UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Estrutura genética, variabilidade morfológica e investimento reprodutivo de *Cattleya liliputana* (Orchidaceae:Laeliinae), endêmica do Quadrilátero Ferrífero, Minas Gerais, Brasil

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Belo Horizonte

2013

Universidade Federal de Minas Gerais

Instituto de Ciências Biológicas

Pós-graduação em Genética

Estrutura genética, divergência morfológica e investimento reprodutivo de *Cattleya liliputana* (Orchidaceae:Laeliinae), endêmica do Quadrilátero Ferrífero, Minas Gerais, Brasil

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Dissertação apresentada ao Programa de Pós-graduação em Genética da Universidade Federal de Minas Gerais como parte dos requisitos para obtenção do título de Mestre em Genética

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AGRADECIMENTOS

Por fim, agradeço à Fundação Boticário e FAPEMIG pelo financiamento das viagens de coleta, experimentos de bancada e bolsa de estudos.

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Introdução

Capítulo 1

Dedico este trabalho à minha mãe e minha avó, que com muito carinho e amor me ensinaram amar a natureza.

The Orchidaceae seems to be a family in a state of active evolution.

Robert Dresller

RESUMO:

A compreensão dos efeitos da paisagem sobre a conectividade e divergência genética de populações naturais é essencial para o entendimento dos padrões biogeográficos de distribuição da diversidade biológica. Esse conhecimento é particularmente importante para regiões como Quadrilátero Ferrífero, um centro de diversidade e endemismo das áreas de altitude do leste da América do Sul. Microssatélites nucleares foram utilizados para averiguar a estrutura genética espacial, estimar a diversidade genética e explorar a relação entre diversidade genética, diversidade morfológica e investimento reprodutivo em sete populações naturais de *Catteya liliputana* (Orchidaceae). A modelagem Bayesiana de agrupamento espacial revelou um padrão de estruturação genético de acordo com a divisão pré-estabelecida de populações naturais. O algoritmo de máxima diferenciação de Monmonier e modelagem Bayesiana no programa STRUCTURE identificaram uma barreira genética localizada entre a Serra do Caraça e o vale do Rio das Velhas. Análises populacionais revelaram uma estruturação espacial moderada (F_{st} = 0.138, p<0.001) e alta diversidade genética e endogamia (H_e = 0.733±0.03; F_{is} = 0.308±0.04). A relação genética entre populações e testes de Mantel indicaram um padrão biogeográfico para distribuição da variabilidade genética e morfológica. Foi encontrado uma correlação positiva entre diversidade genética e morfológica entre populações. Populações pertencentes aos grupos genéticos identificados apresentaram estratégias de investimento reprodutivo diferentes. Dois grupos genéticos distintos foram encontrados em *Cattleya liliputana*, cada um representa uma unidade evolutiva, merecendo atenção no manejo e conservação da espécie. O presente estudo demonstra como a descontinuidade de afloramentos rochosos e características do relevo atuam sobre a estruturação genética, diferenciação morfológica e variação no investimento reprodutivo. O isolamento genético ao longo do tempo pode acarretar em diferenciação morfológica com capacidade de limitar a reprodução entre populações de *C. liliputana*. Esses processos em conjunto com alta heterogeneidade ambiental podem ser um fator importante na irradiação evolutiva de *Cattleya* sect. *Parviflorae*.

Palavras chave: Quadrilátero Ferrífero, genética da paisagem, orchidaceae, campo rupestre, microssatélites, morfometria, estratégia reprodutiva.

ABSTRACT:

Knowledge of the role of landscapes in shaping genetic connectivity and divergence is essential for understanding patterns of biogeography and diversity. This is particularly relevant for the highly heterogeneous Iron Quadrangle region, a major biodiversity hotspot in eastern South America highlands. Nuclear microsatellites were used to assess the landscape genetics, estimate genetic diversity and explore genetic relationships with morphological variability and reproductive strategies for seven natural populations of *Cattleya liliputana* (Orchidaceae). Spatial Bayesian clustering suggested genetic structuring in accordance with pre-established natural populations. Monmonier´s maximum difference algorithm and Bayesian analysis on STRUCTURE identified an major discontinuity between Serra do Caraça and Rio das Velhas valley. Population-based analyses revealed moderate genetic structuring ($F_{ST} = 0.138$, p<0.001) and high genetic diversity and inbreeding (He = 0.733±0.03; $F$is = 0.308±0.04). Interpopulation relationships and Mantel tests supported a biogeographical pattern to the distribution of the genetic and morphological variability. We found significant correlation for genetic and morphological diversity among populations. Populations from of the two main genetic and morphological groups also exhibited different investment in the production of flowers. Two distinct groupings were identified within *C. liliputana.* Each comprises an evolutionarily significant unit, warranting conservation considerations. The current study illustrates that landscape discontinuity drives genetic structuring, morphological divergence and reproductive trade-offs. Isolation over time may lead to morphological changes that can limit reproduction between populations of *C. liliputana*. These processes in conjunction to high landscape heterogeneity can be an important driver of *Cattleya* sect. *Parviflorae* species diversification.

Keywords: Iron Quadrangle, landscape genetics, orchidaceae, *campo rupestre*, microsatellites, morphometrics, reproductive strategy.

INTRODUÇÃO GERAL

A FAMILIA ORCHIDACEAE

Orchidaceae é considerada uma das famílias de plantas mais diversas e amplamente distribuídas. A maior riqueza de espécies da família está concentrada nos trópicos, em especial em regiões montanhosas (Dressler, 1990). A grande variabilidade de caracteres florais e diversificados mecanismos de polinização têm atraído a atenção de biólogos evolutivos desde os tempos de Darwin. Robert Dresller escreve em seu livro *The Orchids: Natural History and Classification* que *"The Orchidaceae seems to be a family in a state of active evolution."* De fato, encontramos na família uma ampla gama de espécies "boas", variedades, complexos de espécies e híbridos naturais sem definição taxonômica clara. A sistemática de Orchidaceae tem enfrentado grandes desafios na classificação de espécie e na proposição de mecanismos evolutivos que expliquem os padrões de diversidade da família (e.g. Chiron & Castro Neto, 2002; C van den Berg et al., 2000; Cássio van den Berg et al., 2009). É importante, portanto, que se tenha cautela na leitura de filogenias. As classificações atuais devem ser vistas como hipóteses de trabalho a serem testadas com incorporação de novos dados e, idealmente, como um guia na procura de novas informações que ajudem esclarecer os padrões evolutivos do grupo.

Segundo Cameron et al., (1999), Orchidaceae pode ser dividida em cinco subfamílias (Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae e Vanilloideae),sendo Epidendroideae a mais diversa, com cerca de 650 gêneros e 18.000 espécies. A subtribo Laeliinae (dentro de Epidendroideae) é a terceira maior de Orchidaceae, possuindo aproximadamente 40 gêneros e mais de 2000 espécies distribuídas no Neotrópico (Pridgeon *et al.*, 2005). Laellinae inclui alguns dos mais importantes gêneros de plantas ornamentais, como *Laelia* e *Catlleya,* e gêneros representativos da flora de orquídeas neotropicais, como *Epidendrum*, *Encyclia* e *Prostechea* (van den Berg *et al.*, 2000). O grande número de espécies, grupos genéricos e infragenéricos indicam que essa subtribo constitui um grupo em processo de irradiação adaptativa recente, atraindo interesse em relação a seus padrões de diversificação (van den Berg *et al.*, 2009).

Segundo (Barros, 1990) "O gênero *Laelia* com 7 seções das quais 5 ocorrentes no Brasil , (hoje reconhecidas como *Cattleya*), apresenta características evolutivas interessantes, principalmente a sect. *Parviflorae,* que ocorre predominantemente em Minas Gerais e que representa um exemplo de "evolução explosiva" ainda em andamento."

A seção *Parviflorae* possui 36 espécies e vários híbridos naturais, ocorrentes principalmente em campos rupestres de Minas Gerais, com algumas poucas espécies na Bahia, Espírito Santo e Rio de Janeiro. Segundo (Pabst & Dungs, 1975), as montanhas de Minas Gerais representam um dos locais no Brasil com o maior numero de espécies de Orchidaceae. Quando comparada com as demais seções do gênero, a seção *Parviflorae* apresenta algumas características muito peculiares: a) possui mais espécies que todas as demais seções juntas; b) quase todas suas espécies são rupícolas, contrariamente às outras seções onde as espécies são exclusivamente epífitas; c) apresenta alta incidência de poliploidia; d) várias espécies apresentam flores amarelas, alaranjadas ou vermelhas, enquanto nas demais seções, as cores predominantes são o lilás e o roxo; e) as espécies são de reconhecimento relativamente difícil (Hoehne 1952), devido à grande semelhança entre si e à grande variabilidade morfológica entre populações; f) suas flores são sempre menores que nas demais seções (com exceção da seção *Microlaelia);* g) híbridos naturais são relativamente comuns entre suas espécies. (Barros, 1990). A situação da seção Parviflorae, do ponto de vista do taxonomista, é a de um grupo muito complexo, devido à variabilidade intra-específica e ao aparecimento de indivíduos com características intermediárias entre as espécies previamente aceitas (Fig. 1).

SISTEMÁTICA E PROBLEMAS TAXONÔMICOS EM *CATTLEYA*:

As espécies de *Cattleya* sect. *Parviflorae* foram primeiramente descritas e por mais de um século classificadas como pertencentes ao gênero *Laelia*. O gênero *Laelia* foi criado formalmente por Lindley, tendo como espécie tipo *Laelia grandiflora*, presente no México. A primeira proposta de classificação para o gênero *Laelia,* elaborada por Lindley, separava as espécies em dois grupos: *Grandiflorae*, expécies mexicanas e Parviflorae, espécies brasileiras. As principais características utilizadas para esta classificação foram peças do perianto, apresentando pétalas maiores que as sépalas no primeiro grupo, e sépalas e pétalas do mesmo tamanho no segundo. Desde então, várias

propostas de classificação vêm sendo sugeridas (Schlechter, 1917; Brieger, 1960, 1961; Pabst & Dungs, 1975; Withner, 1990; van den Berg *et al.*, 2000, 2009; Chiron & Castro Neto, 2002).
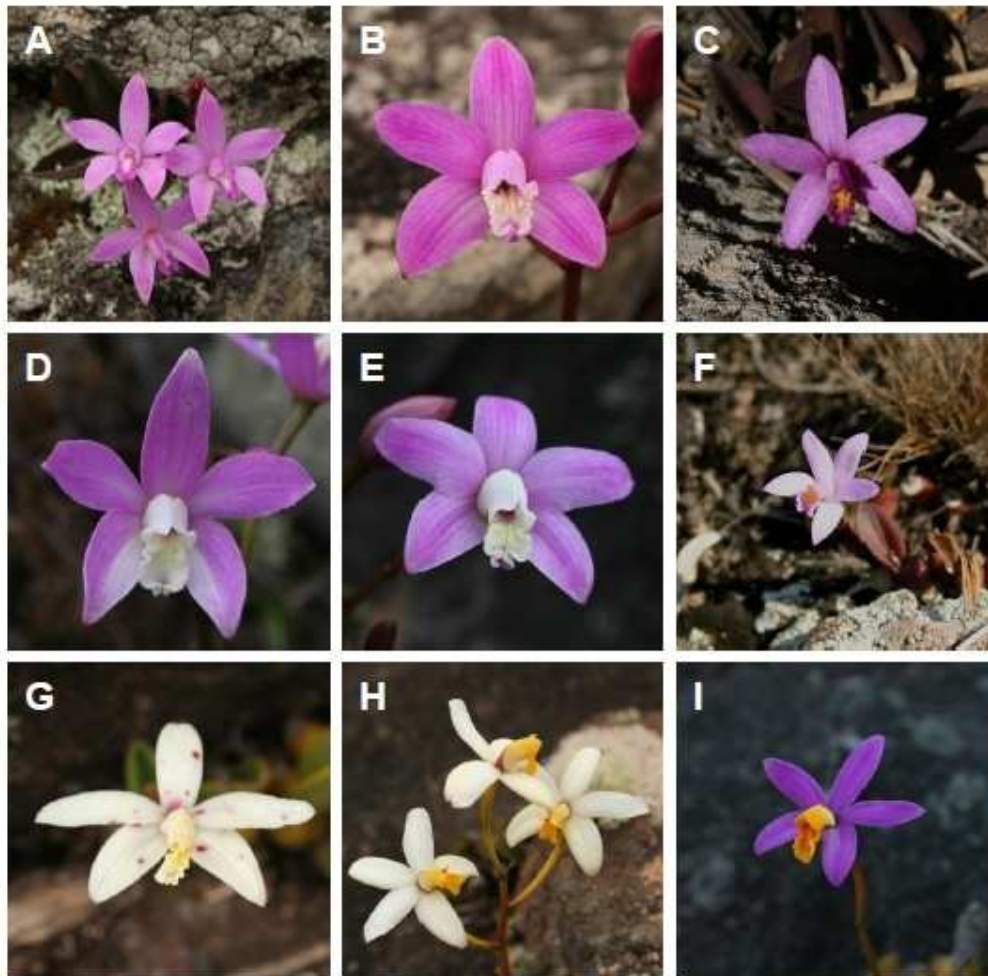
Figura 1. Variabilidade morfológica do complex de espécies da Aliança Liliputana. **(A)** *Cattleya ghilanyi*, **(C)** *Cattleya liliputana*, **(G)** *Cattleya fournieri*, **(I)** *Cattleya longipes.* **(B,D,E,F** and **H)** Intermediários morfológicos entre as espécies.

Pabst & Dungs (1975) fizeram uma tentativa de classificação infragenérica, propondo a estruturação do gênero *Laelia* em quatro seções, sendo estas *Cattleyodes*, *Hadrolaelia*, *Microlaelia* e *Parviflorae.* A seção *Parviflorae* foi estabelecida para acomodar as espécies rupícolas e com pseudobulbos obclavados. De acordo com o autor, a seção foi subdividida em cinco alianças, levando em consideração a altura do escapo floral em relação às folhas e coloração das flores (Fig. 2)

O gênero *Hoffmannseggella* H. G. Jones (Jones, 1968) foi criado como uma tentativa para acomodar as espécies rupícolas brasileiras pertencentes anteriormente a seção *Parviflorae* (Pabst & Dungs, 1975), tendo como espécie tipo *L. cinnabarina* Batem. ex Lindl. A utilização de *Hoffmannseggella* é controvérsia, em razão de sua aceitação levar a divisão do atual gênero Cattleya em uma grande quantidade de gêneros proximamente relacionados à *Hoffmannseggella,* mas de difícil circunscrição morfológica e baixa identificabilidade. No entanto, essa é a taxonomia adotada pela Lista de Espécies da Flora do Brasil (de Barros *et al.*, 2013).

A Aliança Liliputana é um complexo de espécies da seção *Parviflorae* proposto por Pabst (Pabst & Dungs, 1975) e Withner (Withner, 1990). O complexo é reconhecido pelo tamanho diminuto das inflorescências e reduzido número de flores (Fig. 2). Todas as espécies do complexo apresentam distribuição geográfica restrita. Cinco espécies são endêmicas do Quadrilátero Ferrífero (QF) (*C. liliputana, C. kettieana, C. fournieri, C. reginae* e *C. longipes*) e outras duas (*C.duvenii* e *C. ghillanyi*) possuem distribuição deslocada ao norte, abrangendo região da Serra do Cipó e região do Pico do Itambé, município de Diamantina, MG (Verola, 2008; Antonelli *et al.*, 2010; dados não publicados).

Figura 2. Variação na altura do escapo floral em *Cattleya* sect. *Parviflorae*, caractere taxonômico importante para separação das espécies em Alianças segundo Pabst & Dungs (1975) e Withner, 1990. **(A)** *C. cinabarina,* representante da Aliança Parviflorae. Espécie apresenta escapo floral grande, aproximadamente três vezes o tamanho das folhas. **(B)** *C.*

ghillanyi, representante da Aliança Liliputana. Espécie apresenta escapo floral aproximadamente do mesmo tamanho das folhas.

Cattleya liliputana (Past) Van den Berg é uma espécie rupícola encontrada em afloramentos rochosos entre 1400 a 2070m de altitude. A espécie pode ser identificada em campo pela presença de folhas avermelhadas medindo 9±2.5 mm, pedúnculo floral diminuto medindo 23.4±15.9mm e labelo magenta de formato arredondado medindo aproximadamente 10 x10 mm com guias de néctar amarelas de grande destaque na parte interna do lobo medial do labelo (Fig. 3). Indivíduos produzem de 1 a 5 inflorescências que abrigam de 1 a 3 flores de Julho a Outubro.

O gênero *Cattleya* ainda carece de uma revisão infragenérica detalhada que reflita o processo de diversificação das espécies. Entretanto, análises genéticas em *Cattleya* sect. *Parviflorae* têm sido dificultadas pelo baixo polimorfismo encontrado em sequências plastidiais, levando a construção de árvores filogenéticas de baixo suporte (van den Berg *et al.*, 2009; Antonelli *et al.*, 2010; Gustafsson *et al.*, 2010). A baixa informatividade de sequências plastidiais tem comprometido o teste de hipóteses filogeográficas que poderiam esclarecer a evolução e o padrão de diversificação das espécies.

Figura 3. Hábito e variabilidade morfológica de *Cattleya liliputana*.

DESAFIOS PARA CONSERVAÇÃO:

Os afloramentos rochosos do Quadrilátero Ferrífero (QF) habitat de uma grande diversidade de espécies de *Cattleya* sect. *Parviflorae* apresentam também grande heterogeneidade estrutural, dando origem a um relevo peculiar formado por rochas ferríferas (canga), quartzíticas e graníticas (Spier *et al.*, 2003; Jacobi & Carmo, 2008; Carmo, 2010). Cerca de 50% do minério de ferro brasileiro é extraído no QF em aproximadamente 50 minas em atividade na região (DNPM, 2005). Os afloramentos do QF se encontram sob crescente pressão econômica fomentada pelo interesse em investimentos para extração de minério. A degradação ambiental direta, causada pela remoção dos afloramentos para abertura de lavras, e indireta, causada pela crescente urbanização e especulação imobiliária, têm colocado em risco de extinção grande quantidade de espécies. Por exemplo, dados preliminares sugerem que aproximadamente 34% da área de ocorrência de *Cattleya liliputana* foi irreversivelmente perdida e o restante apresenta grande fragmentação e perda da qualidade ambiental (B. Leles, dados não publicados).

Muitas espécies de *Cattleya* sect. *Parviflorae*, em especial as endêmicas do QF, se encontram ameaçadas. Algumas espécies já constam na Lista Vermelha da Flora de Minas Gerais, publicada em 2007 (Tabela 1). Entretanto, pouco se conhece sobre distribuição geográfica, diversidade morfológica, diversidade genética, mecanismo de reprodução, polinizadores, taxonomia e relação filogenética de espécies da seção *Parviflorae* com outras espécies do gênero. A carência de informação contrasta com a rápida perda de habitat e a grande vulnerabilidade das espécies, o que vem tornando o risco de extinção uma ameaça real.

Apesar da acelerada degradação ambiental, há carência de dados que sustentem a proposição de estratégias de manejo que minimizem os impactos ambientais para plantas de campo rupestre. Portanto, a produção de dados de história natural, diversidade genética e reprodução são de grande relevância para o início da aplicação de abordagens orientadas para conservação. A junção desse conhecimento com o trabalho

de licenciamento ambiental e resgate de flora realizado pelas operadoras de minas no QF aumentaria o sucesso de projetos de conservação.

Tabela 1. Orquidaceae do gênero *Cattleya* ameaçadas de extinsão segundo Lista Vermelha da Flora de Minas Gerais, 2007

Espécie	Categoria de Ameaça	Endêmico do QF	Ocorrente no QF
Cattleya brevipedunculata	CR		"
Cattleya auyeenii	CR		
Cattleya fournieri	CR	"	"
Cattleya itambana	CR		
Cattleya jongheana	CR		"
Cattleya longipes	CR	"	"
Cattleya milleri	CR	"	"
Cattleya guttata	CR		
Cattleya amethystoglossa	CR		
Cattleya perrinii	CR		
Cattleya virens	CR		
Cattleya bradei	EN		
Cattleya briegeri	EN		
Cattleya caulescens	EN		"
Cattleya loddigesii	EN		
Cattleya walkeriana	EN		
Cattleya warneri	EN		
Cattleya coccinea	EN		
Cattleya crispata	EN		"
Cattleya tunaii	EN		
Cattleya mirandae	EN		
Cattleya pendula	EN		
Cattleya pumila	EN		
Cattleya cinnabarina	VU		"
Cattleya endsfeldzii	VU		
Cattleya ghillanyi	VU		
Cattleya bicolor	VU		"

CR criticamente ameaçado, EN em perigo, VU vulnerável

A produção de dados ecológicos, em conjunto com sua aplicação em estratégias de manejo, sensibilização de setores empresariais, envolvimento de estudantes, educação ambiental, integração da gestão de RPPNs, maior fiscalização governamental e estímulo a investimentos será o caminho mais próspero para conservação da biodiversidade do Quadrilátero Ferrífero.

OBJETIVOS

Objetivo geral:

Avaliar a estrutura genética de *Cattleya liliputana* e suas relações com variabilidade morfológica e investimento reprodutivo.

Objetivos específicos:

i) Transferir marcadores microssatélites de *Cattleya coccinea* para *Cattleya liliputana*.

ii) Determinar estrutura genética espacial de *Cattleya liliputana*, testar ocorrência de barreiras genéticas entre populações e determinar índices de diversidade genética.

iii) Avaliar variabilidade morfológica de caracteres florais e vegetativos.

iv) Determinar investimento reprodutivo na produção de inflorescências e flores.

v) Testar correlação entre índices de diversidade genética e diversidade morfológica.

CAPÍTULO 1

Genetic structure is associated with phenotypic divergence in floral traits and reproductive investment in a high altitude orchid from the Iron Quadrangle, southeastern Brazil

CAPÍTULO 1

Title:

Genetic structure is associated with phenotypic divergence in floral traits and reproductive investment in a high altitude orchid from the Iron Quadrangle, southeastern Brazil

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Running title: Genetic structure of montane *C. liliputana*

Abstract

Tropical mountains are interesting models to test species radiations and the effects of landscape properties on genetic structure and ecological characters of natural populations. Because of their disconnected geographic nature, mountain tops and rock outcrops are frequently compared with oceanic islands, as most of them display a marked ecological isolation from the surrounding area. Knowledge of the role of montane landscapes in shaping genetic connectivity and local adaptation is essential for understanding evolutionary patterns and development of efficient conservation plans. Nuclear microsatellites were used to assess the landscape genetics, estimate genetic diversity and explore genetic relationships with morphological variability and reproductive strategies in seven natural populations of *Cattleya liliputana* (Orchidaceae). Spatial Bayesian clustering and population-based analyses revealed significant genetic structuring (F_{ST} = 0.138, P<0.001) and high genetic diversity and inbreeding (He = 0.733±0.03; $F$is = 0.308±0.04). Strong differentiation was found between populations over short spatial scales, indicating substantial periods of isolation. Monmonier´s maximum difference algorithm and Bayesian analysis on STRUCTURE identified one major genetic discontinuity between populations. Significant correlation was found for genetic and morphological diversity among populations. Divergent genetic groups showed phenotypic divergence in flower traits and different reproductive strategies, suggesting local adaptations. The results highlight the importance of restricted gene flow, genetic drift and local adaptation as drivers of plant diversification in montane islands such as high altitude rock outcrops.

Keywords: *campo rupestre,* Iron Quadrangle, Espinhaço Range Region, landscape genetics, microsatellites, morphometrics, Orchidaceae, reproductive strategy.

Introduction

Tropical mountains play an unquestionable role in promoting regional and global biodiversity (Burke, 2003). Montane ecosystems contain some of the world´s richest plant communities (Gentry, 1982; Henderson *et al.*, 1991; Barthlott *et al.*, 1996) but our knowledge about species distribution, genetic structure and evolutionary pattern of plant diversity in these regions is still fragmentary (Gentry, 1995; Safford, 2007; Rapini *et al.*, 2008; Echternacht *et al.*, 2011). The risk of habitat loss under climate change predicts high extinction rates for plants and animals in these regions (Thuiller *et al.*, 2005; Ohlemüller *et al.*, 2008; Dirnböck *et al.*, 2011; Gottfried *et al.*, 2012).

Tropical mountains are important models to test species radiations and the effects of landscape properties on genetic structure of natural populations (Hughes & Eastwood, 2006; Antonelli *et al.*, 2009, 2010; Palma-Silva *et al.*, 2011; Amaro *et al.*, 2012; Gutiérrez-Pinto *et al.*, 2012; Caro *et al.*, 2013; Pinheiro *et al.*, 2014). Describing how landscapes and historical changes in landscape properties shape the genetic diversity, gene flow and local adaptation is essential for understanding the patterns of present-day diversity and project future responses in face of increasing climate change (Jump & Penuelas, 2005; Byars *et al.*, 2007; Storfer *et al.*, 2007; Manel & Holderegger, 2013).

Tropical montane landscapes combine discontinuous distribution with edapho-climatic variations resulting from altitudinal gradients. Because of their disconnected geographic nature, mountain tops and rock outcrops are frequently compared with oceanic islands, since most of them display a marked ecological isolation from the surrounding area. A growing number of population genetic studies have explored the effect of landscape on genetic parameters of species' in archipelagos of terrestrial islands' (Trénel *et al.*, 2008; Barbará *et al.*, 2009; Palma-Silva *et al.*, 2011; Gutiérrez-Pinto *et al.*, 2012; Caro *et al.*, 2013; Pinheiro *et al.*, 2014; Chaves et al., in press). Indeed, geographic isolation on islands has proven to be a useful model system for studies of species' radiations (Darwin, 1859; MacArthur & Wilson, 1967; Emerson, 2002; Stuessy *et al.*, 2006). The basic concept and tools developed for island biogeography and new methodological developments for integration of spatial and temporal data on population genetics and genomics can be applied for evolutionary studies providing insights on mechanisms and patterns of species diversification (Manel *et al.*, 2003; Storfer *et al.*, 2007, 2010; Sork & Waits, 2010; Manel & Holderegger,

2013). Understanding the pattern of genetic connectivity, gene flow and local adaptations are crucial, not only for improving ecological knowledge, but also for managing properly the genetic diversity of threatened populations (Segelbacher *et al.*, 2010; Manel & Holderegger, 2013).

The Espinhaço Range Region (ERR) is an orographic formation that runs north-south over 1000 km in the Eastern Tropical South America with high numbers of rock outcrop formations. Espinhaço mountains separates important biomes like the Atlantic Rain Forest, Cerrado and Caatinga. The Iron Quadrangle (IQ) composes the southern end of the ERR and covers an area of approximately 7200km^2 at Minas Gerais state, southeastern Brazil (Fig. 1). The IQ is known for its high geological heterogeneity and represents one of the most important and well-studied geological sites in South America (Spier *et al.*, 2003; Knauer, 2007), being responsible for around 50% of Brazilian iron ore production (DNPM, 2005). Quartzite and iron rock outcrops occur interspersed throughout the IQ mountain tops. Outcrops typically harbor rupicolous vegetation embedded within a landscape composed of contrasting plant communities. Studies of plant community at iron outcrops revealed that most of the species present discontinuous distribution highly structured by the presence of outcrops and availability of specific microhabitat conditions resulting in high indices of α and β diversity (Jacobi *et al.*, 2007). The IQ is recognized as one of the regions with higher floristic diversity and endemism in South America highlands, having over 30 percent of endemic species, most of which are associated with rock outcrops environments (Jacobi & Carmo, 2008; Echternacht *et al.*, 2011).

The isolation of rock outcrops species is expected to constrain the gene flow, enhancing the effects of genetic drift, selection and population divergence (Hutchison & Templeton, 1999; Willi *et al.*, 2007). Indeed, genetic studies of inselberg species have provided support for these expectations (Sarthou *et al.*, 2001; Barbará *et al.*, 2007, 2009; Palma-Silva *et al.*, 2011; Pinheiro *et al.*, 2014). Divergent natural selection based on contrasting environments can promote phenotypic and genetic differentiation among populations (Schluter, 2000; Crispo, 2008), resulting in local adaptative radiation. The edaphic structure (occurrence of iron and quartzite rock outcrops) and climatic heterogeneity of the IQ makes it an interesting model to test whether contrasting landscape and evironmental factors influence population genetic and ecological parameters in eastern South American tropical region.

In the present study, we examined the landscape genetics, morphological variation and reproductive investment of *Cattleya liliputana* (Pabst) van den Berg, a rupicolous orchid restricted to high altitude rock outcrops at the Iron Quadrangle. The objectives were: (i) to test in which extent landscape factors affect genetic structure and the degree of isolation among disjoint populations; (ii) to test whether genetic structure is correlated with divergence in floral and vegetative morphological traits and reproductive investment; (iii) to test whether genetic, morphological and reproductive differentiation is associated with climatic and edaphic factors (iron or quartzite outcrops) and (iv) to analyze the implications for conservation based on the genetic and ecological data. The results showed that genetic divergence is associated with landscape and edaphic factors. The genetically differentiated populations exhibited morphological and reproductive divergence, suggesting the effect of genetic drift and local adaptation. These genetic and ecological divergences are likely to influence the long-term success of conservation projects and may be the basis for understanding evolutionary processes in montane *Cattleya* radiation.

Material and Methods

Study species and population sampling
Cattleya liliputana (Pabst) van den Berg (van den Berg *et al.*, 2009) is a rupiculous orchid with its distribution restricted to the Iron Quadrangle. The species can be identified in the field by small reddish leafs (9±2.5 mm) and small round lip (around 10x10 mm) with a yellow mid lobe (Fig. S1 and S2) (Pabst & Dungs, 1975; Withner, 1990). Individuals produce usually 1 – 5 inflorescences bearing 1 – 3 flowers from July to October. Flowers usually last for several days, about 20% of flowers are pollinated and usually less than 5% of individuals produce fruits (B. Leles, unpublished data). Fruits open around November–December and seeds are wind dispersed.

Samples of leaves for DNA extraction and pseudobulbs bearing inflorescences for morphological measures were collected from seven populations in all major mountain ranges at the Iron Quadrangle (IQ) (Fig. 1). Studied populations of *C. liliputana* are found in iron and quartzite rock outcrops from 1483 to 2055m a.s.l. Five populations (CAL, MOE, OBR, CAP and GAN) are found around the Rio das Velhas river valley forming an U-shaped distribution. This region is characterized by large iron ore deposits and intensive mining activities. Populations grow predominantly on iron outcrops, being rarely found on small quartzite outcrops imbedded in iron deposits. Two other populations (CRP and PSL) are located in a peripheral mountain named Serra do Caraça (Fig. 1, Table 1). This region is characterized by large and massive quartzite outcrops reaching the highest altitudes of ERR mountains. A minimum distance of five meters between individuals was set during population sampling.

DNA extraction, PCR and Genotyping
Total DNA was extracted following the protocol described by Doyle and Doyle (1987). Seven microsatellite markers isolated for *Cattleya coccinea* (Leal, 2013; Novello *et al.*, 2013) were used to analyze genetic diversity and landscape genetics of *Cattleya liliputana* (Table S1).

The amplification reactions were carried out in 25 μl final volume containing 1 unit of Taq polymerase (Phoneutria), $1 \times$ IB Phoneutria buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.04 to 0.16 μM primers, 0.16 μM HEX or FAN-labeled M13 primers and 10 ng of genomic DNA. Reverse amplification primers contained a M13 sequence used for fluorescent label according to Shuelke (2000) (Supplemental Table 1). PCR cycles followed premelting at 94°C for 5 min, 10 cycles with denaturing at 95 °C for 30 sec, an

annealing phase at 56 to 60 °C (depending on the primer) for 1 min, an extension phase at 72°C for 1 min, followed by 25 cycles with denaturing at 89 °C for 30 sec, an annealing phase at 53° C for 1 min, an extension phase at 72°C for 1 min a final extension at 72°C for 45 min. Genotyping was performed on a MegaBACE 1000 automated sequencer, using 0.1% Tween 20 and ROX-500 size standard (GE Healthcare). Alleles were identified using MegaBACE Fragment Profiler version 1.2 software (GE Healthcare). Presence of null alleles and scoring errors at each locus were tested using MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.*, 2004) For microsatellite primers and amplification conditions see Supplemental Table 1.

Genetic diversity and landscape genetics

Observed (H_o) and expected (H_e) heterozygosities, number of alleles and deviations from Hardy – Weinberg equilibrium were estimated for each locus and each population using the software Arlequin version 3.1 (Excoffier *et al.*, 2005). Allelic richness (A_R) with rarefaction and F_{IS} estimates were performed in FSTAT 2.9.3 (Goudet, 1995).

Population structure and landscape genetics were analysed with several methods. Arlequin 3.1 was used to perform Analysis of Molecular Variance (AMOVA). Three AMOVAs were performed. One considered only two hierarchical levels and analyzed the partition of total genetic diversity among and within populations. Two AMOVAs tested for barriers to gene flow. One AMOVA tested differentiation between two groups according to software STRUCTURE 2.3.4 (Pritchard *et al.*, 2000): the Serra do Caraça populations (CRP and PSL) and core IQ populations (CAL, MOE, OBR, CAP and GAN). Other AMOVA tested for groupings according to neighbor-joining analysis, considering three groups: Serra do Caraça populations and division of core populations in a west group (CAL, MOE and OBR) and an east group (CAP and GAN).

Bayesian analysis of population structure was performed using the STRUCTURE (Pritchard *et al.*, 2000), and models consisted of ten independent runs for each K, set from K=1 to K=10, consisting of 1,000,000 Markov chain Monte Carlo (MCMC) iterations, with initial burn-in of 50,000, allowing admixture and assuming correlated alleles frequencies. To infer the best number of clusters, we calculated the average likelihood of each K, 'log of probability' (LnP(D)), through all runs as suggested by Pritchard *et al.* (2000) and the ΔK statistic according to Evanno *et al.* (2005). The occurrence of barriers between populations was tested using the

Monmonier´s maximum difference algorithm implemented by Alleles in Space (Miller, 2005)

The number of populations and the occurrence of genetic discontinuities among them were also inferred by a spatial explicit Bayesian model based on simulations of microsatellite data and geographic information carried out in Geneland (Guillot *et al.*, 2005, 2012). MCMC simulations consisted of 20.000.000 interactions with a thinning of 2000, with correlated alleles frequencies and correction for null alleles. At least ten independent runs were performed and the best model was selected based in posterior probability with burn-in of 1000. In order to characterize the spatial distribution of genetic populations defined by Bayesian simulations, maps of population membership probability for each designed cluster were generated.

Cavalli-Sforza & Edwards pair-wise genetic distances (Dc) were obtained in the software FreeNA (Chapuis & Estoup, 2007). Neighbor-joining clustered dendrogram based on Dc distances was constructed in Mega 5.1 (Tamura *et al.*, 2011). 1000 bootstrap replicates of the Dc matrix were conducted in MSA (Dieringer & Schlötterer, 2003) (Dieringer & Schlötterer, 2003) and used to calculate bootstrap support using PHYLIP 3.69 package (Felsenstein, 2006).

Morphological and reproductive investment analyses

Flowering pseudobulbs were collected in the field and preserved in 70% ethanol. Flowers were dissected under a stereo microscope, mounted on plastic cover slips with reference scale and scanned at 600dpi. Dissected pseudobulbs were measured with digital caliper. Digitalized images of flower parts were measured with software ImageTool 3.0. Thirty two continuous characters, 24 floral and 8 vegetative (Fig. S2), were measured. Measurements were Ln-transformed, and correlation analysis was used to identify autocorrelation between variables. Variables with autocorrelation higher than 85% were excluded from analysis. A discriminant analysis was conducted for 26 remaining characters performed with population as the categorical variable (individuals were grouped according to the population to which they belonged). Cluster analysis of the populations was carried out using the Mahalanobis generalized distance calculation from the pooled residual covariance within group matrix, and clustered with paired group and Manhattan similarity algorithm. Bootstrap support was obtained by 1000 replicates of the distance matrix. Data were analysed using PAST 1.91(Hammer *et al.*, 2001) and Statistica (StatSoft, 2003).

The reproductive investment of each *Cattleya liliputana* population was estimated in the field through establishment of 12 5x2m plots along a transect at the center of studied populations and six 5x4m plots randomly distributed along population area, totalizing 240m^2 per population. The number of pseudobulbs and number of inflorescence of each individual inside the plots were counted during 2012 flowering season. The number of flowers per inflorescence was determined on randomly collected pseudobulbs used in the morphometric analysis. The effects of size and genetic clusters identified by STRUCTURE on inflorescence production were tested using a General Linear Model (GLM) on R software.

Correlation Analyses

The correlations between the matrices of genetic (Dc) distances) and geographic distances, between the matrices of genetic and morphological distances, and between matrices of morphological and geographical distances, were estimated using the Mantel test with 10,000 randomizations in PASSaGE (Rosenberg & Anderson, 2011). A Spearman rank correlation analysis between the morphological (D2m) and genetic (H_e and A_R) variability was carried out using Statistica 6.1.

Climatic Analyses

Random points were created on studied areas above 1300m a.s.l. using ArcGis 10.0. Minimum distance between points was set to 500m, totalizing 438 points. Points were separated according to geographic regions: west region (CAL and MOE), south region (OBR), east region (CAP and GAN) and Caraça region (CRP and PSL). Six low correlated climatic variables and altitude were extracted from WorldClim data: altitude; annual mean precipitation; annual mean temperature; mean diurnal temperature range; minimum temperature of the coldest month; predicted evapotranspiration; and vegetation index at July, the dry season. Climatic structure of areas was analyzed using principal component analysis on Statistica 6.1 (StatSoft, 2003).

Results:

Genetic diversity and landscape genetics

All loci were polymorphic with the number of alleles varying from 15 to 39 per locus (Table S1). MICROCHEKER analysis did not show evidence of stuttering or allele dropout for any loci, but suggested the presence of null alleles. Populations showed high diversity with expected heterozigosity, ranging from 0.712 to 0.793 and allelic richness ranging from 6.66 to 11.47. Populations GAN and CAP had the highest genetic diversity (Table 2). All populations showed deviations of Hardy-Weinberg equilibrium, with F_{IS} ranging from 0.250 to 0.35; at least part of these F_{IS} values may be due to null alleles.

Analysis of molecular variance (AMOVA) indicated significant genetic differentiation among populations (F_{ST}=0.138, p<0.001; Table 3). MCMC simulations on Geneland consistently retrieved seven different genetic populations (Fig. 2a). Maps of population membership probability suggested a moderate genetic differentiation among populations even at close proximity (Fig. 2b-h). The Mantel test relating genetic and geographical distances between populations was not significant ($r = 0.371$, $p = 0.071$), suggesting that isolation by distance is not an important factor to explain the genetic divergence between populations.

Monmonier analysis revealed one barrier separating Serra do Caraça populations (CRP and PSL) from the remaining populations occurring around Rio das Velhas valley (core IQ populations) (Fig.3a). Bayesian simulations carried out on STRUCTURE also suggested a strong differentiation between Serra do Caraça and core IQ populations, consistent with the genetic barrier revealed by Monmonier algorithm (Fig. 3b and 3d). ΔK statistics (Evanno et al., 2005) suggested the occurrence of three genetic clusters (Fig. 3c) split on two geographic groups. A second AMOVA clustering the populations in two groups according to STRUCTURE and Monmonier algorithms resulted in 15% of total variation between groups and 6.3% among populations within groups (Table 3).

The neighbor-joining dendrogram based on Cavalli-Sforsa pairwise distance showed that the Caraça populations constitute a group strongly differentiated from the remaining populations with strong bootstrap support (99.6%), in accordance with previous analysis (Fig. 4A). Additionally, the dendrogram suggested that core IQ populations could be further split in two geographic groups: a west group, including CAL, MOE and OBR populations, and an east group including CAP and GAN

populations, with moderate boostrap support, suggesting a possible other barrier at the Rio das Velhas valley (Fig 1). The AMOVA considering these three genetic groups according to the neighbor-joining dendrogram showed that 7.6% of total variation was found among groups and 7.7% among populations within groups ($P<0.001$, Table 3).

Morphological divergence

The scatterplot of individual scores in the canonical variance analysis (CVA) involving 26 morphological characters revealed in the first axis the separation of the core IQ and Serra do Caraça populations (Fig.4b). PSL population was seen to be separated from CRP population on the second canonical axis. There was an overlap of most of the core IQ populations, with exception of the GAN. CVA showed that the first four axis were statistically significant. The first axis accumulated 56.2% of total variance and was mainly correlated to variables 22, 26 and 30 (Supplemental Fig.2). The second axis accumulated 16.2% of variance and was correlated with variables 8, 10, 15 and 31.

The paired group dendrogram of Mahalanobis generalized distance of population centroids revealed a topology of morphological variability consistent with the genetic findings (Fig. 4c). The Mantel test between genetic and morphological distances was near to significance ($r = 0.422$, $P = 0.053$). The median of the generalized Mahalanobis distance from individuals to their population centroids (D2m), a measure of morphological diversity, ranged from 14.73 (OBR) to 37.92 (GAN). Population GAN had the highest morphological diversity, consistent with genetics results (Table 2). Genetic diversity was positively correlated with the morphological diversity (Spearman correlation for H_e and D2m, $r = 0.306$, $P < 0.05$; and for N_a and D2m, $r = 0.285$, $P < 0.05$). Like genetic distance, morphological distance was not correlated with geographical distance between populations (Mantel test, $r = -0.267$, $P=0.195$).

The adnation of the lip base to the column and the bending of the lateral lobe of *Cattleya* species form a tubular chamber where the pollinator must get access to efficiently pollinate the flower. Density plots of column and lip morphometric characters revealed a phenotypic shift. Plants from the Serra do Caraça group produce flowers with wider and taller chambers than core IQ group (Fig. 4d). Plants from the Serra do Caraça exhibited a larger size for medium lobe base width (MLBL), column width (CW) and lateral lobe height (LLH) than the plants from the core IQ ($P<0.01$). Column length (CL) is shorter in the Serra do Caraça plants (all $P<0.01$).

Reproductive investment

General linear model (GLM) analysis indicated a significant positive correlation between individual size (number of pseudobulbs) and the number of inflorescences produced during a flowering season (Table S2). The mean individual size of Serra do Caraça and core IQ groups was significantly different ($P<0.001$, Table S2). Serra do Caraça individuals bear fewer pseudobulbs than core IQ populations (size = 56.89 ± 8.97, N=87 for Caraça, and 160.0 ± 17.05, N=137 for core IQ individuals). GLM analysis pointed out a strong interaction between individual size (number of pseudobulbs) and genetic group ($P<0.001$, Table S2) on the number of inflorescences suggesting that Serra do Caraça populations produce more inflorescence per individual plant size than core IQ (Fig. 5a). Furthermore, flower production per inflorescence was significantly higher for Serra do Caraça in comparison with IQ populations ($F=5.068$, $P<0.001$) (Fig. 5b). Altogether, these results suggest that Serra do Caraça genetic group have a reproductive trade-off with higher investment on inflorescence and flower production.

Climatic niche analysis

In order to test whether climatic differences may account for the genetic, morphological and reproductive investment differences between Caraça and core IQ groups, we compared WorldClim data of major IQ mountain ranges (Fig 6). Principal component analysis of randomly seeded points at areas above 1300m a.s.l., the occurrence area of *Cattley liliputana,* revealed a climatic differentiation among IQ regions (Fig. 6a). However, this differentiation is not consistent with the genetic differentiation. Serra do Caraça region showed a large overlap with CAP and GAN areas (Fig 6b). The current macroclimatic differences do not support a climatic hypothesis for population divergence. Other microclimatic and edaphic factors may contribute to differentiation between Serra do Caraça and other IQ regions.

Discussion

Genetic diversity and structure

Genetic studies of montane species can provide insights into the combined effects of genetic drift, restricted gene flow and local adaptation on evolution of lineages restricted to disjunct populations (Hughes & Eastwood, 2006; Palma-Silva *et al.*, 2011; Pinheiro *et al.*, 2014). The Bayesian modeling of population membership probability of *C. liliputuna* and AMOVA results showed a significant genetic differentiation in accordance with outcrop and mountain discontinuities suggesting that landscape contribute to genetic isolation at high altitude environments even at short distances. A Mantel test did not support isolation by distance among populations. Phylogeographic studies of rock outcrop species have indeed confirmed some hypotheses associated with such isolated habitats, such as low levels of gene flow and strong genetic drift (Byrne & Hopper, 2008; Collevatti *et al.*, 2009; Barbosa *et al.*, 2012; Pinheiro *et al.*, 2014).

The most outstanding pattern recovered by the use of microssatelite markers was the deep population differentiation between Serra do Caraça and core IQ. A combination of genetic analyses retrieved similar population structure. Bayesian modeling of microsatellite data using STRUCTURE, Monmonier´s maximum difference algorithm and neighbor-joining tree based on pairwise genetic differentiation identified one major barrier separating populations of quartzite outcrops of Serra do Caraça from the core IQ populations predominantly at iron outcrops. A less remarkable barrier separating west from east populations of core IQ was detected by neighbor-joining tree, suggesting that Rio das Velhas valley can constitute a partial barrier to gene flow. AMOVA corroborated these analyses, showing a high differentiation between Serra do Caraça and the core IQ groups (F_{CT} =0.15), and moderate differentiation (F_{CT}= 0.076) among the three groups, Serra do Caraça, west and east IQ. Populations CAP and GAN, in the transition zone between Serra do Caraça and core IQ genetic clusters, showed the highest diversity and admixture, suggesting that gene flow may take place through these locations in a stepwise way before reach western mountains. The significant pairwise genetic differentiation among populations and the absence of a pattern of isolation by distance suggest that genetic drift, as observed in other rock outcrop species (Levy & Neal, 1999; Byrne & Hopper, 2008; Palma-Silva *et al.*, 2011; Mayol *et al.*, 2012; Pinheiro *et al.*, 2014), has been an important force shaping

genetic variation in *C. liliputana*. In finite populations, such as those inhabiting continental and oceanic islands, genetic drift may be the predominant force governing allele frequencies of neutral loci (Hutchison & Templeton, 1999).

Nuclear genetic differentiation of orchids tend to be low when compared with other plant taxa (Phillips *et al.*, 2012). The genetic structure at landscape scale showed by *C. liliputuna* can be considered high ($F_{ST}=0.138$) in comparison with other orchid species at Neotropical mountains. Studies with *Cattleya* species in larger spatial scales resulted in similar (Cruz *et al.*, 2011; Leal, 2013) and lower (Borba *et al.*, 2007) fixation indices. Genetic structure of *C. liliputana* is also considerably higher than the one found for *Laelia* and *Epidendrum* (Pinheiro *et al.*, 2011, 2014; Kartzinel *et al.*, 2013) and higher than the overall F_{ST} values found for the myophilous *Bulbophyllum* and *Acianthera* species at the northern Espinhaço mountains (Borba, 2001; Azevedo *et al.*, 2007).

The limited gene flow across mountain tops showed by *C. liliputana* can be due to low pollinator movement. *C. liliputana* is the smallest species in *Cattleya* genus, what restrict the pollinator size to get access to the flower chamber formed by the bending of the lip towards the column. *C. liliputana* flowers are visited by small solitary bees (Verola, 2008). Small bees are known to have low movement dispersion (Gathmann & Tscharntke, 2002). Moreover, the high endemism exhibited by most of *Cattleya* sect. *Parviflorae* species suggests strong constrains in seed germination and recruitment, possibly associated with local microhabitat demands, what may limit the species dispersion capabilities, reinforcing the effects of isolation.

Morphological divergence and reproductive trade-offs

Pollinator-mediated divergent selection is thought to promote speciation in several specialized plant groups (Armbruster & Muchhala, 2009). However, generalized food-deceptive pollination systems usually attract a wide range of occasional pollinators that, after a few trial visits, avoid the rewardless flowers and switch to rewarding ones (Gumbert, 2000). This insect behavior is found to exert only weak selective pressure on floral traits (Salzmann *et al.*, 2007; Juillet & Scopece, 2010; Zitari *et al.*, 2012) that is unlikely to produce effective premating incompatibilities between species. However, this may not be the case for montane food-deceptive *Cattleya* species, which are pollinated by one or a few pollinators. In this pollinator system, morphometric changes

in flower traits was found to be an important premating barriers for reproductive isolation of sympatric species (Smidt *et al.*, 2006; Silva-Pereira *et al.*, 2007)

Morphometric analysis of *C. liliputana* flower and vegetative traits retrieved a similar grouping pattern to that found with genetic markers, with a remarkable separation of Serra do Caraça from the remaining populations (Fig 4a-c). PSL and CRP populations showed phenotypic shifts in morphometric traits that define the size of flower chamber, a change with potential ecological adaptation. The wider and taller pollinator chambers (Fig. 4d) results in a larger entrance to pollinators. This phenotypic shift may allow these populations to exploit new pollinators. Indeed, changes in the column length are known to change the site of pollinaria deposition and avoid hybridization between sympatric *Cattleya* species (Silva-Pereira *et al.*, 2007). Similar changes found for *C. liliputana* could reduce gene flow and enhance genetic divergence between Serra do Caraça and IQ populations.

Serra do Caraça populations exhibited higher individual investment in production of inflorescences and number of flowers per inflorescence. The increase of sexual reproductive effort can be a response to the lower mean individual size (Fig. 6), that suggests adverse conditions to growth and vegetative reproduction. Investment in reproduction is related to population ability to survive under strong selective pressures. For example, population colonizing new environments are selected for increased reproductive effort (Burton *et al.*, 2010), because even small increases in reproductive rate are able, over time, to result in substantially larger population growth. The variation in reproductive strategy and flower traits suggest that local adaptation could be involved in the observed genetic differentiation.

Our results are consistent with an adaptive scenario, but as with many other field studies, we acknowledge that the morphological and reproductive variations we observed could be the result of phenotypic plasticity and not of evolutionary adaptation. However, flower size was shown to be highly heritable in some plant species and it can thus respond to natural selection (Ashman & Majetic, 2006; Kaczorowski *et al.*, 2008; Gómez *et al.*, 2009; Krizek & Anderson, 2013). The genetic basis of flowering and the balance of growing and reproduction have also been tracked to single genes and QTLs in model plants (Bendahmane *et al.*, 2013; Friedman & Willis, 2013; Gaston *et al.*, 2013), but no information on the genetic basis of flower morphology or reproductive investment is available for our study species. More studies of QTL mapping,

reproductive biology and transplantations experiments are needed to clarify the role of phenotypic plasticity and local adaptation in *C. liliputana* population divergence.

Are populations of *C. liliputana* undergoing speciation?

Population differentiation has long been perceived as an early step during the speciation process (Givnish, 2010). The genetic, morphological and reproductive differences found here open the debate about the degree of divergence between these groups, raising the question whether *C. liliputana* populations are undergoing speciation.

From a broader perspective, the relationship between population divergence and speciation is not always obvious. Indeed, some species can maintain high population differentiation without necessarily splitting into several lineages (Magurran, 1998). Conversely, speciation can sometimes emerge in the presence of gene flow (Nosil, 2008; Pinho & Hey, 2010), in particular when the diverging populations become highly adapted to their respective habitats (Nosil *et al.*, 2005; Papadopulos *et al.*, 2011; Osborne *et al.*, 2013). *C. liliputana* populations from Serra do Caraça was referred as *Cattleya aff. kettiana* by some plant taxonomists (Mota, 2006). Although Serra do Caraça populations are genetically and morphological differentiated, our data does not support that these populations are reproductively isolated, although the shift in floral traits could represent a beginning of pre-mating isolating mechanism. Further research on reproductive biology and reciprocal crossing experiments will help understanding the mechanisms driving speciation in this orchid lineage.

Environmental landscape

We investigated some putative environmental factors that could cause different selective pressures in the IQ landscape (Fig. 6). IQ highlands make up most of the climatic gradient that separates Cerrado biome at west and Atlantic Rain Forest biome at east. The climatic analysis of high altitude areas in the IQ revealed that mountain ranges are heterogeneous and substantially different among each other. However, the genetic and ecological divergence of Serra do Caraça populations cannot be fully explained by landscape climatic differences. Microclimatic differences and edaphic factors could play an important role as selective pressure. For instance, the regions vary greatly in outcrop composition. Iron outcrops are mostly found around the Rio das Velhas valley being almost absent at Serra do Caraça region. Quartzite outcrops at Serra do Caraça are large massive rocks that extend over several kilometers while most of iron outcrop around the

Rio das Velhas valley are small and intercalated by patches of forest and grassland. In accordance with *C. liliputana*, genetic differentiation of *Vellozia compacta* was also found to be associated with the disjunct distribution of populations at different rocky types (iron vs. quartzite) (Lousada et al 2013), suggesting that common environmental factors such as rock type, low gene flow and genetic drift could act broadly in outcrop vegetation community.

Implications for conservation

One environment particularly threatened by climate change is the high altitude mountain area (Beniston *et al.*, 1997; Dirnböck *et al.*, 2011; Gottfried *et al.*, 2012). This environment is locally variable as small changes in altitude can lead to large changes in temperature, humidity and exposure and other environmental factors. It is not yet clear whether the majority of plant species are able to evolve to maintain current distributions under global climate change (Jump & Penuelas, 2005). Moreover there is very little information on adaptive potential in Neotropical mountains. The findings from this study suggest that *C. liliputana* populations are genetically variable and able to vary reproductive traits under contrasting environmental conditions. The genetic and ecological divergence of *C. liliputana* is likely to influence the long-term success of managing efforts, as the genetic differentiation and local adaptation might aid or hinder the survival and reproduction of the plants. Taking into account the genetic, morphological and reproductive divergence of *C. liliputana*, we recommend that Serra do Caraça and core IQ populations should be managed as evolutionary significant units.

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FIGURE LEGENDS:

Figure1. Sampled populations of *Cattleya liliputana* occurring at the Iron Quadrangle, southeast Brazil. (A) Mountain ranges above 1000m at Espinhaço Range Region. (B) Altitude map of the Iron Quadrangle with *Cattleya liliputana* populations included in the study

Figure 2. Bayesian clustering analysis conducted in GENELAND (Guillot *et al.* 2005). (a) Distribution of posterior probability of a number of *K* genetic clusters. (b - h) Maps of population membership probabilities to belong to one of *K* = 7 clusters for 160 individuals of *Cattleya liliputana*.

Figure 3. Genetic structure of *Cattleya liliputana* populations. (a) Genetic barrier (dotted line) estimated by Monmonier´s maximum difference algorithm. (b) Representation of Bayesian clustering analysis of seven populations of *Cattleya liliputana* based on seven microssatelite loci for *K*=2. (c) Delta *K* graphic of average likelihood for each *K* based on ten runs. (d) Representation of Bayesian clustering analysis of seven populations of *Cattleya liliputana* for *K*=3. Different colors represent different genetic clusters. Populations are separated by vertical bars. For population names see Table 1.

Figure 4. Morphological and genetic divergence of *Cattleya liliputana* populations. (a) Dendrogram of Cavalli-Sforsa pairwise genetic distance with supporting values based on 1000 bootstrap replicates. (b) Representation of the scores of first and second canonical axes of CVA using 18 floral and 8 vegetative continuous characters. (c) Dendrogram of phenetic relationships constructed using Mahalanobis generalized distance clustered with paired group and Manhattan similarity algorithm. Bootstrap support was obtained by 1000 replicates. Cophenetic correlation = 0.9703. (d) Density plots of morphometric measurements of *C. liliputana* individuals grouped according to genetic analysis in Serra do Caraça group (back dashed line) and core IQ group (grey solid line) for MLBL, medium lobe base width; CW, column width; CL, column lenth; LLH, lateral lobe high. These characters correspond to numbers 3, 26, 25 and 7, respectively, in Supplemental figure 2. For population names see Table 1.

Figure 5. Reproductive investment of *Cattleya liliputana* individuals grouped according to genetic analysis. (a) Number of inflorescences per size and General Liner Model of inflorescence production for Serra do Caraça group (black) and core IQ group (gray). (b) Mean number of flowers per inflorescence. For GLM analysis see Supplemental Table 2.

Figure 6. Climatic structure of mountain areas above 1300 meters a.s.l. at the Iron Quadrangle, Brazil. (a) Random points sampled for climatic variables colored according to geographic region; Red, west region; Red, south region; Blue, east region; Pink, Serra do Caraça region. (b) Principal component analysis of based on annual mean precipitation; annual mean temperature; mean diurnal temperature range; minimum temperature of the coldest month; predicted evapotranspiration; vegetation index at July and altitude.

Supplemental Figure 1. Representative habitat and individuals of *Cattleya liliputana*. (a) Iron outcrop and (b) quartzite outcrop at high altitude areas of the Iron Quadrangle. (c) Representative *C. liliputana* individual growing on iron outcrop. (d) Representative *C. liliputana* individual growing on quartzite outcrop.

Supplemental Figure 2. Outline of flower and vegetative parts indicating the morphological characters used in the morphometric analysis.
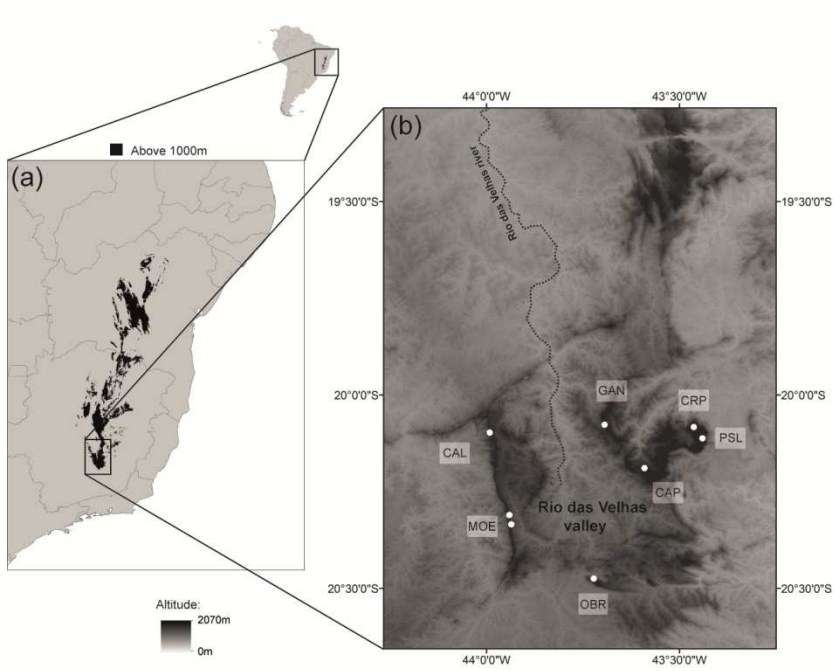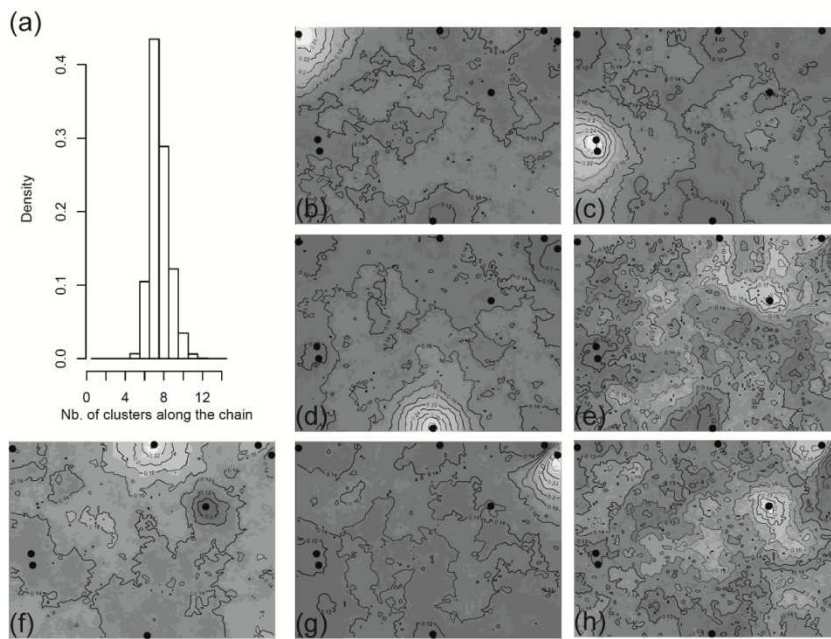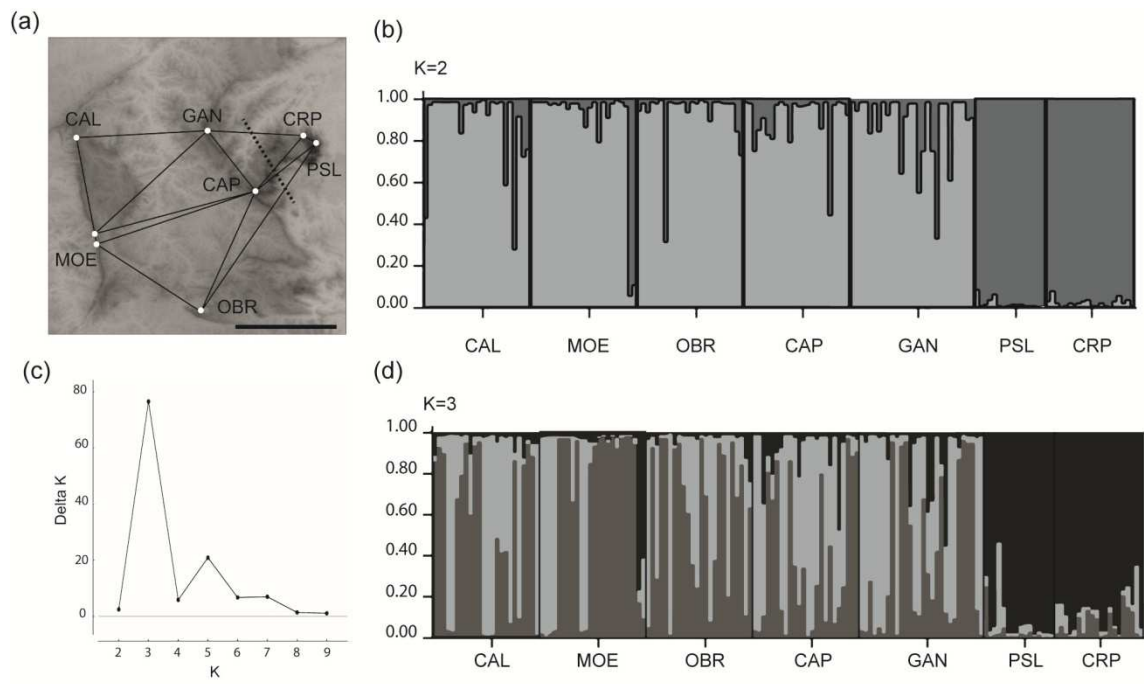
Figure 1

Figure 2

Figure 3

(a)

(b)
K=2

(c)

(d)
K=3

Figure 4

(a)

(b)

(c)

(d)
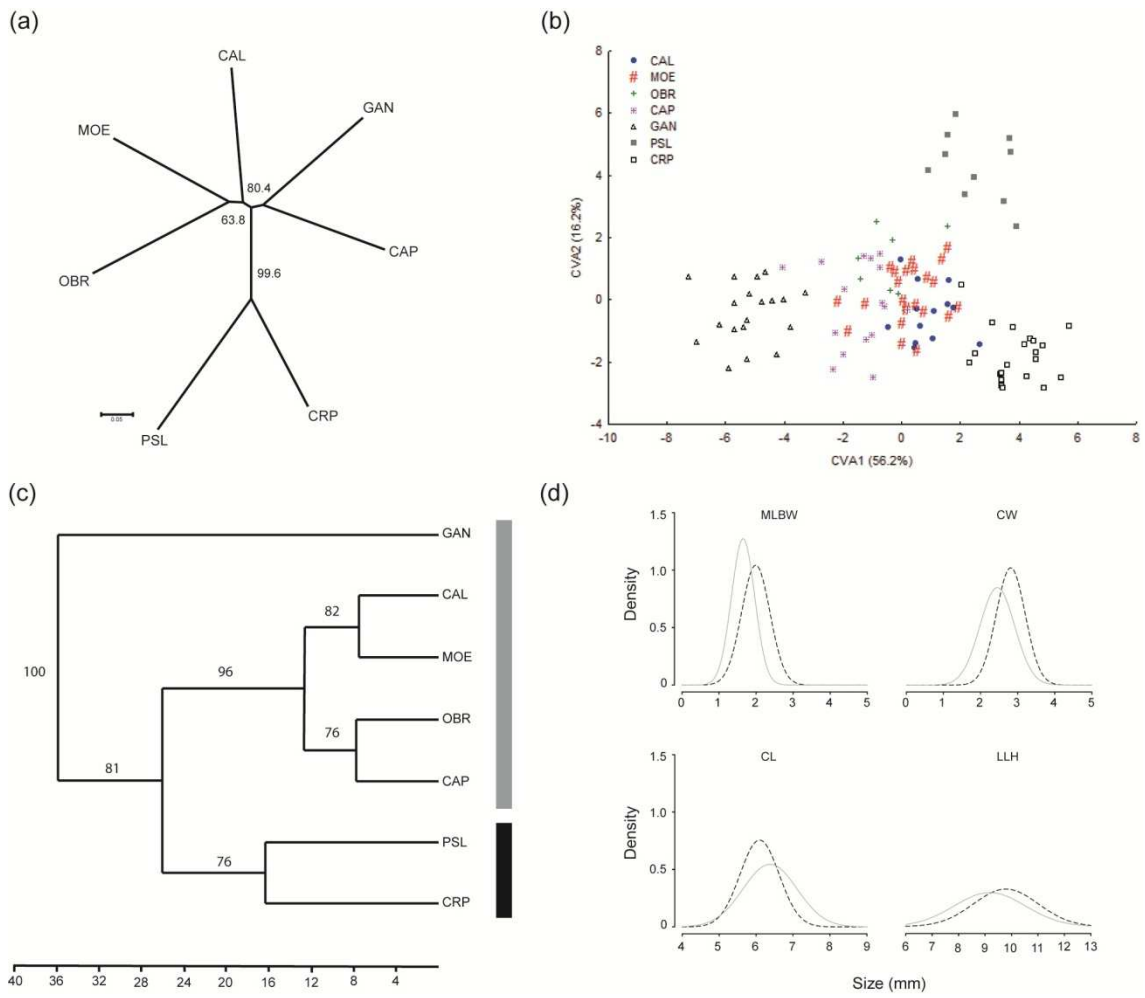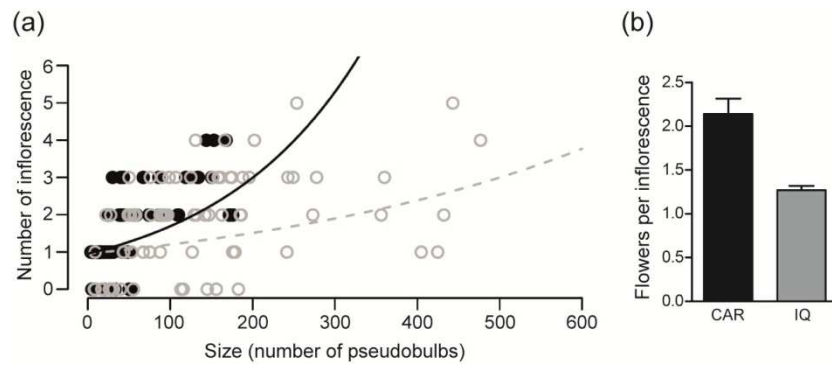
Figure 5

Figure 6

(a)
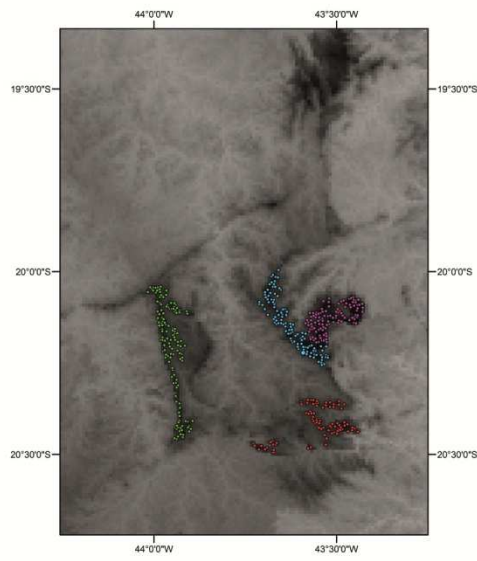
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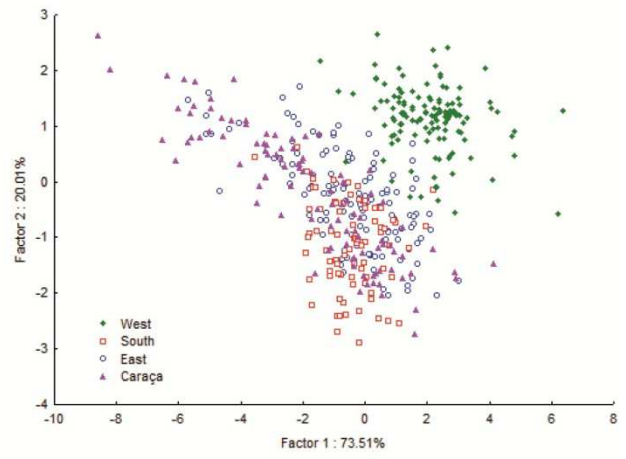
Supplemental Figure 1
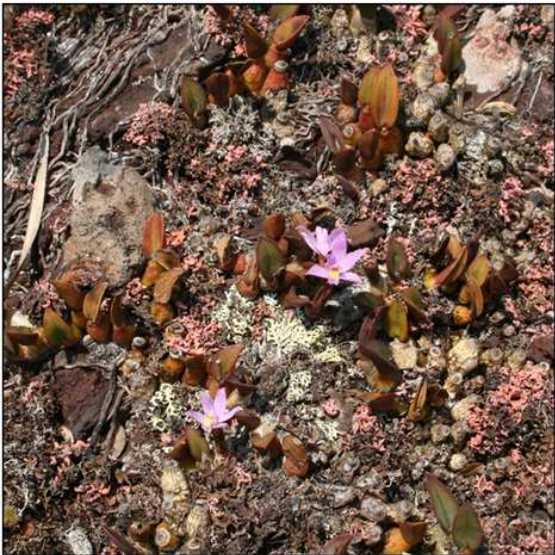
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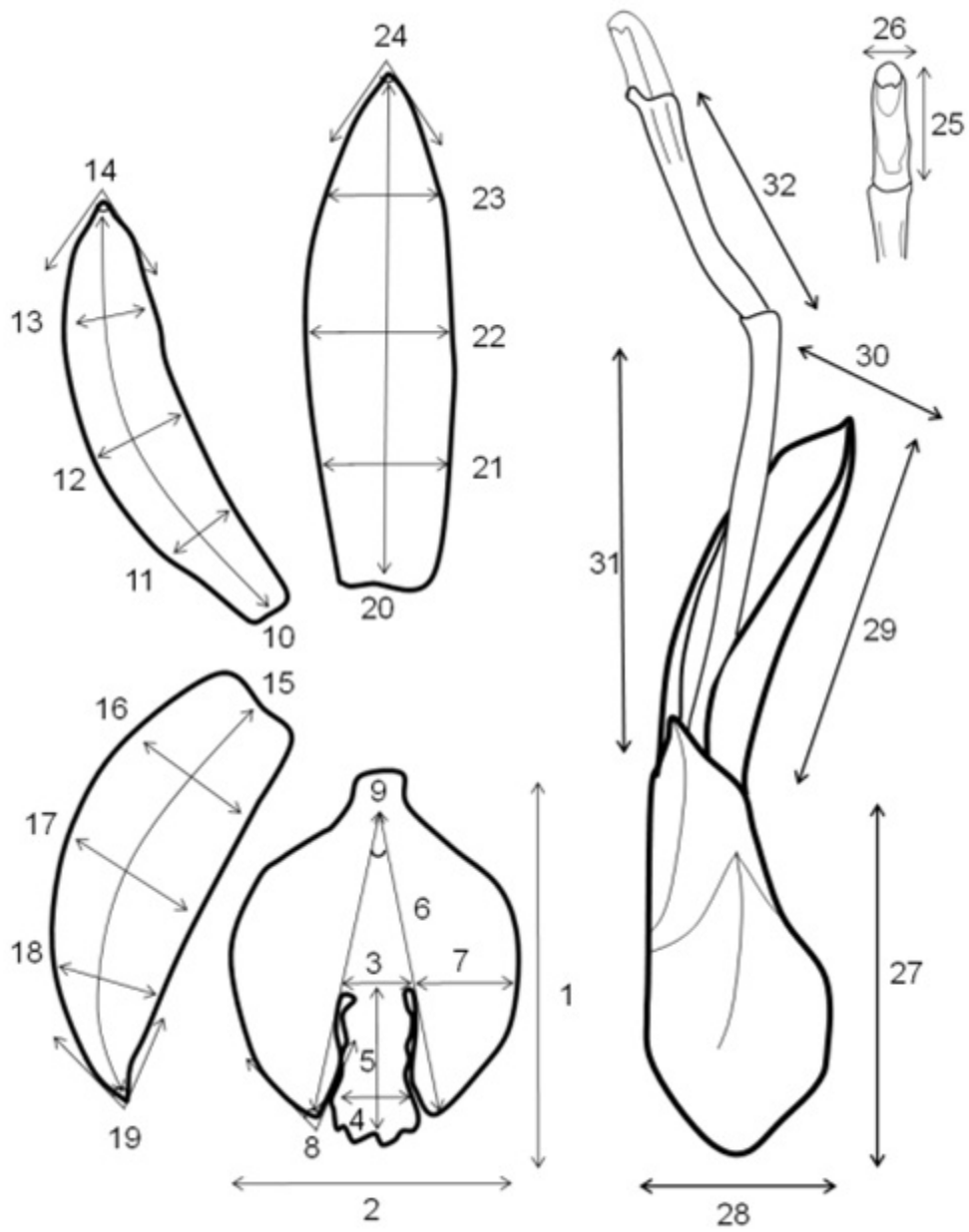
Table 1. Population code, location, type of substrate, sample size and voucher information of studied populations of *Cattleya liliputana* (N = 160 individuals) from the Iron Quadrangle, Minas Gerais state, southeastern Brazil.

Code	Mountain Range	Municipality	Coordinates	Rock outcrop	Altitude	$N_{gentics}$	$N_{morph.}$	Voucher
CAL	Serra da Calçada	Brumadinho	20°05'S/43°56'W	Iron	1483	24	13	BHCB64807
MOE	Serra da Moeda	Moeda	20°20'S/43°56'W	Iron	1530	24	23	B. Leles 002
OBR	Serra de Ouro Branco	Ouro Branco	20°29'S/43°52'W	Quartzite	1513	24	7	B. Leles 082
CAP	Serra de Capanema	Ouro Preto	20°12'S/43°54'W	Iron	1807	24	17	BHCB112460
GAN	Serra da Gandarela	Rio Acima	20°05'S/43°41'W	Iron	1611	28	19	B. Leles 001
CRP	Serra do Caraça	Catas Altas	20°04'S/43°28'W	Quartzite	1637	20	11	B. Leles 057
PSL	Serra do Caraça	Catas Altas	20°06'S/43°27'W	Quartzite	2055	16	21	BHCB47268

Vouchers are deposited in the herbarium BHCB

Table2. Parameters of genetic diversity and morphological variability of seven populations of *Cattleya liliputana*, occurring in the Iron Quadrangle, southeastern Brazil, based on seven microsatellite loci and 32 continuous floral and vegetative characters.

Populations	$A_R(\pm SD)$	$H_o(\pm SD)$	$H_e(\pm SD)$	*D2m*
CAL	9.09 (±3.16)	0.488 (±0.243)	0.753 (±0.210)	19.49
MOE	6.66 (±2.70)	0.537 (±0.227)	0.712 (±0.150)	17.08
OBR	8.23 (±3.25)	0.488 (±0.209)	0.742 (±0.226)	14.73
CAP	9.52 (±3.73)	0.563 (±0.221)	0.781 (±0.189)	19.09
GAN	11.48 (±3.86)	0.534 (±0.175)	0.793 (±0.167)	37.92
CRP	6.93 (±1.75)	0.535 (±0.179)	0.714 (±0.138)	27.02
PSL	7.02 (±3.47)	0.479 (±0.187)	0.714 (±0.169)	21.54

A_R, allelic richness; H_o, observed heterozigozity; H*e*, expected heterozigozity, D2m, median of the squared Mahalanobis distances of individuals to the population centroid. See Table 1 for populations code.

Table 3. Analysis of Molecular Variance (AMOVA) for different hierarchical levels of seven populations of *Cattleya liliputana* occurring in the Iron Quadrangle, Minas Gerais state, southeastern Brazil.

Source of variation	Df	Sum of squares	Variance components	% Total variance	*P*-value
Cattleya liliputana s.l.					
Among populations	6	9964.55	32.09	13.8	< 0.001
Within populations	313	62524.20	199.76	86.2	< 0.001
Two groups according to Barrier and STRUCTURE:					
(I) Serra do Caraça group (CRP and PSL) and (II)					
core IQ (CAL, MOE, OBR, CAP and GAN)					
Among groups	1	5091.65	38.18	15.0	< 0.001
Among populations within groups	5	4763.39	16.07	6.3	< 0.001
Among individuals within populations	313	62633.70	200.11	78.7	0.035
Three groups according to neighbor-joining					
dendrogram: (I) Serra do Caraça group (CRP and					
PSL) and (II) east IQ (CAP and GAN) and (III) west					
IQ (CAL, MOE and OBR)					
Among groups	2	5730.55	18.06	7.6	< 0.001
Among populations within groups	4	4124.50	18.10	7.7	< 0.001
Among individuals within populations	313	62633.70	200.10	84.6	0.07

For groups see Table 1 and Fig. 1

Suplemental Table 1. Microsatellite primers, amplification conditions and allele richness of 160 individuals of *Cattleya liliputana*.

Locus	Primer sequence (5'-3')	Repeat motif	T_a (°C)	Size (bp)	A_R	Source
Cac01	F: M13-TACAACGCCCAATTTGAATG	(GA)17	60.0	108	31	Novello *et al*., 2013
	R: CCATCATTTGCCTTTTCACA					
Cac02	F: M13-CAGGATTTCTCCTCGTGCTC	(AG)18	60.0	173	29	Novello *et al*., 2013
	R:GCAGAGCGGAACAAGGATAG					
Cac11	F: M13-TCAAGGCCTGCACATAGAGA	(AG)8	60.0	167	15	Novello *et al*., 2013
	R: AAGAGGAAGGCTTCGTTGC					
Cac16	F: M13-AACAGGCATTTGGAGCTTTT	(AG)23	60.0	250	39	Novello *et al*., 2013
	R: CCTCATTTCTCTCACCCTCTTT					
Cac18	F: M13-CTGGTGAGGGAGAAGAAAAACA	(GA)11N(AG)26	60.0	224	28	Novello *et al*., 2013
	R: CCCTCTCCCTCTCTTTTCCA					
Cac26	F: M13-TGGCTTGGTGTTGCATTTAT	(GA)6(GT)6	56.0	167	28	Leal, 2013
	R: TGTGAGCCTCAATAAGCCAAT					
Cac27	F: M13-TGGATCTTACCTTGGGCTTCTA	(GT)7GC(GT)4(GA)11	62.0	209	30	Leal, 2013
	R: CCCTGCTCTCTTTCTCACTCAT					

M13-Tail: TTTTCCCAGTCACGAC;

T_a, temperature of annealing; A_R, allelic richness

Supplemental Table 2. General Linear Model for size and number of inflorescence of seven populations of *Cattleya liliputana*, occurring in the Iron Quadrangle, southeastern Brazil.

Variable	Df	Deviance	Residual Df	Residual Deviance	F	p
Size						
Genetic group	1	3971.2	156	9376.6	58.52	<0.001
Number of inflorescence						
Size	1	27.452	156	129.31	47.69	<0.001
Genetic group	1	0.253	155	129.06	0.44	0.5078
Size XGenetic group	1	10.472	154	118.56	18.19	<0.001

CONSIDERAÇÕES FINAIS:

ATUALIZAÇÃO DO ESTADO DE CONSERVAÇÃO DA *CATTLEYA LILIPUTANA* DE ACORDO COM CRITÉRIOS DA IUCN.

A Lista Vermelha da Flora de Minas Gerais (Mendonça & Lins, 2000, 2007), uma boa compilação de dados para conservação de espécies endêmicas de Minas Gerais, indica que 27 espécies atualmente classificadas no gênero *Cattleya* se encontram sob ameaça. *Cattleya liliputana* não pôde ser enquadrada em nenhuma categoria nessa publicação em razão de deficiência de dados de história natural, distribuição geográfica e estrutura populacional.

De acordo com critérios da IUCN, a definição da categoria de ameaça requer dados de tamanho populacional, avaliação da redução populacional e suas causas; distribuição geográfica, fragmentação e singularidade do habitat de ocorrência e probabilidade de extinção na natureza (IUCN, 2012a; b).

Os dados deste projeto permitem enquadrar *Cattleya liliputana* como Em Perigo (EN) em razão de (i) estimativas de redução populacional com perda irrecuperável de habitat maior que 34%, com possibilidade de atingir 50% nas próximas décadas, (ii) espécie com área de ocorrência menor que 500 km^2 (B.Leles, dados não publicados) e (iii) severa fragmentação de habitat e perda da qualidade do habitat em afloramentos de canga (Carmo, 2010). De acordo com a nomeclatura proposta pela IUCN *Cattleya liliputana* se enquadra no critério EN A3cd+ B2ab(ii,iii).

RECOMENDAÇÕES PARA CONSERVAÇÃO E MANEJO DE *CATTLEYA LILIPUTANA:*

Apesar da intensa perda de habitat, as populações remanescentes de *Cattleya liliputana* apresentam boa variabilidade genética. Esse fato permite que a espécies responda melhor a estratégias de manejo. Algumas populações, como as presentes na Serra do Caraça, Serra de Capanema e Serra de Ouro Branco se encontram em unidades de conservação. Entretanto, na prática apenas as populações da Serra do Caraça estão de fato protegidas.

Os principais riscos para extinção de *Cattleya liliputana* são:

i)   Perda de habitat

ii)   Queimadas

iii)   Coleta ilegal

iv)   Baixo sucesso reprodutivo

As principais medidas para conservação da espécie são:

i)   Ampliação de unidades de conservação abrangendo principalmente afloramentos de canga e populações da espécie.

ii)   Monitoramento e controle de focos de incêdio durante a estação seca.

iii)   Restrição de acesso.

iv)   Recuperação demográfica (polinização *in situ,*propagação *in vitro* e reintrodução de resgates de flora).

v)   Educação ambiental e conscientização da população sobre a flora típica de Minas Gerais.

Sugestões para Conservação:

Primeiramente, sugerimos que populações do eixo Gandarela/Capanema, presentes na área proposta pelo ICMBio para criação do Parque Nacional Águas do Gandarela, devem se tornar o foco principal de estratégias de conservação da espécie. Isso ocorre em razão de (i) altos índices de diversidade genética e morfológica, (ii) localização

central na zona de contato entre duas unidades evolutivas, (iii) bons índices de reprodução e estrutura demográfica (dados não publicados). Esses dados demonstram como a criação do Parque Nacional é de grande importância para conservação da espécie. Além disso, resaltamos que é importante manter o perímetro original da proposta de criação do Parque Nacional, incluindo os afloramentos da região Noroeste da serra onde se localizam as maiores populações da espécie. A perda desses afloramentos acarretaria na extinção de uma das mais importantes e bem conservadas populações de *Cattleya liliputana*.
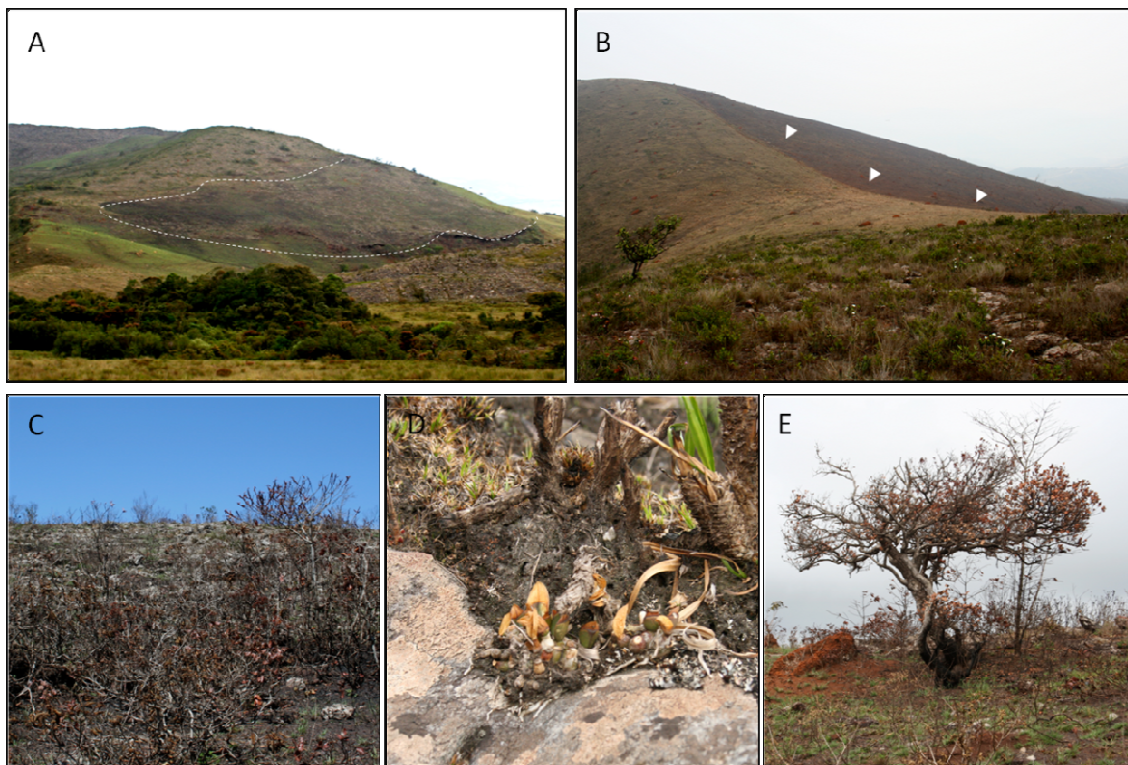

Figura 1. Registros de queimadas em áreas de ocorrência de *Cattleya liliputana*. **(A)** Afloramento de ferro na Serra de Capanema atingido por incêndio durante seca de 2011. **(B)** Linha de fogo de incêndio próximo a campo rupestre na Serra da Moeda durante seca de 2012. **(C)** Área de campo rupestre queimada durante seca de 2012 na Serra da Calçada. **(D)** Detalhe para indivíduo de *C. liliputana* queimado durante incêndio. **(E)** Detalhe para árvore queimada que continha espécies de orquídeas epífitas.

As populações da Serra de Ouro Branco, Serra da Moeda e Serra da Calçada também devem ser foco de estratégias de conservação. Essas estratégias devem visar principalmente o combate aos focos de incêndio, monitoramento populacional e controle de acesso. É comum a ocorrência de coletores ilegais e queimadas na Serra de Ouro Branco, apesar do local ser tombado como Parque Estadual e teoricamente gerido

pelo IEF. A conservação da população da Serra de Ouro Branco é de grande importância taxonômica em razão de ser a localidade tipo da espécie. A manutenção das populações na localidade tipo de sua descrição pode fornecer informações importantes para revisões taxonômicas de *Cattleya* Sect. Parviflorae.

De acordo com as características de cada localidade, sugerimos a seguinte orientação para manejo de *Cattleya liliputana*:
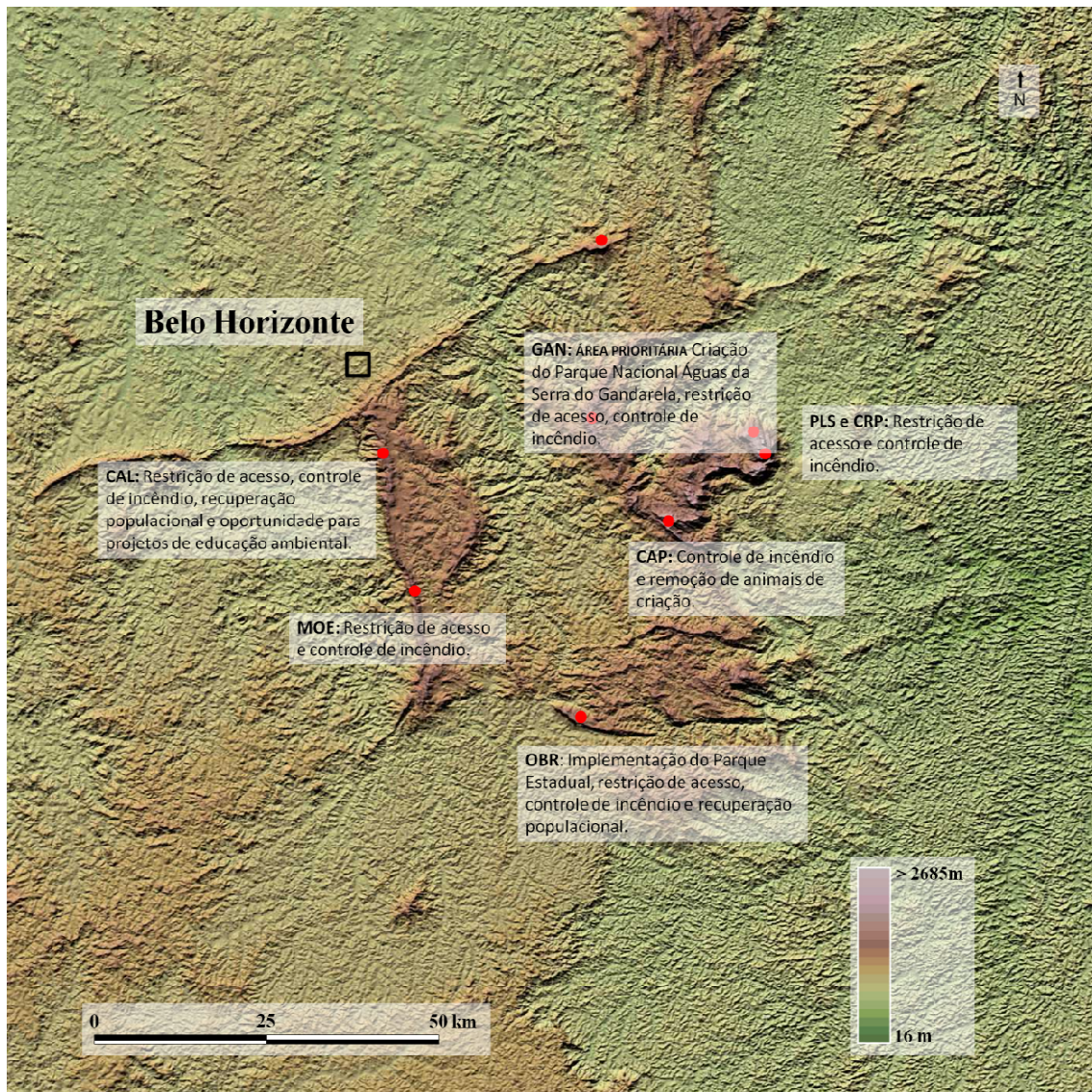

Figura 2. Estratégias de manejo para conservação de *Cattleya liliputana.*

CONCLUSÕES FINAIS:

Apartir da análise de estruturação genética, diversidade morfológica e investimento reprodutivo concluímos que:

1- As populações apresentaram alta diversidade genética e estruturação espacial moderada.
2- Existem dois grupos genéticos separados por uma região de baixo fluxo gênico entre as populações da Serra do Caraça e as populações ao redor do Vale do Rio das Velhas.
3- Os dois grupos genéticos apresentam estratégias distintas de investimento reprodutivo. Populações com menor capacidade de crescimento apresentam aumento no investimento em produção de inflorescências e flores.
4- Índices de diversidade genética e morfológicas são positivamente correlacionados.
5- As distâncias genética e morfológica entre populações são positivamente correlacionadas e não podem ser explicadas por isolamento por distância, sugerindo efeito de deriva genética e adaptação local.

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