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Plant Species Complexes as Models to Understand Speciation and Evolution: A Review of South American Studies

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ABSTRACT

Identifying discontinuous entities within species complexes is a major topic in systematic and evolutionary biology. Comprehensive inventories describing and identifying species rapidly and correctly before they or their habitats disappear is especially important in megadiverse regions, such as South America continent, where a large part of the biodiversity is still unknown and remains to be discovered. Species complexes may account for a substantial number of plant groups in the South American flora, and studies investigating species boundaries in such challenging groups are needed. In this context, multidisciplinary approaches are crucial to understanding the species integrity and boundaries within species complexes. Morphometrics, cytogenetics, anatomy, crossing experiments, and molecular markers have been combined in different ways to investigate species complexes and have helped depict the mechanisms underlying the origin of South American species. Here, we review the current knowledge about plant species complexes on the hyperdiverse South American continent based on a detailed examination of the relevant literature. We discuss the main findings in light of the potential evolutionary mechanisms involved in speciation and suggest future directions in terms of integrating multispecies coalescence methods with several complementary types of morphological, ecological, and geographical data in this research field.

I. Introduction

Species identification, delimitation, and description constitute a long and controversial debate in the fields of systematic and evolutionary biology. Species are the most relevant unit of biodiversity; thus, cautiously deciding how species entities are defined will influence crucial conclusions related to ecological and evolutionary analyses (de Queiroz, 2007; Wiens, 2007; Freudenstein et al., 2017). Furthermore, today's global biodiversity crisis has caused an urgent need for comprehensive inventories describing and identifying species rapidly and correctly before they or their habitats disappear. This effort is especially important in megadiverse regions, such as the South America, where a large part of the biodiversity remains to be discovered, and where the patterns and processes responsible for generating and maintaining the rich biota are poorly understood.

Species delimitation is not always difficult. In cases where speciation occurred a long time ago, newly discovered species will fit into most species concepts. The major difficulty in delimiting species occurs at the beginning of species formation. It is particularly complicated in the following situations: (1) when diversification (or lineage formation) arises with little morphological change, known as cryptic or sibling species (Bickford et al., 2007); (2) when species exhibit extensive morphological diversity (Figure 1) and little genetic divergence, commonly occurring in evolutionary radiations (Shaffer and Thomson, 2007; Barley et al., 2013); or (3) when a subset of populations becomes morphologically and genetically distinct, forming a new lineage from ancestral population types, known as progenitor-derivative speciation (Crawford, 2010; Freudenstein et al., 2017). In these "species complexes," studying species delimitation is most important because we can better understand the first steps of species formation. Furthermore, in species complexes, incomplete knowledge of the biodiversity status of the whole group will lead to erroneous taxon sampling and flawed assessments of biodiversity, biogeography, and speciation processes (Heath et al., 2008).
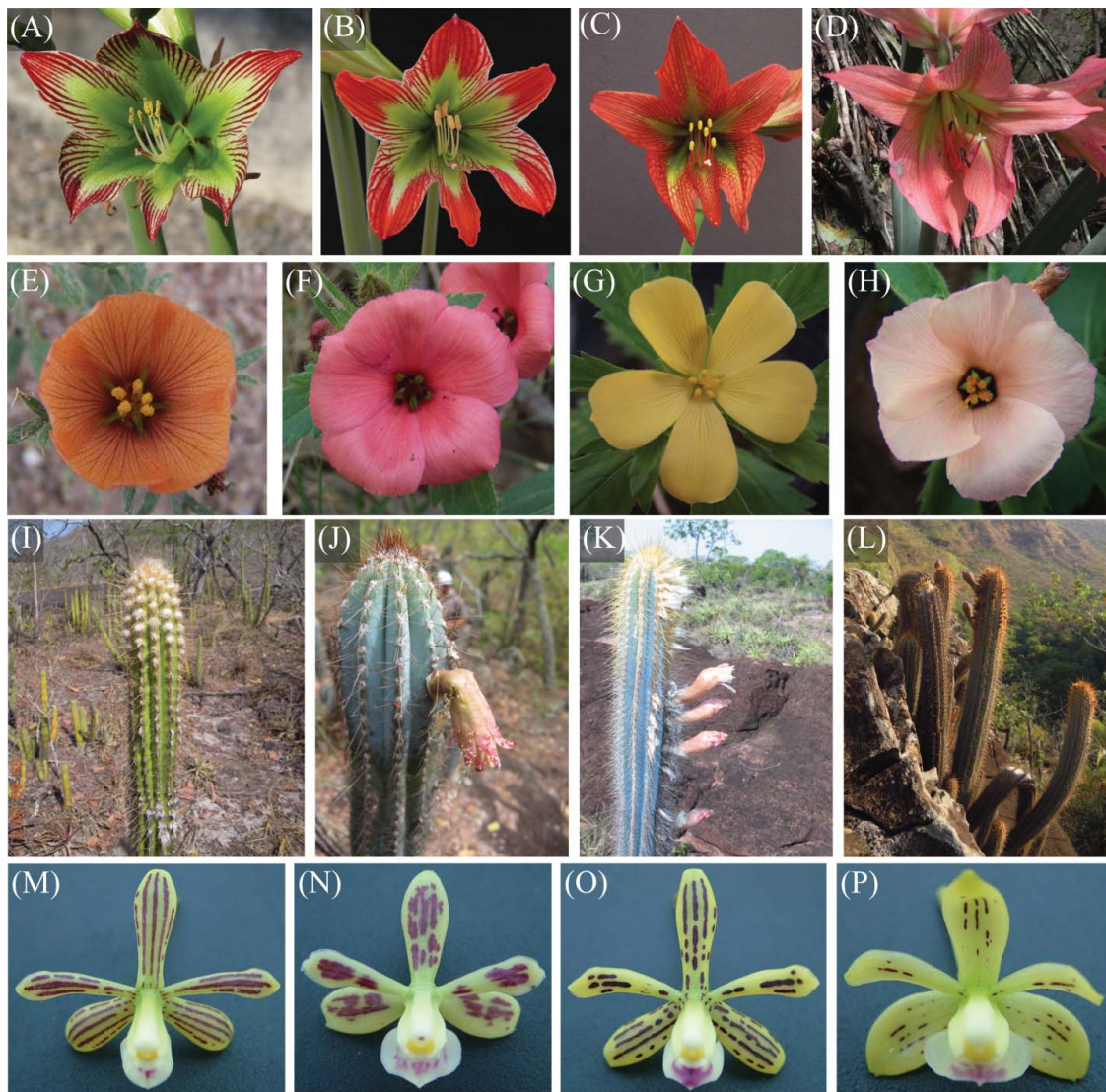
Figure 1. Examples of extensive morphological variability found in plant species complexes occurring within South America. (A–D) *Hippeastrum glaucescens* Herb. complex (Amaryllidaceae), (E–H) *Turnera sidoides* Vell. complex (Passifloraceae), (I–L) *Pilosocereus jauruensis* (Buining & Brederoo) P. J. Braun complex (Cactaceae), (M–P) *Prosthechea vespa* (Vell.) W. E. Higgins complex (Orchidaceae). Photo credits: (A–D) Mauro Peixoto, (E–H) Viviana G. SolísNeffa, (I–L) Evandro Marsola de Moraes, and (M–P) Fábio Pinheiro.

The biological species concept, created at the middle of the 20th century (Mayr, 1942), was one of the most influential ideas regarding species delimitation. The definition of species coined by Ernst Mayr (i.e., groups of actually or potentially interbreeding natural populations that are reproductively isolated from other such groups) was influenced by the work of Dobzhansky (1937), who explored the association between barriers to reproductive isolation and species formation. According to this concept, groups of closely related species do not interbreed when growing sympatrically. If hybridization occurs, low hybrid sterility and/or viability would maintain the integrity of the parental species. The biological species concept strongly influenced scientists for generations. Many evolutionary biologists advocated that total

reproductive isolation is crucial to maintaining species integrity and thus to recognize different taxonomic entities.

Since the biological species concept was proposed in 1942, many other authors have attempted to formulate alternative definitions to answer the same question: What are species? These alternative concepts have emphasized different sources of data (i.e., morphology assessments, DNA analyses, ecological measures, etc.) and different methods (i.e., morphometry, population genetics, phylogenetics, etc.) to delineate different species concepts. Here, we will not describe these alternative concepts, as this subject has been reviewed intensely in the literature (Luckow and Hortorium, 1995; Mayden, 1997; de Queiroz, 1998, 2007; Wilkins, 2009; Freudenstein et al., 2017). Because different species concepts

often refer to different stages of speciation, the concepts *per se* are not mutually exclusive (de Queiroz, 1998). In fact, most of the concepts are complementary, as each has been applied to particular stages of the continuous process of species formation. This idea led the same author to formulate the General Lineage Concept of Species (de Queiroz, 2007), in which species are treated as distinct evolving metapopulation lineages. According to the General Lineage Concept of Species, the former species concepts are treated as operational criteria used to explore the magnitude of evolutionary lineage separation. In this context, there are several advantages to using multiple lines of evidence to explore species delimitation, and some recent reviews on this subject encourage this approach (de Queiroz, 2007; Padial and de la Riva, 2010; Hausdorf, 2011; Carstens et al., 2013; Freudenstein et al., 2017). In addition, few species criteria will support and/ or show full congruence during the early stages of speciation (de Queiroz, 1998), as is often observed in species complexes.

Research in the field of species delimitation and its conceptual changes have been flourishing in the last decades, producing several methods and techniques to achieve this goal (Sites and Marshall, 2003; Knowles and Carstens, 2007; Wiens, 2007; Flot, 2015). Discussion on species delimitation still persists, but today, with the development of informative molecular tools and computational resources capable of processing large amounts of data, the methods have become more meticulous and capable of reconciling the micro- and macroevolutionary dimensions. However, distinguishing among emergent species from populations is especially difficult in species complexes. The line between these two categories is tenuous: divergent species generally do not reach total monophyly, which may be considered gene flow between populations (Naciri and Linder, 2015; Willis, 2017). To overcome such difficulties, multispecies coalescent-based methods have been used, because these approaches do not require reciprocal monophyly of alleles to delimit species, mainly when taxa originate from recent speciation events (Kingman, 1982; Yang and Rannala, 2010). Delimitation methods incorporating multispecies coalescence theory (e.g., BEAST, Heled and Drummond, 2010; BPP, Yang and Rannala, 2010; Yang, 2015; spedeSTEM, Ence and Carstens, 2011; DISSECT, Jones et al., 2015; among others) are already recognized for their empiricism in species complexes (e.g., Carstens and Satler, 2013; Tomasello et al., 2015; Folk and Freudenstein, 2015). Each approach attempts to identify and delimit species via different statistical strategies generally utilizing multiple unlinked loci to reach this goal (for reviews, see Fujita et al., 2012; Camargo et al., 2012).

Using the approach of multiple unlinked loci was important for the development of species tree-based methods and for documenting the important evolutionary role of natural hybridization and speciation with gene flow in species complexes (Nosil, 2008; Jackson et al., 2017). Levin (1978) suggested that biological species are distinct entities generally separated by multiple barriers, which may reduce gene exchange between them. Coyne and Orr (2004) introduced the notion that different species can maintain lower levels of gene exchange without losing their integrity, which was further developed by the idea of "porous genomes" (Wu, 2001). The idea that species can maintain their integrity even in the presence of interspecific gene exchange is crucial in the study of species complexes. In general, incomplete reproductive barriers and gene flow blur species boundaries between sympatric populations, as hybrids may exhibit intermediate morphological characters (Soltis and Soltis, 2009). However, in several cases, parental species maintain their integrity as separate entities, even in cases in which introgression (gene flow) is found. Thus, partial reproductive barriers could be considered the rule, rather than the exception, for most species complexes. Nonetheless, only recently, the coalescent methods for assessing lineage independence have started to explicitly account for gene flow when inferring species boundaries (Chan et al., 2017; Jackson et al., 2017; Morales and Carstens, 2018).

II. Plant diversity in South America

Compared with other megadiverse regions, South America is the most biodiverse region on Earth in terms of the number of angiosperm species (Govaerts, 2001). Moreover, South America harbors different plant diversity centers, which surpass 5000 species/10,000 km^2 (Barthlott et al., 2005). Considering the total number of endemic species observed in 16 megadiverse countries (145,610), 28% occur exclusively in South America (Forzza et al., 2012). This high diversity has attracted the attention of the scientific community, which has launched different hypotheses to explain the origin of this diversity (reviewed by Antonelli and Sanmartín, 2011). Much effort has been expended to investigate broad patterns of diversification, using plant phylogenies at higher taxonomic levels to depict the mechanisms involved in plant diversification (Hughes et al., 2013). On the other hand, population-level aspects have received less attention, and little is known regarding microevolutionary mechanisms occurring during the first stages of speciation. Common methods used to investigate species complexes, such as transplant

experiments (Hagen, 1984) and measures of reproductive isolation in hybrid zones (Baack et al., 2015), have rarely been employed in studies of South American plants. Population-level studies are crucial steps toward understanding speciation mechanisms, as lineages diverge, and incipient reproductive isolation barriers arise, which play an important role in maintaining species diversity (Scopece et al., 2010).

In this context, the study of species complexes plays a crucial role in understanding speciation because our knowledge of this process is incomplete (Via, 2009). Thus, species complexes offer a great opportunity for connecting the micro- and macroevolution scales using coalescent models within a phylogenetic framework (Pons et al., 2006; Knowles and Carstens, 2007; O'Meara, 2010; Yang and Rannala, 2010; Ence and Carstens, 2011). Furthermore, the study of species complexes under an integrative analytical approach will be useful for testing the hypothesis of species delimitation and for better understanding the processes that have promoted neotropical diversification (Padial et al., 2010; Fujita et al., 2012; Guillot et al., 2012). Most studies that have used species complexes as models for understanding speciation originated from temperate countries (Briggs and Walters, 1997), and little is known about this issue in tropical regions, particularly South America.

Species complexes may account for a substantial number of plant groups in the South American flora (Figure 1), and studies investigating species boundaries in such challenging groups are needed. Different South American research teams have been working on species complexes, focusing on different plant groups and using diverse sources of data and analytical tools. The main objective of this study was to investigate the way species complexes have been studied on the megadiverse South American continent. Here, we review the current knowledge about plant species complexes in South America based on a detailed examination of the literature, discussing the main findings in light of the potential evolutionary mechanisms involved in speciation. Specifically, we aimed to (1) review the current knowledge of South American species complexes, (2) examine study distribution among plant groups, (3) investigate the main approaches, sources of data, and analytical tools used to study such groups, and (4) discuss future directions to stimulate this approach as a primary choice for those interested in understanding speciation mechanisms in highly diverse plant groups to guide future studies in the field.

The database used for this review was compiled by conducting searches in the Web of Science® (Institute of Scientific Information, Thomson Scientific). We searched articles published from 1900 (initial coverage of

the Web of Science database) to June 2016, combining the key phrases: 'South America' and "species complex" and/or "cryptic species" and/or "sibling species" and/or "species flock." These phrases needed to be cited in the title, abstract, keywords, or the main body of the article. We filtered only studies performed only with angiosperms and excluded studies of invasive species in their nonnative ranges. In addition, we performed a nonexhaustive Google Scholar database search with the same terms (Table 1). We recorded the following information for all articles retrieved: genus and family names; habit; number of populations sampled; whether a multidisciplinary approach was adopted (i.e., molecular, morphometry, cytogenetics, anatomy, etc.); the inclusion of taxonomic decisions; main research fields involved in the study; and main analytical tools adopted when using molecular markers. Traditional taxonomic methods based mainly on analyses of qualitative diagnostic characters, morphometry using quantitative characters, anatomy, cytogenetics, and transplant and crossing experiments, as well as other ecological analyses, such as pollination, phenology, niche models, and molecular markers, were the main research fields considered in this study. Studies using molecular markers were further classified according to three main analytical strategies of phylogenetic, population genetic, and phylogeographic methods. We used the chi-square test with Yates correction to assess the strength of the associations between adopting a multidisciplinary approach and including taxonomic decisions regarding the species complexes. The same test was also used to explore associations between individual data types (e.g., molecular data alone) and taxonomic decisions. Descriptions of new species and the splitting and/or lumping of current species groups were all interpreted as taxonomic decisions to clarify the taxon delimitation within the species complexes. Associations among different research fields were summarized into a network calculated using Gephi software (Bastian et al., 2009).

The Web of Science® and Google Scholar survey of the literature from 1900 to June 2016 identified 129 articles matching our criteria (Table 1). The first study that used one of the reference phrases was published in 1968. Studies were performed on 44 different plant families and 84 genera. Overall, the studies included a range of plant species, from herbs (89 studies), trees (23 studies), lianas (12 studies), and shrubs (8 studies); yet, they still are not a taxonomically representative sample of plant species from South America. Approximately 70% of the studies were concentrated in 13 families. The seven most studied families were Fabaceae (13), Orchidaceae (13), Poaceae (13), Solanaceae (12), Asteraceae (9), Turneraceae (6), and Bromeliaceae (5) (Figure 2).

Table 1. Summary of studies on plant species complexes occurring within South America.

Reference	Family	Genus	Habit	Number of populations	Multiple methods	Taxonomic decisions	Traditional taxonomic methods	Morphometry/ Anatomy	Cytogenetics	Reproductive/ crossing experiments	Ecological data	Phylogenetic approach	Population genetic approach	Phylogeographic approach	Molecular markers	Nuclear	Plastid
Allred and Gould, 1983	Poaceae	*Bothriochloa*	herb	—	yes	yes		yes	yes	yes							
Alvarez et al., 2008	Solanaceae	*Solanum*	herb	59	yes	no		yes					yes		RAPD, RFLP, AFLP	yes	
Alves et al., 2014	Iridaceae	*Sisyrinchium*	herb	—	no	no						yes			sequencing / barcoding	yes	yes
Ames and Spooner, 2010	Solanaceae	*Solanum*	herb	—	no	no						yes			sequencing	yes	
Ames et al., 2008	Solanaceae	*Solanum*	herb	37	no	no		yes									
Amico and Nickrent, 2009	Loranthaceae	*Tristerix*	herb	30	no	no								yes	sequencing		yes
André *et al.*, 2015	Costaceae	*Chamaecostus*	herb	12	yes	yes		yes				yes				yes	yes
Ballard and Iltis, 2012	Violaceae	*Viola*	herb	—	no	yes	yes										
Barbosa et al., 2012	Velloziaceae	*Vellozia*	herb	25	yes	yes	yes							yes			yes
Berg et al., 1998	Solanaceae	*Solanum*	herb	—	no	no		yes									
Bonatelli et al., 2014	Cactaceae	*Pilosocereus*	herb	33	yes	no					niche models			yes		yes	yes
Borba et al., 2002	Orchidaceae	*Acianthera*	herb	21	yes	yes		yes		yes	flower visitors	yes	yes		allozime loci	yes	
Borges et al., 2012	Fabaceae	*Libidibia*	tree	2	yes	yes		yes	yes	yes							
Borsch et al., 2011	Amaranthaceae	*Pedersenia*	liana	—	yes	yes	yes					yes			sequencing		yes
Bünger et al., 2015	Myrtaceae	*Eugenia*	tree	—	yes	yes	yes	yes									
Caddah et al., 2012	Calophyllaceae	*Kielmeyera*	tree	4	yes	yes	yes	yes					yes			yes	
Caddah et al., 2013	Calophyllaceae	*Kielmeyera*	tree	3	yes	yes					phenology		yes			yes	
Caetano et al., 2008	Anacardiaceae	*Astronium*	tree	7	no	yes							yes		SSR and sequencing	yes	yes
Canela et al., 2003	Bromeliaceae	*Aechmea*	herb	—	no	yes	yes										
Cardim et al., 2001	Orchidaceae	*Oncidium*	herb	5	no	no		yes									
Cardozo et al., 2015	Araceae	*Anthurium*	herb	12	no	yes		yes									
Carlini-Garcia et al., 2002	Orchidaceae	*Miltonia*	herb	8	yes	yes		yes			phenology						

Table 1. (*Continued*)

Reference	Family	Genus	Habit	Number of populations	Multiple methods	Taxonomic decisions	Traditional taxonomic methods	Morphometry/ Anatomy	Cytogenetics	Reproductive/ crossing experiments	Ecological data	Phylogenetic approach	Population genetic approach	Phylogeographic approach	Molecular markers	Nuclear	Plastid
Catling and Porebski, 1998	Rosaceae	*Fragaria*	herb	—	no	yes		yes									
Cavallari et al., 2010	Salicaceae	*Casearia*	tree	9	no	yes							yes		SSR	yes	
Ceolin and Miotto, 2012	Fabaceae	*Galactia*	herb	82	yes	yes	yes	yes			environment characterization						
Conceição et al., 2008	Fabaceae	*Chamaecrista*	shrub	33	yes	yes		yes					yes		15 allozime loci	yes	
Cooley et al., 2008	Phrymaceae	*Mimulus*	herb	6	yes	no		yes		yes							
Costa et al., 2007	Fabaceae	*Chamaecrista*	herb	2	yes	yes				yes	reproductive behaviour, flower phenology, flower visitors		yes		4 allozime loci	yes	
Costa et al., 2009	Bromeliaceae	*Vriesea*	herb	6	yes	yes	yes	yes									
Costea and Stefanovic, 2010	Convolvulaceae	*Cuscuta*	liana	—	yes	yes	yes	yes				yes			sequencing	yes	yes
Costea et al., 2011	Convolvulaceae	*Cuscuta*	liana	—	yes	yes	yes	yes				yes			sequencing	yes	yes
Costea et al., 2015	Convolvulaceae	*Cuscuta*	liana	—	yes	yes		yes				yes			sequencing	yes	yes
Dematteis, 2004	Asteraceae	*Vernonia*	herb	—	no	yes	yes										
Diaz et al., 1996	Convolvulaceae	*Ipomoea*	liana	—	yes	no		yes		yes							
Díaz, 2013	Calophyllaceae	*Calophyllum*	tree	—	yes	yes	yes	yes									
Elías et al., 2011	Turneraceae	*Turnera*	herb	26	yes	no			yes		climatic characteristics						
Esteves and Vicentini, 2013	Rubiaceae	*Pagamea*	tree	3	yes	yes		yes			flower phenology, environmental characterization						
Farinaccio and Mello-Silva et al., 2006	Apocynaceae	*Oxypetalum*	shrub	—	no	yes	yes										
Ferreyra et al., 2013	Fabaceae	*Prosopis*	tree	—	yes	no		yes					yes		RAPD	yes	
Filardi et al., 2013	Fabaceae	*Machaerium*	tree, shrub, and lianas	—	no	yes	yes	yes									
Firetti-Leggieri et al., 2011	Bignoniaceae	*Anemopaegma*	shrub		no	no			yes								

Freitas-Mansano and Tozzi, 2001	Fabaceae	*Swartzia*	tree	—	no	yes	yes										
Gagnon et al., 2015	Fabaceae	*Caesalpinia*	tree	—	yes	yes		yes				yes			sequencing	yes	yes
Gentry, 1981	Passifloraceae	*Passiflora*	liana	—	no	yes	yes										
Giussani et al., 1996	Poaceae	*Poa*	herb	10	yes	no		yes	yes		climatic characteristics						
Gomes-da-Silva *et al.*, 2011	Bromeliaceae	*Vriesea*	herb		no	yes	yes										
Gomes-da-Silva *et al.*, 2012	Bromeliaceae	*Vriesea*	herb	—	no	no		yes									
Gonella et al., 2012	Droseraceae	*Drosera*	herb	1	no	yes	yes										
Gonella et al., 2014	Droseraceae	*Drosera*	herb		no	yes	yes										
Graham and Cavalcanti, 2013	Lythraceae	*Cuphea*	herb	—	yes	yes	yes		yes								
Grombone-Guaratini et al., 2005	Asteraceae	*Bidens*	herb	12	no	no							yes		16 allozime loci	yes	
Grombone-Guaratini et al., 2006	Asteraceae	*Bidens*	herb	—	no	no			yes								
Henderson and Martins, 2002	Arecaceae	*Geonoma*	tree	—	no	no		yes									
Holanda et al., 2015	Humiriaceae	*Humiria*	tree	—	yes	no		yes		yes							
Ispizúa et al., 2015	Solanaceae	*Solanum*	herb	4	yes	no		yes	yes	yes							
Lammers and Hensold, 1992	Campanulaceae	*Lobelia*	herb	—	no	no			yes								
Lima et al., 2015	Myrtaceae	*Myrcia*	tree	17	yes	yes		yes					yes		6 primers and 59 ISSR Loci	yes	
Lombardi, 2006	Celastraceae	*Tontelea*	lianas, shrubs	—	no	yes	yes										
Longo et al., 2014	Solanaceae	*Petunia*	herb	47	no	no								yes	two cpDNA: trnS-trnG and trnH-psbA		yes
López et al., 2002	Asteraceae	*Senecio*	herb	—	no	no			yes								
López et al., 2012	Apiaceae	*Pozoa*	herb	22	no	yes						yes	yes		sequencing / AFLP	yes	yes
López-Sepúlveda et al., 2013a	Asteraceae	*Hypochaeris*	herb	54	yes	no		yes					yes		AFLP	yes	

Table 1. (*Continued*)

Reference	Family	Genus	Habit	Number of populations	Multiple methods	Taxonomic decisions	Traditional taxonomic methods	Morphometry/ Anatomy	Cytogenetics	Reproductive/ crossing experiments	Ecological data	Phylogenetic approach	Population genetic approach	Phylogeographic approach	Molecular markers	Nuclear	Plastid
López-Sepúlveda et al., 2013b	Myrtaceae	*Myrceugenia*	tree	33	no	no									AFLP / SSR	yes	
López-Sepúlveda et al., 2015a	Asteraceae	*Erigeron*	herb	50	no	no							yes		AFLP / SSR	yes	
López-Sepúlveda et al., 2015b	Winteraceae	*Drimys*	tree	44	no	no							yes		AFLP / SSR	yes	
Lousada et al., 2013	Velloziaceae	*Vellozia*	herb	10	no	no							yes		8 primers and 141 ISSR Loci	yes	
Marques et al., 2014	Orchidaceae	*Epidendrum*	herb	10	yes	no			yes	yes	niche models		yes		AFLP/sequencing	yes	yes
Marquete and Mansano, 2012	Salicaceae	*Casearia*	tree	—	no	yes	yes										
Melo and Borba, 2011	Orchidaceae	*Acianthera*	herb	7	no	no		yes									
Meneguzzo et al., 2015	Orchidaceae	*Aganisia*	herb	—	no	yes	yes										
Miller and Spooner, 1999	Solanaceae	*Solanum*	herb	59	no	yes						yes			RAPD / RFLP	yes	
Miz et al., 2008	Solanaceae	*Solanum*	herb	—	no	no						yes			sequencing	yes	yes
Moraes et al., 2012	Cactaceae	*Pilosocereus*	herb	10	no	no							yes		11 SSR	yes	
Moreno et al., 2015	Turneraceae	*Turnera*	herb	—	yes	no		yes	yes	yes			yes		RAPD/RFLP		yes
Norrmann, 2009	Poaceae	*Andropogon*	herb	—	yes	yes	yes		yes	yes							
O'Leary et al., 2012	Verbenaceae	*Lippia*	shrub	—	yes	yes	yes	yes									
Oliveira and Valls, 2002	Poaceae	*Paspalum*	herb	—	no	yes	yes										
Oliveira et al., 2007	Asteraceae	*Vernonia*	herb	8	no	no		yes									
Oliveira et al., 2008a	Poaceae	*Raddia*	herb	14	yes	yes		yes					yes		10 Allozime loci	yes	
Oliveira et al., 2008b	Poaceae	*Raddia*	herb	14	no	yes		yes									
Oliveira et al., 2013	Myrtaceae	*Campomanesia*	tree	—	no	yes	yes										
Oliveira et al., 2015	Poaceae	*Paspalum*	herb	—	yes	yes	yes	yes	yes								

Reference	Family	Genus	Habit	N								Markers		
Palacios et al., 2012	Fabaceae	*Prosopis*	shrub	—	yes	yes	yes	yes			yes	AFLP	yes	
Paula-Souza et al., 2011	Violaceae	*Hybanthus*	herb	—	no	yes	yes							
Pelegrin et al., 2009	Poaceae	*Briza*	herb	—	no	no		yes						
Peraza-Flores et al., 2011	Orchidaceae	*Polystachya*	herb	—	no	yes	yes							
Pereira et al., 2007	Eriocaulaceae	*Syngonanthus*	herb	10	yes	yes	yes				yes	14 allozime loci	yes	
Pessoa et al., 2012	Orchidaceae	*Epidendrum*	herb	20	yes	yes	yes	yes		yes	yes		yes	yes
Pinheiro and Barros, 2007	Orchidaceae	*Epidendrum*	herb	18	no	no		yes						
Pinheiro and Barros, 2009	Orchidaceae	*Brasiliorchis*	herb	30	no	no		yes						
Pinheiro and Miotto, 2005	Fabaceae	*Lupinus*	herb	—	no	yes	yes							
Planchuelo and Dunn, 1989	Fabaceae	*Lupinus*	herb	—	no	yes	yes							
Porter-Utley, 2014	Passifloraceae	*Passiflora*	liana	—	yes	yes	yes	yes		yes		sequencing	yes	
Prohens et al., 2006	Solanaceae	*Solanum*	herb	—	no	no					yes	AFLP	yes	
Ribeiro et al., 2008	Orchidaceae	*Bulbophyllum*	herb	33	yes	yes		yes			yes	9 allozime loci	yes	
Ritz et al., 2007	Cactaceae	*Rebutia*	herb	—	no	no				yes		sequencing		yes
Rivadavia et al., 2014	Droseraceae	*Drosera*	herb	—	no	yes	yes							
Rodrigues et al., 2015	Orchidaceae	*Cattleya*	herb	8	no	no				yes	yes	two cpDNA:rps16-trnKandrpL32-trnL and 295 ISSR loci	yes	yes
Roncal et al., 2007	Arecaceae	*Geonoma*	tree	4	no	yes					yes	ISSR	yes	
Särkinen et al., 2015	Solanaceae	*Solanum*	herb	—	yes	yes	yes			yes		sequencing	yes	yes
Sawyer and Rojas, 1998	Solanaceae	*Deprea*	herb	—	yes	yes	yes	yes						
Schmidt-Lebuhn, 2007	Lamiaceae	*Minthostachys*	shrub	—	no	no				yes		AFLP	yes	
Schulman et al., 2004	Melastomataceae	*Clidemia*	liana	50	yes	yes	yes	yes	soil analysis					
Scotti-Saintagne et al., 2013	Meliaceae	*Carapa*	tree	40	no	no					yes	SSR / sequencing	yes	yes

Table 1. (*Continued*)

Reference	Family	Genus	Habit	Number of populations	Multiple methods	Taxonomic decisions	Traditional taxonomic methods	Morphometry/Anatomy	Cytogenetics	Reproductive/crossing experiments	Ecological data	Phylogenetic approach	Population genetic approach	Phylogeographic approach	Molecular markers	Nuclear	Plastid
Scrivanti et al., 2010	Poaceae	*Bothriochloa*	herb	19	no	no			yes								
Seijo and Fernández, 2003	Fabaceae	*Lathyrus*	herb	37	no	no			yes								
Semir et al., 2014	Asteraceae	*Lychnophora*	herb	—	no	yes	yes										
Silberbauer-Gottsberger et al., 1992	Rubiaceae	*Tocoyena*	tree	—	yes	yes	yes	yes			distribution						
Solís-Neffa and Fernández, 2001	Turneraceae	*Turnera*	herb	48	no	no			yes								
Solís-Neffa and Fernández, 2002	Turneraceae	*Turnera*	herb	12	no	no			yes								
Solís-Neffa et al., 2004	Turneraceae	*Turnera*	herb	41	no	no			yes								
Souza et al., 2012	Alliaceae	*Nothoscordum*	herb	—	yes	no			yes			yes			one cpDNA: trnL-trnF and one nuDNA: ITS	yes	yes
Speranza et al., 2003	Poaceae	*Paspalum*	herb	—	no	no			yes								
Speranza et al., 2007	Turneraceae	*Turnera*	herb	79	no	no								yes	sequencing		yes
Strelin et al., 2013	Calceolariaceae	*Calceolaria*	herb	18	no	no		yes									
Tacuatiá et al., 2012	Iridaceae	*Sisyrinchium*	herb	27	yes	no		yes	yes				yes		ISSR	yes	
Takayama et al., 2015	Asteraceae	*Robinsonia*	herb	28	no	no							yes		AFLP/SSR	yes	
Turchetto et al., 2014	Solanaceae	*Petunia*	herb	64	yes	yes		yes			climatic characteristics				two cpDNA: rnS-trnG and trnH-psbA and one nuDNA: 6 CAPs loci	yes	yes
Urbani et al., 2002	Poaceae	*Paspalum*	herb	32	yes	no		yes	yes								
Vázquez-											Garcidueñas et al., 2003	Fabaceae	*Prosopis*	tree	5	yes	yes
	yes					yes		RAPD	yes								
Vega et al., 2013	Orchidaceae	*Epidendrum*	herb	50	yes	no		yes	yes	yes	phenology		yes	yes	AFLP/sequencing	yes	yes

Versieux and Machado, 2012	Bromeliaceae	*Vriesea*	herb	—	no	yes	yes					
Viruel et al., 2010	Dioscoreaceae	*Dioscorea*	liana	25	yes	yes	yes	yes				
Walter et al., 2015	Portulacaceae	*Portulaca*	herb	178	yes	no		yes	yes			
Welker et al., 2015	Poaceae	*Saccharum*	herb	—	no	no				yes	5 nuDNA: apo 1, d 8, ep 2 -ex 7, ep 2 -ex 8, and rep 1	yes
Wet, 1968	Poaceae	*Bothriochloa*	herb	—	yes	yes		yes	yes			
Zaitlin and Pierce, 2010	Gesneriaceae	*Sinningia*	herb	25	no	no			yes			
Zuntini et al., 2015	Bignoniaceae	*Bignonia*	liana	—	no	yes	yes					
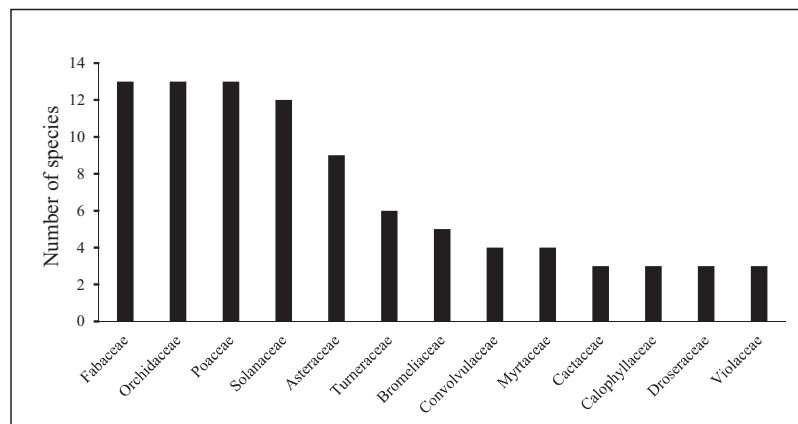
Figure 2. The best represented plant families considering studies of species complexes performed within South America.

Similarly, most of the genera were studied only once, and only 28% were studied more than once (Table 1, Figure 3). The use of molecular markers was the most common approach, adopted by 42.6% of the studies, followed by morphometry (37.9%) and traditional taxonomy (35.6%) (Figure 4). Cytogenetic analyses, mostly meiotic and/or mitotic chromosome counting, were performed in 22% of the studies. Genome sizes estimated using flow cytometry were assessed in four papers. Karyotypes were analyzed in three studies. Cytogenetic molecular techniques (such as *in situ* hybridization) were used in one of the studies retrieved in our review. Ecological data were used in 14 studies, encompassing different methods, such as phenological analyses (5), climatic variables (5), niche models (2), pollination ecology (2), and soil analysis (1). Anatomy was used in 9.3% of the studies.

A large number of the studies used molecular markers (42.6%). Of those, 56.4% employed population genetic methods to analyze the data, whereas 36% used phylogenetic approaches, and only 12.7% used phylogeographic analyses to delimit species (Figure 5A). Eighteen studies combined nuclear and plastid data. Nuclear markers were the primary choice in studies that used molecular

markers (87.3%), and plastid markers were used by 45.4% of the studies (Figure 5B). Among the molecular markers used, sequences from different intergenic regions were used most commonly (23 studies); however, most of these studies used few plastidial regions, usually one or two, and only three studies used four or more plastidial regions (López et al., 2012; Vega et al., 2013; Marques et al., 2014). The majority of studies (11 cases) evaluated only a single-copy nuclear locus, in contrast to three studies that evaluated at least four unlinked nuclear loci (Ames and Spooner, 2010; Andre et al., 2015; Welker et al., 2015). Dominant markers, such as random amplification of polymorphic DNA, amplified fragment length polymorphism, and intersimple sequence repeat, were applied in 20 studies. Co-dominant and unlinked nuclear microsatellite loci and allozyme loci were assessed in 11 and 7 studies, respectively (Table 1).

Less than half of the studies (43.4%) used more than one source of data to analyze species complexes. However, a significant proportion of studies that used a multidisciplinary approach also included taxonomic decisions associated with the species complexes investigated (69.6%, d.f. = 1, χ^2 [Yates corrected] = 8.529, $P <$ 0.01). Considering only studies that adopted only one
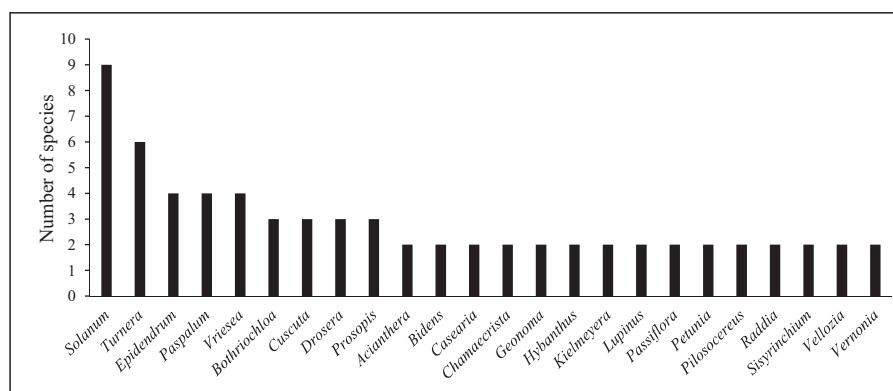
Figure 3. The most well-represented represented plant genera in studies on species complexes in South America.
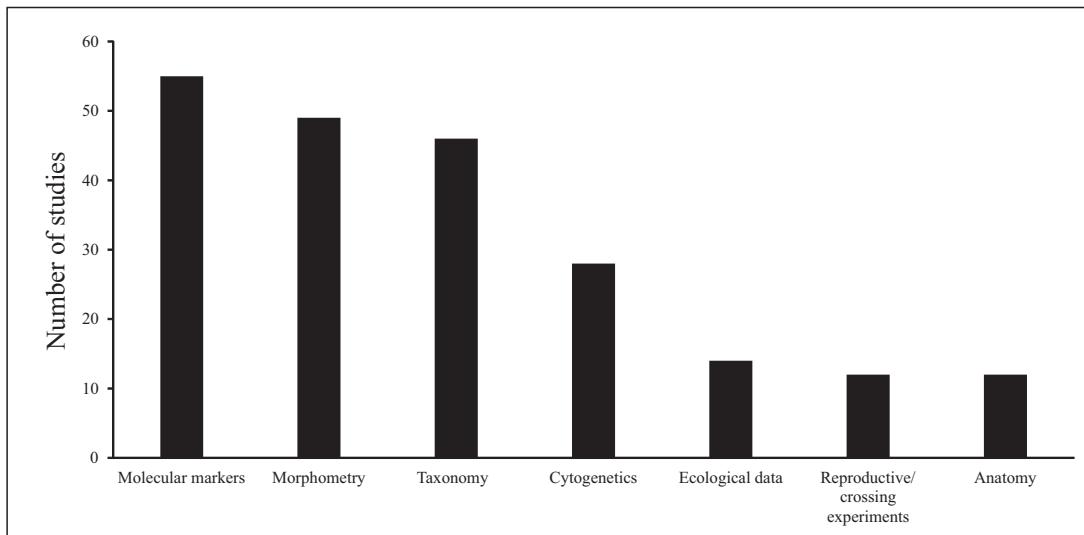
Figure 4. The most widely used methods in studies of species complexes performed within South America.

data type, the use of traditional taxonomic methods always resulted in taxonomic decisions (100%, d.f. = 1, χ^2 [Yates corrected] = 42.476, P = 0.00). The use of molecular markers alone was negatively associated with the adoption of taxonomic decisions [79.1%, d.f. = 1, χ^2 [Yates corrected] = 6.355, $P < 0.05$], and a similar pattern was detected in studies that used only cytogenetics [100.0%, d.f. = 1, χ^2 [Yates corrected] = 9.179, $P <$ 0.01]. Taxonomic decisions were not significantly associated with studies that used morphometry [27.2%, d.f. = 1, χ^2 [Yates corrected] = 0.76, $P = 0.383$]. Associations between the presence of taxonomic decisions and studies using anatomy, reproductive experiments, and ecological data were not assessed due to the low number of studies in these categories.

According to the network (Figure 6), among the studies that adopted a multidisciplinary approach, molecular markers were used 31 times and were combined more often with morphometry (19), morphologic taxonomy (10), and ecological data (7) (Figure 6). Other common

associations between research fields were taxonomy and morphometry (12 studies), taxonomy and molecular markers (10 studies), morphometry and cytogenetics (9 studies), and morphometry and ecology (9 studies, Table 2). In contrast, ecological, anatomical, and cytogenetic studies showed considerably less interaction with other disciplines. Reproductive/crossing experiments and ecological data were never used as a unique source of information to study species complexes and were always combined with other research fields.

The study of species complexes deserves special attention because of the potential to reveal novel and informative results. Most species complexes are potentially composed of recently diverged lineages, which may provide important information regarding the first steps in species radiation, such as colonization of new habitats (Lagomarsino et al., 2016) and selective pressures in different environments (Simon et al., 2011). Owing to their importance, studies of species complexes have guided most of the research on species variation from
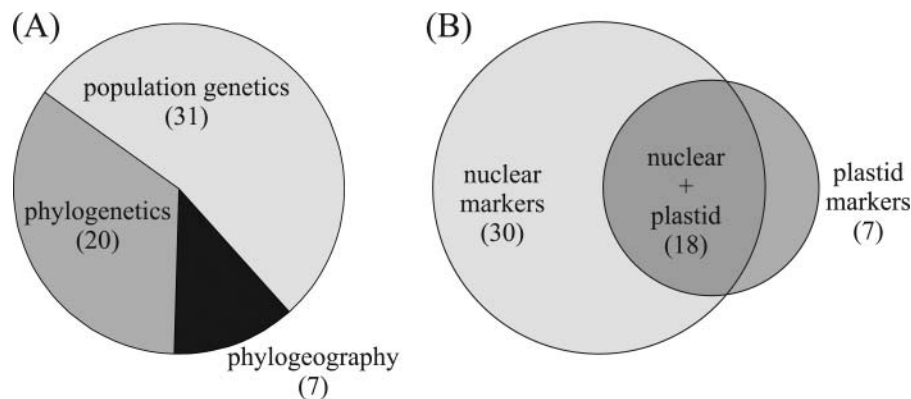
Figure 5. The main methods used to analyze molecular data in studies of species complexes (A) and the types of markers used in these studies (B). Numbers within parentheses indicate the numbers of studies.
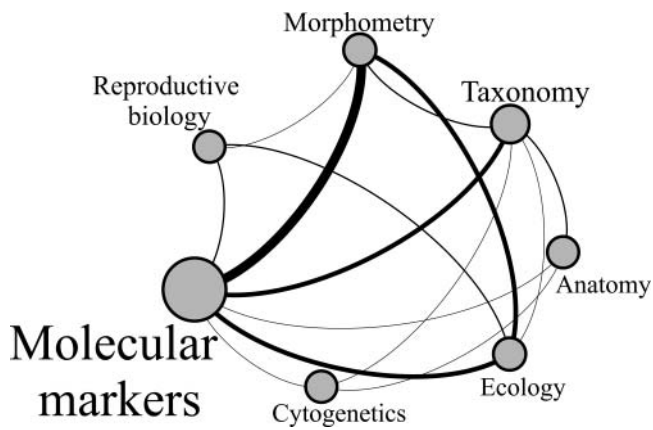
Figure 6. Network showing connections between research fields addressed in studies of species complexes, when adopting a multidisciplinary approach. The size of the circle is proportional to the number of studies in each field, and the line thickness is proportional to the number of connections between fields. See Table 2 for details.

morphological to molecular traits. Thus, these species groups may serve as models to understand speciation events, mainly in the Neotropical region, where mechanisms of species formation remain poorly understood (Antonelli and Sanmartín, 2011). The patterns observed here revealed strong discrepancies among studies of species complexes in South America, showing that such studies have preferentially focused on a few plant families and genera (Table 1). Many studies have been performed on orchids, legumes, and grasses, which are families with great economic importance. Surprisingly, no studies on Euphorbiaceae or Malvaceae species were detected by our survey. Melastomataceae, which is one of the richest plant families in South America, was addressed by only one study (Figure 2). A strong

Table 2. Number of connections between different research fields in studies of species complexes performed in South America. Details are show in Figure 6.

Source	Target	Number of interactions
Anatomy	Cytogenetics	4
Anatomy	Molecular markers	4
Anatomy	Morphometry	4
Anatomy	Reproductive biology	3
Cytogenetics	Molecular markers	5
Cytogenetics	Morphometry	9
Ecology	Molecular markers	7
Morphometry	Ecology	9
Morphometry	Molecular markers	19
Morphometry	Reproductive biology	8
Reproductive biology	Ecology	4
Reproductive biology	Molecular markers	5
Reproductive biology	Cytogenetics	7
Taxonomy	Anatomy	6
Taxonomy	Cytogenetics	3
Taxonomy	Ecology	3
Taxonomy	Molecular markers	10
Taxonomy	Morphometry	12
Taxonomy	Reproductive biology	1

disparity was also observed among the methods chosen by different studies, which focused on molecular markers, morphometry, and traditional taxonomy (Figure 4). Although, most studies were performed using only one line of evidence (Table 1), taxonomic decisions were significantly associated with studies that used a multidisciplinary approach. We explored the patterns identified by our survey to provide a clear picture of studies of species complex and help guide future efforts in this field.

III. Taxonomic bias

Most of the studies on plant species complexes focused on the most diverse plant families found in South America (Figure 2). For example, six of the most studied plant families were also included among the top 10 most diverse families found in Brazil (Zappi et al., 2015). The Orchidaceae family has been a focus of morphological variation studies since Darwin (1882). Large living collections in scientific institutions and of amateur orchidologists have stimulated studies of species complexes worldwide, particularly in Brazil (Pinheiro and Barros, 2007). The same effect is evident in studies on Bromeliaceae (Costa et al., 2009). With some exceptions (Costa et al., 2007; Oliveira et al., 2015), most studies performed on Fabaceae, Poaceae, Solanaceae, and Asteraceae focused on economically important genera. For example, most Solanaceae studies focused on tuber-bearing *Solanum* L. (potato) species (Figure 3). Grasses commonly used for animal pastures, such as *Paspalum* L. and *Bothriochloa* Kuntze, have also been studied extensively. These families show extensive advantages in the study of species complexes, such as short life cycles, ease of cultivation and manipulation in crossing experiments, and high availability of molecular markers.

IV. Use of molecular markers to investigate species complexes

Studies using molecular markers also showed a preference for population genetic methods (Figure 5A). In many cases, these studies commonly reflected associations between the botany and genetics departments of different institutions (Moraes et al., 2012; Caddah et al., 2013). In these studies, genetic differentiation among populations and lineages was crucial to identify the existence of different species. Use of highly informative markers such as microsatellites, coupled with Bayesian assignment methods (i.e., Pritchard et al., 2000), holds great promise for studying species delimitation and speciation processes. For example, Moraes et al. (2012) and Caddah et al. (2013) depicted complex patterns of

variation, enabling recognition of divergent lineages that did not correspond exactly to the species limits traditionally recognized. For instance, hybridization between *Kielmeyera coriacea* Mart. and *K. grandiflora* A.St.-Hil. has blurred species boundaries and challenged the identification of species throughout history (Caddah et al., 2013). The long-term isolation of *Pilosocereus aurisetus* (Werderm.) Byles & G.D. Rowley populations may have promoted divergence of narrow endemic lineages, which occur in a naturally fragmented landscape that imposes strong restrictions on gene exchange and enhances genetic drift (Moraes et al., 2012). Similar patterns were observed in two studies on Asteraceae, which is endemic to oceanic islands, in which multiple events of lineage divergence were driven by strong restrictions on gene exchange (López-Sepúlveda et al., 2013a; Takayama et al., 2015).

Nuclear markers have been highlighted as ideal for interspecific comparisons and have been useful for testing hypotheses of species delimitations (Palma-Silva et al., 2011). Molecular markers with higher levels of gene flow are less prone to introgression and thus more effective for species delimitation (Petit and Excoffier, 2009). In angiosperms, nuclear genomes are usually inherited biparentally and dispersed (from seed and pollen grains), unlike plastidial genomes, which are usually only maternally inherited and dispersed from seeds. This led us to expect that nuclear markers, in angiosperms, would be more suitable for species delimitation than plastidial markers. In fact, most studies that we identified here used nuclear markers (Figure 5B) and conducted better species delimitation in *Sisyrinchium* (Alves et al., 2014) and *Astronium* (Caetano et al., 2008). On the other hand, plastid markers show lower levels of gene exchange, often reflecting patterns of geographic isolation (Bonatelli et al., 2014; Turchetto et al., 2014). Furthermore, using plastidial markers exclusively may be problematic because they have limited utility for many of the coalescent methods, as the chloroplast represents only one locus. In addition, divergence among nuclear and plastidial markers could occur as a consequence of hybridization among species, as reported for two species complexes in the *Cuscuta* genus (Costea et al., 2011, 2015). Thus, despite that only 32% (18) of studies in our review used a combination of both genomes, authors should attempt to combine both nuclear and plastidial markers when studying species complexes to provide a clearer picture of the species boundaries, and processes involved in their diversification.

One interesting approach employed to investigate species complexes using molecular markers is the "blind sampling" strategy, described by Duminil et al. (2006). In special cases, such as species with a short flowering period and/or a lack of morphological diagnostic characters, authors suggest skipping previous morphological identification methods performed by most studies and proceeding directly to molecular genetic analyses when studying species complexes. Some studies identified in our review adopted the same strategy and were able to identify the species effectively *a posterior* (Caetano et al., 2008; Marques et al., 2014; Gagnon et al., 2015). However, it is worth mentioning that without appropriate recording of phenotypes, it is presumably more difficult to identify lineages based on previous hypotheses in the literature or translating results into taxonomic updates.

V. Multidisciplinary approaches

One advantage of adopting a multidisciplinary approach is the possibility of identifying potential mechanisms underlying the diversification and speciation processes within species complexes. In fact, data from multiple sources can form a reciprocal illumination (Hennig, 1966; Blaxter, 2004; Sukumaran and Knowles, 2017), in which different disciplines inform and refine others. In this context, all sources of data are combined to help describe the diversity of life. For example, despite the efficiency of molecular markers in describing levels of biodiversity and evolutionary relationships, studies using this approach exclusively have limited the explanatory power regarding the mechanisms leading to speciation (Lipscomb et al., 2003; Huang and Knowles, 2016). According to Lipscomb et al. (2003), a multidisciplinary approach has the power to expose inconsistencies, which is an important means of identifying important evolutionary processes, such as hybridization and introgression, and phenotypic convergent plasticity. In fact, recent studies have emphasized the importance of integrative approaches in species delimitation (de Queiroz, 1998; Padial and de la Riva, 2010; Puillandre et al., 2012; Zapata and Jiménez, 2012). This trend was detected based on taxonomic decisions, which were significantly associated with studies that employed different methods. Species complexes are often composed of recently diverged lineages, in which the speciation process is incomplete. In such situations, using a single method may not be sufficient to detect the mechanisms involved in lineage divergence (de Queiroz, 1998). On the other hand, different methods increase the power to detect early stages of divergence, improving further attempts to delimit species.

The joint use of molecular markers and morphometry was observed in 15% (19 studies) of all retrieved papers (Figure 6). This pattern may reflect the intention of these authors to investigate the extent to which the morphological similarities reflect the evolutionary relationships

in such groups. This is a fundamental question in species complex studies, as mentioned previously (Briggs and Walters, 1997). In general, the lack of a correlation between morphometric and genetic data is interpreted as evidence of convergent evolution. For example, the results obtained by Borba et al. (2002) support the hypothesis that floral similarities among *Acianthera* Scheidw. species represent convergence driven by flower fidelity to the same pollinators. Indeed, species with similar flowers are allopatric and show extensive genetic differences (Borba et al., 2002). On the other hand, some studies have found strong correlations between observed morphometric patterns and genetic data. The agreement between morphological and genetic differences has been used to recognize different species within the *Chamaecrista cytisoides* (Coll) H.S. Irwin & Barneby complex (Conceição et al., 2008) and within the *Chamaecostus subsessilis* (Nees & Mart.) C. D. Specht & D. W. Stev. complex (Andre et al., 2015).

Crossing experiments were always used in combination with other methods in the studies evaluated in this review (Table 1). Despite the importance of crossability for the biological species concept (Mayr, 1942), such experiments have rarely been used (only 9.3%, 12 papers) to study species complexes in South America. Experimental pollination provides important information regarding mating systems and inbreeding depression (Borba et al., 2011; Sampaio et al., 2012). This information is also crucial to explore the evolution of reproductive barriers among populations and species (Costa et al., 2007; Pinheiro et al., 2013). For example, species with different mating systems may show greater reproductive isolation (Palma-Silva et al., 2015; Pinheiro et al., 2015; Twyford et al., 2015; Neri et al., 2017, 2018). The evolution of reproductive isolation has been central to species recognition since the influential works of Dobzhansky (1937) and Mayr (1942), which articulated the biological species concept. In this context, hybrid zones have been used as natural laboratories to study the evolution of reproductive barriers (Soltis and Soltis, 2009; Baack et al., 2015). However, several studies conducted in hybrid zones have revealed incomplete barriers to gene exchange (Rieseberg et al., 2004), increasing uncertainty regarding species boundaries, mainly in species complexes (Cavallari et al., 2010; Costea et al., 2011, 2015; Vega et al., 2013; Marques et al., 2014). The idea that species integrity is maintained even in the presence of low levels of interspecific gene exchange has altered the concept of "complete reproductive isolation," initially postulated in the biological species concept (Mayr, 1942), to "partially permeable reproductive isolation," known as a porous genome (Wu, 2001). Recent approaches have shown that a combination of multiple

reproductive barriers to gene exchange act in concert, preventing the collapse of species (Palma-Silva et al., 2011; Vega et al., 2013; Marques et al., 2014; Pinheiro et al., 2016; Neri et al., 2017, 2018). Therefore, a multidisciplinary approach is crucial when studying barriers to gene exchange, which in turn will clarify the ecological and genetic mechanisms acting during species and lineage diversification. For instance, interspecific gene exchange may result in transfer of adaptive traits between closely related species, reducing extinction risks during environmental changes (Cannon and Lerdau, 2015).

It is important to highlight that speciation is a population-level process, which involves the evolution of reproductive isolation among different populations (Scopece et al., 2010). The study of reproductive barriers within species, or within species complexes, would help determine the first stages of the speciation process in situations in which species formation is still incomplete. The population-level analysis of the ecological and genetic causes of reproductive isolation was named "the magnifying glass" approach (Via, 2009). This approach is suitable for determining whether ecological and genetic mechanisms are activated before speciation is complete, avoiding confusion with the changes that accumulate after species formation. Very few studies have used this approach in tropical plants as models (but see Pinheiro et al., 2013). One potential reason for this is that most authors still interpret the biological species concept in its original version, in which reproductive isolation would be completely absent among populations within species. A change in this perception and the inclusion of "speciation in a continuum" and "speciation with gene flow" ideas (Wu, 2001; Nosil, 2012; Seehausen et al., 2014) are urgently needed to encourage investigations of reproductive barriers during the early stages of divergence: within species and/or species complexes.

Ecological data were included in 14 of the 56 studies that involved multidisciplinary approaches (Figure 6, Table 1). Eight of these studies included a range of methods, such as the analysis of single lines of environmental data including temperature and/or rainfall, or included more climatic variables to characterize the environments in which the populations were sampled. Actually, the history of species complex studies is strongly associated with characterizing the heterogeneous environments in which the populations were found (Turesson, 1922). The study of ecotypes and races stimulated the use of novel and creative approaches to disentangle the effects of different habitats on variations in morphological characters. For example, transplant experiments are crucial to understand how plasticity and/or heritable characters are responsible for the variation found among populations

growing in divergent habitats (Clausen et al., 1939). In our survey, 38% of the studies applied morphometric analyses to investigate species complexes, indicating potential phenotypic variation within the species complexes. In the studies that employed morphometric analyses, 49 studies in total, it would help to know whether the morphological variation was caused by phenotypic plasticity, local adaptation, or both. Classical reciprocal transplant experiments are not always feasible, but alternatives, such as rearing/raising/growing divergent phenotypes in controlled climatic environments, (e.g., common garden studies) could be implemented more easily (Franks et al., 2014). Nevertheless, Esteves and Vicentini (2013) used morphometry and habitat characterization to explore the existence of plasticity and/or selection for different environments in the *Pagamea coriacea* Benth. complex. They found two discrete morphological groups in this complex, associated with different habitat preferences and reproductive behaviors. Turchetto et al. (2014) reported strong connections among morphological, genetic, and environmental variables, suggesting that selection for different habitats, rather than plasticity, is responsible for the diversification of lineages in the *Petunia axillaris* (Lam.) Britton, Sterns & Poggenb. complex. Salinity, elevation, and climate were important environmental drivers for lineage diversification in the *Hypochaeris apargioides* Hook. & Arn. complex (López-Sepúlveda et al., 2013a).

Phylogeographic studies have played a central role in interpreting how past orogenic changes and climatic fluctuations have affected the diversification of lineages and species in South America (Turchetto-Zolet et al., 2013; Smith et al., 2014; Leal et al., 2016b; among others). Unfortunately, in our review, only seven studies used a phylogeographic approach to evaluate species complexes (Figure 5A). For example, morphological and molecular data have been combined to study the *Vellozia hirsuta* Goeth.& Henrard complex, a highly polymorphic group found among disjointed mountains within the Espinhaço Mountain Range in Brazil (Barbosa et al., 2012). Despite strong morphological differentiation among populations, no associations with the genetic lineages were found, suggesting that *V. hirsuta* should be considered a single polymorphic species. Bonatelli et al. (2014) concluded that diversification of the *P. aurisetus* complex has been affected by long-term isolation in microrefugia composed of xerophytic vegetation, by combining detailed demographic analyses, divergence time estimates, and niche modeling. Integrating different types of data is commonly performed in phylogeographic studies to obtain a clear picture of the processes involved in lineage and species diversification. Considering the growing number of phylogeographic studies in South America (Turchetto-Zolet et al., 2013), several groups remain poorly studied, and many questions remain unanswered. Of special interest are studies exploring how reproductive isolation evolves in a geographic context, and how selective pressures for divergent environments contribute to the formation of barriers preventing gene exchange among populations (Vallejo-Marín and Hiscock, 2016).

Phylogenetic inferences are crucial to recognize broad evolutionary relationships among lineages and species, enabling the recognition of groups that may refer to species complexes. For instance, the phylogeny published by Pinheiro et al. (2009) provided the basic framework for a detailed study of a particular group of *Epidendrum* showing unclear species boundaries (Pessoa et al., 2012). The same strategy is observed in other groups, such as the phylogenetic study published for the tribe Bignonieae (Bignoniaceae, Lamiales, Lohmann, 2006), allowing further population-level studies in species complexes (Firetti-Leggieri et al., 2011; Zuntini et al., 2015). One of the advantages of this approach is to identify cases of trait convergence, to avoid the study of unrelated paraphyletic groups. Rodrigues et al. (2015) constructed a phylogenetic inference for the *Cattleya coccinea* complex, and the recognition of different clades was important for the detailed study of Leal et al. (2016a), using morphometric and microsatellite analyses. The study performed by Welker et al. (2015) also identified several clades within the genus *Saccharum*, allowing future studies to explore particular microevolutionary processes within each species group of this genus. Species complexes belonging to monophyletic groups would facilitate the identification of drivers that fuel diversification events such as lineage dating (Perret et al., 2007; Breitkopf et al., 2015) and trait evolution (Silvestro et al., 2014; Lagomarsino et al., 2016).

In our review, species tree-based methods for species delimitation were limited to the application of a single or few universal markers, thus not incorporating tree-based coalescent methods. However, the accuracy of species delimitation based on a few or single gene trees may be limited and prone to incongruences due to incomplete lineage sorting, hybridization, and introgression (Naciri and Linder, 2015). Thus, unlinked loci and multispecies coalescent methods, which combine concepts from phylogenetic and population genetics, may help to overcome these difficulties. In general, the application of such methods is advantageous because they (1) are highly replicable and capable of being used by researchers in different areas (e.g., ecology, physiology, and systematics) (Flot et al., 2010); (2) are based on falsifiable hypotheses,

removing possible biases inherent to the taxonomist (Fujita et al., 2012); (3) incorporate hypothetical species with incomplete lineage sorting and gene flow (see Camargo et al., 2012, Jackson et al., 2017 and Chan et al., 2017), and (4) have an objective statistical framework (Huang and Knowles, 2016). The study of cryptic diversity in *Cerrado* endemic lizards was a good example of using high-throughput phylogenomic data and coalescent techniques to delimit species and to investigate patterns of species diversity in a geographically widespread animal species (Domingos et al., 2017).

However, the application of multispecies coalescent methods solely to identify evolutionarily independent lineages to delimit species has been criticized (Freudenstein et al., 2017; Sukumaran and Knowles, 2017). Again, the thin line that distinguishes a species population at the beginning of divergence raises the question of the epistemological concept of multispecies coalescence techniques that use only molecular data, showing that this method elucidates the genetic structure, not the delimitation of the species itself (Sukumaran and Knowles, 2017), and the ecological role that a given species plays is crucial for better characterization of the taxa in question (Freudenstein et al., 2017).

Therefore, no method is a panacea, and any approach to species delimitation is unable to distinguish precisely certain taxa given the complexity of evolutionary history (Carstens et al., 2013). Species delimited in multispecies coalescent methods should be considered as hypotheses, and their delimitation should be based on the greatest possible numbers of lines of evidence and methods, as discussed above, with the aim of achieving congruence between them, resulting in a greater robustness of the *a priori* hypothesis (Carstens et al., 2013; Chan et al., 2017; Sukumaran and Knowles, 2017). Thus, methods that incorporate different and independent data, such as ecological, phenotypic, and geographic data, with multilocus analyses and coalescence theory (e.g., iBPP—integrated Bayesian Phylogenetics and Phylogeography, Solís-Lemus et al., 2015; the Guillot model, Guillot et al., 2012) are the new frontier for studies of species complexes. In such studies, these methods may enhance the prediction of species delimitation by contributing independent datasets, seeking the congruence (or the lack thereof) of distinct hypotheses (Sukumaran and Knowles, 2017).

VI. Concluding remarks and perspectives

Our review retrieved 129 studies that explicitly investigated species complexes in South America, which is probably very low considering the levels of plant diversity on this continent. The actual number of biological species is likely to be greater than the current list of nominal species. There is likely a long list of reasons for this, including the fact that species divergence is not always followed by morphological changes, or that morphological and/or ecological variation can occur with low genetic divergence.

Thus, multidisciplinary approaches are crucial to understanding the integrity and boundaries within species complexes (Stebbins, 1950). Integrative taxonomy has fueled discussions about strategies to combine different data sources to achieve comprehensive species delineation (Constance, 1964; Padial et al., 2010). This approach is central for planning future studies of species complexes and may help guide efforts to understand plant speciation in tropical regions. In addition, the creative use of available databases holds great promise for investigating species complexes. Inexpensive approaches, such as crossing experiments and ecological niche modeling, in addition to the data available in public databases, such as TreeBASE (for published phylogenies), GenBank (for molecular data), GBIF (for georeferenced collections), and Dryad (for alignments and morphological matrices), can be associated with multispecies coalescent data, generated from cost-effective sequencing (RADseq, GBS, exon capture, among others; Herrera and Shank, 2016), yielding unprecedented resolution of species boundaries among South American plant species complexes. Furthermore, using genome scanning methods, it is also possible to identify loci and genomic regions under adaptive divergence that may be responsible for reproductive isolation and ecological speciation among populations (Hoban et al. 2016)

We thus believe that the application of new methodologies, such as multispecies coalescence techniques, integrated with different source data (Guillot et al., 2012; Solís-Lemus et al., 2015) in species delimitation of South American species complexes will greatly improve our knowledge of the lineage diversification and speciation processes in this rich biota. In fact, the history of plant variation studies shows that investigating a species complex depends on a creative combination of diverse informative methods. In this context, species complexes should be viewed as important models to understand plant speciation and adaptation, inspiring new generations of botanists, ecologists, and evolutionary biologists.

Acknowledgments

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