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Phylogenetic Analysis of Palisota (Commelinaceae) Using Chloroplast and Nuclear Regions

Alexandra H. Crum
Grand Valley State University

PHYLOGENETIC ANALYSIS OF *PALISOTA* (COMMELINACEAE) USING CHLOROPLAST AND NUCLEAR REGIONS

Alexandra Hazel Crum

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Dedication

To my grandmother, for passing on her love of plants, and my mother and father for passing on their love of biology and listening to me talk about this thesis constantly for the last two years.

Acknowledgements

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Abstract

Palisota (Commelinaceae) differs from other Commelinaceae genera in androecial and pollen characters, a fleshy berry-type fruit, and anatomical characters. *Palisota* has been divided into two sections based on uniseriate vs. biseriate seed arrangement. Molecular phylogenetic analyses in Commelinaceae have placed *Palisota* near the base within the family, although its precise position is unclear. We sequenced chloroplast (*matK, rbcL, rps16*, and *trnL-trnF, psbI-psbK, atpB-rbcL*, *atpF-atpH* and *psbA-trnH* intergenic spacers) and nuclear (*AT103*) regions in 15 of approximately 26 species of *Palisota* and 15 outgroup species. Phylogenetic analyses were performed using maximum likelihood and Bayesian methods, with the goal of resolving the placement of *Palisota* within Commelinaceae and relationships among species. This study represents the first phylogenetic analysis within the genus and the first study to resolve the placement of *Palisota* within the family with strong support. The resulting phylogeny supports a monophyletic *Palisota* as sister to the tribe Commelineae. Sectional divisions within *Palisota* were largely upheld with the exception of *Palisota hirsuta,* a species with biseriate seeds nested within the uniseriate clade.

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Abbreviations

ML – Maximum Likelihood

BS – Bootstrap Support

PP – Posterior Probability

CHAPTER I

INTRODUCTION

Commelinaceae- The monocot plant family Commelinaceae is comprised of 41 genera, containing approximately 650 species (Faden 2012). The family is found in both the Old and New World, mainly occurring in tropical and subtropical regions (Faden 1983). About 17 genera appear in Africa, with 7 genera (*Palisota, Triceratella, Polyspatha, Stanfieldiella, Pseudoparis, Coleotrype,* and *Anthericopsis*) endemic to the continent (Faden 1983).

The family is united by several morphological traits. Commelinaceae species are herbaceous and often succulent, with deliquescent nectarless flowers that typically last only a few hours to a day, earning the family the common name of "Dayflower family" (Hutchinson et al. 2014). Commelinaceae flowers have an androecium of six stamens, although in several genera that count consists of a combination of fertile stamens and sterile staminodes, including *Murdannia*, which displays 3 stamens and 3 staminodes, or *Palisota* with 2-3 staminodes (Faden 2012; Hutchinson et al. 2014). Members of the family also display a differentiated calyx and corolla, swollen nodes, and closed leaf sheaths (Hutchinson et al. 2014).

Commelinaceae species usually produce hermaphroditic or a combination of hermaphroditic and staminate flowers (andromonecious), in rare cases producing pistillate, staminate, and hermaphroditic flowers on the same plant (polygamonoecious) (Faden 2012). Several species in the genus *Aneilema* produce hermaphroditic flowers for about the first five days of flower production in an inflorescence, before switching to staminate flowers (Faden 1991). Whether this progression occurs in other genera is

unknown, although Forman noted that in *Aëtheolirion, Spatholirion,* and *Streptolirion*, perfect flowers typically occurred on the bottom of the cincinnus and staminate flowers on the top (Forman 1962).

History of Classification in Commelinaceae- Commelinaceae has been subdivided several times, using different, mostly morphological characteristics. Early attempts by Meisner (1842), Clarke (1881), Brückner (1926, 1930), Woodson (1942), Pichon (1946), and Rohweder (1956) focused primarily on floral and inflorescence characters and formed mostly unnatural groups. These groups were largely artificial. Brenan (1966) also focused on morphology when he divided the family into fifteen informal groups, although he acknowledged that his groups may well be unnatural and there was a need to incorporate other types of data. Faden and Hunt (1991) did just that, dismantling most of the fifteen groups.

In addition to morphology, Faden and Hunt (1991) used anatomical and palynological data in their classification of Commelinaceae. They divided the family into two subfamilies: the Cartonematoideae (consisting of two genera) and the Commelinoideae (consisting of all remaining genera), on the basis of a lack or unusual placement of raphide canals, lack of glandular hair, and yellow flowers in the Cartonematoideae. The Cartonematoideae was further subdivided into tribes Cartonemeae and Triceratelleae, each containing a single genus. Subfamily Commelinoideae was subdivided into tribes Commelineae and Tradescantieae, with Tradescantieae further subdivided into the seven subtribes Palisotinae, Streptoliriinae, Dichorisandrinae, Cyanotinae, Coleotrypinae, Tradescantiinae, and Thyrsantheminae. Taxa in tribe Tradescantieae were united by 2-4 stomatal cells, spineless pollen exines,

and moniliform hair when hair is present. Taxa in Commelineae were united by 6 stomatal cells, spinulose pollen exines, and non-moniliform hair when hair is present (Faden and Hunt 1991). Some exceptions exist. For example, the genus *Tripogandra,* despite being placed in Tradescantieae, has spinulose pollen exine and non-moniliform hairs, but is maintained in Tradescantieae based on stomatal arrangement and its morphological similarities with the rest of subtribe Tradescantiinae. Similarly, *Geogenanthus* and *Streptoliriinae* are also placed in Tradescantieae even though they have six stomatal cells. In these genera, the terminal pair of cells is larger than or equal the second lateral pair, whereas in tribe Commelineae, the opposite arrangement occurs (Faden and Hunt 1991).

Molecular phylogenetic analyses have shown subtribes Tradescantiinae and Thyrsantheminae to be paraphyletic (Evans et al. 2003; Wade et al. 2006; Hertweck and Pires 2014), and subtribe Dichorisandrinae to be polyphyletic (Evans et al. 2003; Wade et al. 2006). However, with these exceptions, and the uncertainty of the position of subtribe Palisotinae, Faden and Hunt's (1991) subdivision of Commelinaceae has been mostly upheld.

Subtribe Palisotinae consists of the single genus *Palisota*. Faden and Hunt (1991) placed *Palisota* within tribe Tradescantieae due in part to its stomatal type and its lack of spines in the pollen exine. The phylogenetic placement of *Palisota* in Commelinaceae remains unclear, although molecular phylogenetic analyses have consistently placed the genus near the root of the family tree. Evans et al. (2003) and Wade et al. (2006) have placed *Palisota* as sister to a clade containing the rest of Tradescantieae plus Commelineae using morphology and the chloroplast regions *rbcL* and *ndhF*, while Burns

et al. (2011) found *Palisota* to be sister to tribe Commelineae using the chloroplast spacer region *trnL-trnF* and the nuclear ribosomal region 5S NTS. Hertweck and Pires (2014) recovered *Palisota* as sister to the rest of the Tradescantieae using the chloroplast markers *trnL-trnF* and *rpl16*, supporting the classification of Faden and Hunt (1991), but taxonomic sampling was focused on the *Tradescantia* alliance (subtribes Tradescantiinae and Thyrsantheminae). In each case, support for the placement of *Palisota* was low. An analysis of the family based solely on morphological data was largely incongruent with the molecular trees due to a high degree of homoplasy in the observed characters, particularly in characters associated with the androecium (Evans et al. 2000).

Palisota- The genus *Palisota* is comprised of approximately 26 species, all endemic to Africa and mostly found as part of the forest understory. *Palisota* is one of nine predominantly forest genera found in Africa, five of which are endemic (Faden 1983; Faden and Evans 1999). The African forest genera of Commelinaceae tend to have more species with white or nearly white flowers, adaptations for seed dispersal by birds such as berry-like fruits or arillate seeds, and axillary inflorescences (Faden and Evans 1999). All *Palisota* species produce a fleshy berry that may aid in bird-mediated seed dispersal. The genus is polymorphic for flower color and axillary inflorescences, as well as the more weakly forest-correlated occurrence of biseriate seeds (Faden and Evans 1999).

Palisota is concentrated in Western and Central Africa, although three species (*Palisota orientalis, P. manni,* and *P. schweinfurthii*) occur as far east as Tanzania and Uganda (Faden 2012). *Palisota ambigua* and *P. hirsuta* are distributed from west Africa to the Congo (Morton 1967).

Like other members of Commelinaceae, *Palisota* species have short-lived nectarless flowers, closed leaf sheaths, and a differentiated perianth (Hutchinson et al. 2014). The genus differs from the rest of the family in several features. Species of *Palisota* produce a fleshy berry, while the rest of the family, with the exception of a berry-like fruit in *Pollia*, produce a dry capsule (Faden and Hunt 1991). *Palisota* species also display branched and rugose hair types not present in other Commelinaceae genera and two to three bearded anther-less staminodes (Tomlinson 1966). Additionally, members of *Palisota* apparently exclusively possess a base chromosome number of x=20, a condition that is not found in any other member of the family (Faden and Suda 1980; Tomlinson 1966).

Palisota species are perennial plants with a rosette, caulescent, or more rarely, decumbent or climbing habit. Inflorescences can be terminal, axillary, or both, and consist of unpaired cincinni arranged in a thyrse (Faden 2012). In species such as *P. flagelliflora* and *P. ebo,* the inflorescence is reduced to a single cincinnus, while in *P. hirsuta*, the inflorescence is aggregated towards the terminal end of the main stem, forming a dense cluster of thyrses (Brenan 1966; Cheek et al. 2018; Faden 1995). *Palisota* species produce a fleshy berry that is either red or blue/black. Within the berry, seeds may have uniseriate or biseriate arrangement. Blue fruit color is never found without biseriate seed arrangement, but species such as *P. lagopus* and *P. brachythyrsa* produce biseriate seeds in red fruit.

Most *Palisota* species are andromonoecious, or in rare cases such as *P. orientalis,* produce all hermaphroditic flowers (Faden 2012). *Palisota ambigua, P. mannii,* and *P. schweinfurthii* all produce hermaphroditic flowers that are functionally pistillate due to

indehiscent pollensacs (Faden 2012). Staminate flowers are apparently the result of an aborted gynoecium (Faden 2012). It is unknown if *Palisota* follows a similar progression to *Aneilema* in producing first hermaphroditic flowers followed by staminate.

Intrageneric relationships within *Palisota* have not been addressed in a phylogenetic context. The genus was divided into the sections *Monostichos* (uniseriate seed arrangement) and *Distichos* (biseriate seed arrangement) by Clarke (1881). However, the validity of these sections has not been tested as phylogenetic studies of Commelinaceae have typically been concerned with higher level relationships and only used a single representative from *Palisota* (eg. Burns et al. 2011; Evans et al. 2003; Wade et al. 2006).

The current species delimitation for *Palisota bracteosa* is also in doubt. Native to west Africa spanning from Guinea to Gabon, *P. bracteosa* is also becoming established in Hawaii (Faden *personal comm*). Members of this species are characterized by their rosette habit and broad inflorescence bracts. However, both self-pollinating and obligate outcrossing individuals have been observed and may represent two or more cryptic species currently under the *P. bracteosa* name (Faden *personal comm*).

Phylogenetics-Phylogenetic studies can utilize morphological and molecular data to resolve evolutionary relationships. In Commelinaceae, morphological characters have been shown to be highly homoplasious, resulting in unnatural clades when traits that have been gained or lost in multiple lineages are used (Evans et al. 2000). DNA sequences tend to be less homoplasious and more likely to yield an accurate phylogeny (Givnish and Sytsma 1997). DNA sequence data have the additional benefit of substitutions being able

to be modeled mathematically, allowing for consideration of all possible pathways from ancestral sequences to the observed data (Felsenstein 1981).

In plants, DNA sequence data can be obtained from the mitochondria, chloroplast, and nucleus. Mitochondrial DNA, while helpful in animal phylogenetic studies, is not commonly used in plants. The plant mitochondrial genome usually has a very low mutation rate, although some species have been shown to have dramatically accelerated substitution rates, making comparisons across quickly and slowly evolving species difficult (Sloan et al. 2009). Chloroplast DNA has commonly been used for plant phylogenetic studies. Chloroplast regions have the advantage of being easy to work with, as there are many chloroplasts, and therefore chloroplast genomes per cell, as opposed to one nuclear genome per cell. Additionally, unlike nuclear genomes, chloroplast genomes generally do not experience recombination, although exceptions have been noted in some taxa such as *Pinus contorta* and *Nicotiana* hybrids (Marshall et al. 2001; Medgyesy et al. 1985). A drawback of chloroplast DNA, however, is that it is only inherited through the maternal lineage, so instances of introgression can lead to inaccurate tree reconstruction (Soltis and Kusoff 1995).

Nuclear DNA is inherited biparentally and can tell a more complete story of evolutionary history (Álvarez et al. 2008) The occurrence of both introns and exons, and their varying substitution rates also means that the same nuclear DNA region may be useful at shallower and deeper phylogenetic levels (Álvarez et al. 2008). However, particularly in plants, extensive gene duplication has led to independently-evolving paralogs that must be distinguished from orthologs with shared evolutionary history for

correct tree inference (Zhang et al. 2012). Low or single copy genes with few to no paralogs, skirt this issue (Zhang et al. 2012).

Several methods for tree estimation using different optimality criteria are available. While maximum parsimony is useful for morphological data, where models of evolution are somewhat difficult to apply, it is prone to long branch attraction and may not account for unobserved substitutions in a molecular dataset (Felsenstein 1978). The distance-based methods of neighbor-joining and minimum evolution do not utilize all of the information available in a dataset of DNA sequences (Holder and Lewis 2003). Currently, maximum likelihood and Bayesian methods are regarded as the most useful, as both have their basis in statistics and possess the advantage of accounting for possible unobserved substitutions (Felsenstein 1981; Holder and Lewis 2003).

PURPOSE

Phylogenetic relationships within *Palisota* and validity of the sections as currently defined by seed arrangement have never been tested. The placement of *Palisota* within the family overall is also uncertain with different molecular data sets placing it at different locations in the phylogeny. One purpose of this research was to resolve these relationships using DNA sequence data from both chloroplast and nuclear regions. Specifically, my goals were to: 1) determine the phylogenetic placement of *Palisota* within Commelinaceae; 2) determine relationships among representative species of *Palisota*; 3) evaluate the current sectional classification of *Palisota* as defined by Clarke (1881); 4) examine the evolution of several key morphological traits in the context of the

molecular phylogeny; and 5) evaluate monophyly of *Palisota* species that may represent multiple cryptic species, such as *Palisota bracteosa*.

SCOPE

In this study, eight chloroplast regions (*rbcL, matK, rps16,* and the intergenic spacers *psbA-trnH, trnL-trnF, atpB-rbcL, atpF-atpH,* and *psbI-psbK*) and one nuclear region (*AT103)* were sequenced for phylogenetic analysis. Nineteen accessions of 15 *Palisota* species representing both sections were sampled. DNA for the remaining species in the genus was unavailable. Eighteen samples representing seventeen other genera within Commelinaceae were also sampled, with representatives from tribes Tradescantieae and Commelineae, as well as the genus *Cartonema* (subfamily Cartonematoideae), which was used to root the tree.

ASSUMPTIONS

Several assumptions were implicit in the phylogenetic reconstruction of *Palisota*. It is assumed that the combination of chloroplast and nuclear regions used are suitable for resolving not only the relationship of *Palisota* to other Commelinaceae genera, but also the shallower phylogenetic relationships within *Palisota.* Maximum likelihood and Bayesian methods are assumed to accurately reconstruct phylogenetic relationships when an appropriate model of molecular evolution is used. The resulting phylogeny was also assumed to be able to inform our understanding of the evolution of specific character traits in the genus, including stomatal structure, growth habit, fruit color, and seed

arrangement, as well being able to determine if one species actually consists of multiple cryptic species when multiple individuals from the same species are sampled.

Research Questions

This study attempted to answer five questions: 1) What are the intrageneric relationships in *Palisota*, and are the currently defined sections monophyletic? 2) What is the phylogenetic placement of *Palisota* within Commelinaceae? 3) Do the sectional classifications of *Palisota* hold up as currently defined? 4) What can the phylogeny inform us about the evolution of several key morphological traits in *Palisota*? And 5) Is *Palisota bracteosa* one monophyletic species or multiple species currently held under one name?

Significance

Because *Palisota* is the sole genus in a lineage that diverged at a time when Commelinaceae was likely undergoing rapid diversification, determining the placement of *Palisota* within Commelinaceae is important in understanding diversification in the family as a whole. Further, this study will represent the first examination of relationships among species within this genus and the first test of Clarke's (1881) classification of the genus. Despite being endemic to areas threatened by habitat depletion, *Palisota* is little studied in any context. This phylogenetic analysis contributes to our understanding of the diversification and biodiversity of the genus.

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CHAPTER II

CRUM ET AL.: PHYLOGENETIC ANALYSIS OF *PALISOTA* (COMMELINACEAE)

PHYLOGENETIC ANALYSIS OF *PALISOTA* (COMMELINACEAE) USING CHLOROPLAST AND NUCLEAR REGIONS

Alexandra Crum[1], Robert B. Faden[2], Grady Zuiderveen[3], Timothy M. Evans[4]

[1]Biology Department, Grand Valley State University, 3321 P. Douglas Kindschi Hall of Science, 1 Campus Drive, Allendale, Michigan 49401 USA; crumal@mail.gvsu.edu

[2]Department of Botany, MRC 166, National Museum of Natural History, Smithsonian Institution, PO Box 37012, Washington, DC 20013-702 USA; FADENR@si.edu

[3]Department of Ecosystem Science and Management, Pennsylvania State University, 224 Forest Resources Building, University Park, Pennsylvania, 16802

[4]Biology Department, Grand Valley State University, 3321 P. Douglas Kindschi Hall of Science, 1 Campus Drive, Allendale, Michigan 49401 USA; evanstim@gvsu.edu

Abstract-*Palisota* (Commelinaceae) differs from other Commelinaceae genera in androecial and pollen characters, a fleshy berry-type fruit, and anatomical characters. *Palisota* has been divided into two sections based on uniseriate vs. biseriate seed arrangement. Molecular phylogenetic analyses in Commelinaceae have placed *Palisota* near the base within the family, although its precise position is unclear. We sequenced chloroplast (*matK, rbcL, rps16*, and *trnL-trnF, psbI-psbK, atpB-rbcL*, *atpF-atpH* and *psbA-trnH* intergenic spacers) and nuclear (*AT103*) regions in 15 of approximately 26 species of *Palisota* and 15 outgroup species. Phylogenetic analyses were performed using maximum likelihood and Bayesian methods, with the goal of resolving the placement of *Palisota* within Commelinaceae and relationships among species. This study represents the first phylogenetic analysis within the genus and the first study to resolve the placement of *Palisota* within the family with strong support. The resulting phylogeny supports a monophyletic *Palisota* as sister to the tribe Commelineae. Sectional divisions within *Palisota* were largely upheld with the exception of *Palisota hirsuta,* a species with biseriate seeds nested within the uniseriate clade.

Keywords- Commelinaceae*,* molecular phylogeny, *Palisota,* seed arrangement

The plant genus *Palisota* is one of 41 genera in the family Commelinaceae. The family has both Old and New World distribution, primarily in tropical and subtropical regions (Faden 1983). Relatively little is known of the evolutionary history and relationships among *Palisota* species, despite the genus being the largest of the family that is endemic to Africa, an ancient center of diversity for Commelinaceae (Faden 1983). Although 17 genera of Commelinaceae occur in Africa, *Palisota* is one of only seven

endemic to the continent. Currently, one species, *P. preussiana*, is listed as vulnerable on the IUCN Redlist, while the recently discovered *P. ebo* is listed as critically endangered, both in part due to the decline of habitat area. The remainder of *Palisota* species, which share similar habitat requirements, are either unevaluated or lacking sufficient data to assess population status (IUCN 2018).

Palisota, which consists of approximately 26 species, is mostly found as part of the forest understory in Western and Central Africa. Three species (*P. orientalis, P. mannii,* and *P. schweinfurthii*) have been found as far east as Tanzania, likely as relics from a wider historical range (Faden 1983, 2007). *Palisota ebo* and *P. flagelliflora,* are endemic to Cameroon, while other species are relatively widespread, such as *P. schweinfurthii,* which is distributed from Cameroon to Tanzania. (Cabezas et al. 2009; Cheek et al. 2018; Faden 1995).

Palisota shares numerous characters with the rest of Commelinaceae, such as the lack of nectar in briefly open flowers, closed leaf sheaths, and a differentiated calyx and corolla, but the genus differs from the rest of the family in several ways, making it of taxonomic interest (Hutchinson et al. 2014; Panigo et al. 2011). While other genera of Commelinaceae typically produce a dry capsule-type fruit, *Palisota* species produce a fleshy red, blue or black, or (in the case of *P. ebo*) dull yellow berry (Cheek et al. 2018; Faden and Hunt 1991). *Palisota* species also are set apart by unique branched and rugose hair types and antesepalous bearded anther-less staminodes (Tomlinson 1966). While basic chromosome counts in the family are extremely variable, sometimes within the same genus, *Palisota* is the only genus with a single basic chromosome count of x=20, which is also one of the highest observed in the family (Faden and Suda 1980). Its unique

traits relative to the rest of Commelinaceae make it important to understanding diversification in the family as a whole.

Faden and Hunt (1991) examined cytological, palynological, and morphological characteristics of Commelinaceae to divide the family into the subfamilies Cartonematoideae, consisting of the unigeneric tribes Cartonemateae and Triceratelleae, and Commelinoideae, which includes the remaining genera. The Commelinoideae was further divided into tribes Commelineae and Tradescantieae, with Tradescantieae divided even further into seven subtribes. *Palisota* was placed as the sole genus in subtribe Palisotinae within Tradescantieae (Faden and Hunt 1991). *Palisota* shares the defining features of the tribe, including a stomatal structure of 2 lateral subsidiary cells and 2 terminal cells, and spineless pollen exine (Faden 1991). *Palisota* also appears to share moniliform filament hairs with the rest of Tradescantieae when examined at low magnification, although higher magnification reveals the hair cells in *Palisota* to be dumbbell-shaped rather than bead-shaped (Faden 1995; Faden and Evans 1999).

Molecular phylogenetic studies have placed *Palisota* near the base of the family, but they have disagreed in the exact placement. Evans et al. (2003) and Wade et al. (2006) both placed the genus as sister to the rest of the Tradescantieae plus Commelineae based on chloroplast DNA sequences and morphological data. Hertweck and Pires (2014) found *Palisota* as sister to the remainder of tribe Tradescantieae, supporting the classification of Faden and Hunt (1991), but taxonomic sampling in that study was primarily focused on a single subtribe within Tradescantieae. Burns et al. (2011) placed the genus as sister to the Commelineae using both chloroplast and nuclear regions. In

each of these studies, the placement of *Palisota* was only weakly supported, and only a single species of *Palisota* was included.

In addition to the uncertain placement of *Palisota* in Commelinaceae, species relationships within the genus have been little explored. Clarke (1881) divided the genus into sections *Monostichos* (seeds uniseriate) and *Distichos* (seeds biseriate); however, the validity of these sections has never been tested phylogenetically. Phylogenetic studies that have included *Palisota* have been concerned with resolving higher-level relationships in Commelinaceae or have focused primarily on a specific subgroup within the family, and they each only sampled a single species from the genus (Burns et al. 2011; Evans et al. 2003; Hertweck and Pires 2014; Wade et al. 2006). These studies have also incorporated only one or two loci.

Species circumscription has also been largely morphology-based in the genus, and some of the currently-named species in *Palisota* may represent multiple cryptic species. In particular, *P. bracteosa* is suspected of actually consisting of two or more species. Members of this species as currently described are held together by their rosette habit and broad inflorescence bracts that are especially apparent when the plant is flowering. However, both self-pollinating and obligate outcrossing individuals have been observed and may represent two or more cryptic species currently under the *P. bracteosa* name.

The goals of this study are to use molecular data to: 1) resolve the phylogenetic position of *Palisota* within Commelinaceae; 2) resolve interspecific relationships within *Palisota;* 3) test the validity of the currently defined sections; 4) examine the evolution of key morphological traits in the context of the molecular phylogeny; and 5) evaluate the current monophyly of *Palisota* species that may represent multiple cryptic species, such

as *Palisota bracteosa*. Eight chloroplast regions (*matK, rbcL, rps16*, and the *trnL-trnF, psbI-psbK, atpB-rbcL*, *atpF-atpH* and *psbA-trnH* intergenic spacers), and one nuclear region (*AT103)* were sampled and a molecular phylogeny was inferred using Bayesian and maximum likelihood analyses.

MATERIALS AND METHODS

Taxon Sampling -Eighteen accessions representing fifteen *Palisota* species, including one undescribed species, were sampled, as well as eighteen outgroup species representing seventeen other genera within Commelinaceae including *Cartonema,* one of two genera in subfamily Cartonematoideae. *Cartonema philydroides* was used to root the tree based on its position in the family-wide study of Evans et al. (2003). Vouchers are deposited at the United States National Herbarium (US).

PCR amplification and sequencing -DNA was extracted from fresh or frozen leaf tissue and extracted with CTAB as described by Doyle and Doyle (1987) or with the Qiagen DNeasy plant mini kit (Qiagen, Hilde, Germany). We amplified the chloroplast regions *matk, rbcL,* and *rps16,* the chloroplast intergenic spacers *trnL-trnF, psbK-psbI, atpB-rbcL, atpF-atpH,* and *psbA-trnH*, and the nuclear region *AT103*. Primers were taken from Bremer et al. 2002; Li et al. 2008; Manhart et al. 1994; Oxelman et al. 1997; Sang et al. 1997 ; Chiang et al. 1998; Crayn et al. 2000; and the online resources of the Consortium of Life Plant Working Group (CBOL) All reactions took place in a solution of 1.0 μL DNA template, 0.25 mM forward primer, 0.25 mM reverse primer, 3.2 mM dNTP (0.8 mM each), 1X PCR reaction buffer, 2.5 mM $MgCl_2$, and 0.25 units *Taq* polymerase (Sigma Aldrich, Darmstadt, Germany). DMSO (5%) was added to the

reaction when amplifying *matK* and *atpB-rbcL.* Bovine serum albumin (2%) was added when amplifying *AT103.* PCR products were visualized on a 0.7% agarose gel using GelRed stain (Biotium, Freemont, CA). Sanger sequencing was performed in both directions at the University of Michigan DNA Sequencing Core. Sequences were assembled using Geneious v6.1.8 and aligned with CLUSTAL Omega on the EMBL-EBI server, followed by manual adjustment (Biomatters, Auckland, NZ; Li et al. 2015; McWilliam et al. 2013; Sievers et al. 2011). Sequences were trimmed at the 5' and 3' end where there was low confidence in sequence quality and accuracy

Phylogenetic Analysis- An incongruence length difference (ILD) test between the chloroplast regions and the nuclear *AT103* was performed with 1000 replicates. The results were significant (p=0.02), however the ILD test is known to be conservative (Hipp et al. 2004). Only 20 of the 36 taxa sampled were able to be amplified in *AT103*, limiting its ability to resolve relationships, and when only the chloroplast regions were included in analyses, the resulting tree had the same topology as the concatenated dataset, but with lower support at several nodes. Therefore, the complete dataset of nine regions was concatenated and remained unpartitioned. Indels were treated as missing data. Maximum likelihood (ML) analysis with 1000 bootstrap replicates was conducted using RAxML v8 (Stamatakis 2014) on XSEDE's interface on the CIPRES Science Gateway v3.3 (Miller et al. 2010). Bayesian analyses were conducted in MrBayes v3.2.6 also using the XSEDE interface on CIPRES (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The dataset was run over 1,000,000 generations, with sampling occurring every 1000 generations and the first 2500 generations discarded as burn-in. The most likely

model implementable in both programs as selected by Akaike's Information Criterion in jModelTest was GTR+G (Darriba et al. 2012; Guidon and Gascuel 2003). The results were checked for convergence using TRACER v1.7 (Rambaut et al. 2018). Character states for fruit color, growth habit, and seed arrangement were mapped onto the maximum likelihood tree in Mesquite v3.6 using the parsimony ancestral state reconstruction feature (Maddison and Maddison 2018).

RESULTS

The total length of the combined dataset was 6,591 nucleotides (see Table 2 for length of individual regions), with 34.4% of the sites being variable.

Maximum-likelihood and Bayesian analyses yielded trees with similar topologies (Fig. 1 and 2). The Bayesian tree contained polytomies in three clades (*P. thollonii/ P. brachythyrsa/ P. ambigua, P. hirsuta/ P. bracteosa,* and *P. satabiei/ P. bracteosa/ P. bogneri/ P. pynaerti/ P. sp.)* that were fully resolved in the ML analysis with 65%, 52%, and 52% bootstrap support (BS) respectively. Maximum-likelihood analyses recovered a -ln likelihood of 29111.5909. Tribe Commelineae was monophyletic with 100% posterior probability (PP) and 100% BS. Tradescantieae was monophyletic with the exception of *Palisota*, which was placed sister to Commelineae with 100% PP and 100% BS (Fig. 1).

Palisota itself was monophyletic with 100% support from both analyses (Fig 2). The six species belonging to section *Distichos* were recovered as monophyletic with the exception of *P. hirsuta,* which had two accessions united in a clade with one of the accessions of *P. bracteosa* with 100% PP and 100% BS. The clade is unresolved in the Bayesian analysis, but fully resolved in the ML analysis with *P. hirsuta* paraphyletic

albeit with weak support (52% BS) *(*Fig. 1; Fig. 2). The *Distichos* clade without *P. hirsuta* is relatively weakly supported (PP=90%, BS=75%) (Fig. 2). The 11 uniseriate *Palisota* species fall into a single clade with two subgroups, sister to the *Distichos* (excepting *P. hirsuta)* clade (PP=100%, BS =100%).

Apart from the two accessions of *P. hirsuta,* both *P. ambigua* and *P. bracteosa* had multiple accessions included in analyses. The two accessions of *P. ambigua* were sister to each other (PP=100%, BS =100%). In contrast, the two accessions of *Palisota bracteosa* were placed in different lineages within the genus, with one accession occurring in a clade with the two *P. hirsuta* samples (PP=100%, BS=100%) and the other as sister to *P. satabiei* in the ML analysis (BS=52%) and as part of an unresolved clade with *P. satabiei, P. bogneri, P. sp, and P. pynaerti* in the Bayesian analysis (PP=100%, BS=63%) (Fig. 1).

Of the *Palisota* species sampled, three produce blue fruit: *P. tholloni, P. ambigua,* and *P. hirsuta*. All three also have biseriate seed arrangement. *Palisota tholloni* and *P. ambigua* are placed in a clade together (PP=100%, BS =100%) with the biseriate red-fruited *P. brachythyrsa* sister to *P. tholloni* in the ML analysis (BS=88%) and as part of the unresolved biseriate clade in the Bayesian analysis (PP= 100%). *Palisota hirsuta* is within the red-fruited uniseriate clade.

Decumbent, rosette, shrub, and climbing growth habits also occur in the *Palisota* taxa sampled. Within the genus, *P. tholloni* is the only known taxa to have a climbing habit. The decumbent habit is represented by *P. satabiei* and the undescribed *P. sp,* which are placed in two different lineages. The shrub habit occurs separately in three different lineages across the biseriate clade and both subgroups of the uniseriate clade. The rosette

habit occurs in at least two lineages. *Palisota mannii* and *P. barteri,* while typically having a rosette habit, do have populations that may grow as a shrub. Either the herbaceous shrub or the rosette is the ancestral state in the genus.

DISCUSSION

The results presented here represent the first phylogenetic analysis of interspecific relationships in *Palisota*, as well as the first study to resolve the place of the genus in Commelinaceae with strong support. Previous phylogenetic studies have incorporated only one representative *Palisota* species and were based on one to two loci, potentially impacting resolution and leaving the phylogenetic relationships within *Palisota* unknown (Burns et al. 2011; Evans et al. 2003; Hertweck and Pires 2014; Wade et al. 2006).

***Relationship of* Palisota *to other Commelinaceae genera*-**Faden and Hunt (1991) placed *Palisota* within tribe Tradescantieae based on the presence of stomata with four subsidiary cells, the absence of spines on the pollen exine, and moniliform hairs on the staminal filaments (although hairs are actually be dumbbell shaped, as is the case in *P. flagelliflora;* Faden 1995; Faden and Evans 1999). *Palisota* differs from other members of Tradescantieae in its fruit type (berry instead of a capsule), the presence of three antepetalous pollen-bearing stamens, the presence of both rugose and branched hairs, and a basic chromosome number of x=20 (Faden and Hunt 1991).

All molecular phylogenetic studies of Commelinaceae have found Faden and Hunt's (1991) circumscription of Tradescantieae to be non-monophyletic due to the placement of *Palisota* (Burns et al. 2011; Evans et al. 2003; Hertweck and Pires 2014;

Wade et al. 2006), but the position of the *Palisota* has differed in each study. Evans et al. (2003) and Wade et al. (2006) placed the genus as sister to a clade containing both Tradescantieae and Commelineae, while Hertweck and Pires (2014) placed *Palisota* sister to the rest of Tradescantieae. Burns et al. (2011) recovered a sister relationship of *Palisota* to Commelineae. However, in all four studies, the position of *Palisota* received weak support, (≤71% BS). Our results strongly support the sister relationship of *Palisota* with Commelineae with 100% support from both bootstrap and posterior probability measures, suggesting that the current taxonomic circumscription of Commelineae and Tradescantieae requires revision (Fig. 2). The sister relationship of *Palisota* to Commelineae suggests that the traits defining Commelineae, including 6-celled stomatal arrangement and spinulose pollen exine were derived after the tribe and *Palisota* diverged from the rest of Tradescantieae, although *Palisota* shares the characteristic of non-moniliform filament hairs.

Species relationships within **Palisota-** Monophyly of *Palisota* is strongly supported. *Palisota* is distinctly different enough from the rest of the family that Faden and Hunt (1991) included it in its own subtribe. The berry-type fruit, androecial arrangement, branched and rugose hair types, and basic chromosome count of x=20 found in all *Palisota* taxa examined are absent in the rest of the family. Given these uniting features, *Palisota* was expected to be monophyletic, but this study is the first to confirm monophyly by inclusion of multiple *Palisota* species.

Clarke (1881) provided the only monograph of *Palisota*. He included eight taxa, although the *P. prionostachys* and *P. thyrsiflora* he described are now treated as

synonyms of *P. ambigua* and *P. hirsuta* respectively (Faden 2007). This study is the first to test if Clarke's (1881) subdivision of *Palisota* into sections *Monostichos* and *Distichos* on the basis of seed arrangement reflects the evolutionary history of the genus. Clarke's (1881) division holds up with the exception of the strongly supported placement of biseriate *P. hirsuta* within a clade otherwise united by uniseriate seeds (Fig. 2). Biseriate seeds are ancestral within the genus, and *P. hirsuta* may represent a reversal back to this state.

While blue fruit is not found without biseriate seed arrangement, biseriate seed arrangement is also found in some species with red fruit (i.e. *P. lagopus* and *P. brachythyrsa)*. *Palisota lagopus* is sister to the rest of the biseriate species (excluding *P. hirsuta*), while *P. brachythyrsa* was placed in a clade that otherwise would be united by blue berries. The placement of *P. brachythyrsa* in the clade is unresolved in the Bayesian tree and moderately supported in the ML tree. Mildbraed (1925) noted that *P. brachythyrsa* closely resembled a small *P. ambigua* with the exception of fruit color. Both have a bushy habit, ovoid fruits, and biseriate seeds (Faden 2007; Mildbraed 1925). *Palisota ambigua* flowers range from white to blue or violet, while *P. brachythyrsa* and its blue-fruited sister *P. tholloni* flowers are a pale violet (Faden 2007; Hua 1894; Mildbraed 1925). *Palisota tholloni* has also been noted as similar in appearance to *P. ambigua*, being distinguishable by its unique climbing habit and arched anthers (Hua 1894).

Blue berries appear to be homoplasious in *Palisota,* apparently having arisen at least twice. Further resolution of section *Distichos* and inclusion of additional blue-

fruited taxa, such as *P. orientalis,* is needed to understand the evolution of berry color in *Palisota*.

Either the rosette or shrub growth habit is the ancestral state in *Palisota.* The rosette habit arose at least twice in the genus, and possibly arose as many as four times if the shrub habit is the ancestral state. Alternatively, the shrub habit arose one or three times. With the exception of *P. bracteosa*, the clade consisting of *P. hirsuta, P. schweinfurthii, P. preussiana* and *P. alopecurus* all display the shrub habit. *Palisota thollonii* is unique in its climbing habit under ideal conditions, but *P. ambigua* and *P. brachythyrsa* can exhibit a climbing habit. The two decumbent species, *P. satabiei* and the undescribed *Palisota sp.* are not sister to each other, and the decumbent habit either arose twice in the lineage or once, with multiple reversals back to the rosette or shrub habit (Fig. 3).

Multiple accessions of three species, *P. ambigua*, *P. hirsuta,* and *P. bracteosa* were included in analyses. *Palisota ambigua* exhibits considerable variation across its relatively wide range, including variable flower color, leaf size, leaf shape, and the presence of a white pubescence on the underside of the leaf, in some cases making it difficult to distinguish from the species *P. orientalis* (Faden 2007). The two accessions of *P. ambigua,* one from Cameroon and one from the Congo Democratic Republic, were sister to each other. The two accessions of *P. hirsuta*, originally from Ghana and Gabon, were included to confirm its unusual placement in the genus. They were united in a clade with *P. bracteosa. Palisota hirsuta* was weakly supported (BS=52%) as paraphyletic in the ML analysis and part of a polytomy with *P. bracteosa* in the Bayesian analysis (Figs.

1 and 2). Given the weak support, the apparent paraphyly of the species in the ML analysis is suspect, and further resolution of this clade is needed.

The two accessions of *Palisota bracteosa* were placed in two different lineages, one as sister to *P. satabiei* and the other in a separate clade with *P. hirsuta* (Fig. 1). Self-pollinating and obligate outcrossing populations of *P. bracteosa* have been observed (R. Faden, *personal comm*), suggesting that *P. bracteosa* likely consists of at least two cryptic species placed under the same name due to their rosette habit and broad inflorescence bracts (R. Faden, *personal comm*). These two accessions likely represent two different species that are more distantly related than morphology suggests. The accession united with *P. hirsuta* was originally collected in the south of Ghana's Central Province, but the geographic origin for the *P. bracteosa* accession associated with *P. satabiei* is unavailable. This accession was cultivated first at Wageningen Botanic Garden, Netherlands, and later in the Smithsonian Institution greenhouses, and its original field collection source is no longer available. Dismantling of *P. bracteosa* and taxonomic descriptions for the species currently under this name are needed, as well as a better understanding of their distribution and any geographic overlap.

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TABLE 2.1- Taxa sampled from Commelinaceae. DNA was extracted from herbarium or live tissues in the Smithsonian collection. Smithsonian collection numbers of sampled individual are given when available.

Species	**Collection Number**
Palisota alopecurus Pellegr.	Kahn 90/16
Palisota ambigua C.B.Clarke	Poulsen s.n. and Faden 86/55
Palisota barteri Hook	Faden 74/75
Palisota brachythyrsa Mildbr.	Harris 3300
Palista bracteosa C.B.Clarke	SI 80-354 and Faden 86/48
Palisota bogneri Brenan	Bogner 1264
Palisota hirsuta K.Schum	Faden 74/66 and Wieringa s.n.
Palisota lagopus Mildbr.	Wieringa 2833
Palisota mannii C.B.Clarke	Poulsen s.n and Louis s.n
Palisota preussiana K.Schum	Ezavin 330
Palisota pynaerti De Wild.	SI 93-100
Palisota satabiei Brenan	Faden 86/44
Palisota schweinfurthii C.B.Clarke	Keating 90-18
Palisota sp.	Faden 86/59
Palisota thollonii Hua	de Foresta s.n
Aneilema calceolus Brenan	Faden & Faden 77/565
Aneilema clarkei Rendle	Faden & Beentje 85/49
Belosynapsis kewensis Hassk.	s.n.
Buforrestia obovata Brenan	Hahn 6346
Cartonema philydroides F.Muell	Faden s.n.
Coleotrype natalensis C.B. Clarke	Hahn 6352
Commelina congesta C.B. Clarke	Hahn 6350
Dictyospermum conspicuum (Blume) Hassk.	Thitimetharoch 302
Dichorisandra thyrsiflora J.C.Mikan	Hahn 6337
Elasis hirsuta (Kunth) D.R.Hunt	MacDougal and Lalumondier 4953
Floscopa scandens Lour.	Chu 23
Murdannia japonica (Thunb.) Faden	Hahn 14249
Pollia hasskarlii R.S.Rao	Chu s.n
Rhopalephora scaberrima (Blume) Faden	Kress 04-7749
Tradescantia paludosa E.S.Anderson & Woodson	Hahn 6343
Streptolirion volubile Edgew.	Thitimetharoch 576
Thyrsanthemum sp.	Chase 606
Weldenia candida Schult.f.	Chase 592

TABLE 2.2- Taxa sampled and alignment length of individual gene regions.

Region	Number of taxa	Alignment length
matK	36	527
rbcL	34	1371
rps16	36	943
psbA-trnH	33	638
trnL-trnF	33	395
atpB-rbcL	35	1126
atpF-atpH	35	916
psbI-psbK	36	264
AT103	20	411

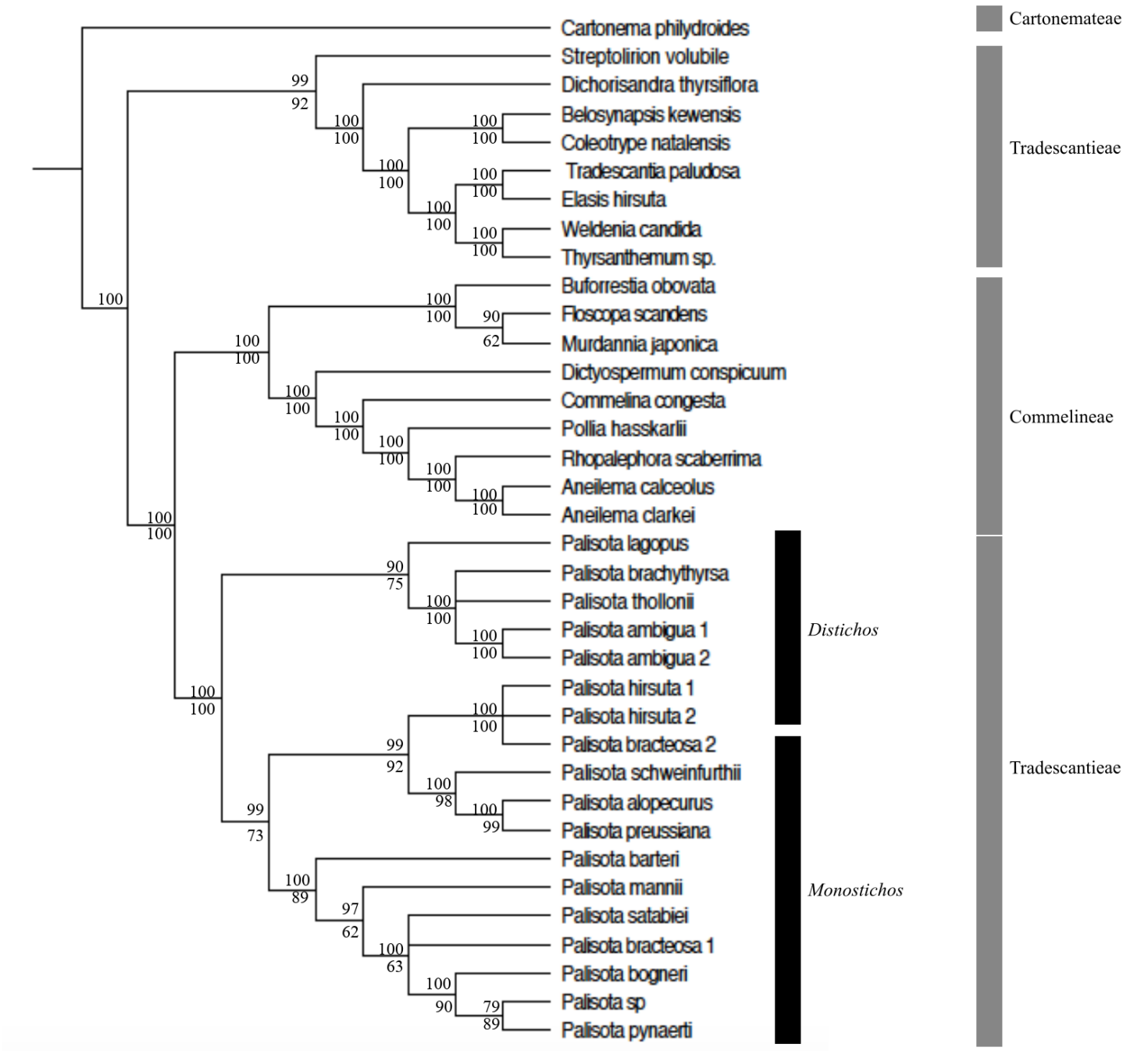

FIG 2.1. Majority rule consensus tree inferred from Bayesian analysis. The most likely tree recovered with maximum likelihood had a congruent topology. Tribe and section classification within *Palisota* is shown to the right. Posterior probabilities are given above each node, with the corresponding bootstrap probabilities from the maximum likelihood analysis given below.

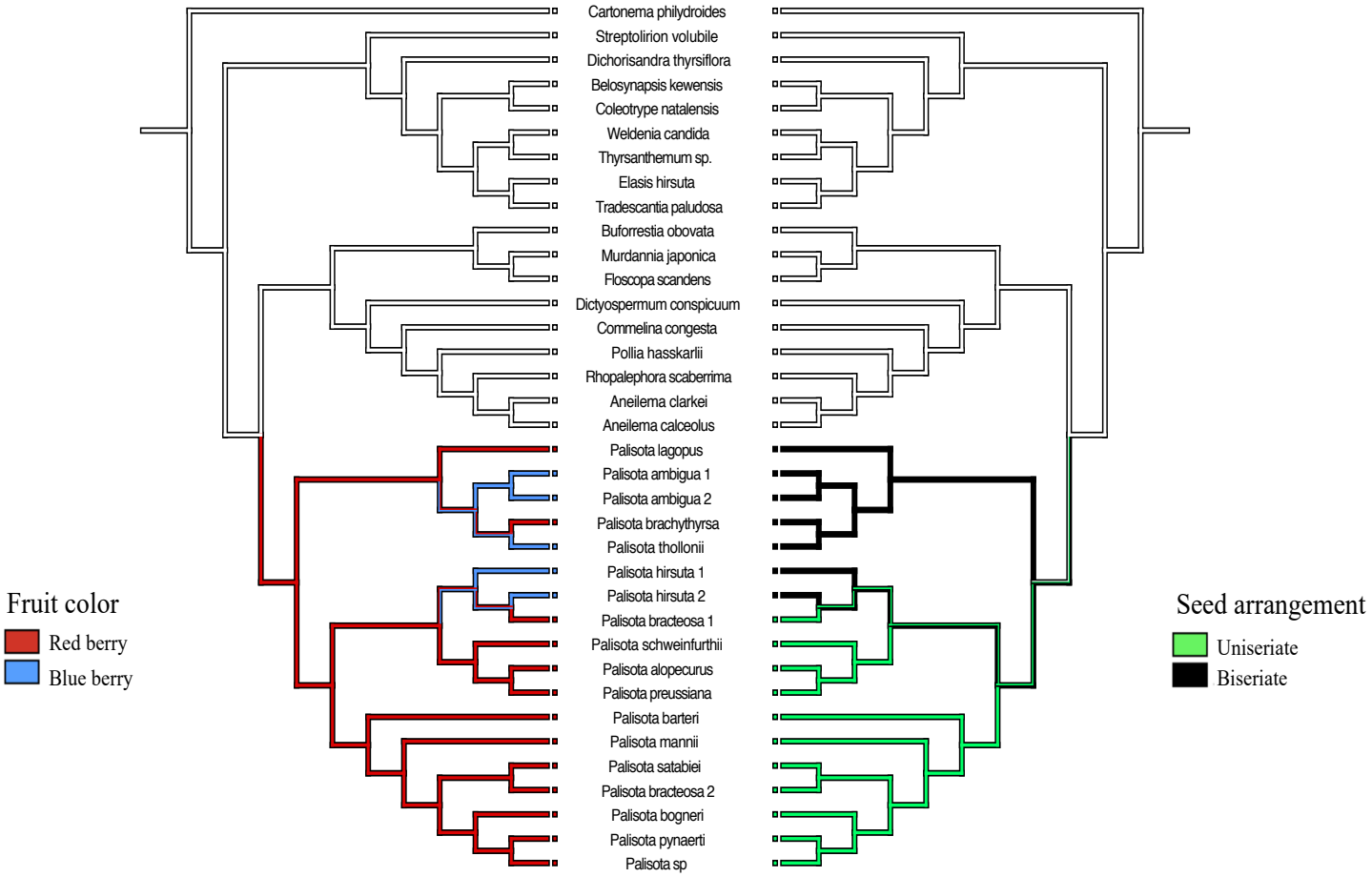

FIG. 2.2. Topology inferred through maximum likelihood methods. Distribution of fruit color (left) and seed distribution (right) within *Palisota* are shown.

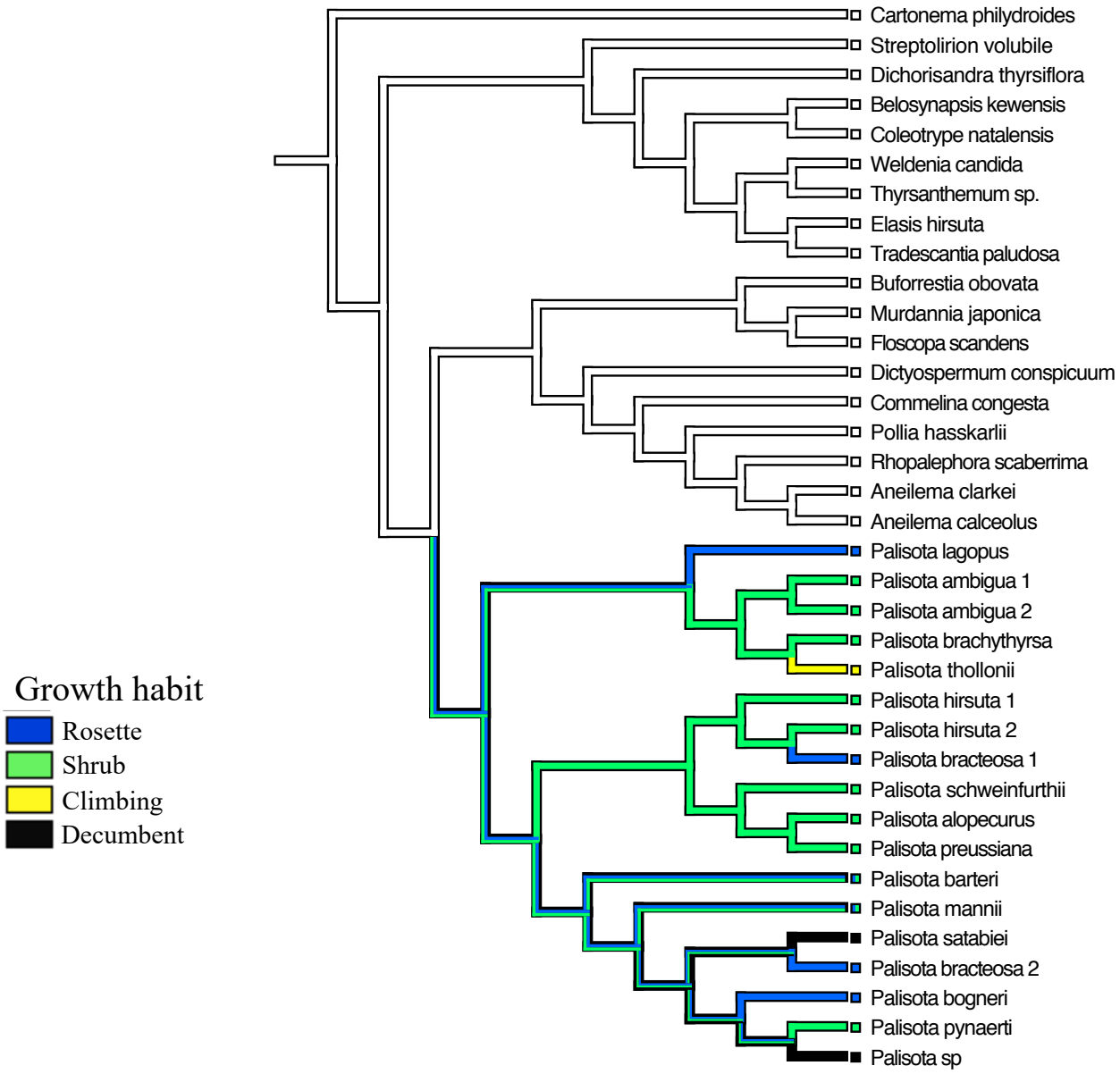

FIG. 2. 3. The maximum likelihood tree with growth habit mapped across *Palisota*.

CHAPTER III

EXTENDED LITERATURE REVIEW

Commelinaceae- The monocot plant family Commelinaceae, sometimes known as the Dayflower or Spiderwort family, consists of approximately 41 genera and 650 species (Faden 2012). The family is characterized by the short-lived, nectar-less deliquescent flowers that have earned the family its common name, as well as a superior ovary, a separate calyx and corolla, and closed leaf sheaths (Hutchinson et al. 2014). Plants are herbaceous and often succulent and terrestrial, although exceptions exist such as the aquatic *Murdannia keisak* (Hutchinson et al. 2014).

Commelinaceae species produce hermaphroditic, hermaphroditic and staminate, or rarely pistillate, staminate, and hermaphroditic flowers on the same individual (Faden 2012). Species in the genus *Aneilema* that produce hermaphroditic and staminate flowers have been observed to produce hermaphroditic flowers for the first ~5 days of inflorescence, then switching to producing staminate flowers (Faden 1991). Floral sex determination in Commelinaceae is generally not well understood, and it is unknown if species in other genera follow a similar progression, although in the subtribe Streptoliriinae (consisting of genera *Streptolirion, Spatholirion,* and *Aëtheolirion*) it has been observed that hermaphroditic flowers typically occurred on the bottom of the cincinnus and staminate flowers on the top (Forman 1962).

Hermaphroditic and staminate Commelinaceae flowers display six hypogynous stamens (Hutchinson et al. 2014). In several genera, such as *Cartonema, Triceratella,* and *Cyanotis* all six stamens are fertile, although in other genera stamens often comprise a combination of two, three or six fertile stamens and zero, three or four infertile staminodes (Hutchinson et al. 2014).

The basic unit of a Commelinaceae inflorescence is the cincinnus, which can be singular or paired (Brenan 1966). Cincinni tend to form into a thyrse with an indeterminate main axis and lateral cincinni (Brenan 1966). Modifications to this basic inflorescence type can be seen in cases such as *Palisota hirsuta*, which has terminal, reduced, and aggregated thyrses, or in the thyrse of one-flowered cincinni seen in *Cartonema* (Brenan 1966).

Commelinaceae is found in tropical and subtropical regions in both the Old and New World. Only a few genera, all in tribe Commelineae (*Aneilema, Commelina, Buforrestia, Pollia, Floscopa,* and *Murdannia*) are found in both, with the rest of the genera being found strictly in either the Old or New World (Faden 1983). The family likely originated in the Old World (Evans 2003). In tribe Tradescantieae, there were one or two introductions to the New World and one or two movements back (Evans 2003). Africa remains a center of diversity of the family, having 17 genera and roughly 40% of the family found there (Faden 1983). Of those 17, seven genera (*Palisota, Triceratella, Polyspatha, Stanfieldiella, Pseudoparis, Coleotrype,* and *Anthericopsis*) are endemic to the continent (Faden 1983).

Classification of Commelinaceae- Commelinaceae has been subdivided several times, using different, mostly morphological characteristics. Early attempts by Meisner (1842), Clarke (1881), Brückner (1926, 1930), Woodson (1942), Pichon (1946), and Rohweder (1956) focused primarily on floral and inflorescence characters and formed unnatural groups. With the exceptions of Clarke (1881) Pichon (1946), all of these early classifications divided Commelinaceae into two tribes or subfamilies. Clarke (1881)

added a third tribe of genera with indehiscent fruit in addition to his two main tribes based on number of fertile stamens, while Pichon (1946) division considered the genus *Cartonema* to belong to its own family and the remaining genera to belong to ten separate tribes.

The two most recent classifications for the family are Brenan (1966) and Faden and Hunt (1991). Brenan (1966) divided the family into 15 informal groups on the basis of morphology, although he acknowledged a need to incorporate evidence from cytology, anatomy, and palynology. Faden and Hunt (1991) later did just that, largely dismantling Brenan's groups in favor of a more natural and formal subdivision of Commelinaceae.

Faden and Hunt (1991) divided Commelinaceae into two subfamilies, the Cartonematoideae and the Commelinoideae, on the basis of glandular microhairs present in the Commelinoideae and largely absent in the Cartonematoideae as well as raphide canals present and not near the veins of the lamina in Commelinoideae and absent or next to the veins in Cartonematoideae. Cartonematoideae was further divided into the monogeneric tribes Cartonemeae and Triceratelleae. The Commelinoideae was divided into the two tribes Commelineae and Tradescantieae, with Tradescantieae divided further into seven subtribes: Tradescantiinae, Palisotinae, Coleotrypinae, Dichorisandrinae, Thyrsantheminae, Cyanotinae, and Streptoliriinae (Fig 1). Taxa in Commelineae are defined by six subsidiary stomatal cells, spinulose pollen exine, and non-moniliform filament hairs. Taxa in tribe Tradescantieae are united by two or four stomatal cells, spineless pollen exines, and moniliform hair when hair is present (Faden and Hunt 1991).

A few exceptions exist to the defining features of Tradescantieae. The genera *Geogenanthus* and *Streptolirion* are placed in the tribe despite having six stomatal

subsidiary cells, although they differ from the six-celled Commelineae tribe in that their terminal pair of cells is larger than or equal to the second lateral pair whereas in Commelineae the terminal pair is smaller (Faden and Hunt 1991). The genus *Tripogandra* is also placed in Tradescatieae despite lacking moniliform hairs and having spinulose pollen exine. However, *Tripogandra* displays morphological similarity to the rest of subtribe Tradescantiinae and has a four-celled stomatal structure. Lastly, while the filament hairs on *Palisota* appear moniliform at low magnification, on closer inspection, the individual cells are actually closer to a dumbbell shape (Faden 1995; Faden and Evans 1999).

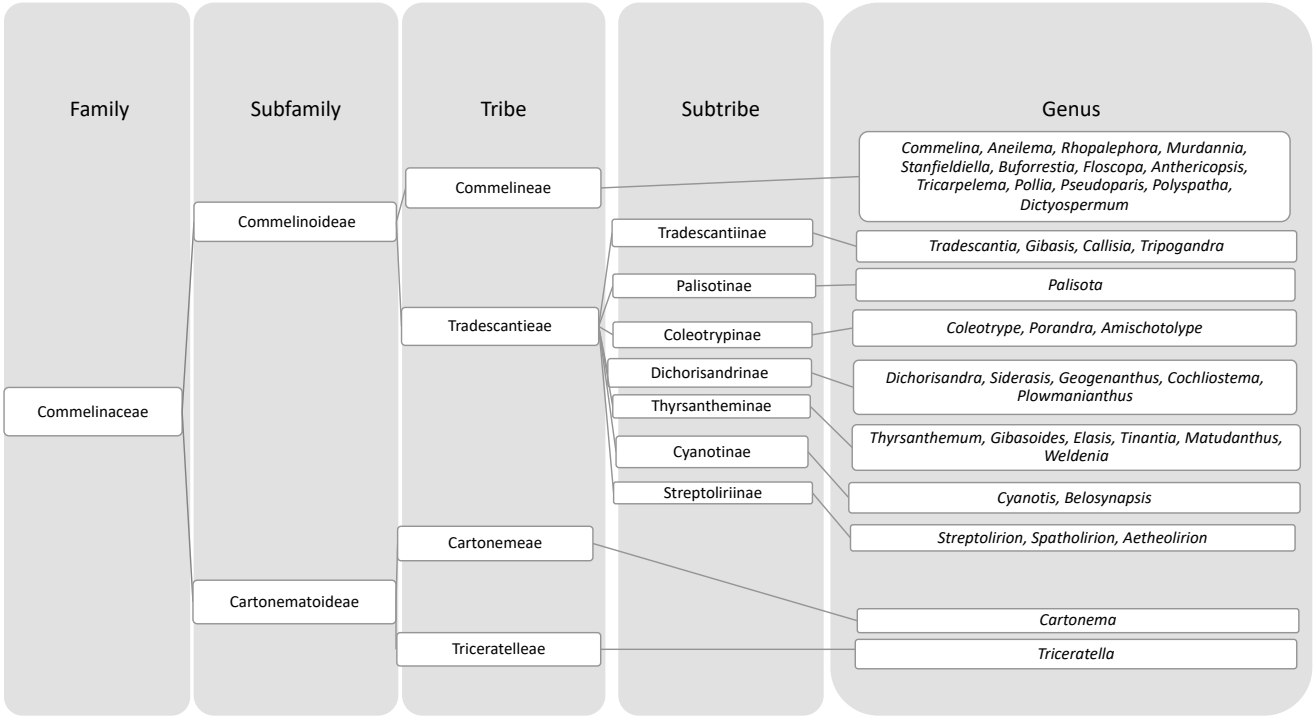

Fig. 3.1- Faden and Hunt's (1991) classification of Commelinaceae.

Plowmanianthus (subtribe Dichorisandrinae) was included as an undescribed genus and named and described later (Hardy and Faden 2004).

A phylogenetic study using a morphological dataset largely disagreed with Faden and Hunt's (1991) classification, but this was due to a high degree of homoplasy in the characters used, particularly in characters associated with the androecium (Evans et al. 2000). Molecular phylogenetic studies since Faden and Hunt (1991) have served to support much of their classification with some notable exceptions. Subtribe Dichorisandrinae was recovered as polyphyletic in two studies of the family using a combined *rbcL*/morphology dataset and a *ndh*F/*rbc*L/morphology dataset respectively (Evans et al 2003; Wade et al. 2006). Subtribe Tradescantiinae is apparently paraphyletic, with *Elasis,* a genus placed by Faden and Hunt (1991) into Thyrsantheminae, nested in Tradescantiinae (Evans et al. 2003; Hertweck and Pires 2014; Wade et al. 2006). Thyrsamtheminae is also either paraphyletic (Evans et al. 2003; Hertweck and Pires 2014) or polyphyletic (Evans et al 2003; Wade et al 2006). Non-monophyly at the generic level has also been found in the *Tradescantia* alliance. *Tripogandra* is nested within *Callisia*, and *Gibasis* and *Tradescantia* are polyphyletic (Bergamo 2003; Hertweck and Pires 2014).

The phylogenetic placement of *Palisota,* the sole genus in subtribe Palisotinae, also remains unresolved. While Faden and Hunt (1991) considered it part of tribe Tradescantieae, molecular phylogenetic studies have disagreed, placing it nearer the root of the family tree. However, there is conflict about what the precise placement is of *Palisota* is. Evans et al. (2003) and Wade et al. (2006) placed *Palisota* as sister to a clade containing the rest of Tradescantieae plus Commelineae, while Burns et al. (2011) found *Palisota* to be sister to tribe Commelineae. Hertweck and Pires (2014) supported the classification of Faden and Hunt (1991), recovering *Palisota* as sister to the rest of

Tradescantieae, but taxonomic sampling was focused on the Tradescantia alliance (Tradescantiinae and Thyrsantheminae), with comparatively little sampling outside the clade. All studies used only one sample from *Palisota* and resolved its placement with relatively weak bootstrap support (<70%).

Palisota- The genus *Palisota* consists of approximately 26 species. The largest Commelinaceae genus endemic to Africa, *Palisota* species are part of the tropical forest understory. The predominantly African forest genera of Commelinaceae, including *Palisota,* display a tendency for a suite of certain traits, including adaptations to make fruits or seeds more attractive to birds, white or nearly white flowers, and axillary inflorescences. The fleshy berry produced by all *Palisota* species is likely to aid in seed dispersal by birds. The genus is also polymorphic for axillary inflorescences and flower color, as well as biseriate seed arrangement, a trait that is weakly correlated with the forest genera (Faden and Evans 1999)

Palisota is concentrated in West and Central Africa, particularly in Cameroon (Faden 1983). Historical aridification across Africa has limited the presence of *Palisota* in eastern Africa, although *P. schweinfurthii, P. orientalis,* and *P. mannii* subsp. *megalophylla* occur as far east as Tanzania, likely having been left behind as part of a wider historical range (Faden 1983; 2007). Species such as *P. ebo* and *P. flagelliflora* have relatively restricted ranges, being endemic to Cameroon, while others are more widespread, such as the spread of *P. schweinfurthii* from Cameroon to Tanzania (Cabezas et al. 2009; Cheek et al. 2018; Faden 1995). Currently, one species, *P. preussiana*, is listed as vulnerable on the IUCN Redlist, while the recently discovered *P. ebo* is listed as

critically endangered, both in part due to the decline of habitat area. The remainder of *Palisota* species, which share similar habitat requirements, are either unevaluated or lacking sufficient data to assess population status (IUCN 2018).

Similar to other members of Commelinaceae, the flowers of *Palisota* are short-lived, deliquescent, nectarless, and have a differentiated perianth (Hutchinson et al. 2014). *Palisota* also features the closed leaf sheaths characteristic of the family (Hutchinson et al. 2014). Faden and Hunt placed the genus in Tradescantieae because of its four stomatal cells, spineless pollen exine, and apparently moniliform filament hairs, although at higher magnification it becomes apparent that the hairs are not moniliform (Faden 1995; Faden and Evans 1999; Faden and Hunt 1991).

Species of *Palisota* are rhizomatous, perennial plants with growth habits varying between decumbent, caulescent, rosette, and in the case of *P. tholloni,* climbing (Brenan 1984; Faden 2012; Faden *personal comm.*). Inflorescences consist of unpaired cincinni arranged in a thyrse that can be terminal, axillary, or both (Faden 2012). The inflorescence may aggregate towards the terminal end of the main stem, forming a dense bunch of pedunculate thyrses, as in *P. hirsuta* (Brenan 1966). In the cases of *P. flagelliflora* and *P. ebo*, the inflorescence is reduced to a single cincinnus (Cheek et al. 2018; Faden 1995). Individual flowers have subequal petaloid sepals and subequal petals ranging from white to maroon or violet in color (Faden 2012).

Most *Palisota* species are andromonoecious, with an aborted gynoecium in the staminate flowers (Faden 2012). *Palisota ambigua, P. mannii,* and *P. schweinfurthii* produce both staminate and functionally pistillate flowers (Faden 2012). In rare cases, such as *P. orientalis,* all flowers produced are hermaphroditic (Faden 2012). In all

flowers, there are 3 antepetalous stamens and two to three antesepalous bearded anther-less staminodes (Faden and Hunt 1991). The upper stamens are sterile and probably an award for pollinators, while the lower stamen is fertile (Faden 2012). The pistillate flowers produce hermaphroditic organs and fertile pollen, but pollen sacs are indehiscent and do not release the pollen, making them functionally female (Faden 2012).

Palisota fruits are a fleshy berry that is either red (or dull yellow in the case of *P. ebo*) or blue to black (Cheek et al. 2018; Clarke 1881). Seeds within the berry locules may have uniseriate or biseriate arrangement (Clarke 1881). *Palisota flagelliflora,* has been observed to produce uniseriate or sometimes biseriate seeds depending on the number of seeds in the locule (Faden 1995). Preserved fruits of the morphologically similar *P. satabiei* have also been observed to have partially biseriate seed arrangement but is generally considered a uniseriate species (Faden 1995).

Although Faden and Hunt (1991) classified *Palisota* as part of Tradescantieae, they recognized that the genus was also different enough from other Commelinaceae genera to merit its own subtribe. *Palisota* has several unique traits relative to the rest of the family that make it of interest. While the rest of the family makes a dry, dehiscent capsule-type fruit (with the exception of *Pollia* which produces a brittle metallic blue to black berry-like fruit), *Palisota* makes a red or blue or black, or in the case of *P. ebo,* dull yellow fleshy berry (Cheek et al. 2018; Hutchinson et al. 2014; Faden and Hunt 1991). *Palisota* is also remarkable for having antesepalous, antherless, bearded staminodes (Faden 2012).

While basic chromosome count varies considerably within Commelinaceae, sometimes within the same genus, *Palisota* is the only genus with a basic chromosome

count of x=20, which is also the highest confirmed within the family (Faden and Suda 1980). *Palisota* has also been noted to have unique branched and rugose macro-hairs not seen in other Commelinaceae genera (Tomlinson 1966). In the mesophyll, palisade cells of *Palisota* taxa have been observed to be lobed, which is rare in the family (Tomlinson 1966).

While floral scent is not entirely unique in the family, it is somewhat uncommon. Apart from *Palisota,* scent has been reported for species of *Callisia, Tradescantia, Cochliostema, Tripogandra, Tinantia, Commelina, Dichorisandra, Aneilema, Pollia,* and *Stanfieldiella* (Faden 1992). Species of *Palisota* that have reported scents are *P. hirsuta, P. alopecurus, P. barteri,* and *P. bracteosa* (Faden 1992). *Palisota hirsuta* is the only member of Commelinaceae noted to have a non-floral scent, instead being reported as mushroom-scented. At least in *P. hirsuta*, this scent comes from the sterile pollen in the upper stamens (Faden 1992).

The unique characteristics of *Palisota,* not shared by other genera in the Commelinaceae, make resolving its place in Commelinaceae important for understanding the patterns of diversification in the family as a whole. Apart from this, relatively little is known about the interspecific relationships of *Palisota* or the evolution of morphological traits like fruit color, seed arrangement, and growth habit across the genus.

Clarke (1881) divided *Palisota* into two sections based on seed arrangement. Section *Monostichos* included species with uniseriate seed arrangement, and section *Distichos* included species with biseriate seed arrangement. The validity of these sections has never been tested phylogenetically, and it is unknown if these sections represent natural groups. However, the pattern of fruit color in *Palisota* has a similar trend to seed

arrangement. Blue fruit color is not found without biseriate seed arrangement, but biseriate seed arrangement is found in some species with red fruit (i.e. *P. lagopus* and *P. brachythyrsa).*

Apart from Clarke's (1881) division of *Palisota,* there has been some speculation on close relationships within the genus based on morphology. In the original taxonomic description of *P. bogneri,* Brenan (1984) suggested that the species was most closely related to *P. barteri*, noting that the primary morphological difference consisted of *P. bogneri* having smaller inflorescences on decumbent peduncles. Faden (1995) suggested that *P. flagelliflora* had a close relationship with *P. satabiei* and *P. bogneri* because the three species had axillary inflorescences, biseriate ovules, and a similar distribution. Further similarities between *P. flagelliflora* and *P. satabiei* were also noted, including a bearded style, an inflorescence of a single cincinnus, and yellow staminode filament hairs (Faden 1995). The more recently described *P. ebo* was suggested to also be a close relative of *P. flagelliflora*, since it also has axillary flagelliform inflorescences composed of a single cincinnus, as well as more distinctive uniting features such as a bearded filament in the unpaired stamen, long pedicels, and vertical flowers (Cheek et al. 2018; Faden 1995).

Both *P. brachythyrsa* and *P. orientalis* have been suggested to share a close relationship with *P. ambigua.* Mildbraed (1925) noted that *P. brachythyrsa* closely resembles a small *P.ambigua* with the exception of fruit color, as *P. ambigua* produces blue fruit and *P. brachythyrsa* produces red. Both *P. brachythyrsa* and *P. ambigua* have a bushy growth habit, ovoid fruits and biseriate seeds (Faden 2007; Mildbraed 1925). *Palisota orientalis,* which produces blue fruit like *P. ambigua*, likely diverged from *P.*

ambigua as a result of dispersal or vicariance at the eastern edge of the range of *Palisota ambigua* (Faden 2007). Although geographically isolated, the two species have a similar growth habit, fruit color and shape, and biseriate seeds. They can be mainly distinguished by floral sex (flowers are functionally male and female in *P. ambigua* and all hermaphroditic in *P. orientalis*), flowering time (the flowers of *P. orientalis* open earlier in the day than the flowers of *P. ambigua*), and whether the cincinni are distally thickened, which occurs in *P. ambigua* and not *P. orientalis* (Faden 2007). Flower color in *P. ambigua* ranges from white to violet, while in *P. orientalis* flower color is exclusively white (Faden 2007).

Palisota ambigua has also been compared to two other species with biseriate seed arrangement, *P. hirsuta* and *P. tholloni.* Although he did not suggest a close relationship between the two, Morton (1967) noted that *P. ambigua* resembled a small *P. hirsuta,* whose flowers also range between white and violet in color. Similarly, Hua (1894) described *P. tholloni* as similar in appearance to *P. ambigua*, mainly being distinguishable by its unique climbing habit and arched anthers. Like *P. brachythyrsa* and some individuals of *P. ambigua, P. tholloni* flowers are a pale violet (Faden 2007; Hua 1894; Mildbraed 1925).

Apart from unresolved infrageneric relationships in *Palisota*, the delimitation of *P. bracteosa* is also uncertain. As currently defined, *P. bracteosa* is native to west Africa spanning from Guinea to Gabon. The species is also becoming established in Hawaii (Faden *personal comm*). Members of this species are held together by their rosette habit and broad inflorescence bracts that are particularly apparent when the plant flowers. However, both self-pollinating and obligate outcrossing individuals have been observed

and may represent two or more cryptic species currently under the *P. bracteosa* name (Faden *personal comm*).

Phylogenetics- While the overarching goal of a phylogenetic study is to resolve the evolutionary relationships of a group, there are a variety of data types and methods that may be used to generate a phylogenetic tree. Prior to technological advancements making generation of molecular datasets possible, morphological data were a common choice. While in some groups, morphological variation has been shown to be consistent with the evolutionary history as inferred by other types of data, in Commelinaceae, morphological characters have been shown to be highly homoplasious, particularly in androecial characters, leading to largely unnatural classifications prior to Faden and Hunt (1991) and a phylogeny incongruent with both the most recent classification of Commelinaceae and later molecular studies (Evans et al. 2000).

Molecular data, and in particular DNA sequence data, have a few advantages over morphological data. DNA sequences tend to be less homoplasious and more likely to yield an accurate phylogeny (Givnish and Sytsma 1997). DNA sequence data can also be modeled mathematically, allowing for consideration of all possible pathways from ancestral sequences to the observed data (Felsenstein 1981).

In plants, the chloroplast, mitochondria, and nucleus all contain DNA. Both the chloroplast and mitochondrial genomes have the advantage a higher copy number than the nuclear genome, making the plastid regions easier to amplify and sequence. However, mitochondrial DNA, while commonly used in animal studies, is comparatively rarely used in plants. The plant mitochondrial genome typically exhibits a low substitution rate,

although significant rate heterogeneity has been observed, making comparisons across slowly evolving and extremely rapidly evolving species problematic (Sloan et al. 2009). Using chloroplast DNA for plant phylogenetic studies has been more common. Apart from being easy to work with, chloroplast DNA has the advantage of generally not experiencing recombination, although exceptions have been observed in a few species, such as *Pinus contorta* and hybrids of *Nicotiana* (Marshall et al. 2001; Medgyesy et al. 1985). A potential drawback of using chloroplast DNA for resolving phylogenetic relationships is that the chloroplast genome, similarly to the mitochondrial genome, is only inherited down the maternal lineage, so incidences of introgression can lead to a phenomenon known as "chloroplast capture", where the chloroplast gene tree is incongruent with the species tree (Soltis and Kusoff 1995).

In cases where a tree based on chloroplast regions does not reflect the true evolutionary history of a group, biparentally inherited nuclear DNA can tell a more complete story (Álvarez et al. 2008). The occurrence of both introns and exons and their varying substitution rates also means that the same nuclear gene may be phylogenetically informative at shallower and deeper levels (Álvarez et al. 2008). However, plant genomes have experienced extensive gene duplication, and distinguishing orthologs with shared evolutionary history from independently evolving paralogs in different species can be difficult (Zhang et al. 2012). Low or single copy genes with few to no paralogs can skirt this issue (Zhang et al. 2012).

Several methods for tree estimation using different optimality criteria are available. While maximum parsimony is useful for morphological data, where models of evolution are somewhat difficult to apply, it is prone to long branch attraction and may

not account for unobserved substitutions in a molecular dataset (Felsenstein 1978). The distance-based methods of neighbor-joining and minimum evolution do not utilize all of the information available in a dataset of DNA sequences (Holder and Lewis 2003). While useful for exploratory analysis given their relatively quick computation times, these methods may not accurately or completely reflect all of the information available in a molecular dataset.

Currently, maximum likelihood and Bayesian methods are regarded as the most useful, as both have their basis in statistics and hold the advantage of accounting for possible unobserved substitutions (Felsenstein 1981; Holder and Lewis 2003). Both methods require a specified model of molecular evolution. Models can range from the relatively simple Jukes-Cantor model, which assumes all substitutions at all positions are equally likely and nucleotide frequencies are all equal, to more complicated models such as the general-time reversible model, which allows for unequal base composition and six different rates of change for different types of substitutions (Jukes and Cantor 1969; Rodríguez et al. 1990). Maximum likelihood methods determine the likelihood of the data given a tree and the parameters of the chosen model and select the tree with the highest likelihood (Whelan et al. 2001). Models can also incorporate a distribution of substitution rates to account for rate variation at different sites (Yang 1994). Bayesian methods similarly use a given model to find the best tree, but take into account prior beliefs about the data and maximize the posterior probability rather than the likelihood (Holder and Lewis 2003). Both have distinct advantages in phylogenetic analyses. Bayesian analysis is implemented with a Markov-chain Monte Carlo (MCMC) algorithm that is computationally more efficient than maximum likelihood analyses (Holder and

Lewis 2003). The posterior probability also offers a measure of confidence for the different nodes on the tree (Holder and Lewis 2003). However, critics argue that the need to specify priors makes it too subjective and that posterior probabilities can be artificially high (Huelsenbeck et al. 2002: Simmons et al. 2004). Maximum likelihood, despite typically taking more computational time (although heuristic programs like RaxML have drastically shortened the time needed), strictly maximizes likelihood based on the model's parameters without the supposed subjectivity of prior beliefs and has its own measure of confidence in a clade in the form of the bootstrap (Felsenstein 1985; Stamatakis 2014).

Conclusion-The evolutionary history of *Palisota,* despite being unusual in Commelinaceae for its androecial arrangement, fleshy berry, high and consistent basic chromosome count, and unique hair types, remains mysterious. Both its phylogenetic placement in the family and infrageneric relationships are unresolved. Shedding light on these will give a greater understanding of diversification in the family as a whole, as well as evolution of traits within the genus like fruit color, seed arrangement, and growth habit. Maximum likelihood and Bayesian phylogenetic methods used together with a molecular dataset have the potential to resolve these relationships, as well as confirm monophyly of *Palisota* species held together by morphology but suspected of being multiple cryptic species.

EXTENDED METHODOLOGY

Taxon sampling-Eighteen accessions representing fifteen *Palisota* species, including one undescribed species, were sampled, as well as eighteen outgroup species representing seventeen other genera within Commelinaceae including *Cartonema,* one of two genera in subfamily Cartonematoideae. *Cartonema philydroides* was used to root the tree based on its position in the family-wide study of Evans et al. (2003). Vouchers are deposited at the United States National Herbarium (US).

PCR amplification and sequencing -DNA was extracted from fresh or frozen leaf tissue and extracted with CTAB as described by Doyle and Doyle (1987) or with the Qiagen DNeasy plant mini kit (Qiagen, Hilde, Germany). Extraction method depended on availability of supplies at the time of extraction. We amplified the chloroplast regions *matk, rbcL,* and *rps16,* the chloroplast intergenic spacers *trnL-trnF, psbK-psbI, atpB-rbcL, atpF-atpH,* and *psbA-trnH*, and the nuclear region *AT103*. Primers were taken from (Bremer et al. 2002; Li et al. 2008; Manhart et al. 1994; Oxelman et al. 1997; Sang et al. 1997; Chiang et al. 1998; Crayn et al. 2000; and the online resources of the Consortium of Life Plant Working Group (CBOL)). All reactions took place in a 50 μL solution with 1.0 μL DNA template, 0.25 mM forward primer, 0.25 mM reverse primer, 3.2 mM dNTP (0.8 mM each), 1X PCR reaction buffer, 2.5 mM $MgCl_2$, and 0.25 units *Taq* polymerase (Sigma Aldrich, Darmstadt, Germany). DMSO (5%) was added to the reaction when amplifying *matK* and *atpB-rbcL.* Bovine serum albumin (2%) was added when amplifying *AT103.* The PCR profiles of all regions amplified are given in Table 3.1. PCR products were visualized on a 0.7% agarose gel using GelRed stain (Biotium, Freemont,

CA). Sanger sequencing in both directions was performed at the University of Michigan DNA Sequencing Core. Sequences were assembled using Geneious v6.1.8 and aligned with CLUSTAL Omega on the EMBL-EBI server, followed by manual adjustment (Biomatters, Auckland, NZ; Li et al. 2015; McWilliam et al. 2013; Sievers et al. 2011). Sequences were trimmed at the 5' and 3' end where there was low confidence in sequence quality and accuracy.

Phylogenetic Analysis- Because of the rarity of recombination in the chloroplast genome, the different chloroplast regions are able to be concatenated (Nie et al. 2009). An incongruence length difference (ILD) test between the chloroplast regions and the nuclear *AT103* was performed with 1000 replicates to determine if the nuclear and chloroplast genes had incongruent topologies. The results were significant (p=0.02), however the ILD test is known to be conservative (Hipp et al. 2004). Only 20 of the 36 taxa sampled were able to be amplified in *AT103*, limiting its ability to resolve relationships, and when only the chloroplast regions were included in analyses, the resulting tree had the same topology as the concatenated dataset, but with lower support at several nodes. Therefore, the complete dataset of nine regions was concatenated and remained unpartitioned. Indels were treated as missing data.

Maximum likelihood (ML) analysis with 1000 bootstrap replicates was conducted using RAxML v8 on XSEDE's interface on the CIPRES Science Gateway v3.3 using the GTRGAMMA model (Miller et al. 2010; Stamatakis 2014). Bayesian analyses were conducted in MrBayes (version 3.2.6) also using the XSEDE interface on CIPRES (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The dataset was run

over 1,000,000 generations, with sampling occurring every 1000 generations and the first 2500 generations discarded as burn-in. jModelTest v2.1.10 selected TPM2uf +G as the best model using Akaike's Information Criterion (Darriba et al. 2012; Guidon and Gascuel 2003). However, as this model is not implementable in MrBayes, the next most likely implementable model, GTR+G, was used. Apart from the selected model, the priors, with the exception of the shape parameter of rate variation's gamma distribution (*shapepr*), were uninformative. The shape parameter, taken from jModelTest, was fixed at 0.73. The results were checked for convergence using TRACER v1.7 (Rambaut et al. 2018). Character states for fruit color, growth habit, and seed arrangement were mapped onto the maximum likelihood tree using the parsimony ancestral state reconstruction option in Mesquite v3.6 (Maddison and Maddison 2018).

TABLE 3.1. Primer Sequences and PCR profiles of regions amplified.

Region	Primer Sequences (5'-3')	PCR profile
matK	matkAnF: CCT ATA TYC RCT TTT CTT matkAnR: AAA GAR GAT TGT TTA CKA A (Crayn et al. 2000)	5 min-95° C 25 cycles (30 s-95° C, 1 min-42° C, 2 min-72° C) 5 min-72° C
rbcL	RH-1: ATG TCA CCA CAA ACA GAA ACT AAA GC rbcL-1020R: ATC ATC GCG CAA TAA ATC AAC AAA ACC TAA AGT (Bremer et al. 2002; Manhart et al. 1994)	5 min-94° C 30 cycles (1 min-94° C, 2 min-48° C, 3 min-72° C) 5 min-72° C
rps16	rpsF: GTG GTA GAA AGC AAC GTG CGA CTT rpsR2: TCG GGA TCG AAC ATC AAT TGC AAC (Oxelman et al. 1997)	5 min-94° C 30 cycles (1 min-94° C, 2 min-55° C, 3 min-72° C) 5 min-72° C
trnL-trnF	trnLF: AAA ATC GTG AGG GTT CAA GTC trnFR: GAT TTG AAC TGG TGA CAC GAG (Sang et al. 1997)	5 min-95° C 25 cycles (30s-95° C, 1 min-50° C, 2 min-72° C) 5 min-72° C
psbK-psbI	psbK: TTA GCC TTT GTT TGG CAA G PsbI: AGA GTT TGA GAG TAA GCA T (CBOL)	5 min-94° C 30 cycles (1 min-94° C, 2 min-45° C, 3 min-72° C) 5 min-72° C
atpB-rbcL	ast-atpB: GCT GTA CCT CAC AAG TCA CAT TAA TTG GTT GAC CA ast-rbcL: GGT TGA GGA GTT ACT CGA AAT GCT GCC AAG ATA TC (Chiang et al. 1998)	5 min-94° C 30 cycles (1 min-94° C, 2 min-58° C, 3 min-72° C) 5 min-72° C
atpF-atpH	atpF: ACT CGC ACA CAC TCC CTT TCC atpH: GCT TTT ATG GAA GCT TTA ACA AT (CBOL)	5 min-94° C 30 cycles (1 min-94° C, 2 min-51° C, 3 min-72° C) 5 min-72° C
psbA-trnH	psbAF: GTT ATG CAT GAA CGT AAT GCT C trnHR: CGC GCA TGG TGG ATT CAC AAA TC (Sang et al. 1997)	5 min-94° C 30 cycles (1 min-94° C, 2 min-48° C, 3 min-72° C) 5 min-72° C
AT103	AT103F: CTT CAA GCC MAA GTT CAT CTT CTA AT103R: TTG GCA ATC ATT GAG GTA CAT NGT MAC ATA (Li et al. 2008)	5 min-94° C 30 cycles (1 min-94° C, 2 min-46° C, 3 min-72° C) 5 min-72° C

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