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Intraspecific Genetic Diversity and Structure of *Prosopis cineraria* (L.) Druce Across Rajasthan, India, Towards Conserving the Species

Myriam Baibout[1-2], Kumar Sambhav Verma[3], Vinod Singh Gour[4] and Shanker Lal Kothari*[5]

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A B S T R A C T

Prosopis cineraria is a nurse tree with foundational properties native to dryland systems of Southern and Western Asia. State tree of the Indian state of Rajasthan, it is essential to arid systems sustainability of the region and Hindu spirituality. Nevertheless, the species is threatened by modern human impact. Because very little was known on *P. cineraria*'s genetic diversity and the distribution of this diversity, this research aims to investigate for the first-time population genetics of this species in a perspective of conservation and restoration, through the conduction of a large sampling campaign. We propose a potential local adaption on a finer scale than that of Rajasthan. High polymorphic ISSR and DAMD markers were used to optimize the cost-effectiveness of molecular analyses. Structural analyses of our data set revealed that genetic diversity of *P. cineraria* in Rajasthan seems to be distributed into 5 clusters. The geographic distribution could reflect a partial human intervention, while genetic distribution appears to be divided by a strong biogeographic barrier, following the circulating corridors of warm pre-monsoon winds as well as a rainfall gradient. Cluster 5 has been found to be the most diverse population located at Ganganagar.

***Key words*:** Intraspecific diversity, Biogeographical pattern, Rajasthan, Dryland, Forest conservation, Dominant markers, Khejri

Drylands cover 41% of the earth's surface where annual precipitation remains below 500 mm [1]. Home to approximately two billion people, they store about 45% of the global terrestrial carbon [2], and host over a third of the global biodiversity's hotspots [3]. The fragile balance of dryland systems is threatened by desertification induced by the mechanization of agriculture, habitat destruction (mainly through the destruction of native vegetation) and pollution which add to direct bioclimatic drivers such as wind erosion, salinization and soil nutrient depletion [4]. Dryland conservation strategies highlight the importance of conserving nurse species [5], and/or foundation species [6-7]. Trees adapted to desert conditions indeed drive and support the diversity of species and interactions in dryland systems [8]. They have strong effects on community structure by modifying the environmental conditions and contribute to landscape connectivity [9]. Leguminous nurse trees positively affect biota and soil composition [10-11]. They form resource islands where soil characteristics are improved and thus become habitats for entire communities of organisms [10]. Nitrogen fixing trees are therefore used in agroforestry and/or restoration programs for drylands [12-17] since they can help to improve provisioning and non-provisioning services of agriculture such as primary production and nutrient cycling [18]. Understanding what structures, the genetic diversity of desert trees would hence be enlightening in a conservation framework. Biodiversity conservation policies for desert trees have long focused their efforts on maintaining species diversity and habitats, overlooking intraspecific genetic diversity [19-20]. However, genetic variation and diversity within populations are prerequisites for species to retain their adaptive potential to face environmental changes [21-22]. Conservation and restoration programs for desert trees (e.g., *Albizia saman* in Colombia) therefore require detailed knowledge of populations' responses to threats and must be supplemented with information on the genetic differentiation of populations across their ranges [23]. Strengthening existing populations by planting or sowing saplings, or the creation of new stands are two options considered in conservation and/or restoration

* **Shanker Lal Kothari**
✉ slkothari@jpr.amity.edu

[1,3-5] Amity Institute of Biotechnology, Amity University Rajasthan, Jaipur, Rajasthan, India

[2] University Bordeaux, BIOGECO, INRA, 33615, Pessac, France

techniques. When new stands are created and if the populations are well structured, the choice of seeds may consider genotypic identity [24].

Native from Southern and Western Asia, *Prosopis cineraria* is a Fabaceaeous tree and dominant species (i.e., foundation species) in the dryland forest assemblages of India, Pakistan, Iran, Afghanistan and the Arabian Peninsula [25]. Not only is *P. cineraria* widely used in agroforestry because it promotes the growth of understorey crops, but also in silvopastoralism since its nutrient-rich forage is preferred by wild and domestic livestock [26-28]. Numerous ethnobotanical studies attest to a panoply of cultural influences of *P. cineraria* in its native range, and have shown various interactions with humans since time immemorial [29]. *P. cineraria* is thus used in Ayurvedic medicine (traditional Indian herbal medicine) and the ancient scriptures of Hinduism are replete with references to the species indicating the significant use of its wood in Vedic times [26]. In Rajasthan, a tragic episode that claimed the lives of 363 unarmed Bishnois, a rural Vishnuit community, occurred in 1730 when people opposed the army against *P. cineraria* felling. This story further inspired the Chipko movement in the 1970s, a movement that was recognized worldwide as a symbol of non-violence and the fight against deforestation, the particularity of which was that it was led by women [30].

It has been shown that plants genetic composition influences the community structure of the dependent communities from micro to macro-organisms [31], and across scales from metres [32] to 720 000 km^2. Genetic variation of dominant trees is even more likely to have important community and ecosystem consequences [31]. Foundational and patrimonial roles played by *P. cineraria* in the semi-arid ecosystem of Rajasthan therefore make it a privileged target for the implementation of sustainable ecological management in this Indian state [8]. Also, there is an obvious and urgent call to conduct population genetics studies on *P. cineraria* in its native range in order to determine the level of gene flow, population connectivity and genetic diversity to estimate adaptability of the species and its potential to track environmental changes.

At this day, relevant studies on the genetic diversity of desert trees are very sparse. A search in the Web of Science database between 1950 and October 2020 (search: ("genetic diversity" or "genetic structur*" or "genetic differenc*") and (conservation or preservation or protection) and ("forest" or tree or shrub or bush) and (dryland or arid or desert), in the fields of ecology, evolutionary biology and biodiversity conservation) indeed indicates 49 results were affiliated with this research, of which only 28 were journal articles and plant-based studies. These 28 articles come mainly from China (10 of 28), Australia (7 of 28) and Mexico (5 of 28) and not a single from South-West Asia, half of which were published before 2016 and 90% after 2019. Thirty-five woody species were examined, but none considered any species of the genus *Prosopis*, even in Mexico where they are well represented.

Over the last few decades, Asia has suffered from extensive forest fragmentation [33], adversely affecting trees population size and dispersal [34]. The tropical thorn forest (also referred as desert thorn forest in the oldest denominations) is one of the seven biomes occurring in India, mostly in the north-western parts of the country in and around the Thar Desert [35-37]. Emblematic of the Indian state of Rajasthan, *P. cineraria* is one of the matrix species of the tropical thorn forest [26]. But recent history has been marked by a deleterious human impact on the tropical thorn forest [35], [38] and scientific evidences from the CAZRI (Central Arid Zone Research Institute) and the AFRI (Arid Forest Research Institute) in Jodhpur, Rajasthan, have highlighted a worrying decline in *P. cineraria*'s population density over the past decades in the Shekhawati region and Nagaur district in Rajasthan [39]. Lowering of the water table, mechanization of farmlands and incidence of pests are the main explicative factors that are put forward to explain the abnormally elevated mortality rates observed.

The first objective of the present study is therefore to estimate the genetic variability of *P. cineraria* for the whole of Rajasthan, using markers already developed for the species. Hence, we hypothesize that *P. cineraria* would be locally structured to a finer geographic scale than that of Rajasthan. If this is the case, it will be necessary to determine what would be the explanatory factors behind a particular genetic structure in this species in the studied area. Indeed, such a structuring could be explained either: by selection pressures on the basis of ecological and agro-climatic indices, which could lead to local structuring [40]; by low natural demographics in the species which could favor significant drift effects which can also lead to the fixation of particular alleles and therefore to local structures [41] or by occasional colonization events in source-sink dynamics [42]. Thus, high polymorphic Inter-Simple Sequence Repeats (ISSR) and Direct Amplification of Minisatellite-region DNA (DAMD) have been selected from existing markers to study the genetic distribution of *P. cineraria* in 10 agro-climatic zones (AGZ) which have been defined in Rajasthan by geographic survey [43]. These zones are more broadly part of agro-climatic regions (AGR), which include more than one agro-climatic zone. We then seek to test the reliability of the AGZ division in the genetic distribution pattern of the species in order to deduce whether there is a genetic structure in *P. cineraria* in Rajasthan and what are the explanatory factors for this potential structuring.

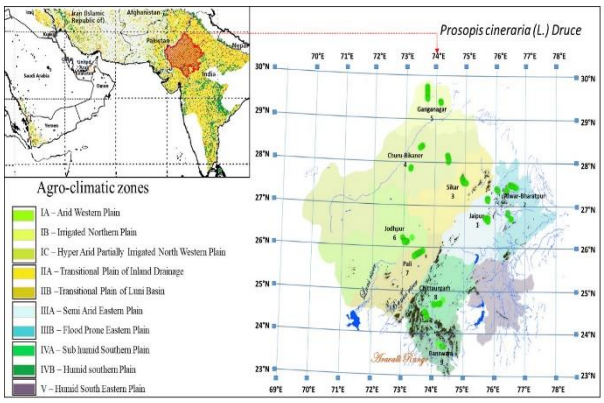

Fig 1 Distribution and sampling of *Prosopis cineraria* in Rajasthan (shown in red on the map) according to Rajasthan agro-climatic zones

MATERIALS AND METHODS

Fresh young leaves from 414 individuals were sampled between May and November 2016 in Rajasthan, India (Fig 1). Trees were randomly selected along transects of 50 km long in the centre of each AGZ defined by geographic survey of Rajasthan [43]. Along each 50 km, 5 trees were sampled every 5 km. Those 5 trees were chosen to be at a sufficient walking distance from one another to avoid

the sampling of clones. For each sample collected, GPS position of the tree was registered using a GPS data logger eTrex®x30 (Garmin, Lenexa, Kansas, USA) (Table 1, supplementary information file 1 (SIF 1)). When population density was high enough, 50 trees were sampled, and less otherwise (zones 8 and 9). Out of the 10 defined zones, we were able to sample trees within 9 of them (Fig 1). We thus numerically named populations from 1 to 9 as per their sampling order and in accordance with the district where they were collected. Leaves were collected on mature trees, wrapped in aluminium foil and then stored at -20 °C before washing and grinding.

Table 1 Number of localities, geographical location, and sampling

Samples identifiers	Number of samples	Locality number	Geographic location	Average latitude (in DD)	Average longitude (in DD)
JP + SJ	57 (48 + 7)	1	Jaipur	26,852	75,953
AB + Alw	75 (45 + 30)	2	Alwar-Bharatpur	27,476	76,885
Sik	50	3	Sikar	27,616	75,135
Chu	50	4	Churu-Bikaner	28,198	74,053
Gan	50	5	Ganganagar	29,687	73,969
Jod	50	6	Jodhpur	26,168	73,086
Pal	50	7	Pali	25,856	73,52
Chitt	25	8	Chittaurgarh	24,519	74,047
Ban	7	9	Banswara	23,575	74,363

Green zones on the inset map represent the suspected distribution of *P. cineraria* within geographic distribution map of Southern and Western Asia dryland forest, based on Global Dryland Assessment [33]. In the enlarged map of Rajasthan, Numbers from 1 to 9 indicate defined population names while green dots show sampled individuals. Agro-climatic regions (AGR) are represented by the same declination of colored surfaces: region I in light green, region II in yellow; region III in blue, region IV in dark green and region V in purple.

DNA extraction

Samples were powdered using an automated grinder (2010 Geno/Grinder, SPEX SamplePrep, Metuchen, NJ, USA) and further used for DNA extraction protocol conducted using DNeasy® 96 Plant Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's protocol. One negative control was set on each plate. DNA concentration and quality (through 260/280 and 260/230 absorbance ratio) were assessed with a UV spectrophotometer NanoDrop 8000 (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). DNA concentrations were standardized to 15 ng/µL using ChemagicSTAR automated workstation (Hamilton, Reno, Nevada, USA) before PCR reactions.

Table 2 ISSR and DAMD primers used in the study

S. No.	Primer identification	Primer sequence, 5'—3' (length)
		ISSR
1	M_1	CACACACACACAGG (14 mer)
2	M_2	AGAGAGAGAGAGAGAGT (17 mer)
3	M_3	GAGAGAGAGAGAGAGAT (17 mer)
4	M_4	CACCACCACGC (11 mer)
5	M_5	GTGTGTGTGTGTGG (14 mer)
		DAMD
1	M_7	CCTCCTCCCTCCT (13 mer)
2	M_8	GAGGGTGGNGGNTCT (15 mer)
3	M_{10}	ACAGGGGTGGGG (12 mer)

DNA amplification and electrophoresis

ISSR and DAMD markers have the advantage of being inexpensive, highly polymorphic and randomly distributed in the genome. PCR-based, they only require small amounts of template DNA. Bands profiles are generated from a single primer PCR reaction [44-45]. Markers were selected on the basis of PCR optimization protocol and primer survey developed by Sharma *et al*. [46]. Retained candidates were 5 ISSR and 3 DAMD markers (Table 2), scoring the highest rates of polymorphism on *P. cineraria* samples while all giving a good amplification rate at a 49°C Tm.

Further reactions were performed in 12.5 µl final volume with 15 ng/µL of template DNA, primer concentration of 0.4 µM and 1x concentration of 5x FIREPol® Ready to Load Master Mix (Solis BioDyne, Tartu, Estonia). Reactions were thermal cycled for PCR with an initiating step at 94°C for 3 min, 35 cycles of denaturation, annealing and extension at 94°C for 1 min, 49°C for 1 min 30 s (common Tm for all selected primers), and 72°C for 2 min, followed by a final extension at 72°C for 7 min in a Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Carlsbad, California, USA). After the amplification, electrophoresis was conducted on 2% (w/v) agarose gel in 1x TAE buffer at 100 V for 40 to 80 min (Fig 2).

All DNA extraction and amplification protocols were performed at the Bordeaux Genome Transcriptome Facility (https://pgtb.cgfb.u-bordeaux.fr/en).

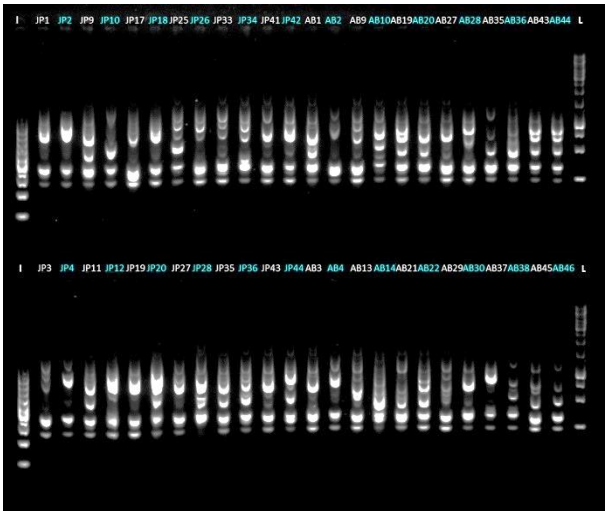

Fig 2 Amplification profile of ISSR primer M1 (as per Table 1) amongst 48 out of 379 *P. cineraria* accessions. Lanes are labelled as per individual identifier in this case either JP (for Jaipur AGZ) or AB (for Alwar-Bharatpur AGZ). L (on the right) represents the 1 kb DNA ladder and l (on the left) the 100 bp DNA ladder

Scoring of the DNA profiles

ISSR and DAMD are dominant markers and polymorphism was recorded in a binary presence /absence matrix (1/0). Each amplification product was considered as a distinct allele. Only bands with a consistent signal across the data set after second control amplification were taken into account for final scoring, whilst the rarest alleles, i.e., those recorded less than 4 times in the final data set, were removed from the analysis. 35 tree samples had to be discarded due to poor DNA quality. Final data was set to 379 individuals screened for each of the 8 markers. Molecular weights of the bands were estimated by using O'GeneRuler DNA Ladder Mix, ready-to-use 100 bp and 1 kb (Thermo Fisher Scientific, Carlsbad, California, USA) as standards.

Data analyses

Descriptive parameters of genetic diversity

GenAlEx 6.5 was used to estimate population's allele frequencies and Nei's genetic distance (SIF 2, 3) [47]. Global and pairwise population F_{ST} were estimated from the software BayeScan v2.1 [48] and AFLP-surv v.1.0 (SIF 2, 3) [49]. After the conversion of genetic data into a pairwise individual-by-individual genetic distance matrix, further genetic analyses were performed, including Analysis of Molecular Variance (AMOVA) and Principal Coordinates Analysis (PCoA) (SIF 2). Population differentiation based on AGZ (Fig 1) was estimated with Φ_{PT} indexes, an analogous statistic to Wright F_{ST} indexes which better suit with dominant markers to estimate population differentiation [50-52]. Population structure was assessed using the Bayesian clustering method implemented in STRUCTURE v2.3.4, running an admixture model with correlated allele frequencies between clusters [53-54]. Burn-in period was set to 300,000 and MCMC length was set to 150,000. The optimal number of clusters was determined with the method Evanno *et al*. (2005) and Puechmaille method (2016) implemented in the SructureSelector web server (SIF 4), using the CLUMPAK software to generate graphs from STRUCTURE outputs (SIF 5) [55-57]. Individuals were assigned to a cluster when the probability of assignation to that cluster was greater than 0.5.

Spatial analyses

Correlation patterns in between geographic distances and genetic distances were tested using Monmonier's algorithm to identify and map genetic boundaries in the landscape, i.e., zones of marked genetic changes [58-60]. Briefly, geographic Euclidean distance matrix was used to define landscape cells around populations with a Voronoi diagram (PASSaGE [61], inputting pairwise genetic distance taking GPS population locations as well as Φ_{PT} generated values as attributes as per the Maximum Difference Barriers (MDB) method. Finally, directional correlograms using the method of Sokal (1986) (Smouse *et al*. 1986), i.e., Windrose correlograms, were computed via PASSaGE software based on Moran and Gary's autocorrelation indexes. A Windrose consists of multiple rings or annuli where each annulus represents a specific distance interval. The outer radius (distance) of the i^{th} annulus is determined by $r_i = C_i^2 + D_i + E$ [61].

RESULTS AND DISCUSSION

Genetic diversity is mainly contained within populations; molecular variance is indeed 87% distributed within population and 13% is amongst populations (SIF 2). Populations are significantly different with a mean Φ_{PT} of 0.125 (95% CI: -0.030; + 0.030). Pairwise differentiation indexes Φ_{PT} are significantly positive (alpha = 0.01) in 33 out of 36 comparisons. The three groups of populations' pairs 6-3, 4-5, and 9-8 are not significantly different (Table 3). The expected number of true populations (i.e., genetic clusters) is 5 (MedMedK, MaxMedK, MaxMeanK, – threshold = 0.05) (Fig 3a).

Table 3 Pairwise Population PhiPT Values from GenAlEx 605 (DATA=379 ind, POP=9)

	Jaipur	Alwar-Bharatpur	Sikar	Churu	Ganganagar	Jodhpur	Pali	Chittaurgarh	Banswara
Jaipur	0,000								
Alwar-Bharatpur	0,029	0,000							
Sikar	0,127	0,082	0,000						
Churu	0,156	0,096	0,144	0,000					
Ganganagar	0,156	0,094	0,137	0,005	0,000				
Jodhpur	0,161	0,104	0,010	0,147	0,128	0,000			
Pali	0,181	0,156	0,195	0,102	0,115	0,191	0,000		
Chittaurgarh	0,198	0,122	0,165	0,183	0,136	0,149	0,252	0,000	
Banswara	0,221	0,141	0,237	0,225	0,163	0,217	0,309	0,015	0,000

Note: Non-significant value is displayed in red

It is possible to determine whether individuals are significantly assigned to a cluster by working from the confidence intervals obtained for the membership coefficients of each individual in each identified group. A first cluster (Fig 3b, in blue, K5-1) includes individuals mainly from populations 1 (Jaipur District) and 2 (Alwar and Bharatpur districts), but is also well represented in populations 8 (Chittaurgarh district) and 9 (Banswara district). A second cluster (Fig 3b, in magenta, K5-2) is only represented in populations 3 (Sikar District) and 6 (Jodhpur District). A third cluster (Fig 3b, in purple, K5-3) groups individuals mostly from population 7 (Pali district), but is also well represented in populations 4 (Churu and Bikaner districts) and 5 (Ganganagar district). A fourth cluster (Fig 3b, in orange, K5-4) is composed of individuals mainly from populations 8 (Chittaurgarh district) and 9 (Banswara district). Finally, a fifth cluster (Fig 3b, in green, K5-5) groups individuals mainly from populations 4 (Churu and Bikaner districts) and 5 (Ganganagar district). Few admixed individuals (Fig 3b, in black) are found in different populations, but they might stand in higher proportion for the population 5.

Windrose correlograms were generated on PASSaGE software to test isolation by distance (IBD) between AGZ as well as amongst AGR, inputting geographic distance data matrices with genetic distance matrices. The pattern given by averaging all the individual correlograms to reflect the gene flux dispersal seems to globally be oriented as per a South-

West/North-East gradient (Fig 3c). MDB analysis reveals the existence of a strong biogeographic barrier isolating populations 8 and 9 from the others (Fig 4).

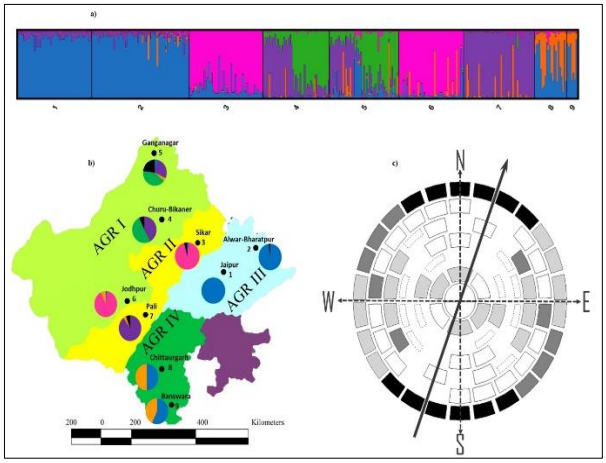

Fig 3 (a) STRUCTURE bar plot generated from CLUMPAK for the most likely number of clusters (i.e. K=5) as a result of the STRUCTURE analysis among ranges; (b) Respective pie charts corresponding to the allocation of populations in Rajasthan for the most likely number of clusters (i.e. K =5) In blue, proportion of individuals significantly assigned to cluster K5_1, in magenta proportion of individuals significantly assigned to cluster K5_2, in purple proportion of individuals significantly assigned to cluster K5_3, in orange proportion of individuals significantly assigned to cluster K5_4 and in green proportion of individuals significantly assigned to cluster K5_5. Percentage of admixed individuals is shown in black and (**c) Average windrose correlogram across all sampled individuals from PASSaGE software based on Moran and Gary's autocorrelation indexes**

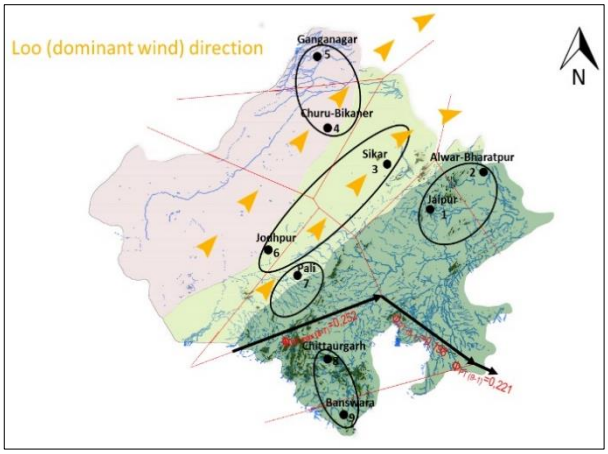

Fig 4 Geographical distribution of the main 5 emerging population clusters from STRUCTURE analysis with Voronoi diagram generated on PASSaGE software

Mean population GPS locations are shown by the black dots. Background map shows the updated Champion and Seth Indian vegetation classification: hyper-arid zones are represented in pink colour, tropical thorn forest is shown in beige and tropical dry deciduous forest is represented by the green coloured zone. Generalized summer wind direction is represented by the black arrow. Main rivers and irrigation systems are indicated in blue colour. Aravalli mountains range is represented in brown colour. Black numbers from 1 to 9 indicate defined population identifiers. Coloured dots represent mean population locations as per UTM WGS 84 (zone 43 North) coordinates. Black arrows form the MDB between Northern and Southern populations as per maximum populations' pairwise ΦPT values.

The genetic diversity of woody species is based on the mechanisms of pollen and seeds dispersal. Gravity, wind, water or transport by other organisms are considered passive mechanisms of dispersal between populations and their variation can significantly shape the dynamics of the range of a species and its responses to different environments [62]. For small populations, maintaining gene flow depends on a sufficient migration rate in source-sink dynamics to offset the effects of drift [41-42]. Because the tree species composing dryland forests are less densely represented than structuring species of temperate forests, and rather form woody patches of open forest [33], such desert trees might be more prone to drift effects due to a reduction in their genetic diversity. Also, in order to understand what structures genetic diversity, it is important to determine the nature of the dynamics involved. Different models have shown that there is an interplay between migration, demography and local adaptation [41], where local adaptation is the result of adaptation conferring on the resident genotype a relatively higher fitness on average than that attributed to a foreign genotype [63]. This latter mechanism is driven by various locally important environmental selection pressures [40]. In the particular case of arid systems, it is questionable whether the trees found in the most arid areas are the result of local adaptation or mishaps where some individuals could locally subsist due to microclimatic conditions in an unfavourable matrix (see *Fagus sylvatica* in the Ciron valley, France). Indeed, if the main dispersal mechanisms are based on dissemination by desert ruminants, it is then to be expected that the dynamics involved would be the result of a greater stochasticity due to the exploratory behaviour of the animals that feed. On the other hand, if anemochory is responsible of the dispersal, the dynamics would a priori be more regular. In the case of hydrochory based on the seasonality of river floods, a certain regularity in the dynamics of dispersion would also be implied. In *P. cineraria*, it is known that different dispersal mechanisms such as pollinating insects, desert ruminants, or the wind may be involved, but the exact dynamics of dispersal remain unclear to date [64-65].

The coupling of analyses based on the study of allelic frequencies with those based on genetic and geographical distance show that *P. cineraria* individuals clustered into 5 groups in Rajasthan. Even if the techniques that we present in this study rely on markers which are not the most resolutive to get a very precise idea of the genetic diversity of the tree, they nevertheless give a good picture of the genetic structure which can help guide the setting of conservation policies for this species. Also, one of the main advantages of the present study lies in the importance of the sampling campaign which was carried out over a large part of the territory of Rajasthan – the most important to date on this species – despite the technical problems which may have been encountered due to very harsh climatic conditions (temperatures up to 50°C at times), short funding, language barriers (when interacting with locals speaking different dialects depending on the district) and all international and local administrative documents and authorizations which took a particularly long time to obtain.

Based on our results, anemochory could be the prime passive mechanism responsible for dispersal. Indeed, results indicate that genetic structure of the species seems to be mainly influenced by climatic belts, through wind dispersal along the circulation corridors of warm pre-monsoon winds,

and along the precipitation gradient (Fig 4). This is consistent with previous genetic studies on other botanical models that have shown that the genetic structure of keystone forest species such as *Fagus sylvatica* or *Cyperus papyrus* is influenced by wind circulation [66-67]. Our results show there is therefore a distinct stand for the hyper-arid zone (populations 4 and 5), a stand for the tropical thorn forest zone (populations 6 and 3), and an emerging stand for the southernmost dry deciduous forest zone (populations 8, 9, 1 and 2). This distribution seems to be divided by a strong biogeographic barrier formed by the Aravalli Range, and this is further confirmed by the MDB (Fig 4). Thus, populations 8 and 9, standing both below the Aravallis, and in a much rainfed climatic zone where forest assemblages are different from those in arid areas in the North, are genetically isolated from other sampled populations. *P. cineraria* seems hence to be genetically structured at a finer geographic scale than the one of Rajasthan, suggesting a potential local adaptation to the same. There seem indeed to be different populations of *P. cineraria* in this state, and these populations seem globally genetically differentiated according to the AGZ in which they are located, and even more precisely, according to AGR.

Water dispersal could be secondarily involved and would affect newly established populations in the alluvial plains. Indeed, one population sampled in the Pali district seems to stand out somewhat while being associated with the group located northward to the Aravalli Range (populations 1 to 6). This population alone contains the majority of individuals associated with the cluster 3 represented in purple colour on the STRUCTURE barplots (Fig 3). Looking at the geomorphology of the area to which this population is affected, it appears that the sampled trees are established in the Luni River watershed, which rises below the state of Rajasthan in Gujarat (Fig 1). The trees sampled in this region were all rather bushy, competing with other species, such as the invasive *Prosopis juliflora*, and had a rather small trunk diameter, suggesting that they formed a recently established stand. It has also been shown that young shoots of *P. cineraria* settle preferentially in the alluvial plains [26]. It is thus possible that this stand is genetically distinct from the other stands in the north of the Aravallis because of its recent establishment, and some seeds may have been transported from the neighbouring state of Gujarat through waters of the Luni River during flooding time. Indeed, a relatively recent study has already documented the hydrochory for another tree of the same genus (*P. Laevigata*) in the Mexican Chihuahuan desert [68], where 50% of the tested seeds floated after 30 days in water, leading to the conclusion that flotation reflects an ability to float in temporary streams and facilitate dispersal in natural habitat. Those first results now need to be verified by further investigations.

In some areas, the genetic structure of *P. cineraria* in Rajasthan may also have been partly influenced by human intervention. It has for instance already been admitted that a thorn-less variety of *P. cineraria* locally called “Thar Shoba” originating from the Thar Desert has been selected over generations by horticultural research of the Central Institute for Arid Horticulture (CIAH) from 1995 to 2002 and is propagated vegetatively to be distributed to farmers [69]. This variety shows significant genetic differentiation from other natural populations[70]. With little or no perspective on the issue at the time of our sampling campaign, we did not perform any special sampling effort on these “thorn-less” individuals, and most of our trees sampled had thorns (408/414 but 4 “thorn-less” individuals from Ganganagar district, in the Thar Desert and 1 from Jaipur area). We noted a posteriori from the sampling campaign that there were, in addition to marked differences in terms of thorns number, important differences at size and shape levels of the individuals, in particular between the arid and semi-arid zones located northward of the Aravallis and the humid plains located southward of the mountain range, in and around Banswara district, where the trees rose much more than usual.

This study serves as a basis for future analyzes which would take into account these considerations, which seem to converge on a potential human influence (or perhaps domestication for some varieties like the “Thar Shoba”), at least on a part of the territory, in particular at the level of the semi-arid central zone extending from Jodhpur districts (Pop 6 sampling area) to Nagaur (between Pop 3 and Pop 6 sampling area). Indeed, it turns out that this area is historically associated with the activity of the Bishnoi community of which *P. cineraria* is the totem tree [29].

Moreover, it is within this same zone, which extends more widely to the Shekhawati region (encompassing the district of Sikar, i.e., Pop 3) that an abnormally high mortality rate has been observed during the past decades. Since it is admitted that artificial plantations can decrease the genetic diversity of wild stands [71], a plausible explanation could come from the fact that there has been domestication and/or artificial selection of *P. cineraria* with potentially over-representativeness of suckering, and that this has led to an impoverishment of the genetic diversity within the varieties domesticated locally because of a “replacement” by a spatially dominant variety. This phenomenon would then correspond to a genetic bottleneck effect (a rapid reduction in genetic diversity) that would affect the adaptive potential of the trees, therefore putting them potentially at risk of inbreeding depression, and at the same time making them more vulnerable to global changes and/or diseases.

Consequently, and for conservation purposes, it would be wise to test for inbreeding depression due to potential bottlenecks using coalescent methods with more resolutive markers in further studies. During the time of our study, new molecular markers were developed on the species like Simple Sequence Repeats (SSR) markers [72-73] but also Start Codon Targeted polymorphism (SCoT) markers [74], while chloroplast genome has just been sequenced for the species [75]. Those could be prime tools to determine the level of gene flow and connectivity of *P. cineraria* populations from different locations. Further research related to the same should now prioritize the elucidation of how does genetic structuring match with phenotypic differences. This could be done by the instauration of a common garden test protocol [40], [76]. The common garden experiment is one of the most useful experimental tools for the study of local adaptation. It overcomes the problems posed by the influence of the environment on phenotypes (due to phenotypic plasticity) by placing all the individuals studied in the same environment. The methodology implies to use the theoretical framework of quantitative genetics. In particular, the comparison between Q_{ST} and F_{ST} indexes is a relatively common test to know if a given trait is subject to local adaptation.

A deeper investigation of *P. cineraria*’s ecology in India will also be necessary so it will help answering what are the main drivers of genetic differentiation at a more local scale. To assess which drivers are responsible for dispersal of the species, a call for a biogeographical study at the distribution range level of the species is also required. Regarding human intervention, the need is to dig out how did

and how much people shaped demography and distribution of the species. For more clarity on the status of the species in Rajasthan, it would be interesting to implement a more systematic method of tree mapping because it lacks an overall vision. This can be done by establishing a monitoring network with regularity in the method so that the population status of the stands can be discussed. For the moment, we do not really know if the observed differences between populations are more related to genetic factors or environmental ones. To elucidate this, it would be necessary to conduct provenance tests (common garden test) or even more accurate tests such as "reciprocal transplant" [63]. On a very large scale, this could be a stepping stone for conserving the species and lead to a breeding program, as it is done for instance for the maritime pine *Pinus pinaster* in southwestern France[19] as part of forestry management.

CONCLUSION

This is a first report on population genetics of *Prosopis cineraria* in Rajasthan on a final data set of 379 individuals from 9 different agro-climatic zones, using ISSR and DAMD highly polymorphic markers. The molecular technique is a single-primed PCR reaction allowing the optimization of the cost/efficiency ratio of molecular analyses. Structural results reveal that genetic diversity of *P. cineraria* in Rajasthan is distributed into 5 clusters. The main outcome is that genetic distribution appears to be divided by a strong biogeographic barrier (Aravalli range) and seems to follow the circulating corridors of warm pre-monsoon winds as well as a rainfall gradient. This distribution seems consistent with the spatial division of Rajasthan into 4 of the 5 existing agro-climatic regions (clusters 1, 2, 4 and 5 in Fig 3b), but a partial human intervention might be reflected in the geographic distribution of the third cluster (Fig 3a-b, in purple, K5-3). For conservation of genetic diversity of *P. cineraria*, the populations of cluster 5 and cluster 6 may be given priority which are located at Ganganagar and Jodhpur. The cluster 5 is having the most diverse population and cluster 6 has the genotypes which are not reflected in cluster 6. Those first results are very valuable considering the population decline of *P. cineraria* due to modern human impact, as well as the small number of published studies to date on the genetic diversity of desert woody species and can be considered as a first step towards the establishment of a conservation program for *P. cineraria* in its distribution range.

Declarations

The authors of the manuscript have no conflict of interest.

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