymorphic loci are usually generated per AFLP primer combination, the odds of finding a diagnostic allele are very good. The odds of finding such alleles however decrease with decreasing genetic divergence between populations (Bensch & Akesson 2005). It is also possible to estimate introgression between potentially hybridizing taxa by using the maximum likelihood method and treating alleles as coming from a given source population based on their relative frequencies in each population (Rogers *et al.* 2001). This allows one to generate a hybrid index score for each individual based on its unique AFLP fingerprint.

AFLPs are also useful in phylogenetic studies involving recently diverged groups and have been used to clarify species boundaries in cryptic species such as Hawaiian crickets (Parsons & Shaw 2001) and recently radiated species flocks such as Lake Malawi cichlids (Albertson *et al.* 1999).

Because of their utility in phylogenetic studies of cryptic species boundaries, AFLPs should be useful in testing the heterospecificity of two potentially hybridizing

taxa, the AFLP data matrix can be used to generate genetic distances between each individual. The genetic distance matrix can then be submitted to a principal component analysis. The results of this analysis can be viewed as a phylogeny or a minimum spanning tree with the individuals treated as terminal branches (Wiens 2000). These trees show, based on clustering, whether or not separated evolutionary units are present in the data set. If hybrids between bimodally separated taxa are present, they will form a third cluster intermediate between two clusters on a minimum spanning tree.

1.5 Taxonomy

1.5.1 Taxonomic characters

Taxonomists use a wide array of characters to identify species and designate higher level taxa. These include behavioral, chemical (Petrakis *et al.* 2003), ecological (Allsopp & Lambkin 2006), morphological, and genetic (Damgaard *et al.* 2005, Gaubert *et al.* 2004, Hodges & Zamudio 2004, Savage *et al.* 2004). Strongly supported conflict between the results obtained from two or more independent character data sets, though rare, can stress the need for more specimens, complementary analysis methods, more characters, or a discrete data set when there is a high degree of intraspecific morphological variation (Hillis & Wiens 2000, Hodges & Zamudio 2004, Leache & McGuire 2006). Incongruence may also be explained by convergence (Gaubert & Veron 2003, Wiens *et al.* 2003), reticulation (Leache & McGuire 2006), or rapid speciation (Althoff *et al.* 2006).

1.5.2 Taxonomic congruence

Congruence is concerned with agreement of analysis method (Chippindale & Wiens 1994, Hillis & Wiens 2000, Levasseur & Lapointe 2001, Mickevich 1978). Taxonomic congruence, or consensus (Bull *et al.* 1993, Miyamoto & Fitch 1995), means agreement in tree topology between separately analyzed data sets. Alternatively, character congruence, or total evidence (Kluge 1989, 1998), requires all characters from independent data sets to be combined before analysis. These methods are used when reconstructing large phylogenies but can also be applied to multiple populations of two different taxa. Multiple data sets and methodological congruence can test weak nodes in a phylogeny (Leache & McGuire 2006) and confirm species status (Gaines *et al.* 2005).

1.6 The *Phanaeus vindex*- *Phanaeus difformis* Study System

1.6.1 Distribution and habits of *Phanaeus*

Phanaeus is a neotropical New World genus of large, robust, often brightly colored scarabaeine dung beetles with a high percentage of species occurring in Mexico (Edmonds 1994). *Phanaeus* with few exceptions are diurnal, generalist feeders of large mammal dung (Edmonds 1994). They exhibit biparental cooperation in nesting and brood ball construction (Halffter *et al.* 1974). Of the 45 described species (Edmonds 1994, 2004, 2006), six species in three infrageneric groups are found in the continental U.S. *Phanaeus quadridens* and *P. adonis* have been documented rarely on the periphery of their ranges in southern Arizona and south Texas respectively (Edmonds 1994, Riley & Wolfe 2003) but the other four species are common in collections and

well established components of many coprophagous insect guilds, especially in the southern and southeastern U.S. The relationships among the members of the genus based on morphology have been elucidated recently by Edmonds (1994).

*1.6.2 The *Phanaeus vindex* - *P. difformis* contact zone*

Phanaeus difformis and *P. vindex* are considered sister species in the *vindex* group of the genus *Phanaeus* (Edmonds 1994). The former ranges throughout most of Texas and Oklahoma with populations in peripheral American and Mexican States. The latter ranges eastward from the Chihuahuan desert and Southern Rocky Mountain foothills to the Atlantic coast and southward from the 43rd parallel to the Gulf of Mexico, being replaced by *P. difformis* in much of central and south Texas. A large zone of potential species contact in the form of a crescent covers southern Kansas, Oklahoma, eastern Texas, and western Louisiana (Figure 2.1).

Fertile F₁ hybrids between the two species have been produced in laboratory crosses. There was some hybrid breakdown going from the F₁ to the F₂ generation with only ~30% of F₁ crosses producing F₂ progeny (Blume & Aga 1978). Hybrid offspring were not compared morphologically to the parentals in the Blume and Aga study. These lab crosses have been called into question based on possible misidentification (Price 2005). Recent detailed characterization of the male aedeagus showed probable mechanical compatibility between species (Price 2005). These taxa can be difficult to differentiate in sympatry because presupposed species specific characters show continuous variation between two extremes. In addition small individuals must be assessed based on a smaller suite of characters due to underdeveloped pronota. This

continuous variation creates the appearance of “morphological intermediates” occurring in the zone of species overlap and raises the possibility of natural hybridization between heterospecifics (Edmonds 1994). It also stresses the need to rule out conspecificity (Price 2005).

1.6.3 Habitat associations in this system and other considerations

Ecological habitat association has been observed within the large zone of overlap with *P. difformis* largely occurring on sandy soil with bunchgrass cover and *P. vindex* largely occurring on clay soil with a vegetative cover of dense sod (Blume & Aga 1976, Blume & Aga 1978, Edmonds 1994). A similar edaphic association is noted in Florida where *P. vindex* and *P. igneus* overlap (Edmonds 1994).

In addition to the reasons listed above, *Phanaeus* dung beetles are appropriate taxa for ecological and evolutionary study for the following reasons. They are locally abundant in the south and easily trapped using bait. They are a recently diversified genus with a present distribution greatly affected by large scale human endeavors, namely deforestation and large domestic animal agriculture (Edmonds 1994, Montes de Oca 2001). They exhibit complex social and reproductive behaviors such as bi-parental cooperative brood care (Halffter *et al.* 1974, Rasmussen 1994) and a “sneaker” or “satellite” reproductive strategy employed by small males (Rasmussen 1994).

1.7 Studies Conducted

In this thesis I discuss the results of two studies using this contact zone system. The first is a molecular analysis of the population genetics of *Phanaeus vindex* and *P. difformis*. The second is a simple congruence test between patterns based on discrete

morphological characters versus genotypes obtained from the first study. The goals of the first study were 1. Use AFLPs to test the currently recognized species status of *Phanaeus vindex* and *P. difformis*, 2. See if hybrids are present within the zone of geographic overlap, 3. Test for genotype/habitat associations across the zone of geographic overlap. The goals of the second study were 1. Conduct a congruence test of morphological data obtained by using a suite of discrete external characters versus the genotypes obtained from the molecular analysis, 2. Perform individual character analysis on the morphological characters to test their ability to accurately predict genotype.

CHAPTER 2

POPULATION GENETICS AND EDAPHIC SELECTION ECOLOGY OF A DUNG BEETLE CONTACT ZONE BASED ON AFLP MARKERS

2.1 Abstract

Natural contact zones provide an ideal context in which to test the existence of reproductive boundaries between populations and to assess the possible causes of those boundaries. Bimodality, which provides evidence for species boundaries in a contact zone, can have multiple endogenous and exogenous causes. Amplified Fragment Length Polymorphisms reveal that *Phanaeus vindex* (MacLeay) and *Phanaeus difformis* (LeConte) are distinct evolutionary units which occur in local sympatry at the Fort Worth Nature Center. The structure of this contact zone is distinctly bimodal with strong segregation of taxa based on soil type. Elsewhere in the zone of geographic overlap, *P. vindex* is an edaphic generalist, being found on several different soil types. In addition, certain allele frequencies within *P. vindex* locations exhibited a significant, linear, non-zero relationship with annual precipitation and other allele frequencies within *P. vindex* locations were significantly different on loam soil than on both sand and clay soils. This allele frequency evidence provides a testable hypothesis of ecological selection acting on AFLP allele frequencies in both species and both results stress the underlying importance of ecology in this system.

2.2 Introduction

Contact zones occur when individuals from two closely related but genetically distinct populations occur sympatrically (Harrison 1990, 1993). The most commonly observed phenomenon in contact zones is bimodality, where parental genotypes dominate, and hybrids are rare (Howard *et al.* 2003, Jiggins & Mallet 2000). Bimodality is an indication that species boundaries are complete, or nearly so. Understanding contact zone dynamics is important to our understanding of both speciation (Seehausen 2004, Turelli *et al.* 2001), and the maintenance of species boundaries (Cicero 2004, Vines *et al.* 2003).

Ecology often plays an important role in structuring contact zones (Anderson 1948, Arnold 1997, Arnold & Emms 1998). For example, habitat preference is often associated with a mosaic pattern zone (Harrison & Rand 1989, MacCallum *et al.* 1998) with parental genotypes dominating in parental habitat types across a patchy landscape and hybrid genotypes present in intermediate habitat types (Rieseberg 1995, Vines *et al.* 2003). Contact zones can also be associated with sharp ecotonal transitions resulting from habitat (Virdee & Hewitt 1994), and/or environmental variables (Cruz *et al.* 2004, Grahame *et al.* 2006). This often produces a relatively narrow cline with parental genotypes dominating in parental habitats, and hybrids and backcrosses present in the contact zone. Whether a researcher detects a mosaic or cline may depend in part on the scale of observation which is important when drawing conclusions about zone structure (Ross & Harrison 2002).

For animals in terrestrial habitats, ecological variables such as soil type (Dayton *et al.* 2004), dominant vegetation (Lee & Rotenberry 2005), and vegetation structure (Patten *et al.* 2004), are often cited as correlates of species occurrence. Several explanations for such associations include selection (Nosil *et al.* 2005), natal experience (Davis & Stamps), competition (Mitchell & Banks 2005, Grant & Grant 2006), and adaptive radiation (Fine *et al.* 2005).

One group of terrestrial animals with strong, well documented ecological links is dung beetles. Ecological associations among dung beetle species and larger dung insect guilds are quite common. Dung insect guilds and individual dung beetle species have been associated with climate, elevation, soil type (Blume & Aga 1976), resource type (Errouissi *et al.* 2004), vegetative cover, seasonal activity (Andresen 2005), and diel activity cycle (Fincher 1971). In addition, ecological associations between sympatrically occurring congeners have also been demonstrated (Nealis 1977; See Hanski & Cambefort 1991 for a detailed review).

In a contact zone, molecular markers provide the ability to ascertain if sufficient genetic separation exists between two populations for them to be considered distinct species (Parsons & Shaw 2001). Molecular markers can also be used to detect recent and historic gene flow between species (Besansky *et al.* 2003). Use of these markers in an assessment of species status can add additional information, either confirming existing taxonomy (Gaines *et al.* 2005), or leading to synonymy of taxa (Benavides *et al.* 2002).

AFLPs or Amplified Fragment Length Polymorphisms (Vos *et al.* 1995) have been widely used in studies of plant genetics but uses among animals are on the rise as zoologists increasingly appreciate the utility of the tool (Bensch & Akesson 2005, Howard *et al.* 2002, Parsons & Shaw 2001). AFLPs are very useful for systems where no prior genetic information is available. It is less expensive and labor intensive than many methods, and allows for the generation of multilocus genotype profiles consisting of hundreds of loci spanning the entire genome (Bensch & Akesson 2005). AFLPs have been used to discriminate cryptic species (Parsons & Shaw 2001) and increasingly in contact zones where they have the ability to detect F₁ hybrids from parent taxa (Bensch *et al.* 2002, Haig *et al.* 2004).

This study is an initial investigation into the population genetics of two closely related species of dung beetles using AFLPs across a potential geographic contact zone. The aims of this study are to assess the heterospecificity of the two taxa; if heterospecific, to determine if habitat type plays a role segregating taxa in sympatry; and if heterospecific, to determine if hybrids are present in the zone of overlap.

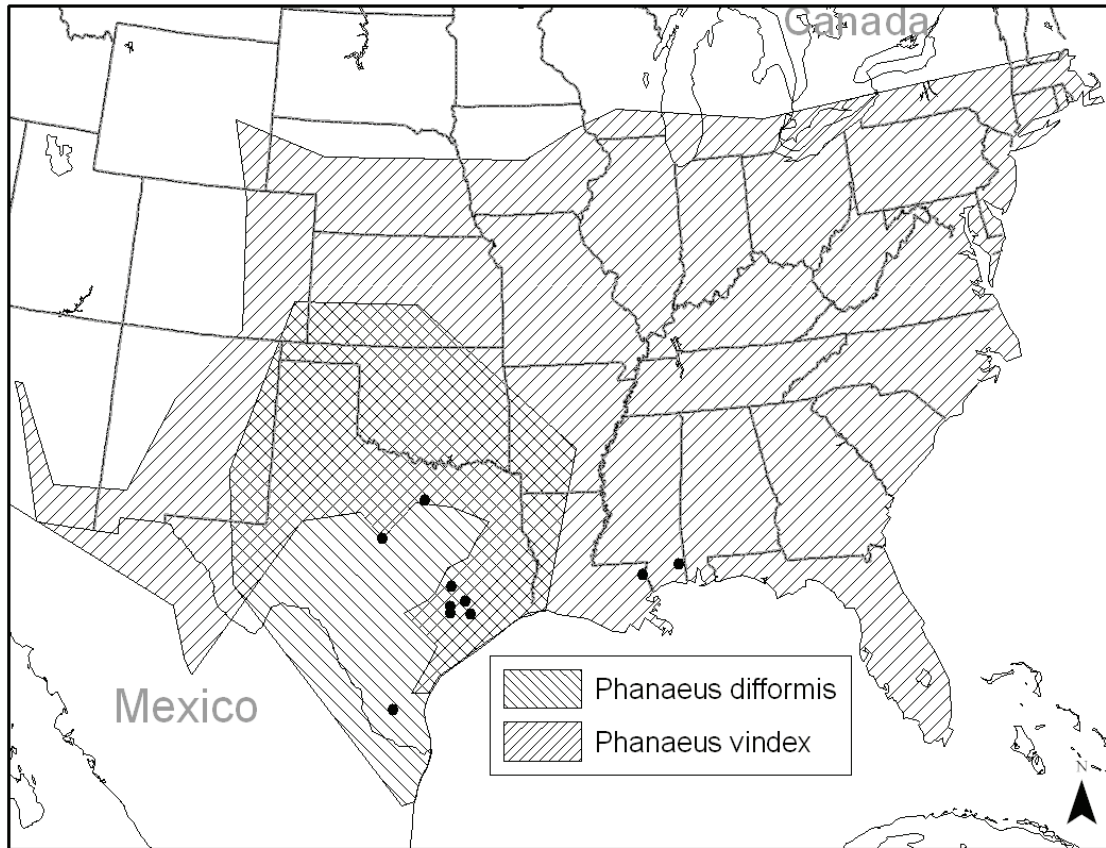


Figure 2.1: Range Map for *Phanaeus vindex* and *P. difformis* with collection localities.
For a list of collecting localities see Appendix A.

2.3 Materials and Methods

2.3.1 Study system

Phanaeus difformis and *P. vindex* are considered sister species in the *vindex* group of the genus *Phanaeus* (Edmonds 1994). The former ranges throughout most of Texas and Oklahoma with populations in peripheral American and Mexican States. The latter ranges eastward from the Chihuahuan desert and Southern Rocky Mountain foothills to the Atlantic coast and southward from the 43rd parallel to the Gulf of Mexico, being replaced by *P. difformis* in much of central and south Texas. A large zone of potential species contact in the form of a crescent covers southern Kansas, Oklahoma, eastern Texas, and western Louisiana (Figure 2.1).

Laboratory crosses have been successful with partial hybrid breakdown between F₁ and F₂ generations (Blume & Aga 1978) and these putative species appear mechanically compatible with respect to male genitalia (Blume & Aga 1978, Price 2005). Recently these hybrid crosses have been called into question because of possible misidentification of the parent species (Price 2005). Some ecological associations have been noted in the large zone of geographic overlap with *P. difformis* collected largely in areas of sandy soil and bunch grass and *P. vindex* collected largely in areas of clay soil dominated by dense sod (Blume & Aga 1976). A similar edaphic association is noted in Florida where *P. vindex* and *P. igneus* overlap (Edmonds 1994). Outside the zone of geographic overlap, both species are more abundant on sandy soils (Edmonds 1994).

In addition to the reasons listed above, *Phanaeus* dung beetles are appropriate taxa for ecological and evolutionary study for the following reasons. They are locally

abundant in the south and easily trapped with bait. They are a recently diversified genus with a present distribution greatly affected by large scale human endeavors, namely deforestation and large domestic animal agriculture (Edmonds 1994, Montes de Oca 2001). They exhibit complex social and reproductive behaviors such as biparental cooperative brood care (Halfpter *et al.* 1974, Rasmussen 1994) and a “sneaker” or “satellite” reproductive strategy employed by small males (Rasmussen 1994).

2.3.2 Animal collection

Dung beetles were trapped live during 2004 and 2005 by using pitfall traps baited with human and swine feces. Beetles were then transported to the lab and frozen (-80°C). *Phanaeus* were trapped primarily at the Fort Worth Nature Center (FWNC), a 3,600 acre nature preserve with several habitat types (Appendix A and Figure 2.1). Within the FWNC, my trap site on clay soil is located approximately 700m from my trap site on sandy soil. In addition, *Phanaeus* were sampled from 8 locations between southern Texas and eastern Mississippi, an area that spans the contact zone as well as parapatric populations of each species under consideration (Figure 2.1). Specimens were also solicited from state and local agencies, universities, zoos in the southern and eastern U.S., and cattle ranches in south Texas. Habitat variables, soil type and vegetative cover were recorded for each trap site in the field. Geographic coordinates for each location were obtained from <http://www.satsig.net/maps/lat-long-finder.htm> and climate data was obtained from <http://www.weather.com/>. A detailed list of geographic, climatic, and habitat variables associated with each collection locality is given in Appendix A. All “major” males were tentatively assigned to species based on

pronotal sculpturing after Edmonds (1994). “Major” males are large males whose prothoracic shield is sufficiently developed for use as a character in this dichotomous key.

2.3.3 DNA isolation and AFLP reactions

Whole genomic DNA was extracted from leg muscle tissue using a DNeasy kit (Qiagen) following manufacturer’s instructions. 1.5µL extracted DNA was digested in 0.1µL MseI and 0.42µL EcoRI and ligated to 1.0µL MseI adapters (0.064 g/L) and 1.0µL EcoRI adapters (0.62g/L) using 0.33µL T4 DNA ligase. Adapters were preheated to 95⁰ C for 5 minutes and cooled at room temperature for 10 minutes prior to adding to reactions. The 11µL total reaction volume included 2.2µL 5X T4 DNA ligase buffer, 1.1µL 0.5M NaCl, 0.55µL BSA (1mg/mL), and 2.8µL water. This reaction was incubated in a thermocycler for 2-3 hours at 37⁰ C, diluted with 60µL TE buffer, cleaned up using a Qiaquick (Qiagen) kit according to clean-up instructions, and diluted again with 60µL TE buffer.

For the pre-selective amplification, 4µL of clean-up product was amplified using 0.5µL each 20µM MseI and EcoRI pre-selective primers with 0.5µL each 10mM DNTPs and Taq DNA polymerase in 4µL buffer E and 10µL water. Total reaction volume 20µL was run under the following thermocycler conditions: 72°C for 2 minutes; 25 cycles of 94°C for 20s, 56°C for 30s, and 72°C for 4 minutes; 60°C for 30 minutes; 4°C hold. The preselective product was then diluted with 40µL buffer TE.

For the selective amplification step, 3µL of a 1:9 pre-selective product-to-water dilution was amplified using 1µL each MseI and EcoRI selective primers with 0.5µL

each 10mM DNTPs and Taq DNA polymerase in 4μL buffer E and 10μL water. Total reaction volume of 20μL was run under the following thermocycler conditions: 94°C for 20 seconds; 50 cycles of 94°C for 20 seconds, 66-56°C for 30 seconds, and 72°C for 2 minutes; 60°C for 30 minutes; 4°C hold. To visualize the AFLPs, 1μL selective product was mixed with 4.3μL each HiDi and water and 0.4μL 400HD ROX standard, denatured for 3 minutes at 95⁰ C and run on an ABI3130XL (Applied Biosystems) sequencer. Oligonucleotide sequences used for AFLP reactions are given in Table 2.1.

Table 2.1: Sequences of primers used in AFLP reactions

Oligonucleotide	Sequence
EcoR1 adapters	5' CTC GTA GAC TGC GTA CC 3'
	5' AAT TGG TAC GCA GTC TAC 3'
Mse1 adapters	5' GAC GAT GAG TCC TGA G 3'
	5' TAC TCA GGA CTC AT 3'
EcoR1 preselective primer	5' GAC TGC GTA CCA ATT CA 3'
Mse1 preselective primer	5' GAT GAG TCC TGA GTA AT 3'
EcoR1 selective primer (labeled)	5' GAC TGC GTA CCA ATT CAG T 3'
Mse1 selective primer	5' GAT GAG TCC TGA GTA ATC A 3'

2.3.4 AFLP analysis

Electropherograms were analyzed using GeneMapper[®] 4.0 (ABI) with peak detection threshold set at 350 RFUs. All alleles between 50 and 400 base pairs were scored. The output allele table was converted to a 1/0 matrix with allele present at a given base pair length scored as a 1 and absence of that allele scored as a zero. One specimen was genotyped twice to test reproducibility of electropherogram generation and subsequent computer analysis.

The 1/0 matrix was analyzed in NTSYS (Rohlf 2000). First, dissimilarity coefficients between each sample were generated. Second, principal component analysis was used to generate a UPGMA tree and a minimum spanning tree from the distance matrix.

Numerical eigenvalues of each sample along the first 3 principal component axes were treated as multilocus genotypes for each beetle since the eigenvalues correspond to loci responsible for the 3 main principal components. The allele table was examined for possible correlation, primarily with principal component 1. The multilocus genotypes were tested for habitat associations. Histograms for axes created in Excel (Microsoft) were visualized relative to location in Arc Map 9.0 (ESRI). Finally, multilocus genotypes were averaged within each population and these population means were analyzed against ecological variables and latitude and longitude gradients to check for correlations.

2.4 Results

2.4.1 Collections and initial AFLP analysis

Beetles were captured from nine locations and four beetles were donated from a tenth location (Appendix A). Three males were assigned to *P. difformis* and ten males were assigned to *P. vindex* based on dorsal pronotal shape. Two male beetles from the FWNC were kept completely intact and remain at the center as voucher specimens.

One hundred eighty eight alleles were generated from genotyping 110 beetles from nine locations with a single AFLP primer combination. One beetle was genotyped twice to verify reproducibility. Alleles represent presumptive loci. Each sample had a unique DNA fingerprint. Number of alleles per sample ranged from 18 to 36 with an average of 29. Forty seven alleles were unique to a single specimen. Two alleles were common to all specimens and an additional two alleles were found in 94% and 96% of specimens respectively. The two genotypes from the same beetle differed by about 2% (samples “1” and “2” in figure 2.2).

2.4.2 Major clades

Two distinct clades resulted from both the UPGMA tree (not shown) and minimum spanning tree (Figure 2.2). Members of both clades were present at the FWNC. Two of three major males initially assigned to *P. difformis* based on the pronotum were among the smaller clade (*difformis* clade) along with all beetles donated from a south Texas ranch. The larger clade (*vindex* clade) contained ten of ten males initially assigned to *P. vindex* based on the pronotum and a single male originally assigned to *P. difformis*. Seven alleles were strongly associated with the *difformis*

clade, being found in at least 80% of all specimens and 20% or fewer of all specimens in the *vindex* clade. Three alleles fit the same criteria for association with the *vindex* clade. Of these associated alleles, alleles at 257 and 150 base pairs were >99% diagnostic for *difformis* and *vindex* clades respectively. Within the *vindex* clade there was distinct population level structuring with all the beetles collected from Sun, Louisiana forming a smaller sub-clade within the larger clade (samples 91-109 in figure 2.2). Genotype differences within clades ranged from 12-22%. Genotype differences between clades ranged from 23-25%.

2.4.3 Bimodality and major ecological associations

Principal component 1 explained 84% of the molecular variance while principal components 2 and 3 explained 1.7% and 1.0% of the variance respectively. Histograms of multilocus genotypes for principal component 1, (PC1), show distinct bimodality at the FWNC and unimodality at all other locations within the zone (Figure 2.3).

Analysis of multilocus genotypes (PC1) for different soil types reveal a strong bimodal association with the *difformis* clade associated with sand and the *vindex* clade associated with clay at the FWNC ($P_{\text{binomial}} < 0.00001$). The *vindex* clade is not associated with a particular soil type elsewhere (Figure 2.4). Within the *vindex* clade, there is an association between annual rainfall and multilocus genotype for PC1 between locations ($r=0.662$, $P=0.05$) with this trend also present for the *difformis* clade (Figure 2.5a). Also within the *vindex* clade there is an association between soil type and multilocus genotype for PC2 among locations (Table 2.2, Figure 2.5b).

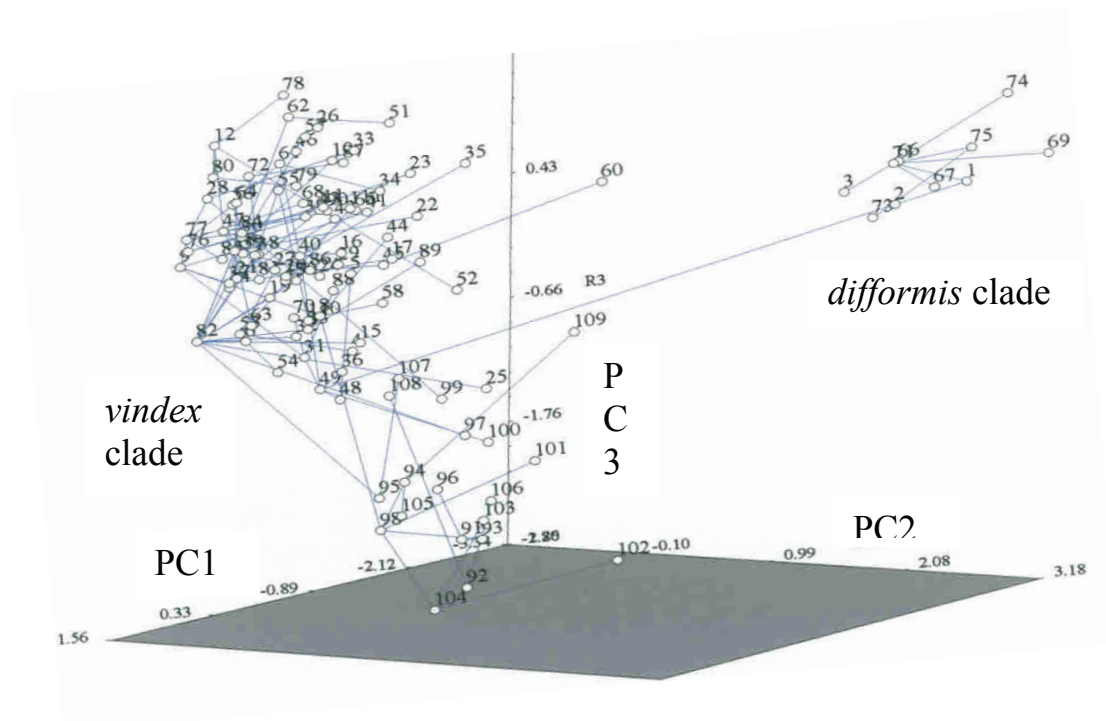


Figure 2.2: Minimum spanning tree (MST) of 111 AFLP genotypes derived from 110 dung beetle specimens showing 2 distinct clades. Axes are the 3 principal components (PC) of genetic variance.

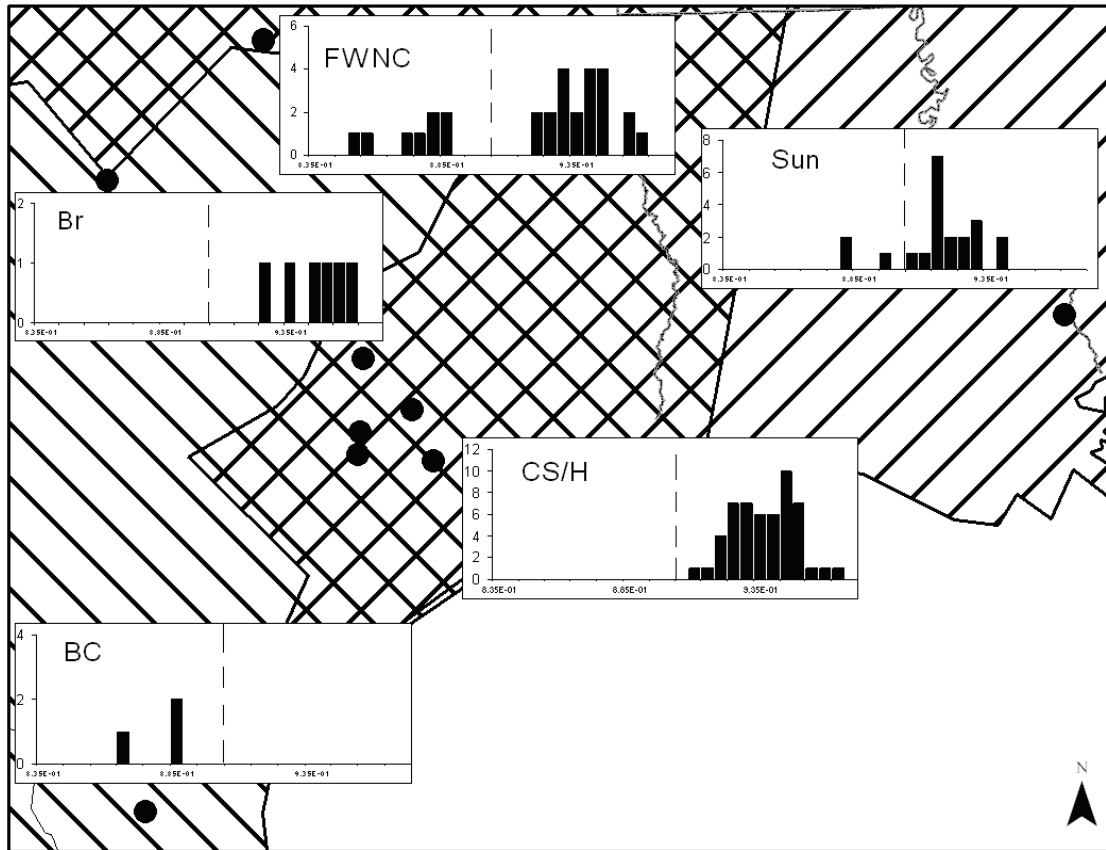


Figure 2.3: Multilocus genotype frequency histograms for principal component 1 eigenvalues by location across a dung beetle contact zone. The five locations between College Station and Houston, TX are consolidated into one histogram. See Appendix A for a list of locations.

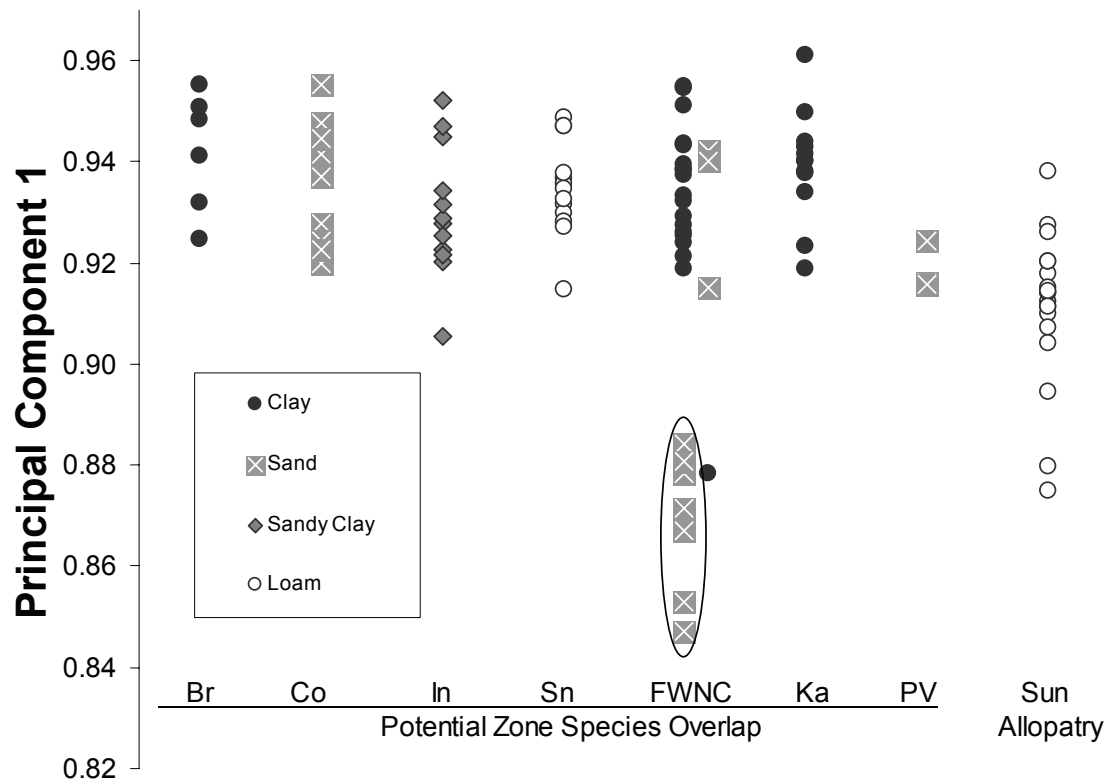


Figure 2.4: Multilocus genotypes for principal component 1 and associated soil types for each collection locality. The *difformis* clade is circled. For localities see Appendix A.

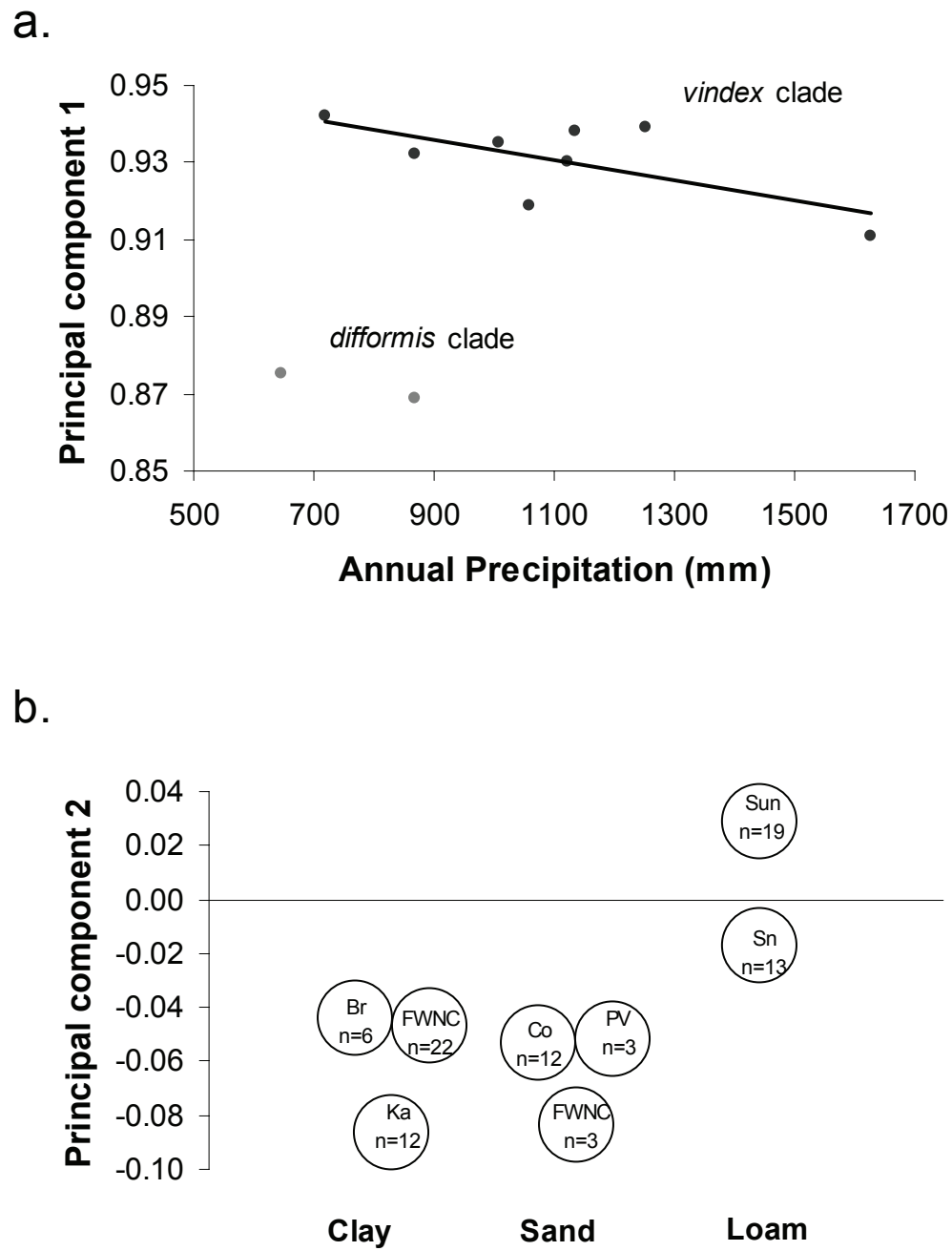


Figure 2.5: Correlations between allele frequencies and (a.) a cline in annual precipitation; and (b.) soil type between locations within *vindex* clade.

Table 2.2: Nested ANOVA of soil associations with frequency of certain PC 2 alleles in the *vindex* clade. Location is nested within soil type. See Figure 2.5b.

Source	DF	SS	MS	F	P	%variance
Soil	2	0.0956	0.0478	7.45	0.0316	35.5
Location (soil)	5	0.0321	0.0064	3.11	0.0129	20
Error	79	0.1631	0.002			44.5

2.5 Discussion

2.5.1 Summary of major findings

The major findings of this study are as follows. First, principal component analysis revealed 2 distinct clusters. These clusters were largely consistent with the current treatment of *Phanaeus vindex* and *P. difformis* based on a single morphological character (Figure 2.2). Second, both species occur in sympatry at the Fort Worth Nature Center and the genetic architecture at this location is distinctly bimodal (Figure 2.3). Third, there were no early generation hybrids in my data set (Figure 2.2). There were no genotypes clustered intermediately between the two clades and those genotypes on the fringe of the *vindex* clade, #s 60, 102, and 109, were characterized by the lack of *vindex* indicated alleles, rather than the presence of indicated alleles of both species as would be expected for hybrids. Fourth, the two species in sympatry segregate

edaphically (Figure 2.4). Fifth, among locations within *P. vindex*, there is a linear correlation between annual rainfall and mean multilocus genotype eigenvalue for principal component 1 (Figure 5a). This means there are differences in the frequencies of certain PC1 alleles between locations and that these allele frequency differences correlate to annual precipitation at each location. This trend interestingly, is also seen for the two *difformis* locations (Figure 2.5a). Sixth, among locations in the *vindex* clade there is a significant difference between certain PC2 allele frequencies associated with loam soil and those associated with both sand and clay soils (Table 2.2, Figure 2.5b).

2.5.2 Bimodality revisited

The Fort Worth Nature Center is only the second location where both species have been reported together. The other location is around Medora, Reno County, Kansas (Edmonds 1994). It is perhaps of interest to note that both locations are situated on the periphery of the rather broad geographical zone of overlap (Figure 2.1). Continuing detailed collection through the center of the zone of overlap between these two locations and a detailed genetic characterization of the Medora contact zone would be informative. The genetic structure of the FWNC contact zone is distinctly bimodal (Figures 2.3 and 2.4).

A central question of interest in evolutionary biology is “what maintains bimodality in contact zones?” Bimodality can be maintained by any number of prezygotic and postzygotic isolation mechanisms alone or in concert (Jiggins & Mallet 2000). For instance, postzygotic isolation can promote bimodality either via endogenous or exogenous selection against hybrids (Bronson *et al.* 2003). Bimodality

can also be attributed to prezygotic isolation or the failure of divergent populations to reproduce (Howard 1999, Peretti 2003, Via & Hawthorne 2002). Interestingly, Jiggins and Mallet (2000) argue that bimodality is associated most strongly with prezygotic rather than postzygotic factors, but see Coyne and Orr (2004) for a counter argument.

My results indicate that one extrinsic factor promoting bimodality in this system is a strong edaphic habitat preference. There is not any evidence of early generational hybrids in my data set and individuals which appeared to possess somewhat intermediate genotypes (see samples 60, 102, and 109 in figure 2.2) showed that signature because they lacked alleles indicative of their own species rather than possessing associated alleles of both species as would be expected with early generation hybrids. While it is possible these three samples reflect a reticulate lineage between both species, the lack of early generation hybrids in my data set indicates that a strong edaphic preference is the primary force maintaining bimodality in this group.

Mitochondrial DNA studies have the potential to detect both past and ongoing introgression as well as the directionality of any such gene flow in this group. Also, detailed hybrid crosses could be employed to test possible intrinsic factors involved in maintaining bimodality and contact zone structure as well as asymmetries in success of reciprocal crosses.

*2.5.3 The role of ecology in the population genetics of *Phanaeus**

Based on my findings, ecology is obviously very important in this system. I found a significant correlation between allele frequency and annual precipitation between locations of *Phanaeus vindex*. This trend interestingly, is also seen for the two

P. difformis locations (Figure 2.5a). This correlation did not correspond to a geographic cline. Although more studies are needed, these results provide a testable hypothesis of selection acting on alleles within PC1 along a gradient of rainfall in both *P. vindex* and *P. difformis*.

Testing hypotheses of selection acting on a genetic polymorphism is important to the growing body of literature in support of selection theory (Li 1997, Freeman and Herron 2004, Huestis 2005, Huestis and Marshall 2006). Geographically wide ranging organisms such as *P. vindex* are quite suitable to testing selection theory because they are found under a broad range of environmental and ecological conditions to which they must adapt. Moreover, Fincher (1973) found that larval survival in *P. vindex* was affected by soil texture and percent-clay-content. All members of the genus encase their brood balls in clay (Edmonds 1994) so there is likely a minimum percent-clay-content requirement for each species to reproduce successfully. Fincher (1973) also found that *P. vindex* was better able to reproduce successfully in several different low-clay-content soils than the two other *Phanaeus* species in the same study.

My data also indicate that *P. vindex* is an edaphic habitat generalist, being found on many different soil types (Figure 2.4). In addition I found a significant difference between the frequency of PC2 alleles and loam soil type within *P. vindex* (Figure 2.5b). This is very interesting because loam is intermediate in particle size between sand and clay but the PC2 allele frequencies associated with sand and clay are very similar to each other (Figure 2.5b). This result also provides a testable hypothesis of selection acting on alleles within PC2 as they relate to soil type within *P. vindex*. It would be of

interest for future researchers to measure percent-clay-content associated with collecting location, rather than the more qualitative designations of “sand”, “clay”, and “loam”. If certain alleles are directly linked to soil type, selection acting on these allele frequencies would be of interest, not only to selection theory, but also to the contact zone structure of this system because *P. vindex* shows distinct soil type preference when occurring with *P. difformis* in local sympatry (Figure 2.4).

2.5.4 The role of habitat preference in contact zone structure.

On a landscape scale, this contact zone is probably a mosaic because sand and clay soil types occur patchily throughout the zone of geographic contact. Three female *P. vindex* specimens were trapped on sand at the FWNC and all were collected with *P. difformis* males in the same trap. This co-occurrence of both species, though infrequent, suggests the potential for these beetles to come into contact in local sympatry. If the sympatric heterospecific taxa in this study do come into contact under natural conditions, what, if anything limits introgression, preserving bimodality?

In a two species, two habitat scenario, prezygotic habitat segregation alone promotes positive assortative mating which in turn preserves bimodality and species boundaries (Via and Hawthorne 2002). An additional component, selection against immigrants from one habitat to the other, may also operate in concert (Nosil *et al.* 2005).

Interspecific habitat segregation should promote positive assortative mating between heterospecifics in sympatry. Assortative mating should in turn preserve

bimodality between heterospecifics (Diehl & Bush 1989; Via & Hawthorne 2002). But this begs the question, what causes habitat preference in this system in the first place?

Traditionally, taxa are thought to be under natural selection in allopatry and that this in turn promotes segregation in sympatry. Alternatively, either one or both taxa could be habitat generalists in allopatry but segregate in sympatry because of local specialization, competition, or to avoid costly interspecific matings. Based on extremely low fecundities (Hinton 1981), reduced ovaries, long ovary development time (Halffter & Lopez 1977), and high degree of parental investment in brood care (Halffter *et al.* 1974), one might expect strong selection against costly interspecific matings in this system. This, along with the strong associations between ecology and genotype demonstrated by this study, suggests that this system may be an appropriate one in which to test these alternatives.

CHAPTER 3

CONGRUENCE OF MORPHOLOGICAL AND MOLECULAR DATA DELIMITING SPECIES BOUNDARIES FOR *PHANAEUS VINDEI* (MACLEAY) AND *P. DIFFORMIS* (LECONTE) (COLEOPTERA: SCARABAEIDAE: SCARABAEINAE)

3.1 Abstract

In this study, I tested the taxonomic congruence of a discrete morphological data set with a genetic data set obtained in a previous study (Chapter 2 of this work). Both data sets were analyzed separately with principal component analysis in NTSYS in order to test for congruence. In addition I performed individual character analyses on the morphological character data set to test the ability of each character to accurately predict genotype. A high degree of congruence was found between data sets and one character in the individual character analysis was found to be ~99% predictive of genotype. I discuss my findings in the context of continuous character states between two extremes exhibited in this group and also the continued use of these beetles in contact zone studies.

3.2 Introduction

Phanaeus is a neotropical New World genus of large, robust, often brightly colored scarabaeine dung beetles with its epicenter in Mexico (Edmonds 1994). *Phanaeus*, with few exceptions, are diurnal, generalist feeders of large mammal dung (Edmonds 1994). They exhibit biparental cooperation in nesting and brood ball construction (Halfpeter *et al.* 1974, Rasmussen 1994). Of the 45 described species

(Edmonds 1994, 2004, 2006), 6 species in 3 infrageneric groups are found in the continental U.S. *Phanaeus quadridens* and *P. adonis* have been documented rarely on the periphery of their ranges in southern Arizona and south Texas, respectively (Edmonds 1994, Riley & Wolfe 2003), but the other 4 species are common in collections and well established components of many coprophagous insect guilds, especially in the southern and southeastern U.S. The relationships among the members of the genus using morphology have been elucidated recently by Edmonds (1994).

Phanaeus difformis and *P. vindex* are considered sister species in the *vindex* group (Blume & Aga 1978). The former ranges throughout most of Texas and Oklahoma with populations in peripheral American and Mexican States. The latter ranges eastward from Arizona and the rocky mountain foothills to the Atlantic coast and southward from the 43rd parallel to the Gulf of Mexico, being replaced by *P. difformis* in much of central and south Texas. A large zone of species overlap in the form of a crescent covers southern Kansas, Oklahoma, eastern Texas, and western Louisiana (Figure 2.1).

Specimens can be difficult to identify in sympatry because morphological characters used to identify species exhibit continuous variation between two extremes. This creates the perception of “intermediates” in the zone of geographic overlap between species (Edmonds 1994). In addition, small individuals must be assessed based on a smaller suite of morphological characters due to underdeveloped pronota. Mechanical compatibility appears probable based on male genitalia (Price 2005). Moreover laboratory hybrid crosses have produced fertile F₁s (Blume & Aga 1978).

Ecological segregation has been observed within the large zone of overlap with *P. difformis* largely being trapped on sandy soil and *P. vindex* largely being trapped on clay soil (Blume & Aga 1976, Blume & Aga 1978, Edmonds 1994). Previously in this work, I provided molecular evidence both of *vindex* and *difformis* as bimodally distinct evolutionary units, and the segregation of these two species in local sympatry based on soil type as designated by previous authors.

Taxonomists use a wide array of characters to separate taxa. These include behavioral, chemical (Petrakis *et al.*, 2003), ecological (Allsopp & Lambkin 2006), morphological, and molecular (Fukami *et al.* 2004, Kawai *et al.* 2006, Verbruggen *et al.* 2005, Wolstenholme *et al.* 2003) characters. Incongruence between two or more sets of characters, though rare, can leave relationships poorly resolved stressing the need for more specimens, complementary analysis methods, more characters, or a discrete data set when intraspecific taxonomic morphologies are highly polymorphic (Baker & Gatesy 2002, Day 2002, Hillis & Wiens 2000, Leache & McGuire, 2006, Masters & Brothers 2002, Wahlberg & Nylin 2003). Incongruence may also be explained by convergence (Chippindale & Wiens 1994, Gaubert & Veron 2003), reticulation (Leache & McGuire 2006), or rapid speciation (Althoff *et al.* 2006). Congruence between multiple data sets provides a robust test of heterospecificity between two species (Gaines *et al.* 2005).

In light of recent molecular data for *Phanaeus vindex* and *P. difformis* (Chapter 2 of this work), a congruence test between genotypes and a suite of discrete morphological characters is desirable. This present study is a simple congruence test

between morphological data and known molecular genotypes to assess the relative strength of individual characters in making species assignment, as well as the strength of a discrete suite of characters as a whole.

3.3 Materials and Methods

3.3.1 Animal collection

Dung beetles were collected live during 2004 and 2005 by using pitfall traps baited with human and swine feces. Beetles were then transported to the lab and frozen (-80°C). *Phanaeus* were collected primarily at the Fort Worth Nature Center (FWNC), a 3,600 acre nature preserve. Within the FWNC beetles were collected from both a sandy soil and clay soil habitat, separated by approximately 700 meters (Appendix A). In addition, *Phanaeus* were sampled from 8 locations between southern Texas and eastern Mississippi, an area that spans the contact zone as well as parapatric populations of each species under consideration (Figure 2.1, Appendix A).

Specimens were also solicited from state and local agencies, universities, zoos in the southern and eastern U.S., and cattle ranches in south Texas. Habitat variables, soil type and vegetative cover were recorded for each trap site in the field. A detailed list of geographic, climatic, and habitat variables associated with each collection locality is given Appendix A. All major males were tentatively assigned to species based on pronota after Edmonds (1994). Legless genotyped and whole beetles trapped on the FWNC were returned to the nature center as voucher specimens. Specimens not collected at the FWNC were retained by the primary author as of the date of thesis submission.

3.3.2 Genetic data used

AFLP multilocus genotypes of 110 specimens from 10 locations were generated using principal component analysis in NTSYS (Rohlf 2000) according to the same analysis method employed in the present study. Results produced two distinct clades with major male pronotal characters largely consistent with Edmonds 1994 description of *difformis* and *vindex* (Figure 2.2). Both species were found in local sympatry at the FWNC and segregated strongly based on soil type (Figure 2.4). The multilocus genotypes used in this congruence test correspond to principal components 1 and 2 (GPC1 and GPC2) in figure 3.4. Also see Figures 2.3 and 2.5 in this work.

3.3.3 Morphological characters used

All previously genotyped specimens from 10 collection localities were assessed based on five discrete morphological characters employed by Edmonds (1994). Character state cut-offs are taken from Edmonds 1994 but several of these character states are more continuous in the revision. For instance, I did not distinguish between weakly serrate and serrate anterolateral pronotal edges (Table 3.1). Figures 3.1 and 3.2 provide color plates of each character under consideration. Table 3.1 provides a complete list of character definitions and cut-offs.

Table 3.1: Character states used in morphological analysis. All characters are taken from Edmonds (1994).

Character	<i>difformis</i> (1)	<i>vindex</i> (0)
1	Costae absent on the first 2 complete interstriae	Presence of at least 1 continuous midlongitudinal costa extending a minimum of 1/2 of the length of the elytra from the base on any of the first 2 complete interstriae on either elytra.
2	Circumnotal ridge irregularly serrate behind anterolateral angle (3 or more indentations)	Circumnotal ridge simple, having a single indentation, or having a single "tooth" (two indentations)
3	Tibia quadridentate	Tibia tridentate
4	Striae 1-2 narrow (<1/5th width of interstriae 2-3 respectively)	Striae broad/flat (>1/5th width of intersriae 2-3 respectively)
5	Male: sides of pronotal disc curved. Posterolateral disc tips not extending laterally beyond the pronotal margin. Female: Transverse pronotal prominence effaced medially and/or bowed posteriorly.	Male: sides of pronotal disc straight. Posterolateral disc tips extending laterally just to or beyond the pronotal margin. Female: Transverse pronotal prominence complete and straight.

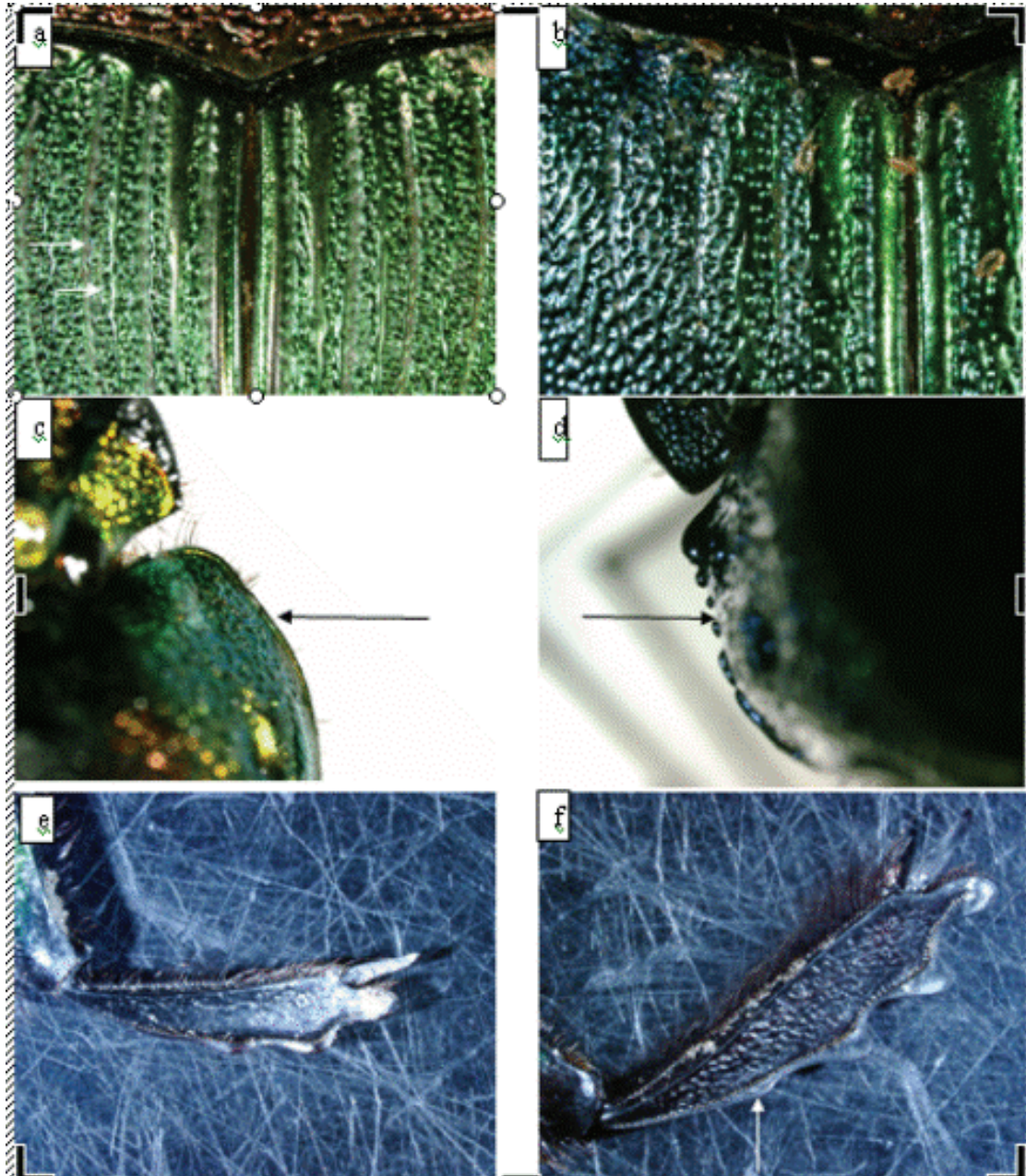


Figure 3.1: Color plates of characters 1-4 under study. Plates a., c., and e. are *vindex* characters and b., d., and f. are *difformis* characters. Plates a. and b. correspond to the two elytra characters. In plate a. the top arrow indicates the stria (character 4) and the bottom arrow indicates the costa (character 1). Plates c. and d. correspond to lateral pronotal serration and plates e. and f. correspond to tibial dentition. The arrow in plate f. indicates the 4th tibial “tooth” in a quadridentate individual. See table 3.1 for a list of characters.

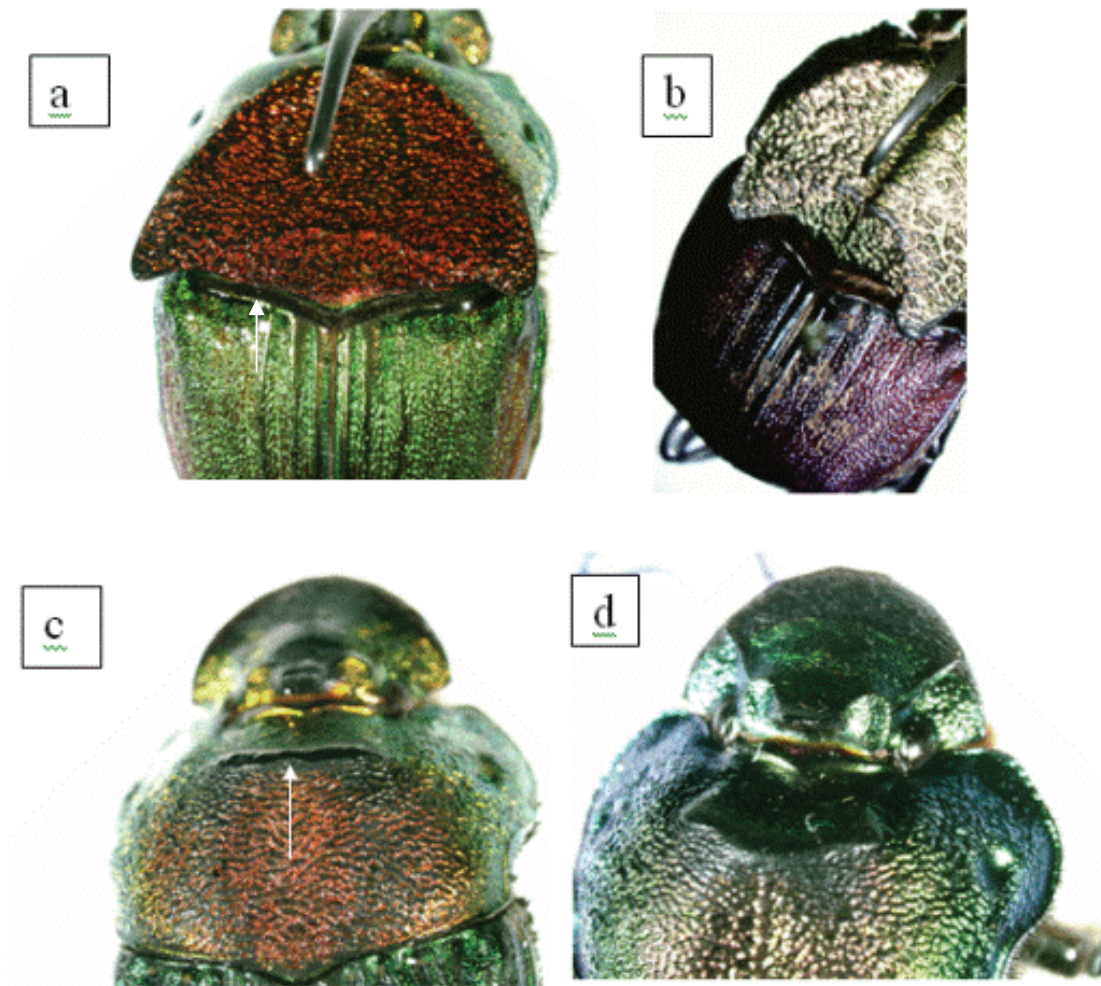


Figure 3.2: Color plates of character 5 under study. Plates a. and c. are *vindex* characters for “major” male and female respectively. The arrow in plate c indicates the pronotal ridge. Plates b. and d. are *difformis* characters for “major” male and female respectively.

3.3.4 Character examination and measurements

Each individual was scored with a 1 or a zero for each character (*difformis* = 1 or *vindex* = 0). Worn tibia and inconclusive pronota for small males were scored as missing data for those characters. Other characters for each specimen that were lost during molecular work were also scored as missing data. Photographs were taken of front tibia prior to molecular work and re-examined during morphological analysis. Specimens were examined using a Nikon SMZ 1500 microscope with lighting provided by a Nikon NI-150 high intensity illuminator under 7.5x, 10x and 20x magnification. Characters 2 and 3 were assessed at 7.5x and 10x power. Characters 1 and 5 were assessed at all three magnifications. Photographs of elytral bases were taken under 20x power. Subsequently stria and interstria widths were measured using Image J 1.36b (National Institutes of Health). The 4 relative stria widths were averaged to get a mean value for each specimen. Means <0.20 were scored as *difformis*, and means >0.20 were scored as *vindex* after Edmonds (1994).

3.3.5 Data analysis

Character 5 was eliminated prior to analysis due to many inconclusive scores for minor males. In addition, all specimens for which there was additional missing data were removed prior to analysis. The output 1/0 matrix of 90 specimens was analyzed using NTSYS. Dissimilarity coefficients were calculated for each sample pair and this output matrix of coefficients was analyzed with principal component analysis to get the 3 major axes of morphological variance. The first 2 principal components were graphed against one another in two dimensions to check for data clustering. Individual specimen

eigenvalues obtained for morphological principal components were then graphed against their respective multilocus genotype eigenvalues.

Individual character analyses were done for each morphological character to determine its ability to predict genotype accurately as well as its availability for score. Individual character analyses were done for both the 90 specimen data set employing 4 characters and the 110 specimen data set with all 5 characters and missing data.

3.4 Results

The resulting eigenvalues from the principal component analysis are given in Table 3.2. Principal components 1 and 2 together accounted for ~87% of the morphological variance within the data set. Graphing the first two principal components (MPC1 and MPC2) together produced only weak clustering. Note that each point may consist of many individuals (Figure 3.3). The 6 genotypic *vindex* males that clustered closer to the genotypic *difformis* in MPC2 all had *difformis* scores for tibia and lateral pronota while the single genetic *difformis* female that clustered closer to the genotypic *vindex* had a *vindex* score for lateral pronota (Figure 4). Graphing MPC1 and MPC2 against the first 2 principal components of genetic variance (GPC1 and GPC2) produced varying degrees of clustering (Figure 3.4). The strongest separation corresponded to GPC2 and MPC1. Clustering for these two principal components was 100% congruent with complete separation of clusters occurring along both axes (Figure 3.4c).

Both individual character analyses gave complementary results with the predictive ability of pronotal sculpturing scoring ranking second in the five character

analysis (Table 3.4). The predictive ability of individual morphological characters ranged from 80-99% (Table 3.4). No single character was 100% diagnostic in predicting genotype but costate/acostate elytra proved the most predictive. A single genotypic *vindex* female lacked mid-longitudinal costae. The character for dorsal pronotal sculpturing was only available for 12 of 39 or 30.8% of all males but was available for 71 of 71 (100%) females. Due to wear, the character for tibial dentition was available for 92 of 110 (83.6%) specimens.

Table 3.2: Results of the Principal Component Analysis of morphological variance conducted in NTSYS (Rohlf 2000). Only the first two principal components are shown.

	MPC1	MPC2
Eigenvalue	68.34	10.14
Percent of variance	75.9%	11.3%

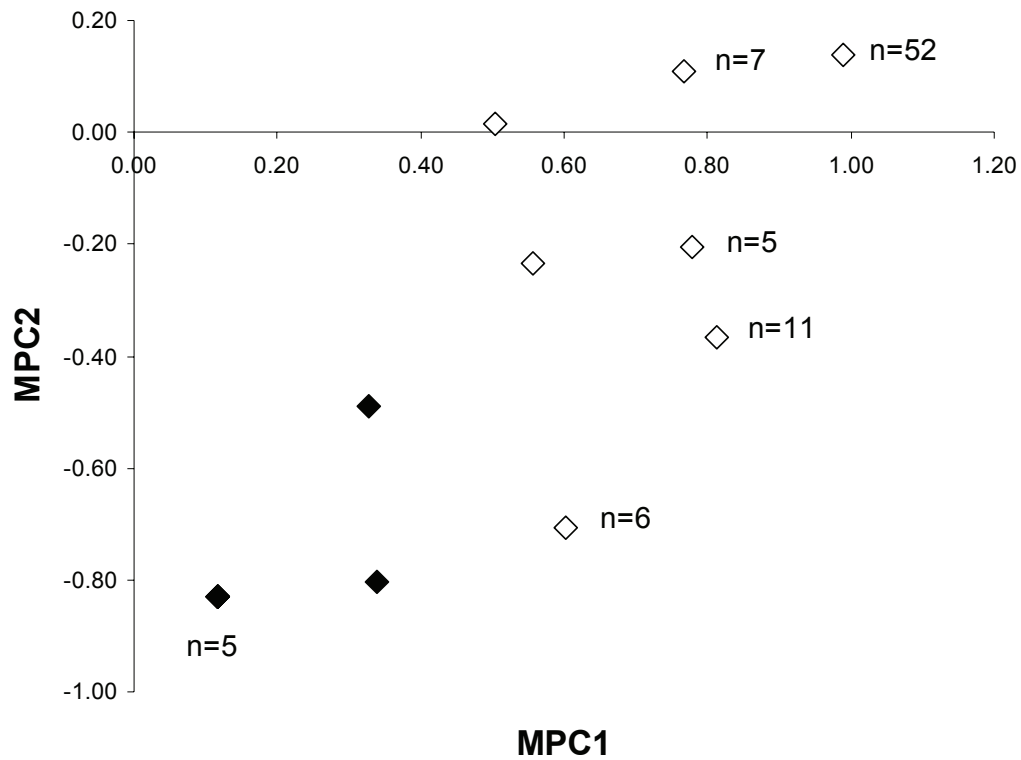


Figure 3.3: Multi-character morphotypes for each beetle graphed against the two major axes of morphological variance. Because multiple individuals shared a common morphotype, each point corresponds to multiple specimens. Where not noted for a point, n=1. Closed diamonds are genotypic *P. difformis* and open diamonds are genotypic *vindex*.

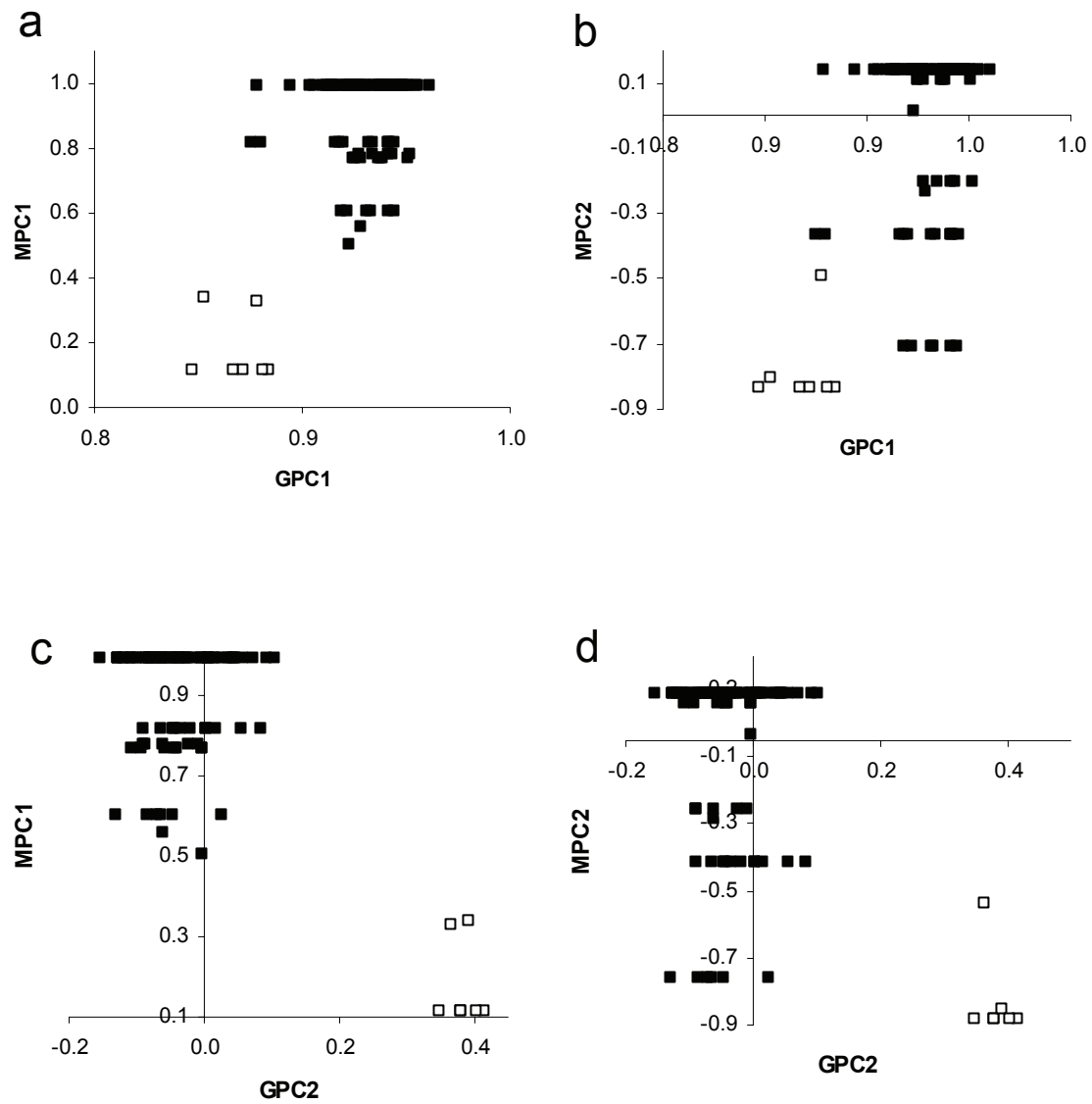


Figure 3.4: Eigenvalues of the 2 major axes of both morphological and genetic variance plotted against each other for 90 *Phanaeus* dung beetles. Open squares are genetic *difformis* and closed squares are genetic *vindex*.

Table 3.4: Predictive abilities of 4 morphological characters in an individual character analysis. This analysis utilized the 90 specimens used in principal component analysis.

Character	Accuracy in predicting genotype	rank
Costae on elytra	89 of 90 = 98.9%	1
Edge of pronotum	77 of 90 = 85.6%	3
Tibial dentition	73 of 90 = 81.1%	4
Relative stria widths	80 of 90 = 88.9%	2

Table 3.5: Predictive abilities and availabilities of 5 morphological characters in an individual character analysis. This analysis utilized all 110 beetles for which genotypes were available.

Character	Accuracy in predicting genotype	rank
Costae on elytra	109 of 110 = 99.1%	1
Edge of pronotum	94 of 110 = 85.5%	4
Tibial dentition	74 of 92 = 80.4%	5
Relative stria widths	94 of 108 = 87.0%	3
Sculpturing of pronotum	79 of 83 = 95.2%	2

3.5 Discussion

3.5.1 Summary of major findings

The results of the morphological analysis are largely consistent with clusters by genotype with some anomalies (Figures 3.3 and 3.4). Figure 3.3 lacks good clustering due to a small number of *vindex* with both serrate pronotal edges and quadridentate tibia. This data as a whole indicates that the character suite is largely predictive of genotype for the majority of specimens. Principal component 1 of morphological variance and principal component 2 of genetic variance were congruent, segregating completely along both axes (Figure 3.4c)

None of the discrete characters in either individual character analysis proved diagnostic but mid-longitudinal costae proved the most reliable (99% accurate) and available (100% available) character in this sample set. The single acostate female molecular *vindex* scored as a morphological *vindex* for 3 of the remaining 4 characters. The character for dorsal pronotal sculpturing was available for only 12 of 39 (30.8%) males but was available for 71 of 71 (100%) females.

3.5.2 Principal component analysis in taxonomy

The use of principal component analysis in this system to address a taxonomic question corresponds well to the “individuals-as-terminals” method described by Wiens (2000). In this method, genetic distances are calculated for each individual such that when a phylogenetic tree is generated, each terminal branch is represented by a single individual. While this is also done when reconstructing a much larger phylogeny from single representatives of each taxa, it appears to be a very powerful approach when

using a large, population-genetics-style sample size to address the question of heterospecificity between two sympatric groups. This method is very important because it accounts for intraspecific as well as interspecific variation between taxa.

The varying predictive abilities of individual characters resulting from individual character analyses indicate that differential weighting of morphological characters (Chippindale and Wiens 1994) may provide greater accuracy in predicting genotype when using discrete characters in this system. Under this system, identification would hinge most heavily upon the presence/absence of some mid-longitudinal costae on the first two elytral interstriae and least on the dentition of the front tibia. Other characters would be weighted intermediately according both to their accuracy and availability for a given specimen.

3.5.3 Continuous character states in *Phanaeus*

While the discrete morphological character “cut-offs” used in this study proved useful to conduct a congruence test with genotypes, the fact remains that the characters in question exhibit continuous variation both within and between species. Thus, specimens are easily misidentified based on a single, or even two morphological characters creating the perception of “intermediates”.

One explanation for the presence of continuous character states and morphological “intermediacy” in this group is historical hybridization (reticulate evolution) between *Phanaeus vindex* and *P. difformis*. Edmonds (1994) observed “intermediates I have seen, however, are *vindex* which resemble *difformis*, never vice versa”. This was also my experience both morphologically and molecularly. Such

morphological variation may simply be intraspecific variation within two phenotypically diverse species but it also may allude to biased gene flow from *difformis* to *vindex*. If the former is true, a diagnostic character, such as a DNA fingerprint is necessary for positive identification in sympatry. If the latter is true, mitochondrial DNA studies could be employed to test this hypothesis in detail. Such mitochondrial studies also hold to the potential to detect both past and ongoing introgression as well as the directionality of any such gene flow. Also, extensive laboratory crosses could be conducted with detailed characterization of the morphological and molecular characters of hybrid and backcross progeny.

3.5.4 Re-evaluating previous hybrid crosses

Fertile F_1 hybrids between the two species have been produced in laboratory crosses. There was some hybrid breakdown going from the F_1 to the F_2 generation with only ~30% of F_1 crosses produced F_2 progeny (Blume & Aga 1978). Hybrid offspring were not compared morphologically to the parentals and these lab crosses have been recently called into question based on possible misidentification (Price 2005). Blume and Aga made their hybrid crosses based on a combination of costate elytra and ventral color. The combination of my molecular and morphological data indicates that crosses made on the basis of presence/absence of mid-longitudinal costae on the interstriae of elytra would have produced a very high percentage of heterospecific pairings. While additional hybrid crosses are beneficial and appropriate, Blume and Aga's hybrid crossing data should be considered largely accurate.

3.5.5 Importance of ecology in the *Phanaeus* system

Ecology plays a strong role both in intraspecific molecular variation within *Phanaeus vindex* and in segregating these two species in local sympatry (Blume & Aga 1978, Chapter 2 of this work). While hybrid crossing data demonstrates that endogenous selection is acting on hybrid genotypes in this system, my genetic data indicate that exogenous selection also plays a role in intraspecific genotype selection within *P. vindex* and in segregating these two taxa based on soil type in a local contact zone. Two alternative explanations for these results are (1) selection acting on genotypes in allopatry (Kleindorfer *et al.* 2006), and (2) selection acting on genotypes in sympatry (Grant & Grant 2006). Does ecology also play a role in structuring morphological variation in this group? If so, the second alternative might produce character displacement of certain phenotypes when beetles occur sympatrically (Brown & Wilson 1956, Grant & Grant 2006, Jang & Gerhardt 2006). Generating more taxonomic characters of all types and the use of data sets where both species are well represented both within and outside the contact zone would both be useful for testing these alternatives.

APPENDIX A

COLLECTION LOCALITIES AND ASSOCIATED ECOLOGICAL AND GEOGRAPHICAL VARIABLES FOR BEETLES USED IN THIS STUDY

Appendix A: Collection localities of variables for beetles used in this study. The Brownwood, TX locality represents a new county record for *P. vindex* for Brown County, Texas.

Locality	Code	Lat/Long	Soil Type	Grass Cover type	Canopy type and density	Annual Precipitation (mm)	Mean Annual Temperature °C	Collection Date	n- collected	n- genotyped	Species expected
Brooks County, TX	BC	27° 3.0' N 98° 25.3' W	-	-		645.5	22.08	Jun-05 (Donation)	4	4	<i>P. difformis</i>
Brownwood, TX	Br	31° 46.2' N 98° 57.2' W	Clay	Sod	Open Pasture	719.2	17.75	12-Oct-04	6	6	<i>P. difformis</i>
Columbus, TX	Co	29° 45.9' N 96° 32.1' W	Sand	Bunchgrass	Moderate Pine	1135.9	20	19-Apr-04	17	12	both
Industry, TX	In	29° 56.2' N 96° 31.6' W	Sandy Clay	Sod	Open Pasture	1121.5	20.75	19-Apr-04	12	12	both
Snook, TX	Sn	30° 28.8' N 96° 29.9' W	Loam	Sod	Open Pasture	1007.7	20.5	19-Apr-04	34	13	both
FWNC	FWNC	32° 50.4' N 97° 28.9' W	Clay	Bunchgrass	Moderate Oak	866.7	18	2004-2005	37	22	both
FWNC	FWNC	32° 50.8' N 97° 28.8' W	Sand	Sod	Open Pasture	866.7	18	2004-2005	11	10	both
Katy, TX	Ka	29° 43.4' N 95° 50.7' W	Clay	Sod	Open Pasture	1253.2	20.67	19-Apr-04	13	12	both
Prairie View, TX	PV	30° 6.1' N 96° 2.5' W	Sand	Sod	Open Pasture	1057.6	19.83	19-Apr-04	3	3	both
Sun, LA	Sun	30° 39.1' N 89° 53.5' W	Loam	Sod	Moderate Pine	1627	19.17	27-May-04	24	19	<i>P. vindex</i>
Lucedale, MS	Lu	30° 52.9' N 88° 36.2' W	Sandy Clay	Sod	Open Pasture	1656.6	18.33	27-May-04	1	0	<i>P. vindex</i>

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BIOGRAPHICAL INFORMATION

Aaron Dickey received his BS in Zoology from Colorado State University in 1997. Since then he has worked mostly in zoos but also performing biology work in the field. Upon completion of his MS in Biology from the University of Texas at Arlington, he plans to attend Texas A&M University and pursue a PhD in Entomology. He has broad interests in natural history, ecology, and evolution. He is happily married to Angie.