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IDENTIFICATION OF PALMS ON SAN SALVADOR ISLAND IN THE GENERA *COCCOTHRINAX* AND *LEUCOTHRINAX* [FAMILY ARECACEAE] USING MOLECULAR METHODS; A PRELIMINARY REPORT

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ABSTRACT

Two species of *Coccothrinax* (*C. argentata*) and (*C. inaguensis*) are reported to occur on San Salvador Island, the Bahamas, but it is not clear whether they are indeed two separate species, or whether they are actually the same species and merely varietal forms or ecotypes. The high degree of morphological variation in the *Coccothrinax* and potential hybridization suggests that a molecular approach is necessary to better understand these palms on San Salvador. Hoping to use the gene PRK (phosphoribulokinase-like protein 2, exons 4 and 5) as a bar code for species identity, we generated sequence data from ten specimens representing what we identified as both typical and atypical morphological forms of both *C. argentata* and *C. inaguensis*. The sequences were compared to previously published *C. argentata* and *C. inaguensis* sequences as well as nine other *Coccothrinax* species or subspecies and specimens from four closely related genera. Using Bayesian analyses we identified the genus *Coccothrinax* as monophyletic, but relationships within the genus were not resolved and the species identities of the *Coccothrinax* specimens we collected was similarly not resolved. Three of the atypical specimens were identified as *Leucothrinax* which we did not recognize from morphology perhaps due to their immature state and their location within a *Coccothrinax*-dominated community. The Bayesian analyses of published data strongly supported *Zombia* as sister to *Coccothrinax*, and *Thrinax* as sister to *Hemithrinax*, both of which were not previously reported. A combined approach of molecular and morphological characters is needed to better identify and discover the relationships among the many species of *Coccothrinax*.

INTRODUCTION

Coccothrinax Sargent (Sargent, 1899) is the most diverse and widely distributed genus of palm in the Caribbean (Roncal et al. 2008). Henderson (1995) recognized 14 species, but 64 species and subspecies are recognized in the World Checklist of Arecaceae (Govaerts et al., 2006, accessed 2014). Evidence of the diversity of the group, and/or difficulty in identifying individuals to the species level lies in the fact that an additional 42 species, subspecies, or varieties were suggested at one time, but are not currently recognized (*ibid.*).

San Salvador Island is home to several dense populations of *Coccothrinax* that are believed to consist primarily of the silver palm, *C. argentata* (Jacquin) L.H. Bailey (Bailey, 1939). A second species, *C. inaguensis* Read (Read, 1966), was reported by Smith (1982, 1993) to grow among populations of *C. argentata* on the southern end of San Salvador Island. However, Kass and Smith (Kass, 1986, p. 66; Kass, 2009, p. 130) hypothesized that *C. inaguensis* may be a "varietal form" of *C. argentata*. Given the lack of clarity of the species occurring on San Salvador,

our goal was to document the occurrence, distribution, and abundance of these two species or varieties across San Salvador.

Coccothrinax individuals dominate Coastal Coppice, *Coccothrinax*-shrub subcommunities, and define these coastal habitats (Kass 2009, p6; Smith 1993 pp. 4, 11). Our initial observations indicated that there were not only several distinct dense populations of *C. argentata* at Rocky Point, Grotto Beach, and Sandy Hook as documented by Kass (2009), but the palms also occurred in low density populations or as isolated individuals across broad regions of the island. Thus the broad distribution of palms suggested that gene flow could be occurring across much of the island and that the populations at Rocky Point, Grotto Beach and Sandy Hook might not be as genetically distinct as we first thought.

When we started measuring densities, we noticed that it was very difficult to determine the species identity of many specimens as either *C. argentat*a or *C. inaguensis* using reported morphological distinguishing characters such as the silvery color of the bottom leaf surface in *C. argentata* and the inverted umbrella-shaped leaves of *C. inaguensis* (Henderson et al., 1995). The variation in silver color was significant not only between individuals but also among leaves in the same individual. Similarly, the leaf shape (inverted umbrella) also varied among geographically close individuals, as well as within individuals. Kass previously questioned the use of overlapping morphological characters to discriminate between these purported species (Kass 1986, p. 66; Kass 2009, p. 130; Kass, unpublished).

The high degree of morphological variation in the *Coccothrinax* may be due to phenotypic plasticity due to local environmental interactions producing ecotypes, or it may be due to distinct genetic lineages (species or varietal types) occurring sympatrically, or possibly due to hybridization among varietal types or species. Potential intrageneric hybridization has been suggested (Henderson et al. 1995) and intergeneric hybridization with *Leucothrinax* has been reported by Nauman (1990) in South Florida. *Leucothrinax morrisii* H. Wendl. (formerly *Thrinax morrisii*, see Lewis and Zona 2008) is broadly distributed from south Florida through the Bahamas, the Greater and, Lesser Antilles and occurs on San Salvador. The genus *Coccothrinax* is broadly distributed throughout the Bahama archipelago, Caribbean, South Florida, and Cuba. Many of the species are more narrowly distributed, being endemic to one or a few islands (Table 2).

Our attempts at field identification to species or varietal forms were frustrating due to the variability we observed in morphological characters. Of course we realized that our frustration may have stemmed from our lack of competence and experience with the systematics of the taxa involved (Cross and Goebel, *pers. comm*.). However, it was also possible that the published systematics (Henderson et al., 1995) of *Coccothrinax* was lacking clear definitive diagnostic sterile characters, or that the current taxonomic status of the group is limited for sterile characters and often overlapping for reproductive characters. Examining reproductive structures such as flowers or fruits may have assisted in accurate identification.

Recognizing limitations in morphological characters used to identify the species, we began to use a molecular genetic approach to identify species of *Coccothrinax* and to determine their abundance and distribution on the island. A molecular analysis might also inform us of good morphological diagnostic characters and we report our first attempt here. A combined morphological and molecular approach will certainly benefit not only our understanding of the group on San Salvador, but of palms throughout the Caribbean.

Molecular Approach

DNA barcoding (Hebert et al., 2003a, Hebert et al., Ratnasingham et al., 2003b) is a molecular approach to identifying species by comparing a genetic marker of an unknown individual to a genetic database composed of organisms whose species identities are known. The rbcL and the matK genes combined have been suggested as appropriate genes to use as barcodes in flowering plants (CBOL Plant Working Group, 2009; Ausubel, 2009) as have the nuclear internal transcribed spacer region and the plastid trnH-psbA intergenic spacer (Kress et al., 2005) or other

combinations of the four genes (Kress and Erickson, 2007). However, only the two genes PRK (phosphoribulokinase-like protein 2, exons 4 and 5) and RPB2, (subunit 2 of RNA polymerase II) have sufficient DNA data within databases (GenBank and EMBOL) to differentiate between multiple species of *Coccothrinax* (Roncal et al., 2008). We chose to look at the PRK DNA region, as a first attempt at species identification because it was technically easier to obtain sequence data from than RPB2.

Roncal et al. (2008) provided a phylogeny of the Caribbean palms (Arecaceae) that included nine species of *Coccothrinax*, as well as individuals of the genera *Hemithrinax, Thrinax, Leucothrinax, Zombia, Schippia* and *Cryosophilia* and seven other genera in the tribe Cryosophileae. We used a subset of their data with the goals of determining the species identity of the palms on San Salvador, as well as identifying whether the palms might be distinct lineages, ecotypes, or possibly hybridizing with other palms on the island.

Roncal et al. (2008) determined relationships of the palms using both the PRK and RPB2 genes and parsimony analyses. Although Roncal et al., (2008) did not identify strong support for relationships among the genera, their analyses did provide strong support for the monophyly of the genera for which they had multiple specimens, with the exception of *Thrinax* which was found to be polyphyletic. Based on this new finding the species *Thrinax morrisii* is now considered a monotypic genus, *Leucothrinax.*

METHODS AND MATERIALS

Sample Selection

We examined 2-5 individuals from three distinct populations of *Coccothrinax* distributed across the island, from Northwest to Southeast [Rocky Point (NW), Grotto Beach (SW), Sandy Hook (SE)] (Table 1). We chose specimens that appeared to be typical *C. argentata* or *C. inaguensis* (Table 1) including two specimens from which leaves had previously been collected as voucher specimens (VCa from Grotto Beach June 2011, and VCi from Sandy Hook June 2011) and placed in the annex herbarium of the Bahamas National Herbarium (BNH) at the Gerace Research Centre (Kass et al. 1998). We also examined five atypical appearing specimens growing among typical appearing *C. argentata* from Sandy Hook and Rocky Point populations (Table 1). Specimens were noted as atypical if they had unusually shaped or unusually large leaves compared to individuals growing within a distance of one meter and/or the length of the inflorescence was unusually long compared to the leaves.

Table 1. New DNA sequences obtained from San Salvador Island.

Sample name	Locality on San Salvdor	lat/long	Sequence info
Coccothrinax argentata:			
1. RP1	Rocky Point	24.10691 74.518582	uni-directional
2. RP30	Rocky Point	24.107487 74.515271	bi-directional
3. G25	Grotto Beach	23.952097 74.563468	uni-directional
4. VCa	Grotto Beach	23.953994 74.561431	bi-directional
Collected as atypical *C. argentata*:			
5. RP29	Rocky Point	24.107129 74.515973	largely bi-directional
Collected as: *Coccothrinax inaguensis:*			
6. VCi	Sandy Hook	23.948117 74.499364	uni-directional
7. SH5	Sandy Hook	23.95221 74.488178	uni-directional
Collected as atypical *C. inaguensis*:			
8. SH 11	Sandy Hook	23.948318 74.499397	bi-directional
9. SH 13	Sandy Hook	23.962913 74.487126	largely bi-directional
10. SH14	Sandy Hook	23.962891 74.487128	bi-directional

DNA extraction, amplification and sequencing

Leaf samples were collected from newly emerging leaves and immediately preserved by drying in silica gel desiccant. Samples were trans-

ported to Florida Gulf Coast University, Fort Myers, USA, for analyses. Total genomic DNA was extracted from dry leaf material using the DNAeasy Plant Mini Kit (QIAGEN, CA, USA).

Table 2. Published DNA sequences from the tribe Cryosophileae. Country of origin from Henderson (1995) and Dransfield et al. (2008).

Species	Country of origin	Genbank/ EMBL Number
Chelyocarpus ulei	S. America	EU215461
Coccothrinax argentata	S. Florida, Bahamas	AM900718
Coccothrinax argentea	Hispaniola	EU215476
Coccothrinax barbadensis	Lesser Antilles, Trinidad & Tobago	EU215472
Coccothrinax borhidiana	Cuba	EU215479
Coccothrinax crinita subsp. *brevicrinis*	Cuba	EU215473
Coccothrinax crinita subsp. crinita	Cuba	EU215475
Coccothrinax inaguensis	Bahamas	EU215471
Coccothrinax miraguama	Cuba	EU215470
Coccothrinax miraguama subsp. *miraguama*	Cuba	EU215470
Coccothrinax salvatoris	Cuba	EU215469
Coccothrinax spissa	Hispaniola	EU215474
Cryosophila stauracantha	Central America	EU215462
Hemithrinax compacta	Cuba	EU215468
Hemithrinax ekmaniana	Cuba	EU215478
Hemithrinax rivularis	Cuba	EU215480
Itaya amicorum	S. America	EU215456
Leucothrinax morrisii	S. Florida, Bahamas, Greater &, Lesser Antilles	EU215463 EU215483
Thrinax excelsa	Jamaica	EU215459
Thrinax parviflora	Jamaica	EU215466
Thrinax radiata	S. Florida, Bahamas, Greater Antilles, Mexico	EU215465
Zombia antillarum	Hispaniola	EU215467
Zombia antillarum	Hispaniola	EU215484

We amplified the phosphoribulokinase-like protein 2 gene, exons 4 and 5 (PRK), as described by Roncal et al. (2008)**,** using the primers prk717f (5'-GTGATATGGAAGAACGTGG-3') and either the reverse of prk969f (prk969r-5'-GCTGCTCATACCCTGGAAT-3') or prk1167r (5'ATGGTYTGRAANARACCNGTNCCRTTGTTGC-3') from Lewis and Doyle (2002). Sequences were obtained by Florida State University DNA sequencing facility using an Applied Biosystems 3730 Genetic Analyzer.

DNA analyses

Sequence data from electropherograms were edited using the program 4Peaks V1.7.2 (Nucleobites.com). Sequences were first aligned with Clustal Omega (Sievers et al., 2011) and then manually edited with SeaView V4 (Gouy et al., 2010). Additional samples from Roncal et al., (2008), (Table 2) were included in the alignments in order to examine all of the taxa closely related to *Coccothrinax* (species of the tribe Cryosophileae found on San Salvador Island and in the Caribbean). *Chelyocarpus ulei* was specified as the outgroup when conducting phylogenetic analyses based on Roncal et al., (2008) and *Itaya amicorum* and *Cryosophila stauracantha* were also included as additional outgroups to the tribe Cryosophileae.

Bayesian methods were used to generate phylogenetic hypotheses. The Akaike Information Criterion (AIC) was chosen as the appropriate model for sequence evolution (MrModeltest, Nylander, 2004; and PAUP*, Swofford 2002) and the K80+I model was used within Bayesian analysis (MrBays 3.2.1, Ronquist et al., 2012). The data were partitioned into indels (presence and absence) and DNA sequence data; for the indel data a single substitution rate (nst – 1) and a proportion of invariant sites (rates=propinv) was used. Two simultaneous runs were conducted from random starting trees using four independent MCMC chains. One million generations were run and trees were sampled every 400 generations. As recommended (Ronquist et al., 2012) iterations were repeated until the average standard deviation of split frequencies was less than 0.01.

The program Tracer 1.4 (Rambaut and Drummond, 2007) was used to assess stationarity and the first 25% of the trees were discarded as burn-in. Trees were manipulated for figures using FigTree 1.4.0 (Rambaut, 2012). Only values with 95% or higher Bayesian posterior probability values were considered credible.

Genetic distances (excluding gaps) were identified among all samples with PAUP* (Swofford, 2002) using the Jukes and Cantor (1969) model.

RESULTS

DNA Data

We obtained sequence data from ten individuals on San Salvador and the fragments ranged from 597 to 626 base pairs in length. Only unidirectional sequences could be obtained from several samples (Table 1) possibly due to an insertion or deletion (indel) event on only one of the two homologous chromosomes resulting in individuals heterozygous for fragment length. Sequence data from a heterozygous individual may be unreadable either before or after the indel event. However, good sequence data can be obtained in both directions up to the indel event as was found in four specimens. In addition, the primers prk717f and prk1167r amplified a much larger fragment (657-678 bp) and although the complete sequence was obtained bidirectionally for only two samples (RP29, SH13) the data were increasingly poor far from the primer. Therefore the sequence data from these samples was considered "largely bidirectional" (Table 1).

Some sites (23 bp) appeared to be heterozygous due to the clear presence of two character states in the two bidirectional sequences. One sample (RP29) had an unusually high number of heterozygous sites all within one gap region (alignment sites 324 to 356) and this could best be explained by the presence of two insertion or deletion events that compensated for each other in length allowing the sequence to have multiple contiguous heterozygous sites between the indels, but clear sequence on either side. Therefore this region was deleted from the analysis in this individual and scored as missing data.

The final alignment of all samples was 663 bp in length and included 17 gaps. Gaps were scored independently as presence (1) or absence (0) data and were included in phylogenetic analyses. Within the alignment 136 sites were variable and 56 were informative (that is, the site identity was shared by more than one sample). Among the 17 gaps, 8 were informative.

We identified variability among *Coccothrinax* on San Salvador; of the seven samples that were confirmed to be *Coccothrinax* we identified four distinct genotypes yet all samples were unique when heterozygous sites were considered. Genetic distances among all species of *Coccothrinax* were as high as 2.2%. Genetic distance among taxa in the tribe Cryosophileae were as high as 6%.

Sample identity

Three samples from Sandy Hook that appeared to have atypical phenotypes (SH11, SH13-14) were found to be *Leucothrinax* (Figure 1) as they were closely related to (were sister to) known samples of *Leucothrinax.*

The placement of two samples (RP29, SH5) was clearly within the *Coccothrinax*, but their relationship to one another and their placement within the group was not resolved.

Five samples from San Salvador, including what we thought were clear representatives of *C. argentata* (RP1, RP30, G25, VCa) and *C. inaguensis* (VCi), differed only by a few bases such as heterozygous sites and gaps. These samples were monophyletic with a sample of *C. crinita* from Cuba; their sequences differed only by unique gap characters in *C. crinita*.

Phylogenetic Relationships

Phylogenetic relationships among the taxa were different from, but did not conflict with, relationships identified by Roncal et al., (2008). We found all *Coccothrinax* to be monophyletic, but relationships within the group were largely unresolved. We found strong support for the place-

ment of *Zombia* as sister to *Coccothrinax*. *Thrinax* and *Hemithrinax* were sister to each other, but their placement deep in the tree was unclear. Analyses using Maximum likelihood rather than Bayesian methods identified the identical tree topology (results not shown).

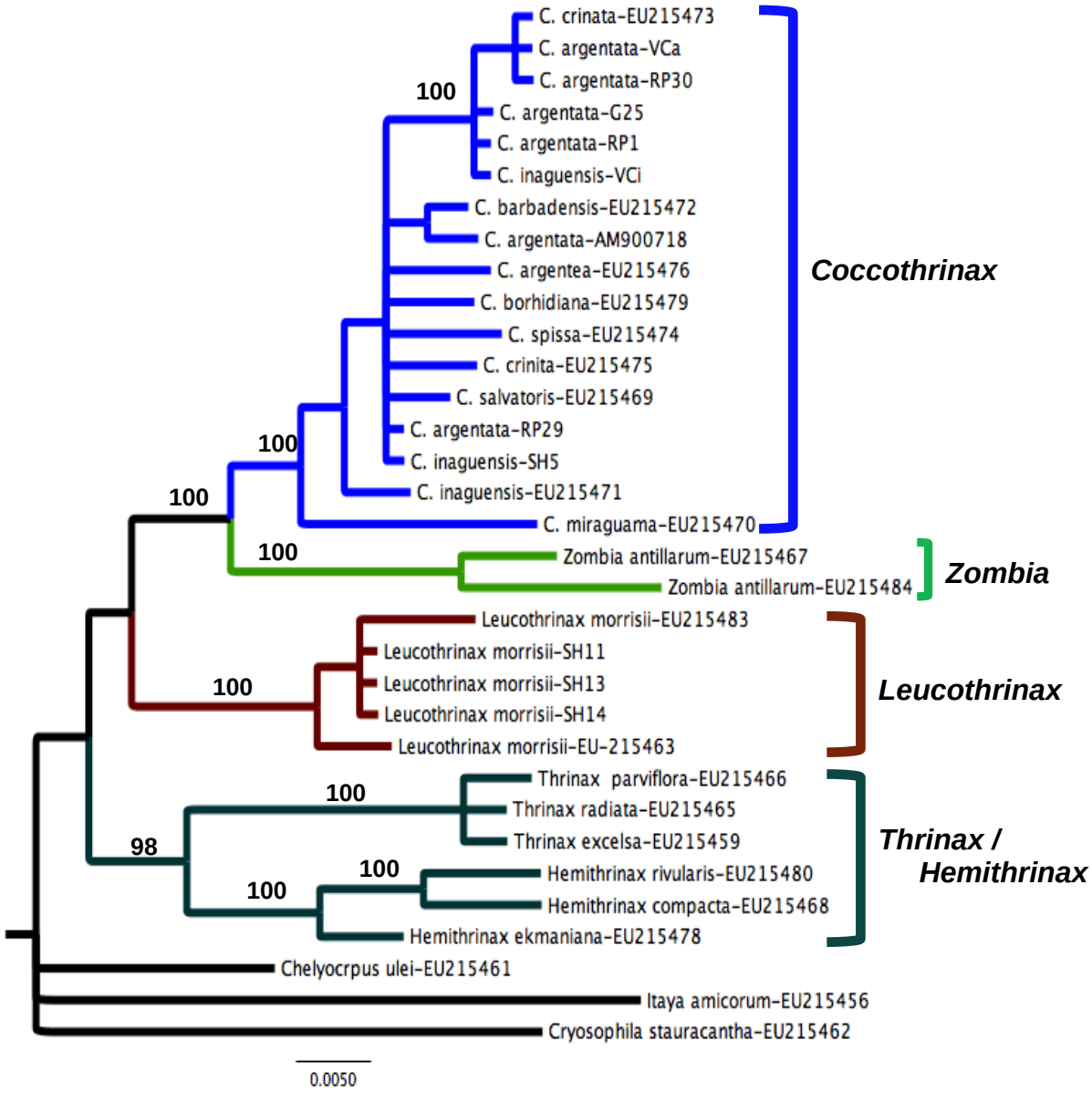

Figure 1. Phylogenetic relationships of new specimens from San Salvador (Table1) and species of the tribe Cryosophileae (Table 2) hypothesized from Bayesian analyses of the nuclear PRK DNA region. Numbers above the lines are Bayesian posterior probability values. Outgroups include Chelyocarpus ulei, Itaya amicorum and Cryosophila stauracantha.

DISCUSSION

Utility of PRK as a Barcode

The PRK gene alone clearly identified the different genera, for example it identified some of our samples as *Leucothrinax*. However, it is unclear whether this gene alone can be used as a barcode for species within *Coccothrinax*. Our samples were sister to *C. crinita*, rather than either published sequences of *C. inaguensis* or *C. argentata*. Since *C. crinita* is considered endemic to Cuba (Henderson et al., 1995) we find the result intriguing. For example, our current data might suggest that some of the palms on San Salvador are *C. crinita*. However, given the low number of variable characters in this gene, and the lack of monophyly of the two *C. crinita* specimens, we believe that more data are needed before firm conclusions are made about the identity of specimens on San Salvador or the relationships among the *Coccothrinax*.

Roncal et al., (2008) determined relationships of the palms using both the PRK and RPB2 (2089 aligned sites). Adding the RPB2 gene to our analysis might provide considerably more information. Alternatively**,** examining genes that have been suggested as useful for bar coding in plants (e. g., rbcL, matK, nuclear internal transcribed spacer region, or the plastid trnH-psbA intergenic spacer) might be useful, but the sequences are currently available for few species of *Coccothrinax*. Given the possibility of gene flow among *Coccothinax* populations across the island, even finer genetic discrimination might be needed with genetic markers such as microsatellites (Namoff et al., 2010) which may still require development for *Coccothrinax*.

Phylogenetic Analyses

We were surprised that a reanalysis of published data (Roncal et al., 2008) identified strong support for new groups such as the sister relationships of *Zombia* and *Coccothrinax*, and strong support for the clade consisting of *Thrinax* and *Hemithrinax*. These relationships were strongly supported by the indels; when we removed the indels from the data set strong support for these relationships was not recovered. Roncal et al. (2008) included indels, so we do not believe that the difference was due to indels alone. Analyses of our data using parsimony analyses (as used by Roncal et al. 2008) did not recover the deeper relationships among genera found here with both Bayesian and Maximum-Likelihood methods. This suggests that a reanalysis of the data with methods other than parsimony and including the second gene (RPB2) might resolve even more relationships among genera.

In the future we hope to examine RBP2 (Roncal et al. 2008) to test its utility as a barcode for *Coccothrinax* species. Examining the PRK in more specimens may also be useful as this DNA region seems to have a high level of variation and may be useful to study the degree of population level genetic variation found across the island.

Morphological Characters

We were not able to identify clear morphological differences between *C. argentata* and *C. inaguensis*. However, we were not able to examine reproductive characters in all specimens nor did we conduct detailed analyses that included examining microscopic characters. Instead we relied on our understanding of their occurrence and distribution on the island. For example we did not expect to find *Thrinax* within a coastal *Coccothrinax* community less than 100 m from the ocean. In hindsight, the three atypical specimens that we identified as *Leucothrinax* based on genes might have been correctly identified as they did have inflorescences longer than the leaves, a key character to their identification. In this case a better understanding of the distribution and community composition would have helped us.

Need for Revision of *Coccothrinax*

The taxonomy and classification of palms in the genus *Coccothrinax* are poorly understood because of the highly variable nature of this group and its broad distribution throughout the Caribbean, Florida, and coastal Mexico (Dransfield et al., 2008, p. 228; Henderson et al., 1995). The taxo-

nomic uncertainty is made less clear by the suggestion that intrageneric hybridization within the *Coccothrinax* may occur as well as intergeneric hybridization with *Leucothrinax*.

Because a combined approach of morphological and molecular analyses is needed, specimens with clear vouchers from confirmed collection sites should be examined. For example, the verification of the locality and species for the published sequence from the *C. argentata* from Kew Gardens is questioned since it is from a cultivated specimen (AM900718) with unknown locality.

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