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Geographic isolation affects inter- and intra-specific seed variability in the *Astragalus tragacantha* complex, as assessed by morpho-colorimetric analysis

Oscar Grillo [a,b,*,1,2], Efisio Mattana [a,1], Giuseppe Fenu [a,1], Gianfranco Venora [b,2], Gianluigi Bacchetta [b,2]

[a] Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISVA), Università degli Studi di Cagliari, V.le S. Ignazio da Laconi 11-13, 09123 Cagliari, Italy
[b] Stazione Consorziale Sperimentale di Granicoltura per la Sicilia, Via Sirio 1, 95041 Borgo Santo Pietro – Caltagirone (CT), Italy

A R T I C L E   I N F O

A B S T R A C T

The effect of geographical isolation on the inter- and intra-specific seed variability of the *Astragalus tragacantha* complex was investigated by using a computer-aided imaging system. In particular, seed morphometric and colorimetric features of *Astragalus balearicus*, *A. tegulensis*, *A. terraccianoi*, *A. thermensis* and *A. tragacantha* were measured and the data were used to discriminate among regions of provenance, taxa and populations. Discriminant analysis of the data supports the accepted taxonomy of the group and the hypothesis that interspecific differentiation resulted from geographical isolation. The results also showed a positive correlation between the wideness of species' distribution and the levels of seed intra-specific variability.

1. Introduction

The genus *Astragalus* (Fabaceae) is estimated to contain 2300 (largely based on currently accepted names in [1]) to 2500 species in 245 sections [2]. The *Astragalus tragacantha* L. complex represents a taxonomically critical group, which deserves in-depth taxonomical investigations [3]. In addition to *A. tragacantha*, the complex includes *A. balearicus* Chater, *A. tegulensis* Bacch. and Brullo, *A. terraccianoi* Vals. and *A. thermensis* Vals. [4,5]. This complex is referred to the sect. *Melanocercis* Bunge of subgen. *Cercidothrix* Bunge [6,7] based on its spiny suffruticose habit, medifixed black and hyaline hairs, imparipinnate leaves with a spine-like rachis, stipules adnate to the petiole, flowers borne in racemes, tubular calyx, and legume exceeding the calyx [4]. These taxa have generally narrow distributions, scattered in the western Mediterranean area, where they grow in rocky or sandy places [4]. In particular, *A. tragacantha* is distributed along the coasts of Provence (France), Catalonia (Spain) and Algarve (Portugal) [5,8]; *A. balearicus* is endemic to the Balearic Archipelago (Mallorca, Menorca and Cabrera; [9]); *A. thermensis* is endemic to N Sardinia [8]; *A tegulensis* is known from a single population in SW Sardinia [4]; and *A. terraccianoi* is endemic to the NW part of Sardinia and southern Corsica [8,10].

The taxonomic treatment of *A. terraccianoi* is controversial. Jeanmonod and Schlüssel [11] suggested that Corsican populations of this *taxon*, should be considered as *A. tragacantha* subsp. *terraccianoi* (Vals.) Jeanm., due to the high similarity of these plants with those of *A. tragacantha* occurring in Provence.

Using seed image analysis techniques, Bacchetta et al. [10,12] were able to confirm the morphological differences between the Cyrno-Sardinian species of the *A. tragacantha*

* Corresponding author. Centro Conservazione Biodiversità (CCB), Dipartimento di Scienze della Vita e dell'Ambiente (DISCIVA), Università degli Studi di Cagliari V.le S. Ignazio da Laconi 11-13, 09123 Cagliari, Italy.
E-mail address: oscar.grillo.mail@gmail.com (O. Grillo).
[1] ccb@unica.it, bg-sar@unica.it.
[2] biologia@granicoltura.it.

complex and in particular, the validity of the new species (*A. tegulensis*) proposed by Bacchetta and Brullo [4], as well as to detect the high variability of seed morphometric and colorimetric parameters among Sardinian and Corsican populations of *A. terraccianoi*.

The aims of the current study were to:

- use discriminant analysis of seed morpho-colorimetric data in the *A. tragacantha* complex to determine if the samples cluster by region of provenance;
- evaluate if differences in seed morphology support the current taxonomic treatment of the complex at issue among all Mediterranean taxa;
- determine the patterns and levels of seed intra-specific variability, with particular focus on the widespread *A. tragacantha* and *A. terraccianoi*.

2. Materials and methods

2.1. Seed lot details

Seeds of two populations of *A. balearicus*, one of *A. tegulensis*, three of *A. terraccianoi*, two of *A. thermensis* and six of *A. tragacantha*, for a total of 1326 seeds, were collected in the field (Table 1 and Fig. 1), stored in the Sardinian Germplasm Bank (BG-SAR), and prepared for image analysis following the protocol of Bacchetta et al. [12].

2.2. Image analysis

Digital images of seed samples were acquired using a flatbed scanner (Epson GT-15000) with a digital resolution of 200 dpi and a scanning area not exceeding 1024 × 1024 pixel. Image acquisition was performed before drying the seeds at 15 °C to 15% of relative humidity in order to avoid spurious variation in size, shape and colour. The scanner was calibrated for colour matching following the protocol of Shahin and Symons [13] before image acquisition, as suggested by Venora et al. [14]. Samples of 100 seeds, randomly disposed on the flatbed tray, were acquired and used for the digital image analysis.

For accessions of fewer than 100 seeds, the analysis was performed on the whole batch.

Digital images of seeds were analysed using the software package KS-400 V. 3.0 (Carl Zeiss, Vision, Oberkochen, Germany). The accuracy and speed of measurements was maximized by running an automated macro specifically developed for the characterization of wild seeds (Bacchetta et al. [12]; with later enhancements by Mattana et al. [15]). Thirty-four morpho-colorimetric quantitative variables, describing seed size, shape and colour, were measured by computer vision. In addition, the mean seed weight of each accession (calculated from 20 seeds, each weighed 10 times, on a four decimal places scale) was included to increase the discriminant power of the statistical analysis [16] (Appendix A).

2.3. Data analysis

Statistical analyses were performed with the software SPSS release 15 [17], applying the same stepwise linear discriminant analysis (LDA) algorithm suggested by Grillo et al. [18]. This approach is commonly used to classify/identify unknown groups characterized by quantitative and qualitative variables [19,20], finding the combination of predictor variables with the aim of minimizing the within-class distance and maximizing the between-class distance simultaneously, thus, achieving maximum class discrimination [21,22]. A cross-validation procedure was applied to test the performance of the classifiers, as reported by Bacchetta et al. [23]. Following this approach, statistical classifiers were developed in order to distinguish sample clusters referred to the provenance regions of the seed accessions and the studied seed lots both at taxa and population level. To graphically highlight the differences among populations, a box plot was drawn using the Mahalanobis' square distance values [24]. This measure of distance is defined by two or more discriminant functions and ranges from 0 to infinite. Samples are increasingly similar as Mahalanobis' square distance approaches zero [12]. A Kruskal–Wallis test was performed to test for the differences in the Mahalanobis' square distance median values among the studied groups. Linear regression was

Table 1
Studied accessions.

| Code	Taxon	Locality	Mean elevation (m a.s.l.)	Geographic coordinates		Seed number
				N	E	
BA1	*A. balearicus*	Cala El Pilar (Balearic Islands)	10	40° 03.043′	03° 58.706′	89
BA2		Cap de Favàritx (Balearic Islands)	14	39° 59.785′	04° 15.481′	100
TEG	*A. tegulensis*	Capo Teulada (Sardinia)	3	38° 53.750′	08° 38.607′	100
TE1	*A. terraccianoi*	Alghero (Sardinia)	45	40° 36.355′	08° 09.062′	39
TE2		Stintino (Sardinia)	37	40° 57.969′	08° 12.189′	100
TE3		Bonifacio (Corsica)	73	41° 22.678′	09° 10.731′	100
TH1	*A. thermensis*	Badesi mare (Sardinia)	5	40° 58.448′	08° 51.245′	100
TH2		Foci del Coghinas (Sardinia)	11	40° 56.336′	08° 49.040′	100
TR1	*A. tragacantha*	Les Goudes (France)	15	43° 12.811′	05° 20.550′	98
TR2		Calanques de Marseilleveyre (France)	5	43° 12.524′	05° 22.389′	100
TR3		Maronaise (France)	10	43° 17.592′	05° 21.236′	100
TR4		Pomegues Island (France)	12	43° 16.269′	05° 17.883′	100
TR5		Mont Redon (France)	180	43° 14.516′	05° 25.919′	100
TR6		Colle Longue (France)	130	43° 38.360′	07° 06.254′	100
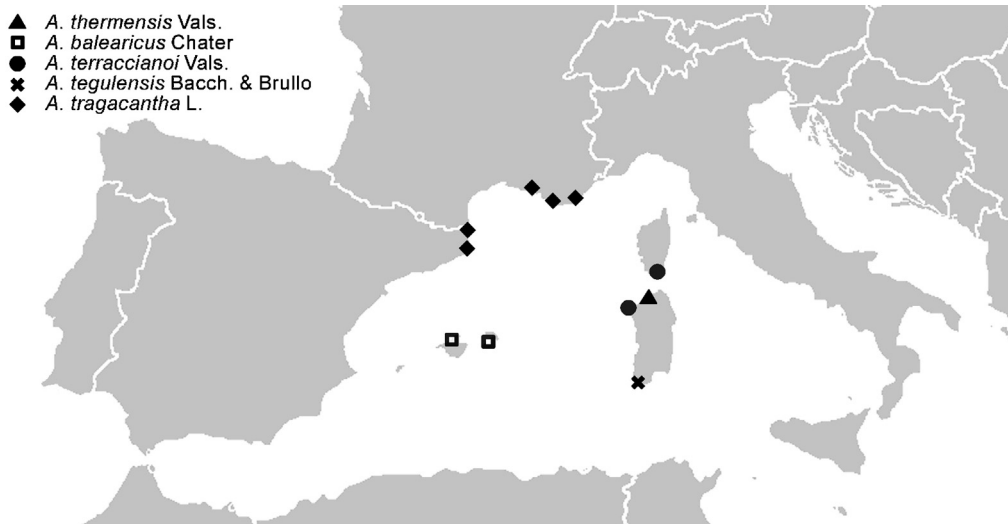
Fig. 1. Distribution of the Mediterranean taxa belonging to the *Astragalus tragacantha* complex (from [8] modified).

modeled considering the number of studied populations and the Mahalanobis' square distance values in order to evaluate if the populations amount affects the intra-specific variability of *Astragalus* species and the number of discriminant steps (and consequently the number of used features).

3. Results

The analysis of seed characters was highly effective in grouping samples by region of provenance, with 95.3% overall accuracy (data not shown). Specifically, all the seeds from the Balearic Islands were correctly assigned, and no seeds from other regions were mistakenly assigned to this group (Fig. 2). Seeds from Provence were correctly discriminated with 98.7% accuracy, while only 1.3% was

wrongly classified as Corsican. Contrastingly, seeds from Corsica showed the lowest percentage of correct assignment (69.0%), due to the assignment of 28.0% of them to Provence and 3.0% to Sardinia regions. Finally, seeds from Sardinia were correctly assigned with 94.6% accuracy, and errors distributed between Corsica (3.2%) and Provence (2.2%).

Applying the same statistical model, the five studied taxa of *A. tragacantha* group were analysed achieving, an overall cross-validated correct identification of 95.5% (Table 2). The seeds of *A. balearicus*, *A. tegulensis* and *A. thermensis* were perfectly identified; those of *A. tragacantha* were correctly recognized in 99.8% of the cases and only one of the 598 analysed seeds was mistaken for *A. terraccianoi*. Finally, the seeds of *A. terraccianoi* were correctly identified in 75.3% of the cases with most misclassifications due to attributions to *A. tragacantha* (23.4%).
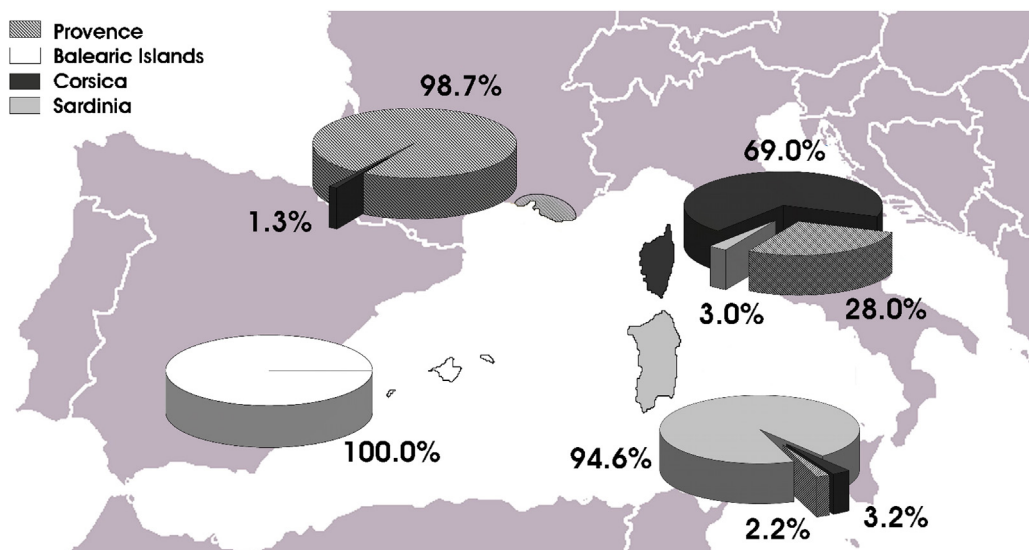

Fig. 2. Discriminant analysis carried out for regions of provenance (without taking into account the taxa).

Table 2
Cross-validated percentages of correct classification for the *Astragalus* taxa classifier.

Taxon	A. balearicus	A. tegulensis	A. terraccianoi	A. thermensis	A. tragacantha	Total
A. balearicus	100.0 (189)	–	–	–	–	100.0 (189)
A. tegulensis	–	100.0 (100)	–	–	–	100.0 (100)
A. terraccianoi	–	0.8 (2)	75.3 (180)	0.4 (1)	23.4 (56)	100.0 (239)
A. thermensis	–	–	–	100.0 (200)	–	100.0 (200)
A. tragacantha	–	–	0.2 (1)	–	99.8 (597)	100.0 (598)
Overall						95.5 (1326)

The number of seeds are given in brackets.

Table 3
Cross-validated percentages of correct classification for the *Astragalus tragacantha* and *Astragalus terraccianoi* populations' classifier.

Population	TE1	TE2	TE3	TR1	TR2	TR3	TR4	TR5	TR6	Total
TE1	61.5 (24)	33.3 (13)	5.1 (2)	–	–	–	–	–	–	100.0 (39)
TE2	10.0 (10)	90.0 (90)	–	–	–	–	–	–	–	100.0 (100)
TE3	–	–	82.0 (82)	6.0 (6)	1.0 (1)	1.0 (1)	6.0 (6)	1.0 (1)	3.0 (3)	100.0 (100)
TR1	5.1 (5)	–	–	89.8 (88)	–	–	–	–	5.1 (5)	100.0 (98)
TR2	–	–	–	–	22.0 (22)	23.0 (23)	20.0 (20)	14.0 (14)	21.0 (21)	100.0 (100)
TR3	–	–	–	–	12.0 (12)	51.0 (51)	16.0 (16)	14.0 (14)	7.0 (7)	100.0 (100)
TR4	2.0 (2)	–	–	–	19.0 (19)	16.0 (16)	30.0 (30)	14.0 (14)	19.0 (19)	100.0 (100)
TR5	4.0 (4)	–	–	1.0 (1)	14.0 (14)	6.0 (6)	9.0 (9)	47.0 (47)	19.0 (19)	100.0 (100)
TR6	–	–	–	1.0 (1)	15.0 (15)	12.0 (12)	12.0(12)	23.0 (23)	37.0 (37)	100.0 (100)
Overall										60.5 (837)

The number of seeds are given in brackets.

In order to compare populations of the two more widespread species and to evaluate their inter-population variability, a separate comparison was carried out among the three populations of *A. terraccianoi* (two from Sardinia and one from Corsica) and the six Provencal populations of *A. tragacantha*. The analysis achieved an overall cross-validated discrimination percentage of 60.5% (Table 3). The seeds of the two Sardinian populations of *A. terraccianoi* (Alghero and Stintino) were correctly identified in 61.5% and 90.0% of the cases, respectively, with misattributions mainly due to the mistakes between them. Sardinian seeds of *A. terraccianoi* were never wrongly assigned to populations of *A. tragacantha*. On the other hand, the Corsican population of *A. terraccianoi* was correctly discriminated at

82.0%, with misidentified seeds being assigned exclusively to the Provencal populations of *A. tragacantha*. The six populations of *A. tragacantha* achieved percentages of correct attribution ranging between 22.0% (Calanques de Marseilleveyre) and 89.8% (Les Goudes) and showed high intra-specific similarity with other populations (Table 3).

To evaluate the inter- and intra-specific morpho-colorimetric variability of the studied taxa, the Mahalanobis' square distance values, among the discriminant functions achieved by the LDA and used to distinguish the analysed seed groups, were used to highlight the spatial dispersion among the compared populations. Fig. 3 shows the graphical representation of the variability of the data points for the analysed species distinguished for population. *A. tegulensis*
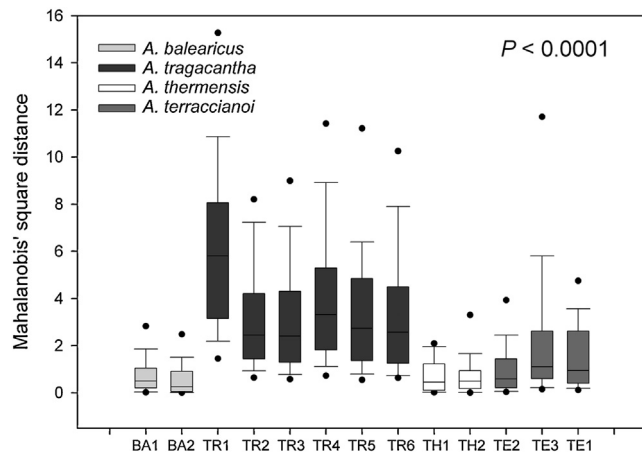
Fig. 3. Spatial dispersion of intra-population data highlighted by Mahalanobis' square distance for each population in comparison with the others of the same species (*Astragalus tegulensis* populations was not analysed). Dots represent 5th and 95th percentile outliers. $P < 0.0001$ by non-parametric Kruskal–Wallis test.

Table 4

Number of populations, discriminant steps and performance of identification. Ranking of the best key discriminant parameters. Number of populations, number of steps required by LDA, the best discriminant parameters and the percentage of correct classification are reported.

	A. balearicus	A. tragacantha	A. thermensis	A. terraccianoi
Number of populations	2	6	2	3
Number of steps	3	14	4	9
1st discriminant parameter	D_{mean} (0.565; 196.279; 0.798)	K (0.882; 16.476; 0.140)	D_{mean} (0.008; 34.215; 0.369)	B_{sd} (0.110; 62.019; 0.056)
2nd discriminant parameter	SqD_{sum} (0.578; 56.437; 0.505)	S_{sd} 0.033; 8.460; 0.132)	B_{mean} (0.173; 32.980; 0.367)	B_{mean} (0.616; 24.015; 0.044)
3rd discriminant parameter	H_{sd} (0.965; 38.003; 0.467)	L_{sd} (0.017; 5.375; 0.128)	S_{mean} (0.010; 19.678; 0.346)	D_{sd} (0.014; 16.479; 0.042)
4th discriminant parameter	–	R_{sd} (0.018; 4.818; 0.128)	Rf (0.964; 3.953; 0.320)	L_{sd} (0.023; 12.877; 0.041)
5th discriminant parameter	–	D_{sum} (0.043; 4.454; 0.127)	–	R_{sd} (0.039; 10.694; 0.040)
Percentage of correct identification between populations	89.9%	51.8%	93.5%	91.6%

For each parameter, the tolerance, F-to-remove and Wilks' lambda values are reported in brackets.

was excluded from this analysis since it was represented by a single population. Intra-specific variability varied among taxa with Mahalanobis' square distance values ranging from 0.0005 to 5.8576 (A. balearicus) and from 0.1010 to 21.5258 (A. tragacantha). The most variable A. tragacantha population was that of Les Goudes (France), with Mahalanobis' square distance values ranging from 0.7648 to 21.5258 (data not shown). A highly significant correlation between the number of populations and the intra-specific variability was found ($R^2 = 0.998$ and $P < 0.001$).

The best discriminating variables chosen by the stepwise method from among the 34 studied variables are shown in Table 4. Discrimination was achieved by colorimetric features (RGB and HLS channels) and densitometric descriptors, except for A. thermensis in which the fourth best discriminating parameter was the roundness factor (Rf), which also displayed the lowest F-to-remove value among the best five-discriminant parameters in the studied species. A significant linear correlation ($R^2 = 0.933$ and $P < 0.05$) was found between the number of discriminant steps (and consequently the number of used features) and the number of investigated populations per species.

4. Discussion

In this study, the analysis of seed features was generally supportive of the taxonomy of the A. tragacantha complex, as proposed by Valsecchi [8]. Moreover, seed morphology likely reflects the geographic provenance of seed collections (without considering taxonomy). Actually, in the western Mediterranean basin, high endemism is related to the age of the geological platform [25] previously reported for Sardinia, Corsica and the Balearic Islands for the Araceae [26]. In the current study, the observed pattern of strong morphological and colorimetric differentiation of the A. balearicus seeds is indicative of the relatively long period of geographic isolation of the Balearic Islands. The observed amount of colorimetric and morpho-metric overlap among the studied populations well explains the distribution of the seed misidentifications. The influence of Provencal populations on the other ones decreases with

latitude, whereas the pressure of Sardinian populations increases with the same criterion, while the influence of the Corsican one seems to be directly proportional to the geographical distances in both directions.

Differentiation in seed characters at inter-specific level is likely driven by geographical isolation. A. balearicus, A. tragacantha, A. thermensis and A. tegulensis are all strongly differentiated, as recently highlighted by Bacchetta and Brullo [4]. The Sardo-Corsican population of A. terraccianoi is less distinct, supporting the findings of Jeanmonod and Schlüssel [11] and suggesting the need of additional taxonomic studies. Populations of this species from Provence and Corsica have recently been found to form two well supported subclades, within the monophyletic clade of A. tragacantha [27].

The wide geographical distribution and relatively larger population size of A. tragacantha and A. terraccianoi with respect to the three narrowly distributed species (A. balearicus, A. thermensis and A. tegulensis), is likely to be the driver of higher intra-specific morpho-colorimetric variability. This relatively high intra-specific variability explains the higher percentage of misassignment of A. terraccianoi seeds compared to those of all the other species in the study. The reduced genetic diversity is often found in populations of endemic and rare plant species [28,29], however, the degree to which phenotypic variability in the seed characters is correlated with genetic diversity remains to be investigated.

This study testifies the usefulness of discriminant analysis, based on morpho-colorimetric seed features, in taxonomic studies and sheds light on the effect of the number of sampled populations on the discriminatory power of the method.

By evaluating the contribution of the variables, using the discrimination algorithm (LDA), it was possible to identify the features that, more than others, were relevant for the discrimination of the Astragalus taxa included in this study, noting that the number of need steps, and consequently, the necessary parameters is proportional to the number of available populations and so the number of analysed seeds, affecting the sample variability and the performance of the identification systems.

Disclosure of interest

Acknowledgements

Appendix A. List of morphometric and colorimetric measured features on seeds.

	Feature	Description
A	Area	Seed area (mm^2)
P	Perimeter	Seed perimeter (mm)
P_{conv}	Convex perimeter	Convex perimeter of the seed (mm)
P_{Crof}	Crofton's perimeter	Perimeter of the seed calculated using the Crofton's formula (mm)
P_{conv}/P_{Crof}	Perimeter ratio	Ratio between convex and Crofton's perimeters
D_{max}	Max diameter	Maximum diameter of the seed (mm)
D_{min}	Min diameter	Minimum diameter of the seed (mm)
D_{min}/D_{max}	Feret ratio	Ratio between minimum and maximum diameters
Sf	Shape factor	Seed shape descriptor = $(4\pi \times \text{area})/\text{perimeter}^2$ (normalized value)
Rf	Roundness factor	Seed roundness descriptor = $(4 \times \text{area})/(\pi \times \text{max diameter}^2)$ (normalized value)
Ecd	Eq. circular diameter	Diameter of a circle with an area equivalent to that of the seed (mm)
EA_{max}	Maximum ellipse axis	Maximum axis of an ellipse with equivalent area (mm)
EA_{min}	Minimum ellipse axis	Minimum axis of an ellipse with equivalent area (mm)
R_{mean}	Mean red channel	Red channel mean value of seed pixels (grey levels)
R_{sd}	Red std. deviation	Red channel standard deviation of seed pixels
G_{mean}	Mean green channel	Green channel mean value of seed pixels (grey levels)
G_{sd}	Green std. deviation	Green channel standard deviation of seed pixels
B_{mean}	Mean blue channel	Blue channel mean value of seed pixels (grey levels)
B_{sd}	Blue std. deviation	Blue channel standard deviation of seed pixels
H_{mean}	Mean hue channel	Hue channel mean value of seed pixels (grey levels)
H_{sd}	Hue std. deviation	Hue channel standard deviation of seed pixels
L_{mean}	Mean lightness channel	Lightness channel mean value of seed pixels (grey levels)
L_{sd}	Lightness std. deviation	Lightness channel standard deviation of seed pixels
S_{mean}	Mean saturation channel	Saturation channel mean value of seed pixels (grey levels)
S_{sd}	Saturation std. deviation	Saturation channel standard deviation of seed pixels
D_{mean}	Mean density	Density channel mean value of seed pixels (grey levels)
D_{sd}	Density std. deviation	Density channel standard deviation of seed pixels
S	Skewness	Asymmetry degree of intensity values distribution (grey levels)
K	Kurtosis	Peakness degree of intensity values distribution (densitometric units)
H	Energy	Measure of the increasing intensity power (densitometric units)
E	Entropy	Dispersion power (bit)
D_{sum}	Density sum	Sum of density values of the seed pixels (grey levels)
SqD_{sum}	Square density sum	Sum of the squares of density values (grey levels)
SW	Seed mean weight	Mean value of seed weight (g)

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