

Surrounded by Concrete: Genetic Isolation of *Tillandsia Recurvata* L. in an Urban Landscape in South-Eastern Brazil

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Abstract

Increasing urban expansion has resulted in the decline of many natural and semi-natural communities globally. However, the connectivity and genetic structure of species that survive in these urban landscapes has received little attention, especially with regard to epiphytic plants. The aims of this study were to 1) investigate how an urban landscape can impact the connectivity and genetic structure of *Tillandsia recurvata*, L., a highly abundant and widely distributed atmospheric epiphyte; to 2) understand what the driving landscape factors are that affects connectivity and structure; and to 3) discuss how urbanisation can affect genetic structure in epiphytes. A total of 288 *T. recurvata* individuals were sampled across 65 trees throughout the city of Alfenas in South-East Brazil. We designed seven novel microsatellite markers and used four cross-amplified loci to determine the basic genetic structure of *T. recurvata*. All populations showed high global spatial genetic structure (SGS) and population differentiation. The high SGS of *T. recurvata* indicated low connectivity between urban populations, with strong evidence to suggest that this was the result of small population size and genetic drift. Pasture trees outside of the urban landscape are likely to be an important source of gene flow to isolated urban epiphyte populations. This study represents an important step towards understanding epiphyte population structure within urban landscapes. Low connectivity across urban landscapes is likely to benefit epiphytes such as *T. recurvata* due to their adaptability and high tolerance; suggesting a bleak future for many other more sensitive epiphyte species under predicted urbanisation globally.

Introduction

Predictions suggest that 66% of the global human population will occupy urban areas by the year 2050, an approximate increase of 2.5 billion people from current levels (United Nations 2018). This predicted expansion of urbanisation has highlighted the need to anticipate and preserve species and habitat connectivity within complex and fragmented landscapes, which are not well understood (Muderere et al. 2018; Lynch 2018; Yang and Sun 2020). This lack of knowledge also presents a barrier to our current understanding of the implications of urbanisation for biodiversity (Newbold et al. 2020). Current evidence suggests that urbanisation may have severe consequences for habitat availability (Gaston 2010; Liu et al. 2016; Kondratyeva et al. 2020). Specifically, landscape fragmentation (i.e. the loss or alteration of habitat spatial configuration) (Fahrig 2003; Zambrano et al. 2019) has been shown to negatively affect the abundance and quality of remnant habitats within a previously continuous landscape, especially in the absence of a wide variety and abundance of urban arborization (Chetcuti et al. 2020). Urbanisation may thus result in the fragmentation of large and interconnected populations (Toledo-Aceves et al. 2014). The probability of a genetic bottleneck and genetic drift may subsequently increase in these smaller populations, as a result of reduced gene pool size (Ackerman and Zimmerman 1994; Ward 2006). Evidence has shown that inbreeding depression and population differentiation then become more likely, under the pressure of sustained dispersal limitations between isolated populations (Wright et al. 2013). As such, low connectivity would be expected in urban landscapes, as well as high spatial genetic structure (SGS) (Chaves et al. 2021).

Plants present an ideal subject to explore the connectivity of urban landscapes, largely due to their strong sensitivity to landscape fragmentation (Fenster and Marten-Rodriguez 2007; Rios and Cascante-Marin 2016). This may result from the slow response of plants to changes in habitat conditions, such as altered pollen dispersal (Llorens et al. 2012), and limited opportunity for colonisation in fragmented landscapes (Bhatt et al.

2015). As such, urban ecosystems typically contain a lower diversity of plant species than primary forests or pasture trees (Poltz and Zotz 2011). Current evidence suggests that low plant diversity in urban landscapes has resulted from the dominance of opportunistic plants, which are better adapted to an urban environment and are able to capitalise on limited establishment opportunities (Williams et al. 2015). Specialist adaptations within synanthropic plants can include self-fertilisation (hereafter referred to as selfing) and rapid growth, which have both been shown to offer reproductive assurance within fragmented landscapes (Agrawal and Whitlock 2012). Specifically, reproductive assurance describes the ability of selfing plants to persist in spite of low genetic diversity within small and isolated populations (Agrawal and Whitlock 2012). In the absence of the selfing strategy, inbreeding depression can reduce population fitness through the accumulation of recessive deleterious alleles in their homozygous state (Agrawal and Whitlock 2012). Selfing plants are able to lower the proportion of these accumulated lethal mutations overtime, as a result of natural selection (Byers and Waller 1999; Bosse et al. 2019). As such, the selfing strategy of plants can prevent population decline (Eckert et al. 2006). This suggests that selfing plants may be able to retain low connectivity across highly fragmented and isolated urban ecosystems, although the extent of this connectivity has not been established.

Similarly low levels of connectivity can also be predicted for plants with an epiphytic life cycle. Angiosperm epiphytes, in particular, frequently express high selfing rates and rapid growth capability (Fenster and Marten-Rodriguez 2007; Rios and Cascante-Marin 2016). Furthermore, epiphytes are mechanically dependent on trees for physical support (Benzing 1990; Zotz 2013) and, as such, are highly susceptible to both direct (deforestation, large-scale disturbance events) and indirect (available colonisation substrate, microclimate alterations- light, relative humidity, temperature) implications of landscape fragmentation (Benzing 1990; Ribeiro 2009; Larrea and Werner 2010; Zotz 2013). However, relatively little has been revealed about the genetic structure of this understudied group of plants within fragmented habitats, and even less so in urban ecosystems (Izuddin and Webb 2015). As a result, epiphytes are ideally suited to the study of plant connectivity in urban landscapes. They are also a globally very taxonomically diverse group of plants, accounting for 10% of all extant vascular plant species (Zotz et al. 2021). In addition, they are capable of reaching high levels of abundance in urban landscapes (Fenster and Marten-Rodriguez 2007; Chaves et al. 2021). Bhatt et al. (2015), for example, found a total of 30 epiphyte species from 12 families on 254 urban host trees in the eThekweni Municipal Area of South Africa. Equally, Alvim et al. (2020) found 56 species of epiphytes, belonging to 22 families, on 827 urban host trees in Juiz de Fora city in Brazil.

Currently, only 30 papers have been published globally on the genetic structure and connectivity of epiphytes. The majority (24 or 80%) of them have discussed epiphyte population genetics in the context of microsatellite marker cross-amplification and species biology. The remaining papers (6 or 20%) investigated the population genetics of epiphytes in relation to fragmented landscapes, such as rural sites (Chaves et al. 2018, 2021; Aoki-Gonçalves et al. 2020), industrial mining sites (Lavor et al. 2014) and modified natural forests or reserves (Alcantara et al. 2006; Goetze et al. 2015). From these six aforementioned papers, contrasting outcomes of landscape fragmentation were reported for epiphyte genetic structure, largely dictated by the variety of taxonomic groups and breeding strategies that have been described. For instance, selfing epiphytes typically showed population differentiation and high inbreeding in fragmented landscapes (Chaves et al. 2018, 2021). In contrast, self-incompatible epiphytes show low SGS and high gene flow, although this connectivity appears to be dependent on an effective pollination strategy (Alcantara et al. 2006; Lavor et al. 2014; Goetze et al. 2015;

Aoki- Gonçalves et al. 2020). Therefore, the genetic structure and connectivity of epiphytes in fragmented landscapes appears to be determined by reproductive strategy and, as such, a similar driving factor can be predicted for epiphyte connectivity in urban landscapes. Although, to the authors' knowledge, there are currently no direct assessments of epiphyte connectivity in planted trees within urban ecosystems.

Furthermore, amongst the identified studies that investigated epiphyte population genetics in fragmented landscapes, several taxonomic groups have received considerably more attention. Bromeliaceae, for instance, has been the focus of the majority of these studies (83% – 5 of 6 studies). This taxonomic bias is likely the result of the wide distribution and high abundance of many bromeliad species throughout the Neotropics (Paula et al. 2016). More specifically, *Tillandsia recurvata* L., has been featured most frequently in fragmentation studies (Chaves et al. 2018, 2021), as well as broader investigations of epiphyte population genetics (Soltis et al. 1987; Solózano et al. 2010).

This concerted focus on *T. recurvata* may be determined by several key factors. For instance, *T. recurvata* has been found in high abundances throughout a wide geographic range, typically located within tropical and sub-tropical Northern and Southern America (Soltis et al. 1987), as well as deciduous forest, coastal sand dune scrub and semi-deciduous forest (Bernal et al. 2011). Moreover, this atmospheric bromeliad has been characterised as one of the most adaptable plants in the Western Hemisphere (Foster et al. 1945), with valuable xerophytic qualities, such as small leaf size, CAM photosynthesis and absorbent leaf scales. *T. recurvata* also exhibits opportunistic traits, such as selfing and high growth rates, which have allowed this species to reach extreme levels of abundance within fragmented landscapes, such as urban areas (Benzing 1980; Soltis et al. 1987; Chaves et al. 2021). As such, these traits make *T. recurvata* an ideal model to investigate the connectivity and genetic structure of epiphytic plants within understudied urban ecosystems.

In the absence of this empirical data to show the connectivity of *T. recurvata* (or other epiphytic species in urban landscapes), we are unable to make wider conclusions about the implications of urban ecosystems for epiphyte connectivity and diversity (Izuddin and Webb 2015). The consequences of this lack of evidence may be most keenly observed in the highly biodiverse tropics (Lewisohn and Prado 2005). Brazil, for example, harbours ca. 9.5% of all species worldwide (Lewisohn and Prado 2005), as well as one of the largest proportions of epiphytes and threatened plant species globally (4,967 taxa in 1,235 genera and 232 families) (Nieder et al. 2001; Moraes et al. 2014; Freitas et al. 2016). In parallel to the current lack of evidence worldwide, the connectivity of Brazil's urban ecosystems have not been studied. This may have direct implications for the proportion of all species that are under threat from urbanisation in Brazil (ca. 10% of 1172 endangered spp.) (Bernard et al. 2019), especially where urban human population is expected to rise by 170% before the year 2050 (Paiva et al. 2020). In order to effectively anticipate the implications of urbanisation for Brazil's species diversity in the future, as well as in urban ecosystems across the globe, an improved understanding of connectivity in urban landscapes will be instrumental.

To improve our understanding of the connectivity of remnant epiphyte populations in urban landscapes, this novel study aimed to, for the first time; 1) investigate how an urban landscape can impact the connectivity and genetic structure of *T. recurvata*, L., a highly abundant and widely distributed atmospheric epiphyte, on planted urban trees; and to 2) understand what the driving landscape factors are that affects connectivity and structure. We also discuss how global urbanisation can affect genetic structure in epiphytes. In addition, this

study tests two hypotheses, based on a previous assessment of *T. recurvata* population structure (non-urban populations) (Chaves et al. 2021); 1) low connectivity across an urban landscape, as well as high SGS, occur as a result of the selfing strategy of *T. recurvata*; and 2) low tree density within an urban landscape directly amplifies differentiation and low gene flow between populations, as a result of genetic drift.

Methods

Study Species

T. recurvata L., an exclusively wind dispersed species, produces a large number of seedlings with the ability to attach to a phorophyte, or non-organic substrates such as power cables (Wester and Zotz 2010). *T. recurvata* shares this ability with other members of the *Tillandsia* genus (Wester and Zotz 2010). This species favours environments defined by low-light, high humidity and low airflow (Caldiz and Andía 1993). Although, the exact limit of this seed dispersal range is unknown, the small, light seeds (approximately 5.0 mm) (Chilpa-Galván et al. 2018) and low seed terminal velocity suggest a high dispersal potential, and likely facilitate its wide geographic range (Victoriano-Romero et al. 2017). This species exhibits both clonal growth and a selfing strategy (Birge 1911).

Study Site

The city of Alfenas (-21°25'59.99" S -45°56'59.99" W) is located in the South of Minas Gerais state in Southeast Brazil, situated 335km from the state capital of Belo Horizonte and 300km from São Paulo. The city covers an area of 849 km² at an elevation of 888m a.s.l. (Fig. 1). The climate is humid subtropical, with an average annual temperature of 21°C. Average annual precipitation is approximately 1252mm. The highest levels of rainfall occur in the summer months (December-February), resulting in a wet and a dry season (Arruda 2013). Average wind speeds range between 5.9km/hr-7.7km/hr; typically moving in a Southeast direction. Alfenas contains large urban developments that produce high levels of human-driven disturbance, resulting in the limited survival of small vegetation patches of seasonal semi-deciduous trees within streets and squares across the city. Urban tree compositions in Alfenas are also quite distinct from natural vegetation, with the marked presence of exotic species (Monalisa-Francisco and Ramos 2019). The functional diversity of Alfenas' urban trees was considered to be low, of small size, and exhibited entomophily, zoochory, evergreen leaves and dry fruits (Monalisa-Francisco and Ramos 2019).

Sample Collection

A total of 288 *T. recurvata* individuals were sampled across 65 trees, within 13 urban spread trees along streets and squares throughout the city of Alfenas (see Fig. 2 for typical example of sampled trees). Fieldwork was conducted between April-June 2021. All of the urban trees were located within an area of approximately 12.4 km² across the city, and the distance between tree patches in streets and squares ranged from 330m-5km. The criteria for plot selection was largely determined by the minimum number of trees per plot (five trees) and the presence of *T. recurvata* individuals (> three individuals per tree; a minimum of three trees in a plot). Twelve of the 25 urban tree plots throughout the city did not conform to the above criteria and were subsequently excluded from the study. Sampling from pasture and forest trees outside of Alfenas city was prevented due to coronavirus restrictions.

Wherever possible, five *T. recurvata* individuals were collected per tree in each plot. To reduce the probability of collecting clones, the distance between each tree within a plot was maximised (> three meters where possible) and samples were not collected when connected to other conspecifics. Samples were oven dried at a 60°C for 48h and stored in individual paper bags, before being transported to Edge Hill University, United Kingdom, for genetic analysis.

Laboratory Procedure

Genomic DNA was extracted from dry leaf tissue, using a modified CTAB extraction method described by Tel-Zur et al. (1999). Purity of the DNA samples was indicated by the A260/A280 ratio (1.56–2.29). Seven microsatellite markers were initially tested for amplification success within the *T. recurvata* populations of this study. These markers were originally designed for other bromeliad species and were reported to have been successfully cross-amplified for *T. recurvata* by Chaves et al. (2018) (CT5, E6B, VGC01, VS10, E6, PAD07, ngFOS_12).

The microsatellite loci were single-plexed and amplified using a PCR reaction volume of 10 µL, containing: 1 µM DNA template, 5.0 µM MyTaq Plant-PCR Kit, 0.2 µM forward primer (tagged with individual fluorochromes-FAM, VIC, PET), 0.2 µM reverse primer, 0.4 µM MgCl and 3.7 µM Nuclease Free Water. A Veriti 96-Well Thermal Cycler (Applied Biosystems) was used to perform a touchdown PCR programme, as described by Palma-Silva et al. (2007). Successful PCR amplification was verified through EtBr gel electrophoresis, using 2% agarose gels.

Of the original seven microsatellites, only four were amplified with sufficiently high levels of success (> 40%). Consequently, seven additional novel, species-specific, microsatellite markers were designed during this study to ensure sufficient levels of polymorphism for the detection of all multi-locus genotypes (MLG). Microsatellite candidate loci were selected from genomic short read sequence data from *T. recurvata* obtained from NCBI (7.2Gb) (Sayers et al. 2022). The BioProject Accession and accession numbers are PRJNA701548 and SRX10089449, respectively.

The microsatellite identification software Krait (0.5.2) (Du et al. 2018) was used to detect suitable microsatellites, both genome-wide and in the noncoding regions of *T. recurvata*. The single sequence repeats (SSRs) were refined to a minimum number of seven di-, tri-, or tetra-nucleotide repeat motifs. These sequences were further limited to SSRs of more than 100bp in length and low GC content (< 50%). Krait (0.5.2) (Du et al. 2018) was also used to design the primers for the selected SSR sequences, in conjunction with the integrated Primer3 software. The criteria for primer selection included: a primer length of 18-26bp, an optimal melting temperature (T_m) of 54–59°C and GC content of < 50%. These designed primers were single-plexed and amplified using the following PCR cycle: initial denaturation (95°C for 3 min), 34 cycles of 95°C for 30s, annealing for 30s (JP01-JP12: 54°C, 4873TD + 35251TD: 56°C, 19286TD: 53°C, 5044TD + 186664TD + 214633TD: 58°C), 72°C for 1 min and a final extension of 72°C for 5 mins.

A combined total of eleven successfully amplified microsatellites were selected from the cross-amplified and designed loci sets (Table 1), which were subsequently fluorescently tagged. The PCR product sizes of these eleven microsatellites (CT5, E6b, VGC01, VS10, JP02, JP03, JP04, JP05, JP09, JP11, TD73) were evaluated through capillary electrophoresis across all 288 *T. recurvata* individuals. This study used an AB3500 Genetic

Analyser (Applied Biosystems, Warrington, UK), 50 cm capillary array (AB). A 10 μ L reaction volume was formulated from 8.9 μ L Hi-Di formamide, 0.1 μ L LIZ500 size standard (Life Technologies, Warrington, UK) and 1.0 μ L of PCR product. Raw scoring data was processed using TANDEM (1.07) (Matschiner et al. 2009) and peaks were binned to integer allele sizes, to ensure results were consistent with the repeat unit of each microsatellite. All primer information will be made available through the Dryad Digital Repository following the publication of this work.

Table 1

Cross-amplified microsatellite markers, originally tested by Chaves et al. (2018) and designed microsatellite loci for *Tillandsia recurvata*. Primer sequence, annealing temperature, repeat motif, base-pair range, alleles and original source are given.

Locus (Primer name)	Primer Sequence 5' – 3'	Annealing Temp. <i>T_a</i> (°C)	Repeat Motif	Base Pair Range (size bp)	Alleles	Reference
CT5	F:AATGAGTTTCAGTTTTAGAAGC R:CCAAGAAAAGAACGGATCA	51	GA ₍₂₅₎	189	5	Boneh et al. (2003)
e6b	F: CGTACGAAGGTAAGCACAA R: CCGTTGAAGAGGTTAGAGG	51	CAA ₍₁₂₎	151	5	Boneh et al. (2003)
VgC01	F: GCTAGGGTTTCACCCCAAAT R: TCAGCCTCTGATCCATCTCC	NA	CT ₍₁₆₎	208–218	6	Palma-Silva et al. (2007)
Vs10	F: GAATCGAGTCGGTGTGACCT R:CCATACCTCAATTCCTCATTCCG	NA	AC ₍₁₀₎ CT ₍₅₎	187–196	12	Neri et al. (2015)
4873TD	F:TCTCGGTAGCTCAGTCCG R:AGAGAACGAGAGAAGAAGGG	58	TC	125	7	This study
JP02	F:AGTTAGCTGTAGTTCTTTCCA R:TGGAAGCAAGTAAAAGGGT	54	ATAG	120–140	NA	This study
JP04	F:CTGGAAGCAAGTAAAGGGT R:TGTAGTTTTCCAGCTTTCCA	54	ATAG	120–140	NA	This study
JP05	F: CGCAATTCAGTAGCTCATG R: TGAGCATGAGTCCGTGAT	54	ATAG	120–140	NA	This study
JP09	F:AGTAGCTCAGTTCTTTCTAGC R:ACGGTATGCCCACTAAGT	54	ATAG	120–140	NA	This study
JP10	F:GCTCATTTAGTTTTCCAGCTT R:CTGGAAGCAAGTAAAGGGT	54	ATAG	120–140	NA	This study
JP11	F:CTCAGTCTCCCCACTAGG R:TGGGCATAGTAGAGTAGTTATTT	54	ATAG	120–140	NA	This study

Data Analysis

Clonal replicates were detected using the R package 'poppr' (version 2.3.0) (RStudio Team, 2021) (Kamvar et al. 2014; 2015). The data was screened for the presence of repeated multilocus genotypes (MLGs). An

unbalanced number of samples and MLGs would indicate the presence of clonal replicates. Standard output for population genetics, to determine genetic variation and distribution within our *T. recurvata* populations, was then produced using the R packages 'gstudio' and 'diveRcity' (RStudio Team 2021). Results included average number of alleles per locus (A), allelic richness (Ar), observed heterozygosity (H_o), expected heterozygosity (H_e), Hardy-Weinberg equilibrium (HWE) and inbreeding coefficient (F_{is}). A chi-squared test (χ^2 -test) with a Monte-Carlo permutation procedure (999 replicates) was used to detect departures from the Hardy-Weinberg Equilibrium. Linkage disequilibrium (LD) was assessed with the 'poppr' package, which compared the relationship between loci ($r r d$) (Agapow and Burt, 2005) against 999 permutations. A distance-based redundancy analysis (dbRDA) was performed using the "vegan" package (Oksanen et al. 2017), version 2.4-2. This analysis was restricted by the geographic location of each sample, in order to calculate the proportion of the variation explained by distance (isolation by distance- IBD).

Spatial patterns of genetic variability were determined through a spatial Principle Component Analysis (sPCA), using package "adeget" (Jombart 2008; Jombart and Ahmed 2011), version 2.0.1. The sPCA scores were then multiplied by Moran's I to form a spatial autocorrelation, which summarised allelic differences between individuals. Global and local genetic structure were calculated through a Monte Carlo permutation, to indicate the degree of genetic similarity between plots. A neighbourhood-by-distance connection network was selected and all axes were retained. This was dictated by the equal variance explained across all axes (Jombart et al. 2008). Ward hierarchical clustering was then used to assign each sPCA axis to a cluster group, using the function `dist()` to generate a distance matrix. The function `hclust()` was applied to build a hierarchy of clusters, with each individual sample allocated to a cluster. Each axis and subsequent cluster group explained a proportion of the variance observed. Cluster groups were then assigned a distinct colour and plotted, relative to each of the five trees within a population, according to their geographic position within a map of the study site (QGIS, version 3.12.0-București (QGIS Development Team, 2020)). A Bayesian clustering method was then used to show this arrangement of spatial genetic structure across individual samples ($N = 282$).

Results

Eleven microsatellite loci revealed moderate levels of genetic diversity within populations, in relation to other assessments of *T. recurvata* genetic structure. Polymorphism within the eleven sampled loci was sufficiently high enough to detect all multi-locus genotypes (MLG) (282) across 282 sampled individuals (Fig. 3), determined through power analysis. The equal number of MLGs and samples indicated an absence of clones within the data set, as anticipated from the experimental design.

The average number of alleles per population ranged from 50–105 (A , Table 2) and mean allelic richness (A_r) ranged from 3.02 to 4.88 (mean = 3.91 ± 0.01). Observed heterozygosity ranged from 0.25 to 0.4 and averaged 62% (± 0.77) across the majority of populations. Expected heterozygosity ranged from 0.59 to 0.74 (Table 2). Inbreeding coefficients (F_{is}) were not significantly different between populations, although the values were relatively high (range of 0.41 to 0.63). A significant departure from Hardy-Weinberg Equilibrium (HWE) was observed across all populations. All populations showed non-significant linkage disequilibrium, with the exception of populations GV ($r d = 0.04$, $n = 25$, $p \leq 0.01$) and U ($r d = 0.025$, $n = 25$, $p \leq 0.01$).

Table 2

Summary of genetic variation in populations of *T. recurvata* in Alfenas city, Southeast Brazil. A, APAE public square; AN, airport; BS, bus station public square; GV, Getulio Vargas avenue; IP, Imesa parking; ID1, industrial district 1; ID2, industrial district 2; MS, Machado de Assis street; MCS, main street square; OT, old train station; PS, Pinheirinho square; SL, São Lucas; U, unifal-MG. *N*, sample size; *A*, average number of alleles per locus; %, percentage of total alleles observed across population samples per locus; *A_r*, Allelic richness; *H_o*, observed heterozygosity; *H_e*, expected heterozygosity; *F_{is}*, relatedness of individuals under a model of random mating.

Population	<i>N</i>	<i>A</i>	%	<i>A_r</i>	<i>H_o</i>	<i>H_e</i>	<i>F_{is}</i>	<i>F_{is}</i> _{Low}	<i>F_{is}</i> _{High}
A	21.55	83	28.85	4.04	0.35	0.64	0.45*	0.39	0.51
AN	6.73	50	20.45	3.51	0.37	0.67	0.45*	0.25	0.57
BS	18.55	69	24.34	3.45	0.33	0.65	0.48*	0.38	0.58
GV	20.73	105	37.99	4.88	0.38	0.74	0.48*	0.40	0.54
ID2	20.45	81	29.7	3.7	0.32	0.66	0.51*	0.38	0.62
ID1	18.73	82	28.81	3.95	0.25	0.68	0.63*	0.55	0.69
IP	17.55	63	21.92	3.02	0.25	0.59	0.57*	0.45	0.67
MCS	9.09	65	22.93	4.21	0.4	0.68	0.41*	0.28	0.51
MS	19.09	72	29.37	3.63	0.36	0.62	0.42*	0.33	0.49
OT	16.64	86	31.52	4.32	0.37	0.73	0.49*	0.40	0.57
PS	19.45	91	32.07	4.07	0.37	0.69	0.46*	0.35	0.54
SL	18.64	87	32.79	4.07	0.37	0.69	0.45*	0.36	0.55
U	20.45	84	30.28	4.03	0.38	0.72	0.47*	0.41	0.53

*Significant Hardy-Weinberg Equilibrium *P*-value from the χ^2 goodness-of-fit test.

Distance-Based-Redundancy-Analysis (dbRDA) indicated a non-significant and weak isolation-by-distance (IBD) pattern between populations ($R_{2adj} = 0.17$; $F = 1.08$, $df = 2$, $p = 0.16$). Significant global structure was detected through a sPCA ($p \leq 0.0004$, $\lambda = 0.00016$). No significant local structure was detected ($p = 1.0$, $\lambda = 0.0000309$), meaning that the frequency of alleles within populations were more similar at neighbouring sites. Eight distinct clusters of spatial genetic similarity were identified. These coloured cluster groups are illustrated in Fig. 4 and Fig. 5, showing each of the five trees per population and the arrangement of SGS across individual samples.

Discussion

This study applied empirical evidence to assess how an urban landscape has impacted the connectivity and genetic structure of *T. recurvata*. In accordance with the hypothesis of this study, the urban *T. recurvata* populations exhibited high global SGS within Alfenas city. However, this was not the result of isolation by distance (IBD). All populations showed genetic differentiation, as well as relatively high levels of inbreeding and moderate genetic diversity. A significant departure from the Hardy-Weinberg Equilibrium (HWE) was

observed across all populations. According to the HWE, several elements may have interacted to determine this outcome, including: small population size, genetic drift, gene flow, breeding system, natural selection and mutation (Lachance 2016).

Population size can function as a baseline for the aforementioned conditions required for the maintenance of HWE (Lachance 2016). In this study, *T. recurvata* was found in small populations, isolated to multiple clusters of low tree density throughout Alfenas city. This species has also been known to inhabit power cables and non-organic substrates, although these populations are found in much smaller quantities (Wester and Zotz 2010). The low tree density in Alfenas city was driven by land-use change and deforestation for urban expansion, which likely led to the fragmentation of a previously continuous landscape (Defries et al. 2010), and also by the low number of planted trees in its streets and squares (Monalisa-Francisco and Ramos 2019). This trend has been mirrored across many urban areas (Arshad et al. 2020). Evidence suggests that this fragmentation may have reduced the size of a previously large and interconnected population of *T. recurvata* (Toledo-Aceves et al. 2014), primarily due to dispersal limitations (Cascante-Marín et al. 2009). A genetic bottleneck most likely occurred within these condensed *T. recurvata* populations, which translated into reduced genetic diversity as a result of small gene pool size (Ackerman and Zimmerman 1994). Genetic drift then possibly ensued from this bottleneck and altered the frequency of alleles overtime within the finite *T. recurvata* populations (Chaves et al. 2021). This resulted in the genetic differentiation and high SGS that were observed in this study (Nussbaum 2016) and have indicated that genetic drift has dictated the connectivity between urban *T. recurvata* populations. Furthermore, the short life span of *T. recurvata* (approx. six years; Chaves et al. 2021) and the extended period of urban expansion in Alfenas (founded in 1805), both suggest that multiple generations of this epiphyte have experienced genetic drift overtime and amplified the differentiation between populations (Nussbaum 2016).

The role of low tree density and small population size on genetic differentiation, as well as genetic drift, were reinforced through a recent simulation of *T. recurvata* spreading dynamics in an orchard landscape (Chaves et al. 2021). Chaves et al. (2021) generated a TREC model that used an individual-based approach, with the simulated spreading dynamics of *T. recurvata* adjusted relative to empirical genetic data. The findings of Chaves et al. (2021) revealed genetic differentiation and high SGS between *T. recurvata* populations, which the TREC model attributed to low tree density. A greater differentiation was also observed at the stage of early colonisation (Chaves et al. 2021). Specifically, they found that each tree formed individual habitat units, which encouraged distinct groups of multi-locus genotypes (MLGs) through genetic drift, resulting from founder events on each host tree (Chaves et al. 2021). The random combination of alleles that formed these MLGs were sufficiently diverse to maintain moderate genetic diversity and mitigate strong evidence of IBD (Chaves et al. 2021). The aforementioned findings of Chaves et al. (2021) were supported by the observations of this study and have reinforced the influence of genetic drift, after the founder events on each studied population, on the connectivity of urban *T. recurvata* populations.

Genetic drift has also been recognised as a key factor in the differentiation of *T. recurvata* in the wider literature (Soltis et al. 1987), as well as in another species of *Tillandsia* (González-Astorga et al. 2004). Similarly, several other species of plants show evidence of differentiation as a result of genetic drift in isolated populations, many of which share comparable characteristics to those of *T. recurvata* (selfing, rapid and clonal growth, epiphytic life cycle, high abundance) (Trapnell et al. 2004; Vekemans et al. 2004; Barluenga et al. 2011;

Pettengill et al. 2016; Atwater et al. 2018; Torres et al. 2018; Mota et al. 2020; Chaves et al. 2021). The evidence presents, therefore, a strong case that genetic drift has driven the random differentiation and high SGS of *T. recurvata* populations in this study.

Genetic drift, high SGS and population differentiation all indicate low gene flow between the *T. recurvata* populations in this study (Ramos et al. 2016; Chaves et al. 2021). *T. recurvata* maintains gene flow exclusively through wind dispersed seeds (Chilpa-Galván et al. 2018), since it has spontaneous self-pollination on its cleistogamous flowers (Bianchi and Vesprini 2013). The exact range of *T. recurvata* seed dispersal remains unquantified, although the small, light seeds of this species and low seed terminal velocity are both suggestive of a high dispersal potential (Chilpa-Galván et al. 2018). Nonetheless, the limitations of wind dispersal, specifically restricted movement within dense and enclosed landscapes, have frequently been documented over long distances (Barrett et al. 1991; Nathan et al. 2002; Vergara-Torres et al. 2010; Belinchón et al. 2017). As such, the confined and complex landscape of urban architecture within Alfenas may have limited *T. recurvata* seed dispersal in this study (Ramos et al. 2016); amplified by the range between the urban tree patches (330m-5km) and the low density of planted trees in the streets and squares. For instance, the air currents surrounding moving traffic and buildings may have dictated the flow of wind dispersed seeds (Lippe et al. 2013). Equally, human-mediated mechanical removal of this opportunistic epiphyte may have influenced population size and subsequent gene flow (Chaparro et al. 2011). Under the pressure of sustained dispersal limitations, evidence has dictated that isolated species become more likely to experience inbreeding depression and IBD, as well as a subsequent reduction in genetic diversity and population fitness (Wright et al. 2013). In contrast, the moderate genetic diversity observed in this study has contradicted this predicted outcome, and suggests that an alternative factor has influenced the gene flow and connectivity of this urban landscape.

Further contemplation of the spreading dynamics of *T. recurvata* may offer an insight into this controlling factor, which has determined the connectivity of these urban populations. Specifically, the adaptability and dominance of *T. recurvata* within fragmented landscapes (Orozco-Ibarrola et al. 2014), and its ability to grow in isolated trees (Chaves et al. 2021) or in “harsh” habitats, such as pastures (Elias et al. 2021), enables the accumulation of dense populations. These areas of high density then act as sources for the spread of *T. recurvata* propagules to the surrounding landscape, facilitating the transmission of several multi-locus lineages (MLLs) (Chaves et al. 2021) and gene flow to more isolated populations. In view of these spreading dynamics, the pasture trees and forest fragments that surround Alfenas city must be incorporated within our current perspective of this urban landscape. Trees in pasture fragments are common in the surrounding land around Alfenas, in particular, and have been shown to host a high abundance of *T. recurvata* (Carvalho et al. 2015; Monalisa-Francisco 2015; Elias et al. 2021). This would create a metapopulation dynamic, with external pasture trees facilitating gene flow to isolated populations across the city and preventing IBD. Under a metapopulation structure, and in combination with the previously described influence of small population size and genetic drift, the results of this study conform to the expected findings of high SGS and population differentiation (Chaves et al. 2021). We were prevented from collecting *T. recurvata* samples from pasture fragments outside of Alfenas city by coronavirus restrictions and were; therefore, unable to test this hypothesis within this study.

Although, in contrast to the findings of this study, low genetic diversity was also anticipated within populations, as a result of genetic drift from consecutive founder events on each isolated urban host tree (Ward 2006). The

size of population required to mitigate this genetic drift has not been quantified in the *Tillandsia* genus, although work on other species of similar selfing plants have proposed a local effective population size of 150–200 individuals (Goldringer et al. 2001; Siol et al. 2007). The local urban *T. recurvata* populations in this study were substantially smaller than this predicted value, which has reinforced the expected outcome of low genetic diversity within these populations.

In order to maintain genetic diversity within a fragmented landscape or urban ecosystem, *T. recurvata* must be adaptable to the consequences of small population size and genetic drift- namely inbreeding as a result of low gene flow (Agrawal and Whitlock 2012). Inbreeding depression has the capacity to reduce population fitness through the accumulation of harmful homozygous recessive mutations, amassed across multiple generations of inbred progeny (Agrawal and Whitlock 2012). However, *T. recurvata* and other selfing plants are able to lower the proportion of accumulated lethal mutations, through the purging of recessive deleterious alleles in their homozygous state- as a result of natural selection (Byers and Waller 1999; Bosse et al. 2019). As such, the self-fertilisation strategy of *T. recurvata* gives reproductive assurance (Ingvarsson 2002), despite the constraints of potentially consecutive founder events (Eckert et al. 2006). A number of papers have reinforced this concept across several species of selfing plants (Lloyd 1992; Johnston and Schoen 1996; Aguilar et al. 2008; Honnay and Jacquemyn 2007; Vandepitte et al. 2010; Cutter 2019; Lander et al. 2019). Thus, the ability of selfing plants to moderate the potentially lethal consequences of isolation, offers a partial justification for the moderate diversity that was maintained within the urban *T. recurvata* populations of this study.

The reproductive assurance of self-fertilisation has made this strategy a common and valuable characteristic amongst angiosperm epiphytes (Fenster and Marten-Rodriguez 2007; Rios and Cascante-Marin 2016). Thus, the response of several selfing species to urban ecosystems, especially those that share a similar life history to *T. recurvata*, can be inferred from the data of this study. For example, the maintenance of genetic diversity within isolated landscapes through reproductive strategy, as was previously discussed, suggests that selfing species are more likely to dominate epiphyte communities in urban areas- allowing greater reproductive success in fragmented landscapes (Herlihy and Eckert 2002; Goodwillie et al. 2005; Bhatt et al. 2015; Furtado and Neto 2015; Prather et al. 2018). The wider literature also suggests that outcrossing epiphytes, in contrast, will be unable to maintain sufficient genetic diversity or persist in isolated urban landscapes, especially in the absence of an efficient pollination strategy (Ksiazek-Mikenas et al. 2019).

Although self-fertilisation offers reproductive assurance in fragmented landscapes, this study has highlighted, although not tested, the potential importance of external pasture trees for sustained connectivity between isolated urban epiphyte populations. The removal of these pasture trees, through land-use change or urban expansion, may eradicate any connectivity between populations (Ingvarsson 2002). Evidence has shown that this can result in population decline of selfing epiphytes or more substantial evolutionary consequences (Wright et al. 2013). Moreover, pasture trees contain a greater diversity of epiphytes than urban landscapes (Poltz and Zotz 2011), largely due to limited opportunity for effective colonisation in the fragmented habitat of urban ecosystems (Bhatt et al. 2015). The loss of pasture trees through urbanisation; therefore, also has direct implications for broader epiphyte diversity, as opportunistic epiphytes that are better adapted to capitalise on limited establishment opportunities within urban landscapes (such as *T. recurvata*) outcompete species that do not possess these same advantages (Poltz and Zotz 2011).

Conclusion

This study has shown, for the first time, how an urban landscape has impacted the connectivity and genetic structure of an epiphyte in planted urban trees, with specific focus on *Tillandsia recurvata*. *T. recurvata* experienced low connectivity between urban populations in Alfenas, with strong evidence to suggest that small population size and genetic drift have dictated this outcome (Soltis et al. 1987; González-Astorga et al. 2004; Chaves et al. 2021). The self-fertilisation strategy of *T. recurvata* gives reproductive assurance (Ingvarsson, 2002), despite the constraints of low connectivity and genetic drift (Eckert et al. 2006).

The findings of this study have also implied the importance of pasture trees and planted trees in urban streets and squares as a source of gene flow to isolated urban epiphyte populations (Carvalho et al. 2015; Monalisa-Francisco, 2015; Elias et al. 2021). The removal of pasture trees, through urban expansion or land-use change, may eliminate this connectivity (Ingvarsson 2002; Barrett 2015; Picó et al. 2009) and result in population deterioration (Wright et al. 2013). In the absence of reproductive assurance from self-fertilisation or an efficient pollination strategy, the wider literature suggests that outcrossing epiphytes are more likely to decline in these conditions (Ksiazek-Mikenas et al. 2019). An empirical assessment of gene flow between urban and pasture trees would be a wise step towards a greater understanding of urban epiphyte connectivity, both in selfing and outcrossing species. This has direct implications for epiphyte diversity, especially as pasture trees typically contain a greater variety of epiphytic species than urban landscapes (Poltz and Zotz 2011). The lower epiphyte diversity in urban landscapes has largely been driven by the dominance of self-fertilising epiphytes (such as *T. recurvata*), which possess a competitive advantage for limited establishment opportunities (Poltz and Zotz 2011).

This study represents a novel and important first step towards understanding the connectivity of increasingly common urban landscapes, in the context of epiphytes on planted urban trees. The authors also present novel microsatellite markers that can help other researchers to investigate the genetic structure of *T. recurvata* elsewhere. According to these findings, low connectivity across urban landscapes; dominated by opportunistic species like *T. recurvata*, suggests a bleak future for epiphyte diversity under predicted urban expansion (Newbold *et al.*2020).

Declarations

Data Availability

All data presented in this study are available from the first author upon reasonable request. All primer information will be made available through the Dryad Digital Repository following the publication of this work.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Authors S.B, F.N.R, and M.R.Q contributed to the study conception and design. Material collection was performed by F.N.R. Data collection and analysis were performed by M.R.Q. Authors T.D and J.P designed novel microsatellite markers for this study. Authors J.C.B and P.A provided advice and guidance during data collection and analysis. The first draft of the manuscript was written by author M.R.Q and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

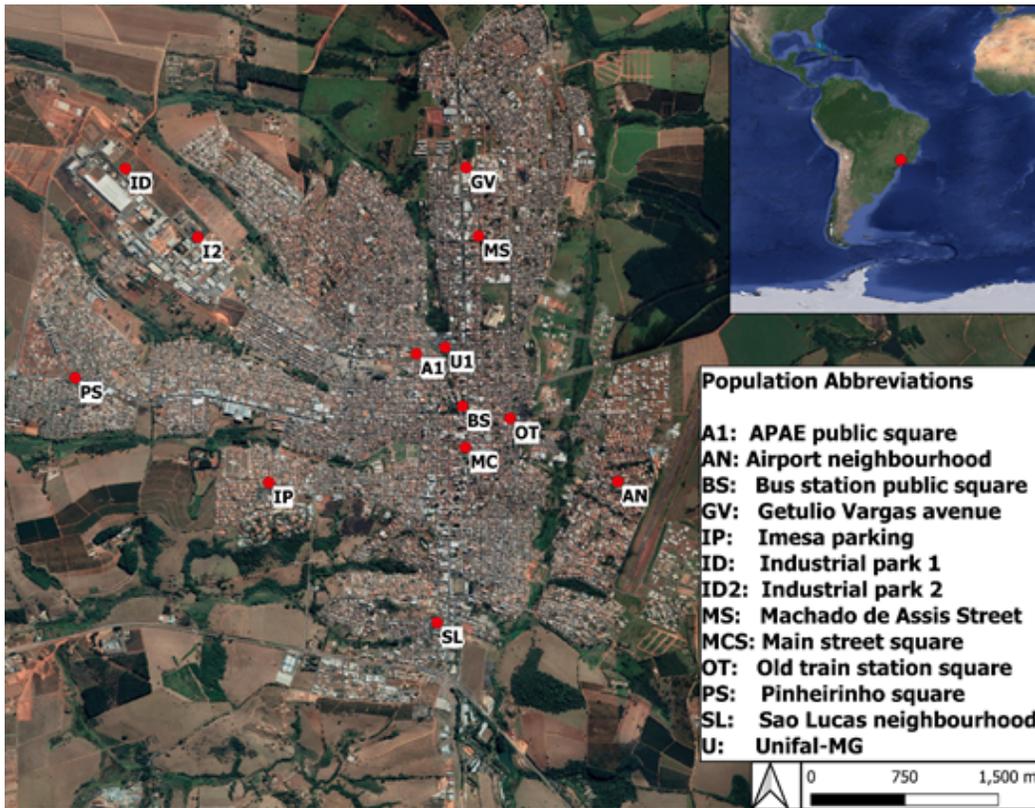


Figure 1

Position of each sample site across the city of Alfenas, Minas Gerais state, Southeast Brazil. Each site was marked by an abbreviation, which have been defined in the within figure legend.

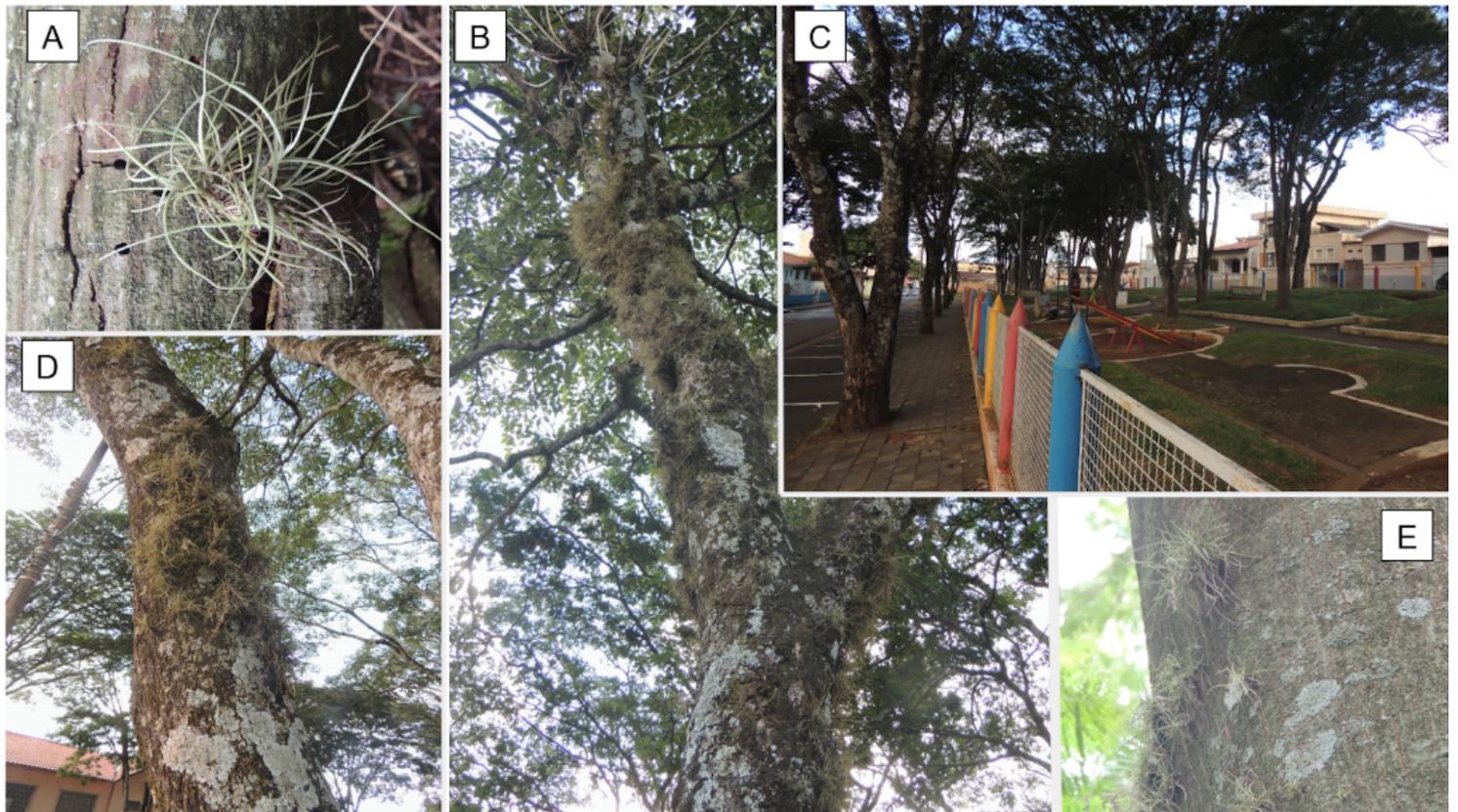


Figure 2

Tillandsia recurvata in Alfenas city. A single *T. recurvata* individual (A), an urban tree with a larger colony of *T. recurvata* individuals (B), an urban square (A1) presenting many large trees, almost all colonized by *T. recurvata* individuals (C), and trees with smaller number of *T. recurvata* individuals (D and E) in Alfenas city. Photos by Flavio N. Ramos

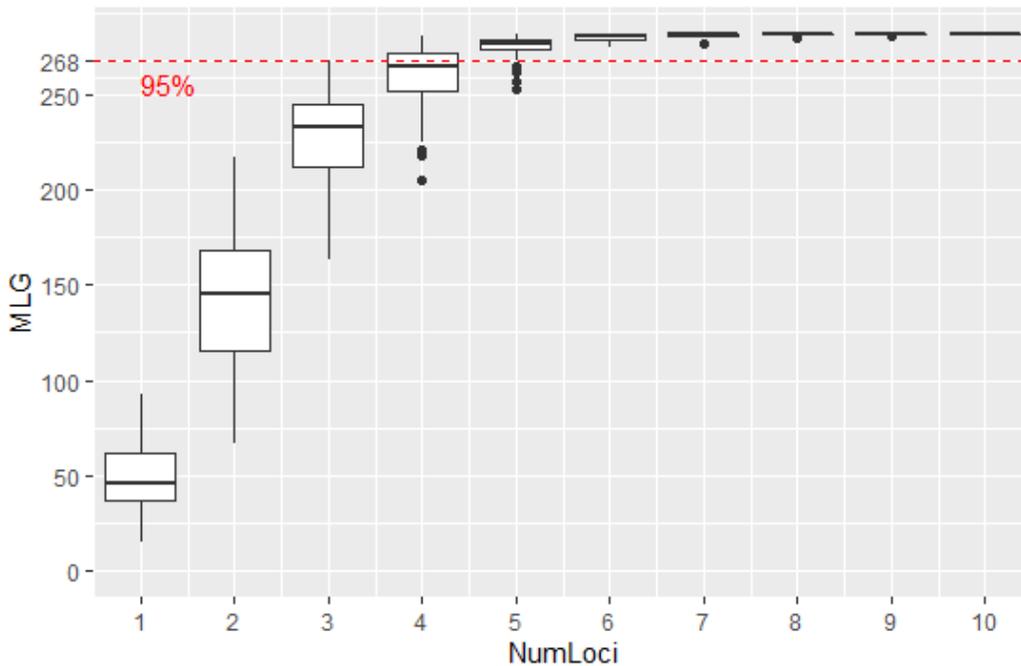


Figure 3

Genotype curve, assessing the ability of sampled loci to identify all multi-locus genotypes (MLGs) within the population. Confidence cut-off point (95%) has been indicated on the graph as a dashed red line. NumLoci (number of loci) ranges between one and eleven. A minimum of five loci were required to identify all MLGs.

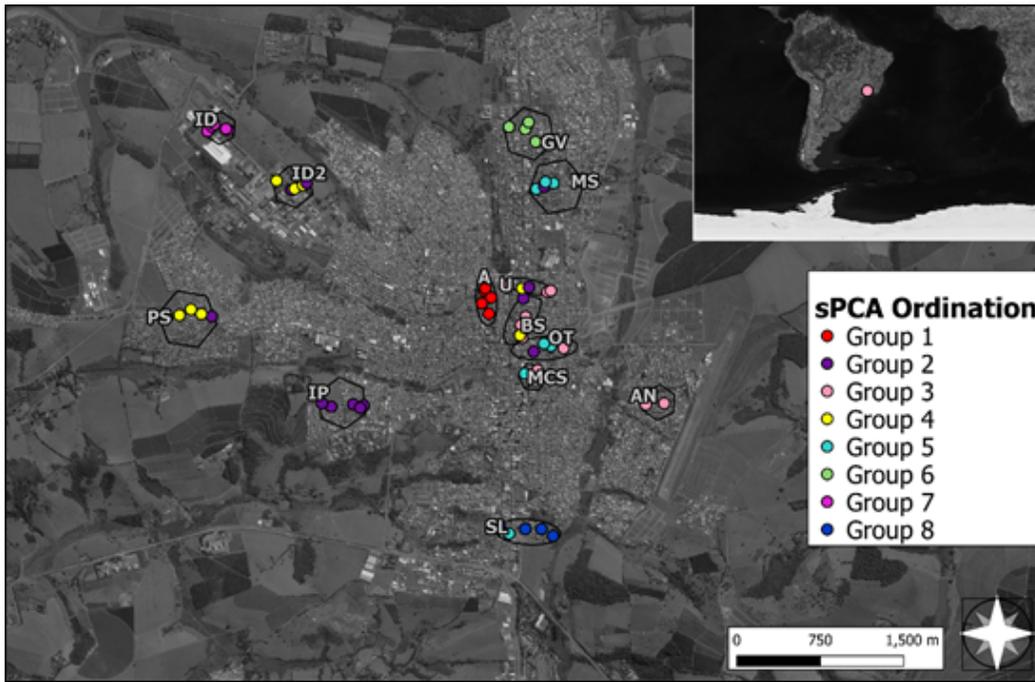


Figure 4

sPCA analysis showing the respective cluster group for each population. The cluster group has been represented by the colour of each point. Each of the thirteen populations has been indicated using a polygon and name label. The five points located within each polygon represent the five trees sampled per population. Each point has been plotted relative to geographic location- Alfenas city, South-east Brazil. Populations: A, APAE public square; AN, airport neighbourhood; BS, bus station public square; GV, Giulio Vargas avenue; IP, I mesa parking; ID1, industrial district 1; ID2, industrial district 2; MS, Machado de Assis street; MCS, main street square; OT, old train station public square; PS, Pinheirinho square; SL, São Lucas neighbourhood; U, unifal-MG

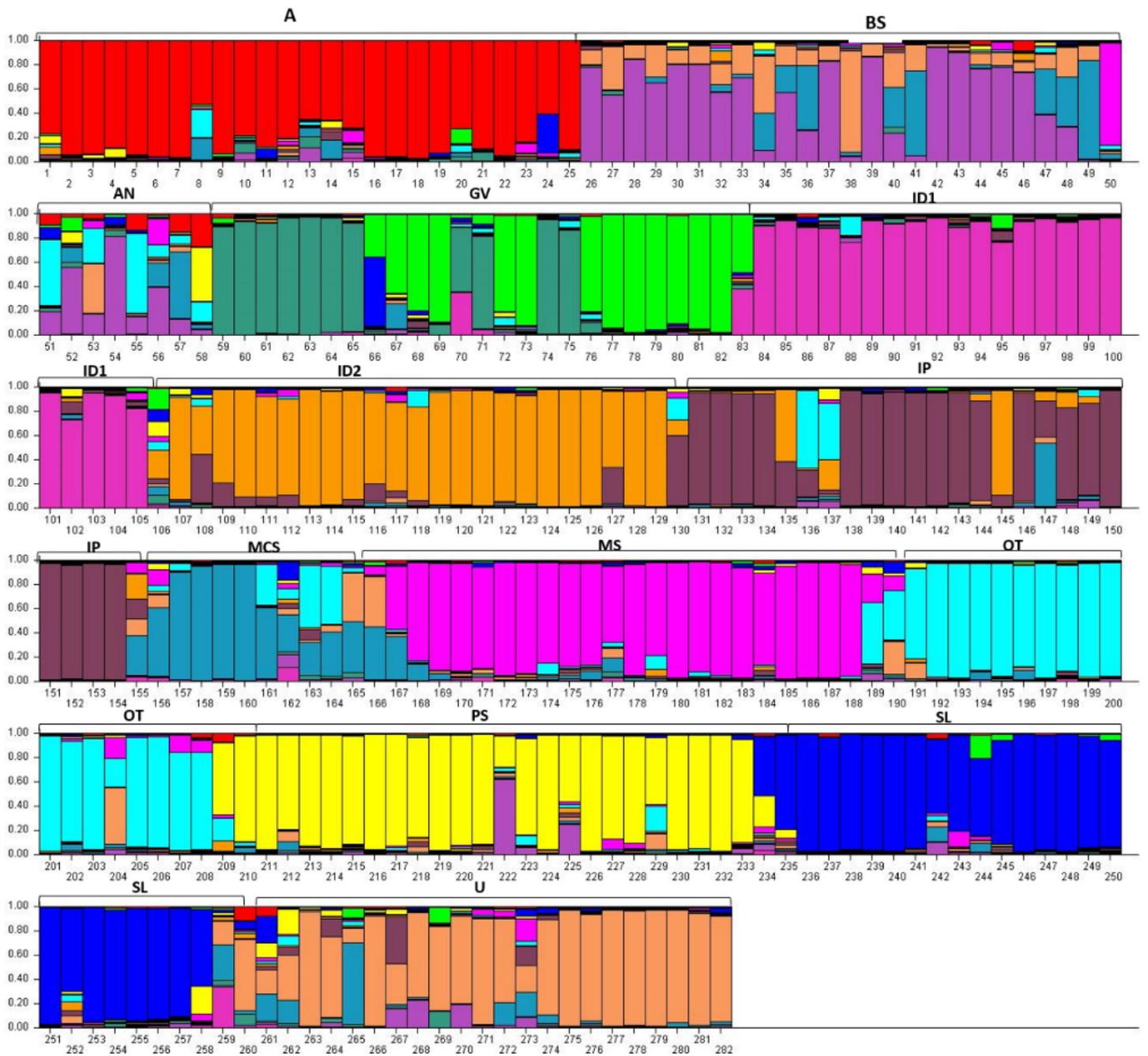


Figure 5

Bayesian clustering method, showing the arrangement of spatial genetic structure across individual samples (N = 282). Each cluster has been represented by colour. Populations are indicated through the labels along the top of each row. Individual sample numbers are shown along the bottom of each row.