Rev. Int. Contam. Ambie. 38 (Environmental Science and Policy for Sustainable Development) 63-68, 2023
https://doi.org/10.20937/RICA.54614

SHORT COMMUNICATION

CLONING AND ANALYSIS OF THE SEQUENCES OF THE SUPER-SELENIUM-RICH PLANT *Cardamine hupingshanensis*

Análisis y clonación de la planta súper rica en selenio *Cardamine hupingshanensis*

Sheng CAI, Ping YANG, Chen XU and Xiaoning CAI*

School of Food Science, Nanjing Xiaozhuang University, Nanjing 211171, Jiangsu, China

*Author for correspondence: bioxncai@163.com

(Received: October 2021; accepted: March, 2022)

Key words: Internal transcribed spacer, genetic, evolution, hypothesis.

ABSTRACT

We employed a pair of universal primers for PCR amplification of *Cardamine hupingshanensis* leaf DNA collected from the soil of a unique selenium-rich deposit in Yutangba, Enshi, China. Then the PCR products were sequenced to obtain the 852-bp DNA sequences containing internal transcribed spacers (ITS). The ITS DNA sequences of different *Cardamine* species were aligned and spliced to obtain a "reference" sequence. Next, the base differences were compared between the ITS sequences of different *Cardamine* species and the "reference" sequence. Our results showed that *Cardamine hupingshanensis* had the greatest differences (17) compared with the "reference" sequence, much more than those between the ITS sequences of other *Cardamine* species and the "reference" sequence and 3.2 times higher than the average. It is speculated that some *Cardamine* species growing in selenium-rich regions of Enshi for a long time produce genetic variations that can adapt to high selenium soil stress and develop a super selenium enrichment capacity, thus evolving the new species *Cardamine hupingshanensis*. Therefore, we proposed the hypothesis of accelerated evolution of *Cardamine hupingshanensis* under high selenium soil stress.

Palabras clave: espaciadores transcritos internos, genética, evolución, hipótesis.

RESUMEN

Empleamos un par de cebadores universales para la amplificación de PCR del ADN foliar de *Cardamine hupingshanensis* recolectada del suelo de un depósito único rico en selenio en Yutangba, Enshi, China. Luego se secuenciaron los productos de PCR para obtener las secuencias de ADN de 852 pb que contienen espaciadores transcritos internos (ITS). Las secuencias de ADN de ITS de diferentes especies de *Cardamine* fueron alineadas y empalmadas para obtener una secuencia de "referencia". A continuación, se compararon las diferencias de base entre las secuencias ITS de diferentes especies de *Cardamine* y la secuencia de "referencia". Nuestros resultados mostraron

que *Cardamine hupingshanensis* tenía las diferencias más altas (17) en comparación con la secuencia de "referencia", mucho más que las que había entre las secuencias ITS de otras especies de *Cardamine* y la secuencia de "referencia" y 3.2 veces más alta que la media. Se especula que algunas especies de *Cardamine* que crecen en las regiones ricas en selenio de Enshi durante mucho tiempo, producen variaciones genéticas que pueden adaptarse al estrés del suelo con alto contenido de selenio y desarrollar una capacidad de enriquecimiento de súper selenio, evolucionando así a la nueva especie *Cardamine hupingshanensis*. Por lo tanto, proponemos la hipótesis de una evolución acelerada de *Cardamine hupingshanensis* a causa de un elevado estrés de selenio en el suelo.

INTRODUCTION

Cardamine hupingshanensis, a new species discovered in China (Bai et al. 2008), is a super-selenium-rich plant that can tolerate high selenium content in the soil to accumulate selenium in large quantity. Yuan et al. (2013) measured 1965 mg/kg dry weight of selenium in the *Cardamine hupingshanensis* leaves, and Shao et al. (2014) also measured 1427 mg/kg dry weight of selenium in these leaves. It is the only super-selenium-rich plant discovered in China. Its ability to accumulate such a high content of selenium indicates that it has a unique enrichment mechanism. Studies on the physiological, biochemical and molecular mechanisms of selenium enrichment as well as the scientific principle of selenium enrichment of this species may contribute to the genetic regulation of selenium content in agricultural products and provide selenium-rich agricultural products for people in need of selenium (Wang et al. 2021, Zuo et al. 2020).

Cardamine hupingshanensis germplasm resources collected from the field were previously identified by traditional taxonomy, so professional identification requirements were higher and identification by molecular biological means will provide convenience for researchers who lack experience (Ma et al. 2021, Raza and Su, 2020). With the advancement in molecular biology, scientists have developed bar code recognition technology to identify the species (Geng et al. 2018, Zhao and Li 2020). For example, determining plants' internal transcribed spacer (ITS) sequences provides convenience for scientific and technical personnel (Chen et al. 2010, Liu et al. 2022).

In this study, we extract DNA from *Cardamine hupingshanensis* collected from the soil of a unique selenium-rich deposit in Yutangba, Enshi, and amplify the ITS sequences of the plant by PCR with universal primers to obtain the corresponding reference sequences, laying a foundation for the accurate identification of *Cardamine hupingshanensis* germplasm resources. Additionally, the differences in ITS sequences of different *Cardamine* species were analyzed, providing clues for further study.

MATERIALS AND METHODS

Test materials

Cardamine hupingshanensis, collected from selenium-rich soil in Yutangba, Shuanghe Community, Xintang Town, Enshi, Hubei Province, was transplanted into a pot in our laboratory. After three months of growth, fresh young leaves were collected for DNA extraction.

DNA extraction and PCR amplification

DN14 kit produced by Aidlab Biotechnologies Co., Ltd. was used for DNA extraction; 2 × F8 FastLong PCR Master Mix produced by Aidlab Biotechnologies Co., Ltd. was used for PCR amplification (denaturation temperature: 94 ºC, renaturation temperature: 56 ºC, extension temperature: 72 ºC,).

Selected primer sequences

The primers were synthesized by Nanjing GenScript Biotechnology Co., Ltd., and codes, positions and sequences of the primers are listed in **table I**.

TABLE I. PRIMER INFORMATION.

Primer codes	Primer sequences	Primer position	Primer direction
ITC-5	TCGTAACAAGGTTTCCGTAGGTG	18S gene	Forward
ITC-3	CCTCGTGGTGCGACAGGGTC	28S gene	Reverse

PCR product sequencing: after being detected and identified by agarose gel electrophoresis (AGE), the PCR products were sequenced by Nanjing Qingke Biotechnology Co., Ltd., and the DNA sequencing map was displayed by Chromas software.

Sequence analysis

Poor quality parts of the sequences obtained at both ends were removed, and the rest was spliced using Contigexpress software; ITS sequences of other *Cardamine* species required were obtained in NCBI website; variations of ITS sequences of different *Cardamine* species were analyzed using SeqMan, the sub-software of Lasergene 7.1. A phylogenetic tree was constructed using Clustalx1.83 and MEGA6 software.

RESULTS AND ANALYSIS

Acquisition of ITS sequences of *Cardamine hupingshanensis*

A combination of ITC-5 and ITC-3 (primer sequences are listed in **Table I**) was used for PCR amplification of DNA of ITS sequences of *Cardamine hupingshanensis*, and DNA products with a length of about 900 bp were obtained. Results of 1.5% agarose gel electrophoresis of PCR products are shown in **figure 1**.

Then the same primers were used for the sequencing of PCR products to obtain the original DNA sequences. Unreliable parts of the obtained sequences at both ends were removed, and the rest was spliced using Contigexpress software to obtain the 852-bp DNA sequences (He et al., 2020). DNA sequences containing part of 18S gene sequence, complete ITS1 (internal transcriptional spacer 1) sequence, complete 5.8S gene sequence, complete ITS2 (internal transcriptional spacer 1) sequence and part of 28S gene sequence have been submitted to the GenBank, with the sequence ID (Accession number) of MW282045.

Phylogenetic analysis of ITS2 sequences among different species

A phylogenetic tree was constructed for the obtained ITS sequences of *Cardamine hupingshanensis* and the ITS sequences of other *Cardamine* species by neighbor-joining (NJ) using Clustalx1.83 and MEGA 6 software, with the ITS sequence (Accession number: AJ232900) of *Arabidopsis thaliana* as the reference sequence. Results are shown in **figure 2**.

According to **figure 2**, *Cardamine* species were divided into three classes:

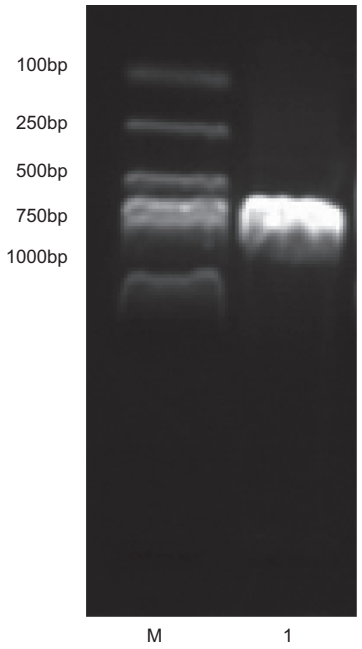

Fig. 1. Agarose gel electrophoresis map of PCR products.

The first class included *Cardamine digitata* (Accession number: EU819328, the same below), *Cardamine purpurea* (EU819358), *Cardamine blaisdellii* (EU819312), *Cardamine cutat* (EU819356), *Cardamine umbellate* (EU819379), *Cardamine victoris* (EU819382), *Cardamine nuttallii* (EU819350), *Cardamine rupicola* (EU819368) and *Cardamine macrophylla* (EU819344).

The second class included *Cardamine amporitana* (AY260598), *Cardamine amara* (KF988052), *Cardamine hirsute* (MH808258), *Cardamine flexuosa* (DQ268409), *Cardamine scutata* (DQ268475), *Cardamine niigatensis* (DQ268493) and *Cardamine clematitis* (EU819318).

The third class included *Cardamine hupingshanensis* (MW282045), which had the longest genetic distance from other *Cardamine* species.

Comparative analysis of ITS sequences of different Cardamine species

To further analyze and compare the variation in the evolutionary process between *Cardamine hupingshanensis* and other *Cardamine* species, these ITS sequences were spliced using SeqMan, the sub-software of Lasergene, to obtain a 617-bp "reference" DNA sequence containing ITS1 sequence,

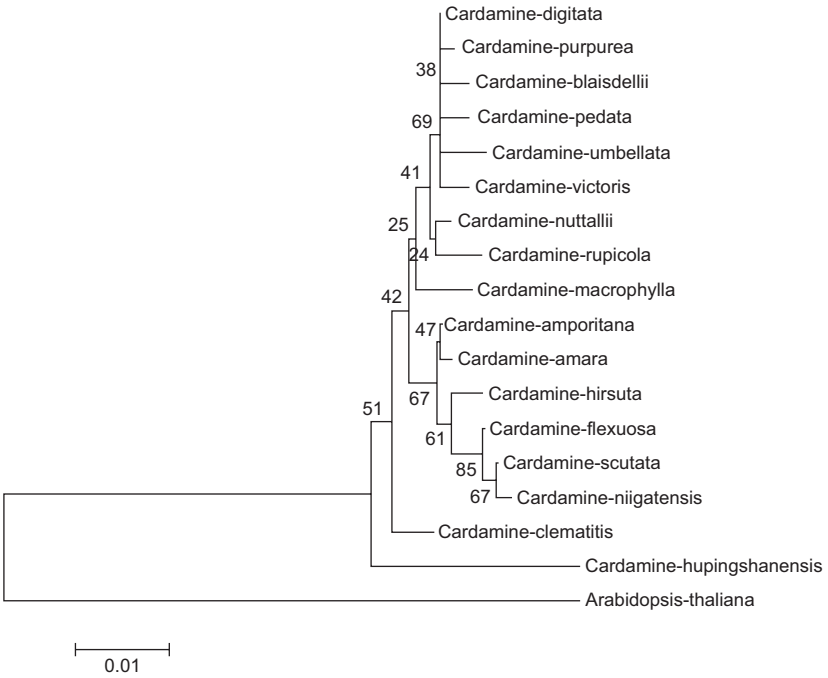

Fig. 2. Phylogenetic tree of ITS sequences of *Cardamine* species by neighbor-joining (NJ).

```
TCGTATCCTGTCCAAAACAGAACGACCCGCGAACCAAAGATCATCACTCA    50
CGGTGGGCCGGTTTCTTAGCTGAGATCGTGCCTGCCGAATCCGTGGTTTC   100
GCGAACAATCCTTACCGGGAGCTCTATCTCTGTTTGGGTTGTGCGCGTTG   150
CTTCCGGATATCACAAAACCACGGCACGAAAAGTGTCAAGGAACATGCAA   200
TTGAACAGCCAGCCTTCGCCTCCCCGGAGACGGTGTGTGTGCGGATGCTG   250
CGCTGCGATCTAAAGTCTAAAACGACTCTCGGCAACGGATATCTCGGCTC   300
TCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAG   350
AATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCCAAGCCTTC   400
TGGCCGAGGGCACGTCTGCCTGGGTGTCACAAATCGTCGTCCCTCTCATC   450
CTTTCAGGACGTGGGACGGAAGCTGGTCTCCCGTGTGTTACCGCACGCGG   500
TTGGCCAAAATCCGAGCTAAGGACGCCGAGAGCGTCACGACATGCGGTGG   550
TGAACTAAAGCCTCTTCGTATCGTCGGTCGTTCTTGTCCATAAGCTCTCG   600
ATGACCCAAAGTCCTCA                                    617
```

Fig. 3. Reference sequence of ITS sequences of *Cardamine* species.

5.8S gene sequence, and ITS2 sequence (**Fig. 3**). Then, the ITS1 sequences and ITS2 sequences of different *Cardamine* species were compared with the "reference" sequence to investigate the variation of the base at the corresponding position.

Table II shows that there were 5.2 variations on average in each of the sequences of ITS1 and ITS2 regions, compared with the "reference" sequence. *Cardamine digitata* had the least variations (2), and *Cardamine hupingshanensis* had the most variations (17). The variations were also 2.1 times those of *Cardamine niigatensis* that had the second most variations.

DISCUSSION

Cardamine hupingshanensis has the potential to tolerate and accumulate selenium and can grow well even on the soil of selenium deposit in Yutangba, Enshi, the only sedimentary independent selenium deposit in the world (Dai 2013, Luo et al. 2020, Wang et al. 2010), suggesting that it has unique genetic characteristics. To the best of our knowledge, this is the first study that reported the extraction of DNA from *Cardamine hupingshanensis* collected from the soil of a unique selenium-rich deposit in Yutangba, Enshi, laying a foundation for the accurate identification of

TABLE II. VARIATION OF ITS1 AND ITS2 SEQUENCES OF DIFFERENT *Cardamine* species.

Species name	Accession number	ITS1 variation position and base	ITS2 variation position and base	Variation
Cardamine hirsuta	MH808258	56G/A, 124C/T, 138G/A, 139T/A	455C/T, 597C/T	6
Cardamine hupingshanensis	MW282045	36A/G, 63T/C, 83T/A, 95G/T, 95-96 inserted with T, 100C/T, 106C/T, 126-127 inserted with A, 131T/A, 201T/A, 205A/G, 214C/T	491C/T, 496C/T, 498C/T, 506C/T, 603G/T	17
Cardamine niigatensis	DQ268493	56G/A, 124C/T, 138G/A	453T/C, 455C/T, 572C/T, 589C/T, 603G/T	8
Cardamine victoris	EU819382	134T/C	564C/A, 580G/A	3
Cardamine clematitis	EU819318	23C/T, 106C/T, 251C/T	599C/T, 602T/C	5
Cardamine rupicola	EU819368	71T/C	445-446T deletion, 527C/A, 580G/A	4
Cardamine blaisdellii	EU819312	250G/T	446T/C, 518T/C, 580G/A, 602T/C	5
Cardamine macrophylla	EU819344	124C/T, 214C/T, 257G/A	463G/T, 602T/C	5
Cardamine umbellata	EU819379	77C/T	520A/T, 560G/T, 580G/A, 602T/C	5
Cardamine scutata	DQ268475	56G/A, 124C/T, 138G/A	453T/C, 455C/T, 572C/T, 589C/T	7
Cardamine pedata	EU819356	171A/C, 260C/T	580G/A	3
Cardamine flexuosa	DQ268409	56G/A, 124C/T, 138G/A	453T/C, 455C/T, 589C/T	6
Cardamine amara	KF988052	56G/A, 138G/A, 214C/T	455C/T	4
Cardamine amporitana	AY260598	56G/A, 138G/A	455C/T	3
Cardamine purpurea	EU819358	122C/T	580G/A, 602T/C	3
Cardamine nuttallii	EU819350	251C/T	580G/A, 602T/C	3
Cardamine digitata	EU819328		580G/A, 602T/C	2

Cardamine hupingshanensis germplasm resources. Additionally, the differences in ITS sequences of different *Cardamine* species were analyzed for the first time, providing clues for further study. The analysis above showed that *Cardamine hupingshanensis* has significant differences in reference sequences compared with other *Cardamine* species, suggesting that it evolved much faster than other *Cardamine* species (**Table II**). Moreover, the ability to tolerate and accumulate selenium of other *Cardamine* species was much lower than that of *Cardamine hupingshanensis*. Therefore, we speculated that some primitive *Cardamine* species growing on the selenium-rich soil in Enshi were exposed to a high selenium environment for a long time, causing mutations. After years of selection, mutants tolerant to high selenium soils and their offspring survived, multiplied and evolved into a new species-*Cardamine hupingshanensis* (Bai et al. 2008). In other words, high selenium soil environment accelerated the genetic evolution of *Cardamine hupingshanensis*, causing much more variations in DNA sequences than other *Cardamine* species. We can identify genes related to selenium tolerance and enrichment in future studies and study their functions and mechanisms. We can also horizontally compare genes of various *Cardamine* species and study the evolutionary pathways of genes related to selenium tolerance and enrichment (Feng et al., 2020).

In addition, we can intuitively observe the evolution speed of species by establishing a "reference" sequence and comparing other sequences with it for the variation of DNA base. This method can also be applied to similar studies. If we develop the corresponding software, the comparative efficiency will significantly improve.

CONCLUSION

To sum up, we extract DNA from *Cardamine hupingshanensis* collected from selenium-rich deposits in Yutangba and Enshi. We amplify the ITS sequences of the DNA using PCR with universal primers to obtain the corresponding reference sequences, laying a foundation for the accurate identification of *Cardamine hupingshanensis* germplasm resources. *Cardamine hupingshanensis* showed the greatest difference in reference sequences compared with other *Cardamine* species. In addition, the differences in ITS sequences of different *Cardamine* species that were analyzed may be helpful for future investigations.

Funding

National Natural Science Foundation of China, Number: 31400567, Recipient: Chen Xu.

REFERENCES

Bai H.F., Chen L.B., Liu K.M. and Liu L.H. (2008). A new species of Cardamine (Brassicaceae) from Hunan, China. Novon: A Journal for Botanical Nomencalture 18(2), 135-137. https://doi.org/10.3417/2006129

Chen S.L., Yao H., Han J.P. and Liu C. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS One 5, 370-375. https://doi.org/10.1371/journal.pone.0008613

Dai G.Z. (2013). Analysis on the progress of selenium-rich agrogeological environment survey in China. Anhui Agricultural Sciences 41(30), 12140-12143. https://doi.org/10.13989/j.cnki.0517-6611.2013.30.012

Feng S.S., Lu H.W., Tian P.P., Xue Y.X., Lu J.Z., Tang M. and Feng W. (2020). Analysis of microplastics in a remote region of the Tibetan Plateau: Implications for natural environmental response to human activities. The Science of the Total Environment 739, 140087. https://doi.org/10.1016/j.scitotenv.2020.140087

Geng H., Gu X. and Zhang Y. (2018). Characteristics of genetic mineralogy of pyrite and quartz and their indicating significance in the Gaosongshan Gold Deposit, Heilongjiang Province, NE China. Earth Sciences Research Journal 22(4), 301-318. https://doi.org/10.15446/esrj.v22n4.57512

He L., Shao F. and Ren L. (2020). Sustainability appraisal of desired contaminated groundwater remediation strategies: An information-entropy-based stochastic multi-criteria preference model. Environment, Development and Sustainability. https://doi.org/10.1007/s10668-020-00650-z

Liu H.P., Wang Y.F., Li Q.L., Yang N.J., Wang Z.C. and Wang Q. (2022). Research on the evolution characteristics of oxygen-containing functional groups during the combustion process of the torrefied corn stalk. Biomass and Bioenergy 158, 106343. https://doi.org/10.1016/j.biombioe.2022.106343

Luo J.L., Yang G., Chen H.H., Zeng W.C., Wang L. and Chen D.Q. (2020). Study on responses of *Cardamine hupingshanensis* to sodium selenate stress. Hubei Agricultural Sciences 59(11), 47-50. https://doi.org/10.14088/j.cnki.issn0439-8114.2020.11.010

Ma S., Wang Z., Tian X.L., Sun B.H., Huang J.H., Yan J.Y., Bao Q.D. and Wang X.X. (2021). Effect of synergistic fermentation of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* on thermal properties of wheat bran dietary fiber-wheat starch system. Food Chemistry 373(Pt A), 131417. https://doi.org/10.1016/j.foodchem.2021.131417

Raza M.F. and Su S. (2020). Differential roles for dopamine d1-like and d2-like receptors in learning and behavior of honeybee and other insects. Applied Ecology and Environmental Research 18(1), 1317-1327. https://doi.org/10.15666/aeer/1801_13171327

Shao S.X., Deng G.D., Mi, X.B., Long, S.Q., Zhang, J. and Tang, J. (2014). Accumulation and speciation of selenium in Cardamine sp. in Yutangba Se Mining Field, Enshi, China. Chinese Journal of Geochemistry 33(4), 357-364. https://doi.org/10.1007/s11631-014-0698-7

Wang Y.B., Chen F.J. and Liang H.W. (2010). A new record species of *Cardamine* from Hubei Province- *Cardamine hupingshanensis*. Hubei Agricultural Sciences 49(9), 2160-2161. https://doi.org/CNKI:SUN:HBNY.0.2010-09-041

Wang Z., Ma S., Sun B.H., Wang F.C., Huang J.H., Wang X.X. and Bao Q.D. (2021). Effects of thermal properties and behavior of wheat starch and gluten on their interaction: A review. International Journal of Biological Macromolecules 177, 474-484. https://doi.org/10.1016/j.ijbiomac.2021.02.175

Yuan L.X., Zhu Y.Y., Lin Z.Q., Banuelos G., Li W. and Yin X.B. (2013). A novel selenocystine-accumulating plant in selenium-mine drainage area in Enshi, China. PLoS One 8(6), 1-9. https://doi.org/10.1371/journal.pone.0065615

Zhao C. and Li J. (2020). Equilibrium selection under the Bayes-based strategy updating rules. Symmetry 12, 739. https://doi.org/10.3390/sym12050739

Zuo X., Dong M., Gao F. and Tian S. (2020). The modeling of the electric heating and cooling system of the integrated energy system in the coastal area. Journal of Coastal Research 103(sp1), 1022. https://doi.org/10.2112/SI103-213.1