Taxonomy and Conservation: A Case Study from *Chamaedorea alternans*

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● *Background and Aims* The establishment of justified recommendations in conservation biology requires robust taxonomic treatments for the group(s) being considered. Controversial or poorly developed taxonomies can have a negative impact on conservation assessments. One example of a taxonomically difficult and controversial species complex that is important in conservation involves two species of Mexican palms, *Chamaedorea tepejilote* and *C. alternans*. The goal of this study was to investigate whether *C. alternans* and *C. tepejilote* are genetically distinct within the Los Tuxtlas region of Veracruz.
● *Methods* Individuals corresponding to the morphology of *C. alternans* and *C. tepejilote* were collected from sympatric and allopatric regions within the Los Tuxtlas Biological Station. Eighty-eight samples were genotyped using amplified fragment length polymorphism (AFLP) markers. Cluster and ordination analyses were used to investigate patterns of differentiation.
● *Key Results* UPGMA and PCO analyses of AFLP profiles recovered two divergent clusters corresponding to morphologically defined *C. tepejilote* and *C. alternans*. No intermediate genotypes were observed and five of the 45 loci were fixed for either the presence or absence between the species. The patterns of divergence observed do not identify a role for sympatric speciation.
● *Conclusions* The observed patterns of differentiation support the recognition of *C. alternans* as distinct from *C. tepejilote*. A suite of vegetative and reproductive morphological features can be used to help distinguish these taxa in the field, but they can be difficult to differentiate from preserved material. Previous treatments of the variation found within the complex (*C. alternans* and *C. tepejilote*) as intraspecific variance is unjustified. Given that the Los Tuxtlas region has suffered from historical and ongoing deforestation and that *Chamaedorea* includes numerous endangered taxa, retaining conservation status for *C. alternans* serves to help safeguard individuals of the species as well as a region and larger taxonomic group all under considerable threat from human activities.

Key words: Species boundaries, Palmae, Arecaceae, AFLP, *Chamaedorea alternans*, *Chamaedorea tepejilote*, endemics, conservation, Los Tuxtlas, Mexico.

INTRODUCTION

Over the past 15 years, phylogenetics has become the primary focus for much of systematic botany, and results from these studies have prompted major advancements towards our understanding of higher level relationships (e.g. Chase *et al.*, 1993; Soltis *et al.*, 1999; Qiu *et al.*, 2000; Soltis and Soltis, 2004). Progress in higher level phylogenetics has been made in Arecaceae (Palmae), and many relationships among subtribes and genera are now robustly supported (Asmussen *et al.*, 2000; Baker *et al.*, 2000*a*,*b*; Lewis and Doyle, 2001; Hahn, 2002*a*,*b*; Lewis and Doyle, 2002; Gunn, 2004; Dransfield *et al.*, 2005; Roncal *et al.*, 2005).

Despite these gains, many issues at lower taxonomic levels continue to hinder complete understanding of palm systematics. Inadequate understanding of both the limits between closely related species and relationships among them have been attributed to a growing crisis in the field of taxonomy that directly impinges on the development of justified recommendations in conservation (e.g. Tautz, 2002, 2003; Gower and Wilkinson, 2003). In fact, the limited progress toward the understanding of species limits in most plant groups may be accredited to both recent focus on phylogenetic research as well as the scale of the problem (Scotland, 2003). In-depth evaluation of hundreds of thousands of species is far more demanding than broad estimation of phylogenetic relationships. Nevertheless, as the global biodiversity situation continues to worsen, accurate estimates of species numbers and limits are important components in the development of logical approaches to conservation (e.g. Golding and Timberlake, 2003; Gower and Wilkinson, 2003).

Palms constitute an important component of tropical forests because of their conspicuous nature, abundance, and impact on forest structure and dynamics. Among some of the least understood groups of Arecaceae are the approx. 10 % of Neotropical palms that have been described as components of species complexes. These include groups of organisms that exhibit diverse forms, and the boundaries between putative species are often obscured by a lack of identified fixed morphological differences, potential hybridization and a paucity of informative collections (Henderson *et al.*, 1995; Borchsenius, 1999). Disputes over whether local forms should be classified as species, infraspecific taxa or natural intraspecific variation have led to varying estimates of species numbers for the family.

The largest genus of Neotropical palms, *Chamaedorea*, serves as a prime example of a group containing intricate species complexes, many of which also include putatively endangered taxa (Mont, 1994; Johnson, 1996). Estimates of the number of species in *Chamaedorea* vary with species concept and researcher. For example, as recently as 1995, Henderson *et al.* recognized 77 species, whereas in 1992 Hodel identified 96 species. A clear understanding of

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species limits within *Chamaedorea* has been hampered by a combination of complex vegetative and reproductive morphology (including dioecy), insufficient material/data and extensive sympatry (Uhl and Dransfield, 1987; Henderson *et al.*, 1995; Hodel, 1999).

One example of a taxonomically controversial species complex that impinges on conservation biology involves *C. tepejilote* Lieb. and *C. alternans* Wendl. *Chamaedorea tepejilote* is a widespread morphologically diverse taxon distributed from southern Mexico to northern South America (Uhl and Dransfield, 1987). For those who recognize *C. alternans*, it is an endemic known only from the Los Tuxtlas region of Mexico where it occurs sympatrically with *C. tepejilote* (e.g. Hodel, 1992). Authorities on *Chamaedorea* (Guillaumin, 1923; Burret, 1933; Hodel, 1990, 1992; Henderson *et al.*, 1995; Thomas *et al.*, 2006) and authors of regional treatments alike (Hemsley, 1885; Standley, 1920; Glassman, 1972; Ibarra-Manriquez, 1988; Quero, 1992) have differed on whether or not to treat *C. alternans* as distinct from *C. tepejilote*.

A need for the critical assessment of *C. alternans* is noted by the fact that the taxon is currently listed with the Mexican Government as federally threatened (Mont, 1994). Nevertheless, recent taxonomic treatments have differed on whether or not even to recognize *C. alternans*. The most recent monograph of *Chamaedorea* (Hodel, 1992) considered *C. alternans* to be distinct based on a number of morphological features (discussed below). In contrast, a more recent morphological treatment of New World palms placed *C. alternans* in synonymy with *C. tepejilote* (Henderson *et al.*, 1995). These conflicting contemporary works clearly place *C. alternans* in a delicate position with respect to conservation. With the latest treatment rejecting recognition of *C. alternans* (Henderson *et al.*, 1995), the taxon is currently at risk of losing its threatened listing.

The uncertain taxonomic status for *C. alternans* has implications that reach beyond basic taxonomy and conservation. Numerous ecological studies focusing on this complex have considered *C. alternans* to be synonymous with *C. tepejilote* (Bongers *et al.*, 1988; Oyama and Dirzo, 1988, 1991; Pompa *et al.*, 1988; Oyama, 1990, 1991, 1993; Oyama and Mendoza, 1990; Oyama *et al.*, 1992; Gonzalez Soriano *et al.*, 1997), whereas others have recognized *C. alternans* as distinct from *C. tepejilote* (Otero-Arnaiz and Oyama, 2001). If *C. alternans* is distinct, some of the aforementioned studies are likely to have inadvertently combined accessions of divergent species into the study of one putative species, which violates fundamental assumptions associated with such analyses. Alternatively, treating *C. alternans* as distinct may result in an unjustified segregation of intraspecific variation. The generation of a more comprehensive understanding of this putative complex should identify which approach is more appropriate and the potential pitfalls of adopting the alternative methodology.

Furthermore, *C. alternans* is endemic to the Los Tuxtlas region, making the putative species a representative of one of the most threatened natural areas in Mexico (Dirzo and Garcia, 1992; Ramirez, 1999). The Los Tuxtlas region spans 1500 square miles and is of broad phytogeographical interest because it combines elements of Caribbean, Central American and mainland Mexican floras within a relatively restricted geographic area (Ibarra-Manriquez, 1988). Los Tuxtlas is located at the northern limit of the tropical rainforest ecosystem (Martinez-Garza and Gonzalez-Montagut, 1999) in the state of Veracruz, southeastern Mexico. Within this region is the Los Tuxtlas Biological Station, which covers 644 ha (at 95 °04–09′W, 18 °34–36′N) between 150 and 700 m in altitude with dominant vegetation classified as tropical moist forest (Holdridge, 1967).

The goal of this study was to investigate whether *C. alternans* and *C. tepejilote* are genetically distinct in the Los Tuxtlas region in order to identify if *C. alternans* should continue to be listed as threatened. Both putative species were analysed using amplified fragment length polymorphism (AFLP) markers (Vos *et al.*, 1995). Prior use of AFLPs within Arecaceae has primarily been limited to the detection of genetic variation within and between populations of major industrial crops such as the coconut and date palms (Perera *et al.*, 1998; Cardoso *et al.*, 2000; Purba *et al.*, 2000; Teulat *et al.*, 2000; Matthes *et al.*, 2001; Diaz *et al.*, 2003; Adin *et al.*, 2004; Kjær *et al.*, 2004; Neumann *et al.*, 2004; Sezen *et al.*, 2005; Clement *et al.*, 2006). More recently, AFLPs have been extended to study sympatric speciation in island palm endemics (Savolainen *et al.*, 2006).

MATERIALS AND METHODS

Materials and sampling

The Los Tuxtlas Biological Station (Fig. 1) has been arbitrarily divided into five consecutive research plots that average 128.84 ha in area (M. Ricker, Los Tuxtlas Biological Station, pers. comm.). Together these span the landscape from west to east towards the Gulf of Mexico (Fig. 1). Accessions of both taxa were sampled along transects that followed approx. 10 m inside the perimeter of each plot as well as across both diagonals. Samples of leaf tissue were collected from sympatric and allopatric regions, and accessions were ascribed to one of the two putative species using morphological features outlined by Hodel (1992). A total of 249 samples was collected, 127 of *C. tepejilote* and 122 of *C. alternans*. Leaf samples for DNA extraction were dried and stored in silica gel. Voucher specimens of exemplar material were collected and deposited at the National Herbarium at the Universidad Nacional Autónoma de México (MEXU).

DNA isolation and AFLP fingerprinting

DNA samples from leaf tissue collected from populations of both putative taxa were analysed using AFLP markers. Forty-four samples of each potential species were selected to represent the geographic distribution across which all samples were collected. Plant material was ground using liquid nitrogen, and total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen). DNA concentrations were estimated by comparison with a
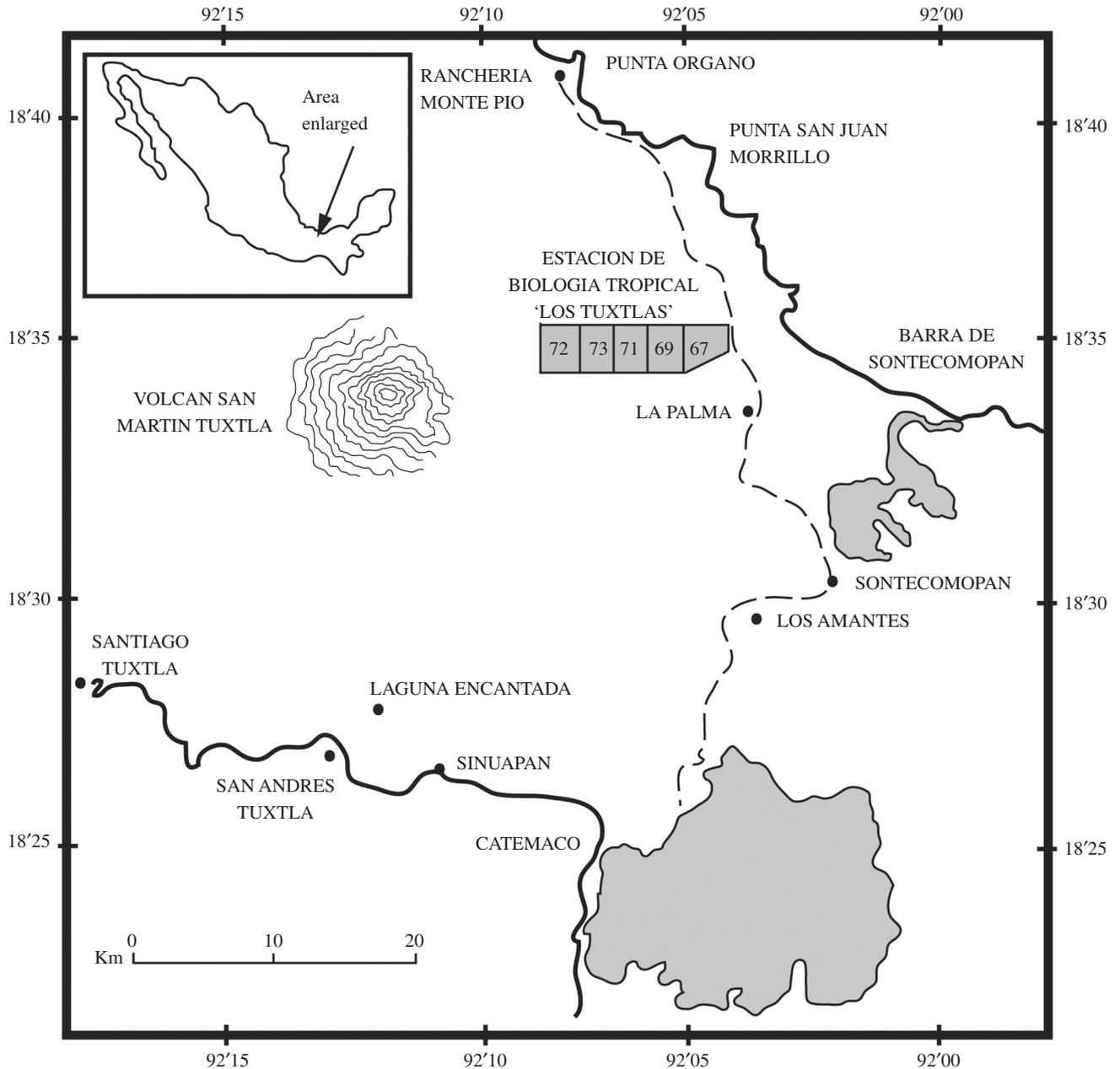
F**IG**. 1. Map of the Los Tuxtlas Biological Station, adapted from Lot-Helgueras (1976).

100 bp DNA mass ladder (New England BioLabs) on 1 % TBE–agarose gels. Following the manufacturer's instructions (Applied Biosystems), approx. 50 ng of genomic DNA was digested with *Mse*I (New England BioLabs) and *Eco*RI (New England BioLabs), and ligated (T4 DNA ligase; New England BioLabs) to double-stranded adaptors through incubation at 37 °C for 12–16 h. Pre-selective and selective primers were based on primer core sequences *Eco*RI 5′-GAG TGC GTA CCA ATT C-3′ and *Mse*I 5′-GAT GAG TCC TGA GTA A-3′. Pre-selective primer combination *Eco*RI -C/*Mse*I -AC and selective primer combination *Eco*RI -CAC/*Mse*I -ACTA were used subsequent to the restriction ligation reaction. Fluorescently labelled products from the selective amplification with internal size standards (GeneScan-500 ROX, Applied Biosystems) were analysed on an ABI 3100 sequencer (Applied Biosystems).

AFLP data analysis

GeneScan version 3.1 (Applied Biosystems) was used to normalize and size fragments, and Genotyper version 3.7 (Applied Biosystems) was used to compare all samples, define loci and output the data matrices for analyses. A locus was defined as any single fragment with a minimum amplitude of 300 fluorescent units occurring in at least one

accession. Loci were scored between 75 and 500 bp. All samples were scored for the absence or presence of an allele at each locus, and pairs of loci with overlapping positions were discarded.

Two distance-based approaches were used to analyse and visualize genetic similarity among accessions in the Multi-Variate Statistical Package version 3.13 (Kovach Computing Services). First, a UPGMA dendrogram was generated using Jaccard's coefficient (Jaccard, 1901) due to its differential weighting on shared presence rather than shared absence and relatively simple interpretation (e.g. Legendre and Legendre, 1998; Duarte *et al.*, 1999). Secondly, a principal coordinates analysis cluster (PCO) was constructed using Euclidean distance. The PCO was primarily used to help screen for potential intermediate genotypes that might be indicative of hybridization or incomplete differentiation. Such patterns are more easily observed in a PCO cluster than in strictly divergent UPGMA dendrograms. Interspecific F_{st} values were calculated in TFPGA (Miller, 1997) treating dominant data according to Lynch and Milligan (1994).

RESULTS

From the 44 accessions analysed per species, reactions for 35 and 39 successfully amplified fragments from representatives of *C. alternans* and *C. tepejilote*, respectively. The average number of fragments recovered from each sample was 55.7 ± 14.4. A total of 69 loci were observed, but this number was reduced to 45 after those with overlapping positions were excluded. Five of the 45 loci exhibited fixed differences (present in all accessions of one putative species cluster and absent in the other), and two loci were monomorphic across all accessions (Table 1). Of the 45 loci scored, 96 % of the loci were polymorphic between two or more samples (Table 1).

Both the UPGMA (Fig. 2) and PCO (Fig. 3) analyses recovered two distinct clusters. With the exception of two samples, these divergent groups correspond to the morphologically defined *C. alternans* and *C. tepejilote*. The exceptions are samples representing *C. alternans* (Bacon 142 and 198), which both had AFLP profiles consistent with the *C. tepejilote* cluster (Figs 2 and 3). The genetic distance between the two clusters was 0·37 in the UPGMA analysis and interspecific F_{st} values averaged 0.42 ± 0.34. Both species were represented by accessions that were collected in each of the five research plots. In the PCO analysis, 60 % of the variation is accounted for by the first two axes in the diagram and 79·5 % of the molecular variation was explained by the two major clusters in this analysis.

Chamaedorea alternans is more abundant in the eastern part of the station. Moving from east to west, eight, 12, eight, two and five accessions were sampled from plots 67, 69, 71, 73 and 72 (Fig. 1). In contrast, *C. tepejilote* had a more even sampling distribution, with four, nine, 11, six and nine accessions sampled from plots 67, 69, 71, 73 and 72. In general, within each species cluster, relatively little local geographic variation partitioned into more similar clusters within a species.

TABLE 1. *Summary of genetic and morphological characteristics of* C. alternans *and* C. tepejilote

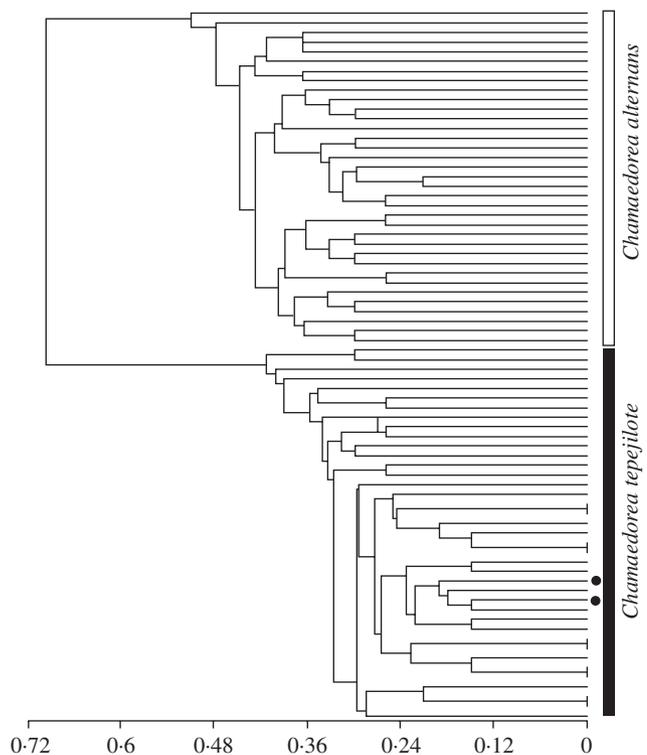|	Chamaedorea tepejilote	Chamaedorea alternans
AFLP fragments*	39	35
Fixed presence fragments*	3	2
Coloration of leaf sheath	Green	White
Venation in leaf sheath	Vague	Distinct
Inflorescences per node	Singular	Multiple
Life form	Multiple stem	Solitary
Habitat	Rocky substrate	Rich, developed soil

*Excludes samples Bacon 142 and 198.

FIG. 2. UPGMA dendrogram using Jaccard's coefficient. *Chamaedorea alternans* is indicated as the top cluster and *C. tepejilote* as the lower cluster. The circles identify the two presumably mislabelled accessions of *C. alternans*.

Eight accessions per species were rerun from the preselective amplification stage to test for reproducibility of AFLP fingerprints. In a replicate UPGMA analysis, including both independently derived profiles for the same sample, 14 of the 16 grouped with their duplicate. Two accessions failed to group with their replicate but each remained within their respective UPGMA cluster.

DISCUSSION

Genetic differentiation

Accessions representing the two divergent clusters recovered in both UPGMA and PCO analyses (Figs 2 and 3)
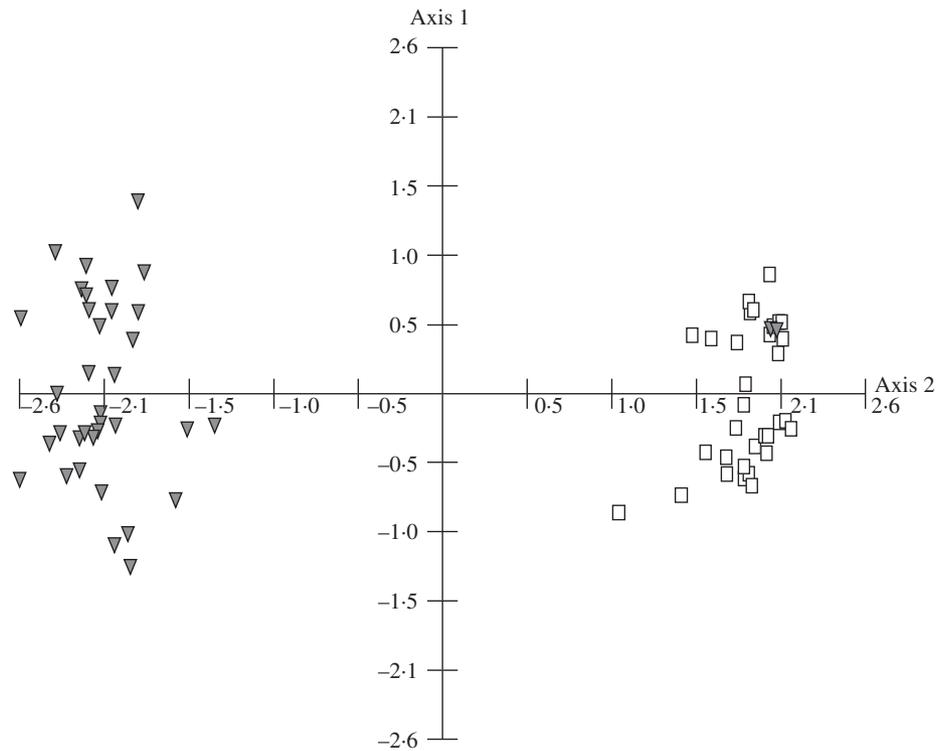
FIG. 3. Principal coordinates analysis diagram based on Euclidean distance. *Chamaedorea alternans* is indicated by triangles and *C. tepejilote* by squares.

almost exclusively correspond to the morphologically defined *C. alternans* and *C. tepejilote* (*sensu* Hodel 1992). The two samples labelled '*C. alternans*' that grouped with the *C. tepejilote* cluster had AFLP profiles entirely consistent with *C. tepejilote*. Given that we observed no evidence for either genetic or morphological intermediates, we propose that these two leaf samples were most likely mislabelled either in the field or in the laboratory. These DNA samples were reanalysed using AFLPs and subsequent data analysis, producing the same results. If these accessions indeed represented the morphology of *C. alternans*, unprecedented levels of introgression would have to be invoked to explain the complete incongruence between numerous morphological characters (see below) and the genetic background. These analyses provide results that are consistent with genetic differentiation between taxa and no evidence of hybridization. With relatively little partitioning of local geographic variation within each species cluster, it appears that each of the two species are effectively outcrossing within the Biological Station.

Recent phylogenetic studies by Thomas *et al.* (2006) provide further support for the distinctiveness of these taxa relative to one another. Using data derived from phosphoribulokinase and one accession of *C. alternans* and *C. tepejilote*, and 56 other species of *Chamaedorea*, the authors report that these two taxa are resolved within the same clade but that they are not sister to one another. This result is consistent with our finding of a higher levels of divergence than would be expected for recently speciated

or introgressing populations (e.g. Linder *et al.*, 1998; O'Hanlon *et al.*, 1999).

Morphology

Using data derived from field and herbarium observations, Hodel (1992) concluded that *C. alternans* represents a distinct taxon. *Chamaedorea alternans* was distinguished from *C. tepejilote* primarily by differentiation in leaf sheath colouring and number of inflorescences per node. *Chamaedorea alternans* has leaf sheaths that are whitish and exhibit distinct green venation, whereas *C. tepejilote* has green leaf sheaths with no obvious venation. Also, *C. alternans* has multiple inflorescences per node, whereas *C. tepejilote* has singular inflorescences per node (Fig. 4). In addition to these more persistent features, Hodel (1992) also used ephemeral floral characteristics, including the number and orientation of branches in pistillate rachillae and the spacing of pistillate flowers, to help differentiate these taxa.

More recently, Henderson *et al.* (1995) addressed the status of *C. alternans* using observations from available herbarium specimens. These authors concluded that specimens of putative *C. alternans* were insufficiently distinct from those of *C. tepejilote* to justify recognition of the former. Subsequently, Henderson *et al.* treated all representatives of this group as *C. tepejilote*, which has nomenclatural priority over *C. alternans*.

Both the genetic data and field-based morphological observations generated in this study confirmed the utility

Fig. 4. Morphological features of *C. tepejilote* (pictured in the left panels) and *C. alternans* (depicted in the right panels). (A) A single inflorescence per node on *C. tepejilote*; (B) several inflorescences per node on *C. alternans*; (C) leaf sheaths of *C. tepejilote*; (D) leaf sheaths of *C. alternans*; and (E) the difference of both fruit size taxa and maturation; immature green samples on the left are *C. tepejilote* and mature black fruits on the right represent *C. alternans*.

of the morphological characters used by Hodel (1992). In the present study, observations were made in the field concerning morphological and ecological differentiation between putative taxa. Although anecdotal, these novel observations consistently identified three additional distinguishing characters: life form, fruit size and timing of fruit maturation. Within the area of study, *C. alternans* was observed to have single stems, whereas *C. tepejilote* has multiple stems. Additionally, *C. alternans* has larger fruits that mature later than those of *C. tepejilote* (Fig. 4).

Despite general sympatry, localized allopatric areas identify partial ecological differentiation between putative species. Within the Los Tuxtlas reserve, a gradient can be observed across which substrates with poorly developed soils and considerable exposed rock in the west give way to more developed soil profiles in the east (Gonzalez Soriano *et al.*, 1997). *Chamaedorea alternans* is common in the eastern half of the reserve in association with the well developed soils, whereas *C. tepejilote* is uncommon on the eastern side and dominates the rocky forests in the central and western half.

Some of the past debate over the recognition of *C. alternans* appears to be caused by the paucity of quality

herbarium specimens available. Most botanical collections from the Los Tuxtlas region are at the Mexican National Herbarium (MEXU). Many of these lack informative characters facilitating positive identification, and numerous collections at MEXU are thus likely to be misidentified. The herbarium at the Los Tuxtlas Biological Station confirms the long-term taxonomic confusion associated with preserved material. Specimens have been annotated numerous times and generally alternate between designations.

These results identify that researchers conducting field-based ecological studies (or any other research relying on accurate taxonomy) on this complex should incorporate the distinguishing features of *C. alternans* and *C. tepejilote*, and that incomplete voucher material should not be relied upon as concrete representatives of each taxon. The historical treatment of *C. alternans* and *C. tepejilote* is unjustified.

Maintenance of species limits

Sympatric distributions can arise from sympatric speciation (Johnson and Gullberg, 1998; Dieckmann and Doebeli, 1999; Kondrashov and Kondrashov, 1999) or secondary sympatry of allopatrically derived species (Wolf *et al.*, 1997; Soliva and Widmer, 2003). Despite being considered theoretically possible, sympatric speciation has generally been thought to be of little importance in the diversification of most groups. However, recent interest in this area is being driven by strong evidence for sympatric speciation among island palms (Savolainen *et al.*, 2006) and cichlid fish (Barluenga *et al.*, 2006).

Given the results presented herein, secondarily derived sympatry is the most probable explanation for the current overlapping distributions of *C. alternans* and *C. tepejilote*. Unlike the AFLP results of Savolainen *et al.* (2006), in which F_{st} values (0.30 ± 0.020) showed a strong L-shaped distribution with few loci correlating to speciation, our F_{st} values represent a more normal and wide distribution (0.42 ± 0.34) with 11 % of loci at fixation and a number of others nearing fixation between species. In addition, ancillary studies (Thomas *et al.*, 2006) suggest that these taxa are not sister, which further discredits the potential involvement of sympatric speciation (e.g. Barluenga *et al.*, 2006).

Species limits within regions of secondary sympatry are maintained by a variety of mechanisms (e.g. Gaiotto *et al.*, 2003). Reproductive isolation can be preserved by differences in pollination syndrome (e.g. flower form, colour or scent), flowering time and/or compatibility system (Eguiarte *et al.*, 1993). Pollination biology is not well studied in *Chamaedorea*; however, in the palm genus *Geonoma*, reproductive isolation among different sets of sympatric species has been directly attributed to differences in both flowering time (Borchsenius, 2002) and pollinator as a result of variation in floral scent (Knudsen, 1999, 2002). It is probable that the observed differences in fruit maturation time between *C. alternans* and *C. tepejilote* reflect differences in flowering times, which may directly confer the reproductive isolation identified

by the AFLP data. Future studies on the general ecology, including pollination biology, of *Chamaedorea* are imperative to identify mechanisms retaining differentiation.

Conservation biology

The Los Tuxtlas region contains an estimated 2695 plant species, at least 10 % of which are thought to be endemic (Wendt, 1993). Unfortunately, the area has been heavily deforested for local agriculture and ranching, and as a result Los Tuxtlas represents one of the most threatened natural areas in Mexico (Dirzo and Garcia, 1992). The area encompassed by the Biological Station contains the remaining large stand of primary vegetation, but it includes a small fraction of the land area and just 940 plant taxa, ten of which are thought to be endemics (Ibarra-Manriquez, 1988).

With high numbers of relatively unstudied endemic species in both the biological station and the region, exactly how many taxa are at risk of extinction remains unclear (Gonzales Soriano *et al.*, 1997). Currently the Mexican government recognizes five plant species found within the region as critically endangered and four others as threatened (Ibarra-Manriquez *et al.*, 1997). Three of these are *C. alternans* (threatened), *C. tenella* (endangered) and *C. tuerkheimii* (endangered). However, the genus *Chamaedorea* is not even listed on the World Conservation Union (IUCN) list (www.redlist.org; Rodrigues *et al.* 2006). The IUCN Action Plan has identified 13 *Chamaedorea* spp. as endangered (Johnson, 1996; S. Zona, pers. comm.) In 1994, a total of 19 *Chamaedorea* spp. had been driven into an *in situ* conservation status, indicating that populations were unlikely to survive within their native range (Mont *et al.*, 1994). A formal IUCN assessment of *C. alternans* is being prepared, which will assist in conservation and management programmes in Mexico and affiliated international organizations (C. D. Bacon, unpubl. res.).

The results presented herein confirm that *C. alternans* is both morphologically and genetically distinct from *C. tepejilote* within the Los Tuxtlas Biological Station. Given that the area represented by *C. alternans*s is under the same threat as the species, conservation status in this case serves to help protect specific threatened organisms (*C. alternans*), as well as a region (Los Tuxtlas) and a larger taxonomic group (*Chamaedorea*) all under significant threat. These results also highlight a need for critical evaluation of species limits when taxonomic controversies encroach on conservation (e.g. Golding and Timberlake, 2003; Gower and Wilkinson, 2003).

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