

DOI: [http://dx.doi.org/10.21123/bsj.2021.18.1\(Suppl.\).0708](http://dx.doi.org/10.21123/bsj.2021.18.1(Suppl.).0708)

## Diversity Assessment by Molecular Barcoding and Seed Morphology in *Ricinus communis* L.

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Received 5/4/2020, Accepted 31/1/2021, Published 30/3/2021



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### Abstract

Fourteen morphologically varied *Ricinus communis* L. seeds were collected from different localities in Egypt, El-Sudan and Saudi Arabia. Seed morphology and ITS barcoding analysis were performed to assess their diversity and phylogenetic relationship. Sequence's alignment of nrITS region from different accessions display high levels of genetic similarities. Cluster analysis could not group different accessions according to their geographical distribution. Nevertheless, the genetic barcodes are interestingly matched with the morphological features of the *Ricinus* seeds. In conclusion, seed morphology proved to be a valuable tool in evaluating biodiversity and phylogenetic relationship in plant species with different locations having low levels of genetic diversity such as *R. communis* L. The molecular assessment of morphologically varied *R. communis* L seeds will help breeders for better utilization of germplasm for the variety development and would support the genetic resources management and conservation of castor beans.

**Key words:** Castor, Genetic diversity, ITS-rDNA, Seed size, Seed color.

### Introduction

Castor (*Ricinus communis*) is a dicot tropical perennial shrub plant of family Euphorbiaceae with endospermic seeds which produced within a schizocarpic fruit. Each fruit produced three oblong seeds with distinct convex dorsal side and flat ventral surfaces containing shallow ridge on the testa, called raphae. Castor oil is extracted mainly from the seed endosperm. The seed is externally protected by hard, brittle shining testa mottled brown in colour. The narrow end of the seed where the hilum and the micropyle exist is covered by white spongy bilobed outgrowth called caruncle. The plant is believed to be originated in Africa, however; it has been widely cultivated in tropical and subtropical countries, especially in India, Brazil, and China (1,2). Castor oil is mainly composed of ricinoleic acid (85%) with traces of palmitic acid, oleic acid and linoleic acid that is economically valuable in cosmetics industry, lubricants and biomedical applications (3,4). Those fatty acids give it a potential to be used in biodiesel production (5,6). In addition, the *Ricinus* leaves are

commonly used to feed the Eri silkworm which provides Eri silk fabrics (7).

The *Ricinus* genome has been revealed a complex genetic background of 325 Mb in size distributed in 10 chromosomes. In 2010, a draft genetic map was constructed for castor bean, however; genomic mutation and gene cloning studies are still lacking (8). The castor bean oil is economically valuable, nevertheless; it has a safety concern raised from its high ricin content and a highly toxic ribosome inactivating proteins (3,9).

Therefore, there is an elevated demand to develop new castor cultivars with enhanced yield and yield contributing traits (10). This wouldn't be achieved without improved knowledge of the genetic diversity and molecular biology of the species. Until recent, morphological character and isozymes-based approach were predominated in assessing diversity in castor bean (2). Although these markers suffer from limitation and inconsistency due to their sensitivity to environmental fluctuations. Nowadays, several DNA-PCR based molecular markers are successfully prevailed for assessing

genetic diversity in different plant species. Many DNA markers span genome-wide to constitute a framework for high-throughput analyses of genetic diversity in plants such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphisms (SNP) (1,11–14). Other DNA based markers span only a specific locus within conserved regions of the genome regarded as fingerprint. Therefore, they were perfectly used for plant barcoding at generic level, species levels and sometimes even discriminate varieties (15). Nuclear Internal Transcribed Spacers (nrITS) region encodes rRNA poly-cistronic transcript that will splice down in to mature transcript of three exons in eukaryotes; 18S rRNA ITS1 and 28S ITS2 interspaced by 5.8S rRNA cistron (16). ITS barcode has efficiently boosting the biodiversity evaluation and estimates phylogenetic relationships among different plant species showing high levels of interspecific divergence (17).

The objective of the current study is to assess the genetic diversity and elucidate the phylogenetic relationships of 14 *R. communis* accessions with different seed morphology.

## Materials and Methods

### Sampling and Location

Seeds from fourteen *R. communis* accessions were collected from different geographical localities in Egypt, El-Sudan and Saudi Arabia. The accessions were labelled R1 to R14 and their geographical locations were determined using Google Earth maps (Fig. 1).

### Seed Morphology

About 25 seeds from each accession were imaged using NIKON-COOLPIX L820 digital camera and used to measure seeds area; as an indicator for seed size, and seed color. Seed area was measured as an area of seed image (mm<sup>2</sup>). While, seed coat color was measured as a mean value for greyscale color intensity. Both parameters were measured using imageJ software (18). Their cluster analysis was performed using PAST software Version 3.24 (<https://folk.uio.no/ohammer/past/>) (19).

### DNA Isolation and Gel Electrophoresis

Total genomic DNA was isolated from decoated seeds according to a modified protocol of Harju et al. (2004) (20). The total isolated DNA was column purified using DNA Purification MiniSpin Kit (VIOGENE cat# PF1001) according to manufacturer's protocol. The purified DNA was

resolved on 1% agarose gel prepared in 1× TAE (Tris-acetate-ethylenediamine-tetra- acetic acid) buffer containing 0.5 µg/ml ethidium bromide. Ethidium bromide-stained gel was visualized using UV-transilluminator (Vilber Lourmat-Germany).

### PCR and Sequencing.

Polymerase chain reaction (PCR) was performed using Mytaq Red DNA polymerase master mix (BIOLINE cat # BIO-21108) according to manufacture instructions. Briefly, the reaction contained 1× PCR red master mix buffer, 2.0 µl of 10 pm/µl of each primer [ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3')], 1.0 µl of DNA template (~30 ng), 0.25 µl of MyTaq™ DNA polymerase (5U/µl), and then the total volume was adjusted to 50 µl using sterile water. The amplification reactions were performed in thermal cycler (Biometra, Germany) as follow: 1<sup>st</sup> cycle of 3 min at 95°C for initial denaturation, followed by 35 cycles of 20 sec at 95°C (denaturation), 20 sec at 55°C (annealing), 30 sec at 72°C (extension), then a final extension was carried out for 10 min at 72°C and the reactions were held at 4°C. The PCR products were separated on 1% agarose gel and were purified using PCR-M clean up system (VIOGENE cat# PF1001) according to manufacturer's protocol. The purified samples were sequenced by GATC company using ABI 3730xl DNA sequencer and ITS4 primer.

### Sequencing Data Analysis.

The obtained nucleotide sequences were aligned to the total nucleotide collection of NCBI using Basic Local Alignment Search Tool for nucleotide blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic tree based on nrITS sequences was constructed using UPGMA tree build method in Geneious 8.1.9 software (21).

## Results



**Figure 1. Geographical locations of the 14 *R. communis* accessions using google earth. R1 to R14 accessions: R1 and R14 in Saudi Arabia, R2-R5 and R7-R13 in Egypt and R6 in El-Sudan.**

### Seed Morphological Features of *R. communis*

Seed area for the 14 *Ricinus* accessions was ranged from small to large. Small sized seeds were observed for R4, R5, R8, R12 and R14, medium seeds were noticed for R2, R3, R6, R7, R9 and R10 while, large seeds were observed for R1, R11 and R13. Wide range of seed coat color was demonstrated. White small seeds with less

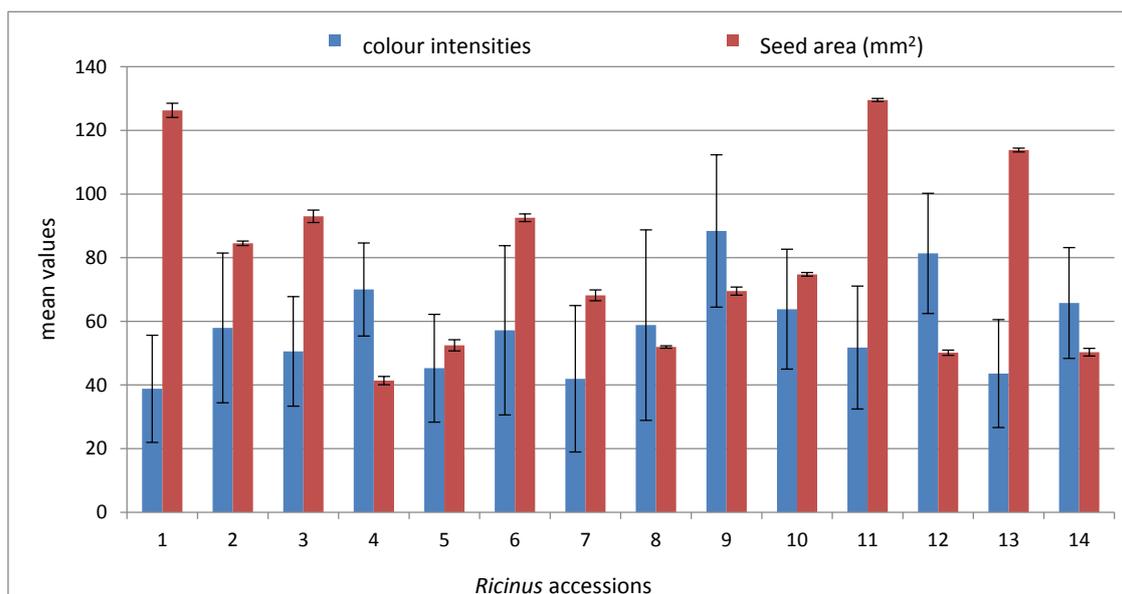
prominent caruncle were observed for R4, R12 and R14, brownish red medium to large seeds with more prominent caruncle and mottle was observed for R2, R3, R6, R11 and R13. Dark chocolate large round seeds with less prominent caruncle and mottle were characteristic for R1 from Abha "Saudi Arabia", similar color was observed for R5 and R8 from Aswan however, the seeds were much smaller and more mottle with less prominent caruncle. Black seeds with less mottle and caruncle were characteristic for R7 from Qena-Egypt. Brown medium size seeds with different mottle pattern was observed for R9 and R10 (Fig. 2).



**Figure 2. Morphological features of the 14 *R. communis* accessions seeds.**

The seed coat color measurements showed that R1 has the lowest greyscale color value of 38.79 indicating less mottled pattern while the accessions R9 and R12 have the highest values of 88.39 and 81.34 respectively indicating high mottled pattern

with large variation between colors on the seed. Large amount of variation was observed for seed area ranged from 41.4 mm<sup>2</sup> to 129.5 mm<sup>2</sup> in R4 and R11 respectively (Fig. 3).

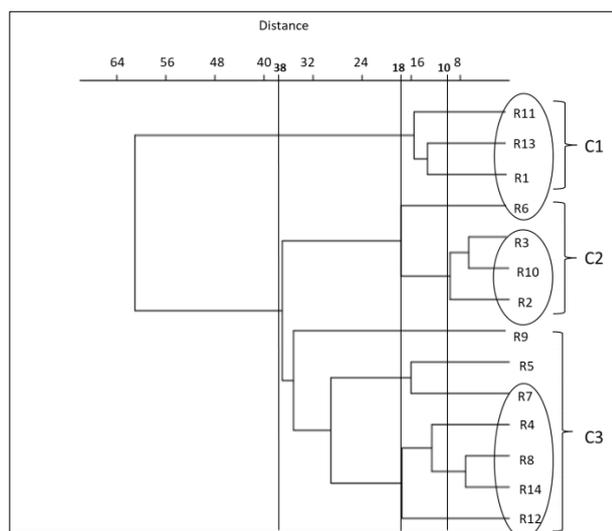


**Figure 3. Average seed coat color intensity and seed area in (mm<sup>2</sup>) of the 14 *R. communis* accessions using imageJ software. Error bars indicating  $\pm$  SD of the replica.**

The cluster analysis based on the average seed coat color and seed area using Euclidean similarity coefficient and paired group algorithm showed three subclades at inter cluster distance of 38. R1, R11 and R13 were grouped in one clade; C1 while R2, R3, R6 and R10 accessions were close to each other

and were separated in the second clade; C2 and the remaining accessions were clustered in the third clade; C3. It worth to note that both accessions collected from Saudi Arabia were not assigned to the same clade; R1 was assigned to C1 clade while R14 was in C3 clade. In contrary, R5 and R8

accessions collected from Aswan were assigned to the same clade; C3 (Fig. 4).



**Figure 4. Cluster analysis based on seed morphological features of the 14 *R. communis* accessions. The encircled groups were performed to easily identify the corresponding groups of the ITS based clustering.**

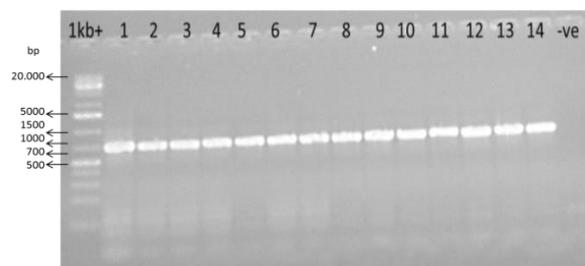
**Table 1. *R. communis* accessions' IDs, their deposited accession numbers in the NCBI gene bank and their locations with coordinates.**

ID	Deposited accession numbers	Location	Coordinates
R1	MN880878	Abha "Saudi Arabia"	18°13'00"N 42°30'00"E
R2	MN880879	Km.47 Alexandria-Matrouh road	31°08'08.2"N 29°49'05.6"E
R3	MN880880	Km.88 Alexandria-Matrouh road	30°50'44.0"N 28°56'46.6"E
R4	MN880881	El-Khanka "Qalyubia"	30°14'01.5"N 31°23'13.2"E
R5	MN880882	Aswan	24°05'20"N 32°53'59"E
R6	MN880883	Shendi-Sudan	16°40'13.9"N 33°27'28.5"E
R7	MN880884	Qena	26°10'12"N 32°43'38"E
R8	MN880885	Sawari- Aswan	24°05'20"N 32°53'59"E
R9	MN880886	El Katameya-Ain El Sokhna road	29.6°N 32.3167°E
R10	MN880887	Cornish El Nile- El-Giza- Cairo	30°00'45.6"N 31°13'02.7"E
R11	MN880888	Tanta	30°47'00"N 31°00'00"E
R12	MN880889	North coastal road	31°01'27.8"N 28°31'02.9"E
R13	MN880890	Fifth Settlement	30°00'27.7"N 31°25'51.6"E
R14	MN880891	Altaaf Saudi Arabia	21°17'39.32"N 40°22'14.39"E

ITS sequences alignment showed strong similarities between all accessions exceeding 82% (Table 2). The highest degree of genetic similarity by value  $\geq 98\%$  was observed between (R2 & R3)

### ITS Barcoding of the *R. communis* Accessions

ITS barcodes showed a fragment length of about 700 bp from all accessions. Negative refers to negative control of the PCR showed no band indicates no PCR contamination (Fig. 5).



**Figure 5. Agarose gel electrophoresis for PCR products amplified from 14 *R. communis* seed accessions (lanes 1 to 14). 1Kb<sup>+</sup> refers to the DNA ladder. -ve refers to negative control of the PCR.**

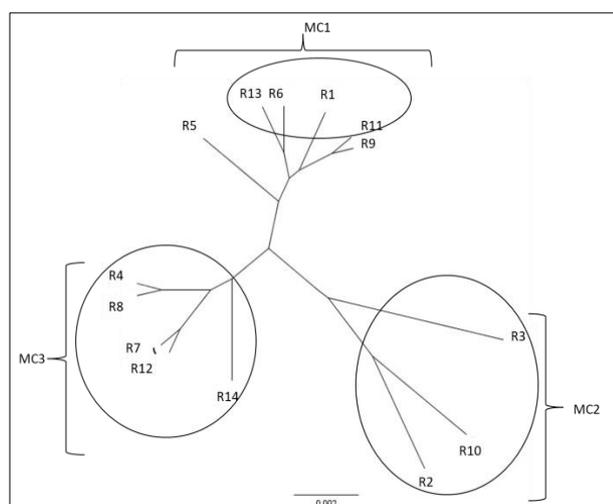
The nucleotide sequences were validated through the National Centre for Biotechnology Information (NCBI) site. All the sequences were deposited into NCBI database and the acquired accession numbers were listed in Table 1.

both from Alexandria-Matrouh road-Egypt, (R9 & R13) from different localities of Lower Egypt region, and (R4 & R8, R12, R13, R14) from different localities of Egypt and Saudi Arabia.

**Table 2. Showing the percent of bases which are identical for ITS sequence alignments of the 14 *R. communis* accessions. Shading indicating the highest degree of similarities.**

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1													
2	96.39	1												
3	95.94	98.13	1											
4	96.82	95.68	95.52	1										
5	83.59	96.10	95.95	97.40	1									
6	84.85	95.53	95.38	97.83	82.98	1								
7	82.84	96.67	96.66	97.40	85.52	84.88	1							
8	84.80	95.81	95.65	98.26	97.28	84.56	84.96	1						
9	84.76	97.11	96.95	97.68	88.99	85.97	90.57	87.44	1					
10	95.81	96.39	96.24	95.82	96.10	95.82	96.53	95.66	97.25	1				
11	84.93	95.53	95.37	97.68	84.28	88.33	83.60	85.32	84.51	95.67	1			
12	82.92	95.82	95.66	98.70	86.25	82.31	85.02	91.16	87.26	96.10	83.94	1		
13	96.97	96.83	96.67	98.26	97.11	82.55	97.96	97.11	98.12	96.54	97.83	97.55	1	
14	96.53	95.54	95.24	98.28	96.82	97.54	97.68	96.67	97.83	95.82	97.11	98.12	96.97	1

The constructed UPGMA phylogenetic tree generated three main clades not in full agreement to their geographical locations. R5 from Aswan, R13, R11, R9 from different locations of Lower Egypt, R6 from El-Sudan and R1 from Saudi Arabia are grouped in clade 1 (MC1), R2, R3 both from Alexandria-Matrouh road (Egypt) and R10 from Cornish El Nile- El-Giza (Egypt) are grouped to the second clade (MC2), while R4, R7, R12 from different locations in Egypt, R8 from Aswan and R14 from Saudi Arabia are grouped together in clade 3 (MC3) (Fig. 6).



**Figure 6. UPGMA phylogenetic tree based on ITS sequences of the 14 *R. communis* accessions. The encircled groups were performed to easily identify the corresponding groups of the seed morphology-based clustering.**

## Discussion

Exploiting the magnitude of diversity among different *R. communis* accessions is a prerequisite for effectual breeding and conservation programs (11). *Ricinus* diversity is broadly explored, however; the current findings still lacking a rational outcome. In the current study, evaluation of *Ricinus* diversity at morphological and molecular levels was achieved.

Seed color and area are essential features used to describe seeds. These parameters are distinguishing features of *Ricinus* seeds that could be employed as a good taxonomic tool to distinguish different species (22–24). Increased greyscale values indicate more variegated colored seeds. However, collected from different habitat of Qalyubia, Aswan, North coastal road and Saudi Arabia, small seeds of accessions R4, R8, R12 and R14 are also the more variegated colored seeds; larger greyscale values. This could be attributed to an increased levels of total phenolics that magnitude seed adaptation against hot climate habitat (23,25,26), or it may be adopted to increase seed persistence in the soil (27). Seeds collected from Upper Egypt region; R5, R7 and R8 displayed relatively closer seeds area and greyscale values, this similarity may be enforced by ecological constraints.

Unexpectedly, seeds collected from Saudi Arabia; R1 and R14 displayed different size and seed coloring pattern that hinder the ecological constraints stimulus. It is worth to note that the clustering analysis based on the compiled seed morphology data generated clustering pattern that unfollow the geographical distribution of the

accessions. At inter cluster distance of 38, the accessions were subdivided into three clades. The first clade includes R1 collected from Abha (Saudi Arabia), R11 from Tanta-Egypt and R13 from Fifth Settlement-Egypt. Careful inspection of accessions clustering in C2 cluster shows accessions of the same geographical origin in the main group of the accessions as R2, R3 both from Alexandria-Matrouh road, and R10 from Cornish El Nile-Giza, all from Lower Egypt region are closer to each other at inter cluster distance of 10 while R6 from Shendi-Sudan belong to this clade at distance of 18. Similar observation was demonstrated in C3 as R5 and R8 accessions collected from Aswan were assigned to the same clade, R7 collected from Qena showed close affinity to R5, interestingly, both located in the Upper Egypt (see Fig. 1). R9, R4 and R12 all from Lower Egypt are assigned to C3 clade, R14 from Altaaf Saudi Arabia was also assigned to this clade. Seeds lean towards altering shape and size under domestication due to fluctuations in developmental condition associated with genomic modification in response to changes in environment (12).

ITS region offers a powerful molecular marker that is used to evaluate phylogenetic relationship among different plant species (15). Various morphological characters were observed among different *Ricinus* accessions although, they are accompanied by limited genetic variation (22, 23). In accordance, 82% up to ~99% of nrITS bases are identical for ITS sequence alignments of the 14 *Ricinus* accessions used in the current study. This may reflect a communal gene pool for the 14 *Ricinus* accessions (28). A cluster analysis of ITS based relationship generates a pattern that does not follow the geographical distribution, which is in accordance with the outcomes generated based on morphological data. In contradiction to the current results, Enan et al., 2012 successfully used the Chloroplast (matK) and (nrITS) sequences to discriminate *Ricinus* accessions to their different geographical distributions (29). Similarly, Manjunath and Sannappa (2014) employed nrITS sequences to distinguish between different *Ricinus* accessions collected from different habitat in India (30). In accordance with the current finding Salihu and his coauthor on 2019 could not find a direct correlation between genetic diversity among 20 Nigerian castor genotypes and their geographical distribution (13). The genetic similarities of *Ricinus* accessions from different locations may be owed to the domestication and immigrants from each other, in addition to random mating within population (12).

The clustering of the 14 *Ricinus* accessions in the Euclidean similarity coefficient fitted with paired group algorithm of seed morphology (Fig. 4) generated three clades; C1, C2 and C3 were in agreement with their clustering using the UPGMA tree based on ITS sequences (Fig. 6), the shared similarities between both trees were encircled, see (Fig 4 and 6). C1, C2 and C3 grouping were compatible with MC1, MC2 and MC3 of the ITS - UPGMA grouping respectively with few exceptions. MC1 clade contains the R1, R11 and R13 accessions that were assigned to C1, in addition R6 from C2, R5 and R9 from C3 were grouped together in the MC1 clade. Genomic adaption associated with developmental changes that may arise due to cultivation could explain these exceptions (12).

A combination of morphological and molecular based parameters may provide a coherent awareness of different *Ricinus* accessions diversity. Seed morphology proved to be an un-neglected parameter in assessing *Ricinus* diversity.

#### Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

#### Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours.
- Ethical Clearance: The project was approved by the local ethical committee in Helwan University.

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## تقييم التنوع في الخروع بواسطة الباركود الجزيئي ومورفولوجيا البذور

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### الخلاصة:

تم تجميع بذور أربعة عشر صنف من أصناف نبات الخروع المتنوع في الشكل من مناطق مختلفة في مصر والسودان والمملكة العربية السعودية. تم فحص مورفولوجيا البذور وتحليل الباركود (ITS) (الفاصل الداخلي المنسوخ لشريط الرنا الريبوسومي) لتقييم تنوعها والعلاقة بين الأصناف. النتائج الوراثية "منطقة الفاصل الداخلي المنسوخ لشريط الرنا الريبوسومي" تُظهر مستويات عالية من التشابه الوراثي. لم يتمكن تحليل شجرة النسب من تجميع المُدخلات المختلفة وفقاً لتوزيعها الجغرافي. ومع ذلك، يتطابق الباركود الجيني بشكل مثير للاهتمام مع السمات المورفولوجية لبذور الخروع. أثبتت الدراسة ان مورفولوجيا البذور أداة قيمة في تقييم العلاقة بين التنوع البيولوجي والتطور الوراثي في الأنواع النباتية ذات المستويات المنخفضة من التنوع الجيني والمنتشرة جغرافياً مثل الخروع، سيساعد التقييم الجزيئي لبذور الخروع المتنوعة شكلياً المرين على الاستخدام الأفضل للبذور لتطوير الأصناف، كما سيدعم إدارة الموارد الوراثية والحفاظ على بذور الخروع.

الكلمات المفتاحية: حجم البذرة - لون البذرة - الخروع- الفاصل الداخلي المنسوخ لشريط الرنا الريبوسومي - التنوع الوراثي