Phylogeographic studies of Brazilian "campo-rupestre" species: *Wunderlichia mirabilis* Riedel ex Baker (Asteraceae)

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Resumo

Estudos filogeográficos de espécies brasileiras de "campo rupestre": *Wunderlichia mirabilis* **Riedel ex Baker (Asteraceae).** A espécie *Wunderlichia mirabilis* Riedel ex Baker, uma árvore dispersa pelo vento, possui um padrão de distribuição geográfico disjunto. Essa espécie ocorre em ambientes de campo rupestre na Cadeia do Espinhaço e nas montanhas de Goiás. A filogeografia de *W. mirabilis* foi investigada com base na variabilidade genética e na filogenia das regiões dos espaçadores não codificadores nuclear (ITS) e de cloroplasto (*trn*L). As taxas de fluxo de sementes são maiores do que as de pólen, e não há uma correlação significativa entre variação genética e geográfica. A radiação de *W. mirabilis* provavelmente ocorreu a partir da Serra do Cabral para outras serras da Cadeia do Espinhaço e do Espírito Santo, em sentido leste, e também para as montanhas de Goiás, Serra da Canastra (Minas Gerais) e São Paulo, em sentido oeste.

Unitermos: campos rupestres, filogeografia, *Wunderlichia mirabilis*, Cadeia do Espinhaço, Serras de Goiás

Abstract

Wunderlichia mirabilis Riedel ex Baker, a Brazilian wind-dispersed tree species, has a disjunct pattern of geographical distribution. This species occurs in 'campo rupestre' locations of the Espinhaço Range, and Goiás Mountains, Brazil. The phylogeography of *W. mirabilis* was reconstructed on the basis of the genetic variability and phylogeny of the nuclear (ITS) and chloroplast (*trn*L) non-coding spacer regions. AMOVA and Mantel analysis revealed for both markers that genetic variability is high within populations and moderate-to-high among populations. Rates of seed flow are higher than those of pollen flow, and there is a non-significant genetic x geographic correlation. The spread of *Wunderlichia mirabilis* probably occurred from Serra do Cabral through other serras in the Espinhaço Range of Minas Gerais and Espírito Santo, in an easterly direction, and through Goiás Mountains, Serra da Canastra in Minas Gerais and São Paulo in a westerly movement from the Serra do Cabral, Minas Gerais.

Key words: "campos rupestres", phylogeography, *Wunderlichia mirabilis*, Espinhaço Range, Goiás Mountains

Introduction

The Brazilian genus *Wunderlichia* (Asteraceae-Mutiseae) has six species confined to the rocky lands of Goiás, Minas Gerais, Rio de Janeiro, Espírito Santo and Bahia states. All *Wunderlichia* species are deciduous and adapted to the xeric and rupestrial environments. The leaves of all species are initially densely pubescent, and after they become glabrescent. At flowering time, the plants become conspicuously naked as soon as the flowers develop (Barroso and Maguire, 1973), helping the achene dispersal.

The species studied, *Wunderlichia mirabilis* (Figure 1), has a disjunct pattern of geographic distribution, and occurs mainly on the quartzite rocky lands of the Espinhaço Range, but also may occur on the sandy-rocky 'cerrado' and 'campos rupestres' in Goiás, Minas Gerais and Espírito Santo (Roque and Pirani, 1997). This species has been recorded in important checklists and floras, such as the Flora of the Serra do Cipó (Roque and Pirani, 1997), and the checklist of the Morro do Ambrósio (Pirani et al., 1994), both in Minas Gerais.
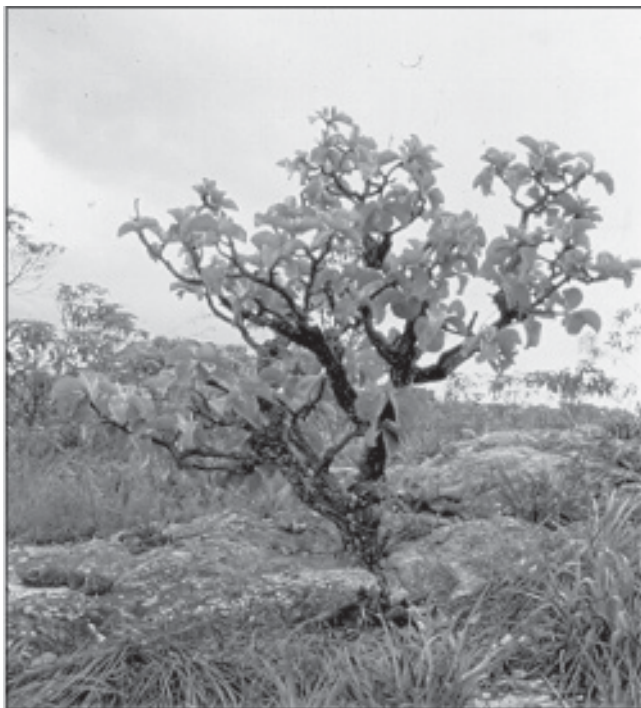

FIGURE 1: *Wunderlichia mirabilis* – individual from Serro (population 83), Minas Gerais (F. Feres et al., 1983).

This study is part of the phylogeographic investigation of "campo rupestre"' species with a disjunct distribution in the Espinhaço Range and Goiás Mountains, in Brazil. The Espinhaço Range comprises a set of mountains (800 to 1800m above sea level on average) with approximately 1100km of extension between the limits of 20°35' and 11°11'S (Magalhães, 1954). The southernmost limit of the Espinhaço Range is the Serra de Ouro Branco in Minas Gerais, and the northern limit is in Bahia (Moreira, 1965). The several elevated regions of the Espinhaço Range are usually called "serras" and are commonly interrupted by river valleys. The main "serras" in Minas Gerais are the Serra do Cipó, Serra do Cabral, Serra da Piedade, and Serra do Caraça. In Bahia, there is the Serra do Sincorá, Serra do Rio de Contas, Morro do Chapéu, and Jacobina (Giulietti and Pirani, 1988).

While in Africa the isolated table mountains are called "Bowals", and in northern South America they are called "Tepuis" or "Sabanas", in Brazil they are known as "campos rupestres", with a savanna-like vegetation. Those mountains represent isolated islands, frequently rich in endemic species (Alves and Kolbek, 1994). The 'campo rupestre' habitat has a lithosolic composition of soils where quartzites are greatly predominant. These quartzite plateaus rise more or less abruptly from a landscape of red-yellow distrophic latossols with cerrado vegetation, and are referred locally to as the "mar de morros" (sea of hills) (Alves and Kolbek, 1994).

The Espinhaço Range is the longest continuous pre-cambrian orogenic belt of Brazil (Almeida-Abreu and Renger, 2002). At the end of the Proterozoic Era, folds gave rise to the Espinhaço Range, and after, in the Silurian Period, folds gave rise to the high lands of Goiás, like the Serra dos Pirineus (Adas, 1985). In Brazil, Proterozoic lands have great economic importance, because in these lands there are important mineral sources, and precious and semi-precious stones (Adas, 1985), especially in the Espinhaço Range, where many mineral sources such as gold, diamond, emerald, iron and topaz are found (Biondi, 1999).

In Minas Gerais, mainly in Diamantina, diamonds were discovered at the beginning of the 18th century (Chaves and Chambel, 2004). Since then, the region

has been exploited for diamonds for more than three hundred years. The most important diamond sources, beyond Diamantina, are situated in Grão-Mogol and Serra do Cabral. These places are historically the greatest Brazilian diamond producers (Chaves et al., 1998). Local communities have also exploited these regions intensively by the manual mining of ornamental stones (Laschefski et al., 2005). Thus, areas of campos rupestres are constantly disturbed by mining activities, both on an industrial and a subsistence scale. Environmental impacts caused by mineral exploitation, such as that of diamonds and semi-precious stones, are very difficult to control because of the amount and the dispersal of mines (Macedo, 1998).

In their study of the geographic patterns of plant species in the Espinhaço Range, Giulietti and Pirani (1988) proposed five main patterns of distribution. One of the most remarkable patterns is a disjunct distribution, where some species (such as *W. mirabilis*) occur in the Espinhaço Range and in the Serras de Goiás, and they suggested that this pattern was evidence of former epochs when there were major links between the floras of these regions.

The goal of our work was to reconstruct the phylogeography of *Wunderlichia mirabilis* Riedel ex Baker, with the intention of revealing the colonization routes of this species, and possibly ancient connections between the Goiás Mountains and the Espinhaço Range. The phylogeography of *W. mirabilis* was investigated on the basis of the genetic variability and phylogeny of the non-coding spacers ITS (nuclear rDNA) and *trn*L (cpDNA).

Materials and Methods

Sample collection

Ninety-seven individuals were collected under license from nine populations of *Wunderlichia mirabilis* on several mountains of the Espinhaço Range in Minas Gerais and in the Goiás Mountains. Despite Barroso and Maguire's (1973) report on the occurrence of *W. mirabilis* in Espírito Santo state also, no populations of this species were found at the localities cited by these authors for Espírito Santo. The sample localities, numbers, sizes and geographical coordinates are in Table 1. The sampled populations are shown in Figure 2.

TABLE 1: *Wunderlichia mirabilis* sampled populations. Localities, sample numbers, sizes and coordinates are indicated.

| Localities	Nº	Coordinates	Sample Size
Grão-Mogol, MG	33	16°35'S, 42°54'W	10
Cristalina, GO	43	16°48'S, 47°34'W	10
Joaquim Felício, MG (Serra do Cabral)	45	17°41'S, 44°11'W	10
Joaquim Felício, MG (Serra do Cabral)	47	17°41'S, 44°17'W	10
Cardeal Mota, MG (Serra do Cipó)	54	19°20'S, 43°38'W	14
Diamantina, MG	76	18°18'S, 43°49'W	11
Serro, MG	83	18°33'S, 43°33'W	12
São Roque de Minas, MG (Serra da Canastra)	109	20°10'S, 46°42'W	10
Estreito, SP	111	20°09'S, 47°17'W	10
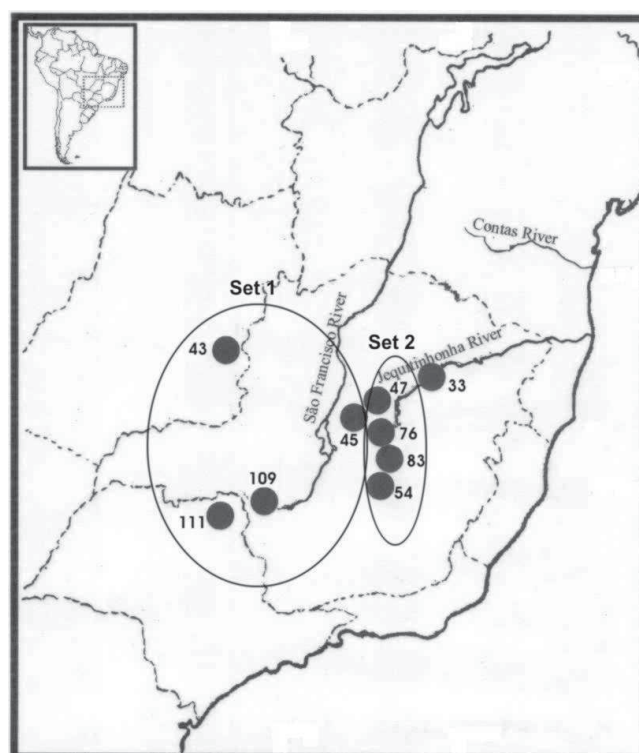
FIGURE 2: Map showing the localities of *Wunderlichia mirabilis* sampled populations. Two main arrangements are indicated.

The leaves from all the sampled individuals were packed with a paper towel and stored in a zipped plastic bag at the field (Feres et al., 2005). On arrival at the laboratory, all the samples, after being ground with liquid nitrogen, were stored at –70°C until their DNA isolation. All herbarium exsiccates (vouchers) are deposited at UEC (Universidade Estadual de Campinas).

DNA extraction, polymerase chain reaction, and DNA sequencing

One hundred mg of ground leaf tissue was used for DNA isolation, with the Wizard® Genomic DNA Purification Kit (Promega Corporation), according to the manufacter's protocol. After the DNA isolation, aliquots of 10μL of each sample were quantified in a 0.8% agarose gel, for evaluation of the amount and integrity of the DNA obtained, using the lambda DNA as a molecular weight pattern. The nuclear ribosomal DNA ITS was amplified in a 25μL final volume of the polymerase chain reaction, using 10ng of template DNA, 2.5μL of 10x reaction buffer, 2.7μL of Mg Cl$_2$ (27mM), 1μL dNTP mix (100mM each dNTP), 1.2 pmole of each primer (92 and 75, Desfeux and Lejeune, 1996), 2μL of 1% PVP (Koonjul et al., 1999), 5μL of betaine 5M (Henke et al., 1997), and 2 units of *Taq polymerase* (Invitrogen). The reaction was performed on a MJ Thermal Cycler (PTC 100) as one cycle of denaturation at 94°C for 1min, followed by 30 cycles of 45s denaturation at 94°C, 1min annealing at 55°C, and 1min30s extension at 72°C, followed by 7min extension at 72°C. The non-coding *trn*L spacer (C and F, Taberlet et al., 1991) was amplified in a 20μL final volume of the polymerase chain reaction, using 10ng of template DNA, 2μL of 10x reaction buffer, 2μL of Mg Cl$_2$ (25mM), 0.8μL dNTP mix (100mM each dNTP), 2pmole of each primer, 2μL of 1% PVP, 4μL of betaine 5M, and 2 units of *Taq polymerase* (Invitrogen). This reaction was also performed on a MJ Thermal Cycler (PTC 100) as one cycle of denaturation at 94°C for 2min, followed by 30 cycles of 1min denaturation at 94°C, 1min annealing at 48°C, and 1min30s extension at 72°C, followed by 2min extension at 72°C.

After the DNA amplification, the PCR products were checked in a 1% agarose gel. PCR products were then purified and concentrated with the QIAquick PCR Purification Kit (QIAGEN), according to the manufacter's protocol.

The purified DNA was sequenced in both directions by the *Taq* dyedeoxyterminator cycle sequencing kit (Perkim Elmer) on an Applied Biosystems 377 automated sequencer. In a 20μl reaction volume we used 2μl of Big Dye®, 2μl of 10x reaction buffer, 2μl from each primer (final concentration 5pmoles), and 5 to 10μl of purified DNA. The sequencing reaction performed on a MJ Thermal Cycler (PTC 100) consisted of 40 cycles of 20s denaturation at 96°C, 10s annealing at 50°C, and 4min extension at 60°C.

Sequence and phylogenetic analysis

For phylogenetic analysis, all sequences were analyzed with the Phred/Phrap/Consed software (Ewing et al., 1998; Ewing and Green, 1998; Gordon et al., 1998) on the Linux operating system. Nucleotide divergence was checked, and the variable positions were chosen (Table 2). Phylogenetic analysis of the ITS and trn*L* data matrices was performed using the heuristic search, the exhaustive search, the branch-and-bound feature and the genetic distance (neighbor-joining) facilities of PAUP 4.0 (Swofford, 1998). In both analyses, confidence in the clades was tested by bootstrapping with 1000 replicates on the 50% majority-rule consensus trees.

TABLE 2: ITS sequenced size and selected characters of *Wunderlichia mirabilis* sampled populations.

Population Nº	ITS size	Position							
		113***	153*	214**	230*	552*	741*	752*	753*
33	680	C	C	C	T	C	G	C	T
43	630	C	C	C	C	C	C	T	A
45	630	A	C	-	T	T	T	C	A
47	620	C	T	C	T	C	C	C	T
54	760	-	T	C	C	T	C	C	A
76	630	-	T	-	C	C	C	T	T
83	650	C	C	C	C	C	C	C	A
109	680	A	C	C	C	C	G	T	A
111	580	-	C	-	T	C	T	T	T

* substitution; ** insertion; *** substitution and/or insertion.

For population genetic analysis, all sequences were first edited using the EditSeq and Seqman programs (DNAStar-Lasergene), and then aligned using the MegAlign program (DNAStar-Lasergene). After the alignment, they were adjusted visually. The program

Modeltest 3.06 (Posada and Crandall, 1998) was used to check the evolutive model of the nucleotide substitutions for all ITS and *trn*L sequences.

Population genetic analysis of the nuclear ribosomal DNA and cpDNA sequence variation

Levels of genetic diversity within and between populations were estimated by the analysis of molecular variance, AMOVA (Excoffier et al., 1992), performed using the pairwise difference feature of Arlequin software ver 2.000 (Schneider et al., 2000). Gene flow within and among populations was approximated as *Nm*, the number of migrants per generation between populations, and was estimated using the expression

$$Nm = 1/4\left(\frac{1}{F_{ST}} - 1\right),$$

where *N* is the elective population size and *m* is the migration rate (Wright, 1951).

Direct estimates of seed and pollen flow by F_{ST} values comparison for marks with contrasting inheritance patterns are given by:

$$\frac{Nm_{pollen}}{Nm_{seeds}} = \frac{\left(\frac{1}{F_{ST_p}} - 1\right) - 2\left(\frac{1}{F_{ST_{mp}}} - 1\right)}{\left(\frac{1}{F_{ST_m}} - 1\right)}$$ (Ennos, 1994).

This can be estimated by:

$$\frac{Nm_{pollen}}{Nm_{seeds}} = \frac{\left(N_e m_b\right) - 2\left(N_e m_m\right)}{\left(N_e m_m\right)},$$

where $N_e m_b$ is maternally inherited, and $N_e m_m$ is biparentally inherited. This equation is used to estimate the biparental (nuclear) fixation index, and the maternal fixation index (organellar) (Ennos, 1994).

Genetic and geographical correlation analysis

Mantel tests were performed on NTSYS-PC 2.0 software (Rohlf, 1997) for genetic and geographical correlation analysis. The genetic distance matrix was generated using PAUP, and the geographical distance matrix was constructed with the aid of Earth Distance (Byers, 1999).

The patterns of spatial variation in a multivariate context were analyzed by obtaining the estimate of the Pearson (*r*) correlation coefficient between the matrices generated on PAUP software, the GTR distance obtained from the Model Test, and the geographical distance between the populations constructed with the aid of the software Earth Distance (Byers, 1999). The significance of this matricial correlation could not be tested by usual statistic tests due to the presence of independence problems among the elements in the matrices. Thus, Z statistics by Mantel (1967) were used. In this work, 1000 random exchanges were used to test the significance of the matrix correlations. Mantel tests were performed on NTSYS-PC 2.0 software (Rohlf, 1997) to check the spatial distribution of the haplotype frequency.

Results

Polymerase chain reaction, DNA sequencing and nucleotide diversity

From the 97 collected samples of *Wunderlichia mirabilis*, 56.7 % amplified the ITS and 59.7 % amplified the *trn*L chloroplast region. Probably, the great number of samples did not amplify either of these markers due to the presence of phenolic and/or polysaccharide compounds in the leaves (Feres et al., 2005).

The sizes and variations of all sequenced populations are listed in Table 2 for ITS and Table 3 for trn*L*. The ITS region size varies from 580 to 760 bases, and the *trn*L sizes vary from 780 to 840 bases. For ITS, eight variable sites were found (Table 2), and for trn*L*, 12 variable sites were found (Table 3).

ITS and *trn*L sequences were registered at the genbank with accession numbers of DQ414741 and 414742 for ITS, and DQ414743 and DQ414744 for *trn*L non-coding spacer.

TABLE 3: trnL sequenced size and selected characters of *Wunderlichia mirabilis* sampled populations.

Population N°	*trn*L size	Position											
		120-121*	187-188*	250*	322*	395*	440***	488*	547***	657-573**	700*	723*	738-745**
33	830	TC	AT	A	T	A	A	A	T	------	T	A	AAATTATA
43	820	TC	GG	A	T	A	A	A	T	------	A	A	AAATTATA
45	810	TC	GG	A	T	A	A	A	T	------	A	A	AAATTATA
47	860	GA	AT	C	G	A	C	G	C	TTTCAC	T	G	--------
54	780	GA	AT	C	T	A	A	A	T	TTTCAC	A	A	AAATTATA
76	800	GA	AT	A	G	C	C	G	C	TTTCAC	T	G	--------
83	830	TC	AT	C	T	C	A	A	T	------	A	A	AAATTATA
109	840	TC	GG	A	T	A	-	A	T	------	A	A	AAATTATA
111	820	TC	GG	A	T	A	-	A	-	------	A	A	AAATTATA

* substitution; ** insertion; *** substitution and/or insertion.

Phylogenetic analysis

The nucleotide substitution model found for all the ITS and *trn*L sequences of *W. mirabilis* was the GTR model (Lanave et al., 1984; Tavaré, 1986; Rodriguez et al., 1990). In this evolutive model the base frequency is variable, and the substitution matrix is symmetrical, which means that the substitution rate of a nucleotide X to Y is associated with the reverse substitution rate (from Y to X).

The ITS/*trn*L data matrix had 35 characters, and 19 were parsimony-informative. The ITS/trnL phylogeny for *W. mirabilis* sampled populations is shown in Figures 3 and 4.

The parsimony analysis, followed by a heuristic search, exaustive search and branch-and-bound search, resulted in identical trees (Figure 3) with 31 steps. The genetic distance analysis (neighbor-joining) resulted in a tree (Figure 4) which was similar to the trees obtained with the parsimony analysis.
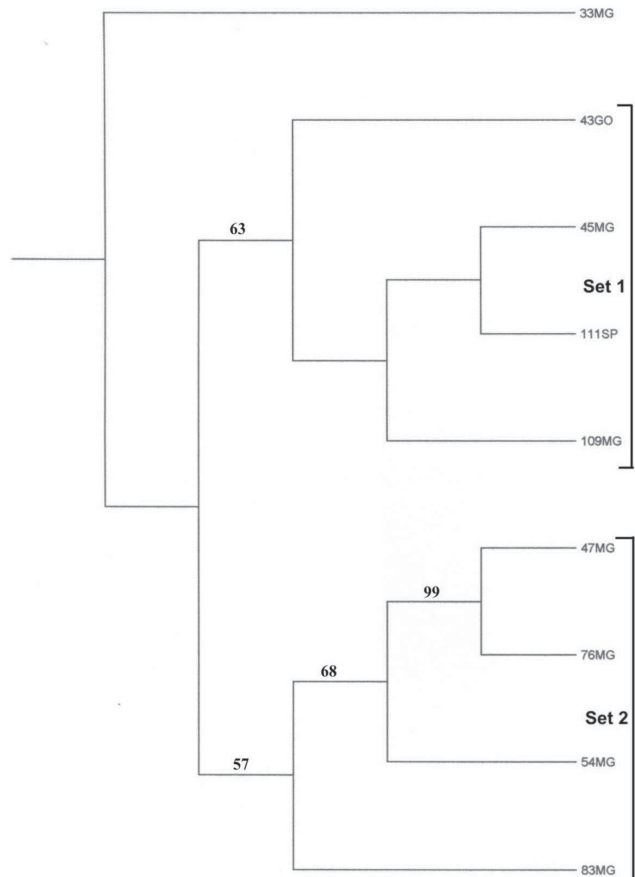

FIGURE 3: Heuristic search/branch and bound/exaustive search tree of nuclear rDNA ITS and cpDNA *trn*L of *Wunderlichia mirabilis*. Bootstrap values are given above branches. Two main arrangements (Set 1 and Set 2) are indicated.
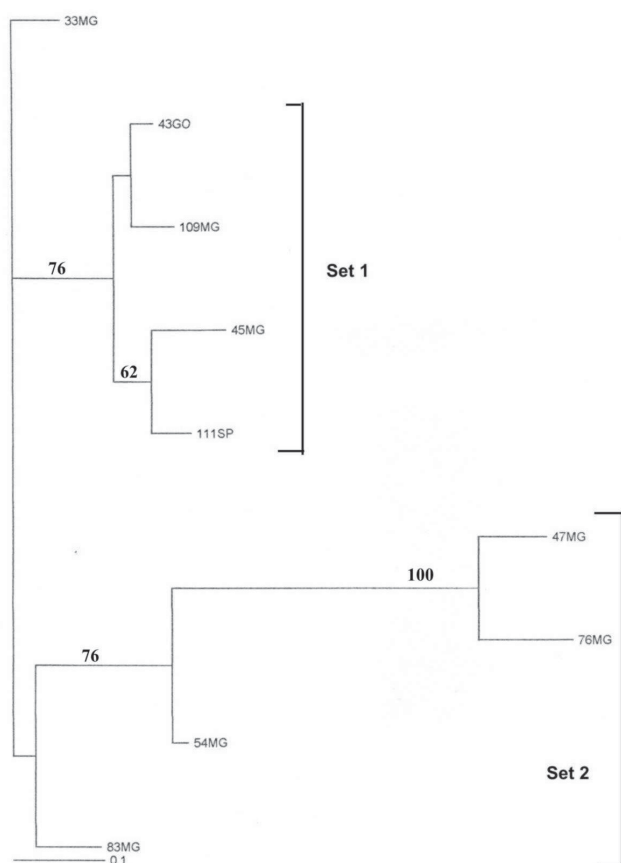
FIGURE 4: Neighbor-joining tree of rDNA ITS and cpDNA *trn*L of *Wunderlichia mirabilis*. Bootstrap values are given above branches. Two main arrangements (Set 1 and Set 2) are indicated.

Population genetic analysis

AMOVA results are listed in Table 4. Genetic variability is higher within populations for ITS (81.73%) and also for *trn*L (88.65%). Seed flow is higher than pollen flow among *Wunderlichia mirabilis* populations (-1.42, Table 4).

The rate of variation within populations found for ITS is lower than for *trn*L. This was an unexpected result, because generally chloroplast markers are more conservative than nuclear markers (Ennos, 1994; Austerlitz et al., 2004). But, seed migration rates are higher than pollen migration rates in this species, and this explains the greatest genetic variation for *trn*L.

The 18% average of variation among populations, found in *Wunderlichia mirabilis,* was an unexpected result, considering its isolated mountain habitats and the great geographical distance among sampled populations in this

work, because it is believed that the greater the geographic distance between sampled populations, the higher the variation among populations found (Nybom, 2004).

TABLE 4: Nucleotide diversity within and among populations of *Wunderlichia mirabilis* based on cpDNA and nuclear rDNA sequences (AMOVA analysis) and Mantel test results. *Nm* and F_{ST}, and gene flow ratio were deduced from nucleotide sequences. Nm_b: bipzzarental inheritance. Nm_m: maternal inheritance.

Pairwise difference	Percentage of variation within populations	F_{ST}	Nm_b	Nm_m	Gene flow pollen/ seed	Mantel *r*	test *p*
ITS	81.73	0.1826	1.1187			0.17001	0.1658
*trn*L	88.65	0.1134		1.9528	- 1.42	0.21547	0.1758

Genetic and geographical correlation analysis

Mantel test results are in Table 4. The genetic and geographic correlation found with both markers (ITS and *trn*L) is not significant.

Discussion

Gene genealogies and population differentiation

Theoretically, *Wunderlichia mirabilis* populations would be isolated from each other, due to the fragmented geographic pattern of distribution in which the mountains of the Espinhaço Range and Goiás would feature as "island habitats" (Spellerberg and Sawyer, 1999). The results from the AMOVA analysis revealed that there is a high seed flow rate among populations, which can explain the high genetic variation within populations and the high trn*L* variation rate. Apparently, there is no geographical barrier to gene flow among *W. mirabilis* populations, at least according to the Mantel result, where there is no geographical x genetic correlation.

High genetic variability levels have been reported for some Brazilian angiosperm species (Borba et al.,

2001; Moraes and Derbyshire, 2002; Telles et al., 2003; Souza et al., 2004). Also, genetic variability, higher within populations than among populations, has been reported (Moraes and Derbyshire, 2003; Botrel and Carvalho, 2004; Gusson et al., 2005), together with the absence of geographical and genetic correlation (Yanaka et al., 2005) for native species. All results for Brazilian species, including *Wunderlichia mirabilis*, suggest that for the majority of native species, genetic variability is higher within populations than among populations.

High rates of pollen flow have been frequently reported for many plant species, but only rarely have high levels of seed flow been reported, such as in the research of El Mousadik and Petit (1996), where they explain the high level of gene flow of the argan tree by seeds as being to the activity of goats and camels in disseminating the fruits. In the case of *W. mirabilis*, the fruits (achenes) are wind dispersed, and probably the ancient *W. mirabilis* populations were much less fragmented than in the present.

Field observations and analysis of the sampled populations revealed that in Set 1 (Figures 2, 3 and 4) the flower size is smaller than in Set 2 (Figures 2, 3 and 4), mainly for population 43, from Goiás. Set 2 may be characterized by having the largest fruits and greatest flower diameter, and these features were found mainly in populations 47, 54 and 83, from Minas Gerais. Also in Set 2, population 83 revealed smaller leaves among all sampled *W. mirabilis* populations.

Phylogeography of *W. mirabilis*

Figure 3 shows the locations of the sampled populations and their spatial arrangement into Sets 1 and 2, based on the results for parsimony (Figure 3) and genetic distance (Figure 4) analysis. From the Serra do Cabral (Joaquim Felício, Minas Gerais), all populations in the west were grouped together (Set 1), and the same was true for eastern populations (Set 2). In the Serra do Cabral, we sampled two populations of *W. mirabilis* (populations 45 and 47), and these populations are arranged separately in the parsimony tree (Figure 3) and also in the genetic distance tree (Figure 4). This result shows that probably the ancestor population of *W.*

mirabilis lived in the Serra do Cabral region, and the fact that the populations 45 and 47 were grouped separately from each other may be a consequence of the ancestral polymorphism (Schaal et al., 1998).

Thus, the possible colonization route for *W. mirabilis* may have had as a starting point the region of Serra do Cabral, through other serras in the Espinhaço Range of Minas Gerais and Espírito Santo, today represented in this work as the populations grouped in Set 2 and population 33. Also, this species moved through the Goiás Mountains and the Serra da Canastra in Minas Gerais and São Paulo, represented by the populations grouped in Set 1. In Set 1, the genetic differentiation found for population 45 remained, and in Set 2, there remained the genetic differentiation found for population 47.

In this scenario and with the morphological observations cited above, probably the individuals that gave rise to the populations belonging to Set 1 had smaller flowers, and the individuals that originated the populations belonging to Set 2 had greater flowers and fruits.

Acknowledgements

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