Pedigree reconstruction and genetic analysis of major ornamental characters of ornamental crabapple (*Malus* spp.) based on paternity analysis

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Ornamental crabapple is an important woody ornamental plant in the Northern Hemisphere. Its flowers, fruits, leaves and tree habit are all important ornamental characters. As there has been no research on the selection of superior parents and phenotypic variation, new varieties of ornamental crabapple are mainly selected from open-pollination progeny. In order to explore the transmission rule of ornamental traits between parents and offspring of crabapple, and to provide a basis for the selection of hybrid parents for directional breeding, 14 pairs of SSR markers were used in this study for paternity analysis of 384 offspring from 4 female parents crossed with 91 candidate male parents. And 273 offspring (71.1%) were matched with only the father at a 95% strict confidence level. We reconstructed 7 full-sib families (number of progeny ≥ 10) on the basis of the paternity analysis results. Genetic analysis of characters in the full-sib families revealed that green leaves and white flowers were dominant traits. All the hybrid offspring from the white flower (♀) × non-white flower (♂) cross produced white flowers, while 7.04% produced non-white flowers when both parents had white flowers. The results showed that white flowers might be a dominant qualitative trait in crabapple, while the depth of red was a quantitative trait. The genetic characteristics of green and non-green leaves and the depth of red of the peel were similar to flower color. Compared with the upright and spreading traits, the weeping trait was recessive. Some progeny showed an earlier blooming period, indicating the possibility of breeding for blooming period. Our findings are important for parent screening and improving the breeding efficiency of new varieties in ornamental crabapple hybridization.

Ornamental crabapples (*Malus* spp.) are plants in the Rosaceae with a fruit diameter of less than 5 cm and flowers, fruits, leaves and other traits of ornamental value. As important woody ornamental plants in temperate regions of the Northern Hemisphere, they are widely used in landscaping and landscape design1,2. There are many varietes of ornamental crabapples. Among the more than 200 varieties reported at present, only slightly more than 60 have known parents2. Most of the varieties were selected from the progeny of natural hybrids, with complex genetic backgrounds and unclear genetic relationships. The breeding of crabapple varieties is still achieved by selecting the offspring of natural hybrids on the basis of excellent ornamental traits and retaining them through vegetative propagation. For example, the new cultivars *M. FengHong NiChang*3 and *M. Fen Balei*4 were selected from *Malus halliana* and *Malus micromalus*, respectively. As it is difficult to identify the male parent in this process, it is impossible to breed a large number of new varieties with high efficiency by artificial crossbreeding for traits, which restricts the directed breeding of ornamental crabapples. Our laboratory aimed to use a variety of traditional methods to conduct artificial hybridization of ornamental crabapples. Due to the low fertility of

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different varieties and the self-incompatibility of gametophytes in *Malus* spp., the success rate was very low, and it was difficult to obtain enough hybrid offspring for effective selection, which limited the efficient advancement of artificial hybridization for ornamental crabapples.

El-Kassaby et al. (2009) presented a strategy for forest breeding called ‘breeding without breeding’ (BWB). This method did not require any controlled pollination or experimental field testing, which are considered to be the most resource-consuming steps in breeding. The method involved using paternity analysis to create a full-sib family and performing quantitative genetics analyses for further genetic improvement or selecting the parents in cross-breeding which could greatly improve breeding efficiency. The key step in BWB was to use parentage analysis to reconstruct the family, that is, to use genetic markers to trace the male parent of half-sibling families. The markers used for paternity analysis mainly include random amplification polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs). With the development of molecular marker technology, the improvement of analysis methods and the development of analysis software, the efficiency and accuracy of paternity analysis have been improved. Among these markers, SSRs are short tandem repeat sequences with 1–6 nucleotides as the repeat unit. They are commonly used in paternity analysis because they are widely distributed throughout the genome of eukaryotes, with the advantages of codominance, high polymorphism, and high stability.

The BWB strategy provides a new approach for improving the current breeding methods of ornamental crabapples. In this study, we surveyed the traits of existing half-sib families and used SSR molecular markers for parentage analysis. Then, we analyzed the crossbreeding compatibility between varieties, the genetic variation of the traits between parents and offspring, and the efficiency of different hybrid combinations to produce qualified ornamental characters. This study is expected to provide a basis for parental selection of artificial hybrids in directional breeding of new varieties of ornamental crabapples.

**Methods**

**Plant materials.** The materials used in the experiment were obtained from the national repository of *Malus* spp. Germplasm (Nanjing Forestry University), which is located in Jiangdu District, Yangzhou City, Jiangsu Province (119°55′E, 32°42′N). There were 105 kinds of crabapple cultivars collected domestically and internationally, all of them comply with relevant institutional, national, and international guidelines and legislation, and there is no intellectual property issue. Thirty clones of each variety were planted in a 2 × 3 m plot, and all of them were between five and eight years old, i.e., in the full bloom phase. In Fall 2013, seeds of *M. ‘Darwin’, M. ‘Red Sentinel’, and M. ‘Rainbow’ were collected from the germplasm. The male parent of these materials was unknown and might be one of 91 cultivars in the nursery with overlapping flowering (Table S1 in Supplementary Information 1). A total of 221, 450, 218, and 206 offspring were obtained from four half-sibling families in 2019, and 96 individuals were randomly selected from each family. The young leaves of 384 offspring and 91 candidate parents were collected, placed in iceboxes, and quickly transferred to the laboratory for preservation in a −80°C freezer in 2019.

**Trait investigation and statistics.** Ten traits were investigated to analyze the degree of trait separation and variation between parents and offspring in 2019. The methods of investigation and classification were as described by Liu (Table 1).

**DNA extraction.** Total genomic DNA was extracted using BioTeke Rapid Plant DNA Extraction Kit (BioTeke, Beijing, China). DNA concentrations were estimated with a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the qualified DNA was normalized to a concentration of 50 ng/μl for polymerase chain reaction (PCR).

**Genotyping with SSR markers.** Fourteen pairs of SSR primers were used in this experiment, 11 were from previous studies and the other 3 were developed by our laboratory based on transcriptome data (Table 2).

PCR amplification with all primers was carried out using an ABI Veriti 96 PCR system (Thermo Fisher Scientific, MA, USA). The reaction mixtures of 15 μl contained 1× buffer, 6 mg/l genomic DNA, 0.25 μmol/L each SSR forward and reverse primer, 0.25 mmol/L dNTPs, 2 mol/L MgCl2 and 1.25 U of Taq polymerase, all of which were obtained from Takara (Takara Biomedical Technology, Dalian, Co., Ltd.). The PCR system was adjusted according to Wang et al. and the program involved an initial denaturation step of 4 min at 94°C, followed by 32 cycles at 94°C for 45 s, the appropriate annealing temperature for 30 s, 72°C for 40 s, and an extension cycle of 1 min at 72°C. The PCR products were separated on an ABI 3730XL instrument (Thermo Fisher Scientific, MA, USA).

**Data analysis.** Genotyping results were analyzed by Peak Scanner V1.0 software (Thermo Fisher Scientific, MA, USA). The segments were arranged in order from smallest to largest as A, B, C,..., the segments with the same base size were represented by the same capital letter, and missing data were indicated by “-”. The number of observed alleles (Na), observed heterozygosity (Ho), and Nei’s diversity index was determined using POPGENE version 1.32 software. Paternity analysis was performed based on the maximum likelihood method by comparing genotypes of known maternal origin and offspring against candidate paternal materials using Cervus 3.0 software with a genotyping error rate set to 0.01.
### Table 1. Ornamental trait classification. The varieties with an early blooming period bloomed between March 31 and April 4, and those with a medium blooming period bloomed between April 5 and April 9. Varieties that entered the blooming period after April 10 were recorded as having a late blooming period, and those that still had not bloomed in May were considered non-blooming. These categories were based on the blooming period of *M. ‘Pink Spires’* (March 31), which began blooming earliest. The color of flowers, leaves and fruit was checked on the sunny side using the Royal Horticultural Society Standard Color Chart (RHSCC). The leaf base was wide, the length was approximately twice the width (ovate-round), and the length and width were similar to those of broadly ovate leaves. In contrast to ovate-round leaves, the narrow leaf base led to an obovate shape. When the middle part of the leaf is the widest and the length is approximately 1.5–2 times the width, the leaf is recorded as broadly elliptical; when the length is 3 to 4 times the leaf width, the leaf is recorded as long elliptical.

| Characters | classification |
|------------|----------------|
| Blooming period | None | Early | Medium | Late | – | – |
| Flower color | – | White | Pink | Red | Light purple | Purple |
| Leaf color | – | Green | red | Purple | – | – |
| Leaf shape | Ovate-round | Broadly ovate | Obovate | Broadly elliptical | Long elliptical |
| Leaf surface | Sparsely | Medium | Thick | – | – | – |
| Fruit color | – | Green | Yellow | Red | – | – |
| Fruit size | Very small (<6mm) | Small (6–13mm) | Medium (13–25mm) | Large (25–50mm) | – |
| Fruit calyx | – | Absent | Always present | – | – | – |
| Glossiness to skin | – | Strongly expressed | Weakly expressed | Absent | – | – |
| Tree habit | Upright (<30°) | Spreading (30°–70°) | Drooping (70°–90°) | Weeping (>90°) | – | – |

### Table 2. SSR-PCR primers. *Indicates an EST-SSR primer developed by our laboratory based on the transcriptome data.

| Code | Sequence | Tm | PIC |
|------|----------|----|-----|
| GD142<sup>14</sup> | GGCACCACAAGCCCTAATAAGGACCTACGCACAGCAAGTTACACG | 62 | 0.828 |
| CH03d07<sup>17</sup> | CAAATCAATGCAAATGCTGCAAGGCTTCTGGCATTTTA | 60 | 0.858 |
| GD96<sup>46</sup> | CGCCGGAAGCATACTCACC | 60 | 0.864 |
| ME52<sup>18</sup> | CACCACAAGCAGCAAGCAGCAAG | 60 | 0.695 |
| gssr-11<sup>*</sup> | GAGAAATGAGTTGTCAGGATGTCAGGAT | 60 | 0.859 |
| Ht02b06<sup>19</sup> | TAAATACGATGAGTGATGAAACC | 62 | 0.877 |
| gssr-2<sup>*</sup> | TCGTGAGAGATGAAACCAT | 52 | 0.898 |
| gssr-21<sup>*</sup> | AGGGAAATGAGTTGTCAGGATGTCAGGAT | 62 | 0.679 |
| Ht02c07<sup>19</sup> | AGAGCTACGGGAGTTGCAATACTG | 59 | 0.864 |
| CN44794-SSR<sup>19</sup> | CATGGCAGTGCTGCTGCAATACTG | 56 | 0.877 |
| CH01f09<sup>20</sup> | ATGCACATCAAGTTGATGAGTTGATG | 56 | 0.784 |
| CH01h10<sup>20</sup> | TGCCAGATGATGATGCTGCAATACTG | 62 | 0.870 |
| CH04b10<sup>20</sup> | AGCCAGCAAAGTCTGCAATACTG | 62 | 0.851 |
| CH05g03<sup>20</sup> | GCTTTGAATGATACTGCAATACTG | 60 | 0.818 |
Results

Genetic diversity. A total of 216 alleles were obtained for the 14 pairs of SSR primers based on 91 candidate parents. The number of alleles per SSR locus ranged from 8 to 24, with an average of 15.4 alleles per locus. In general, the average Ne and He were 6.10 and 0.81, respectively. In addition, 180 alleles were obtained based on 384 offspring. The number of alleles per SSR locus varied from 7 to 20, with an average of 12.9, and the average Ne and He were 4.87 and 0.76, respectively (Table 3). Since half of the genetic variation of offspring comes from only 4 mothers and half comes from the part of the 91 candidate parents. Although there were far more offspring than candidate parents, the genetic diversity of the progeny was still lower than that of the candidate parents (except for gssr-21). All the genotypes from the 14 pairs of SSR primers were available in Supplementary Information 2.

Paternity analysis of half-sib families. Of the 384 offspring from the 4 half-sib families, 273 (71.09%) were matched to a unique paternal tree at a 95% strict confidence level. The average matching success rate of the 4 families was 71.09%, with the M. ‘Sweet Sugartyme’ family being the highest at 75% and the M. ‘Darwin’ family being the lowest at 66.67% (Fig. 1a). Table S2 in Supplementary Information 1 shows the correspondence of paternal materials and offspring at a 95% strict confidence level. Only 44 of the 91 candidate parents produced progeny, and the number of progeny produced by the 44 male parents ranged from 1 to 72. In addition, as the male, M. ‘Red Sentinel’ (No. 6) produced 72 progeny with other ornamental crabapples, showing the highest reproductive contribution rate. M. ‘Winter Red’ and M. ‘Sweet Sugartyme’ ranked second and third, producing 29 and 23 offspring, respectively. Fourteen, 13 and 3 progenies were produced by M. ‘Weeping Madonna’ (67), M. ‘Louisa Contort’ (68) and M. ‘Rainbow’ (12), respectively. M. ‘Darwin’ (25) did not produce offspring when used as the father. In addition, 15 paternal parents produced only one offspring (Fig. 1b).

Family reconstruction and trait variation analysis of the full-sib families. According to the paternity analysis, we reconstructed the full-sib families of ornamental crabapples and selected 7 hybrid combinations with more than 10 progenies from these families (Table S3 in Supplementary Information 1). The characters of 7 parents are shown in Table 4. A summary of the results from flower color, leaf color, fruit color, leaf shape, fruit size and tree habit is shown in Table 5. When the flower color was divided into white and non-white (pink and red), white was dominant over non-white. The hybrid progenies of white flower (♀) × non-white flower (♂) crosses all had white flowers. When both parents had white flowers, 92.92% of the offspring had white flowers and 7.04% had non-white flowers, indicating that the production of white flowers was not recessive. The offspring with green leaves accounted for 90.76%, and only 4 red and 6 purple progenies resulted from red leaf (♀) × green leaf (♂) crosses. There were only 2 red-leaved individuals among the hybrids, and both parents had green leaves. These results prove that green leaves were dominant over red leaves. The ratio of red to non-red (yellow and green) offspring was near 1:1 in red fruit (♀) × non-red fruit (♂) crosses. The red-fruited offspring accounted for 80% when a red-fruited female was crossed with a green-fruited male. The other two combinations mainly produced non-red and red peels. Overall, the ratio of red to non-red fruits was close to 1:1, and red and yellow were the main fruit colors, accounting for 45.38% and 40.00%, respectively.

Spreading was the most common habit among the offspring. Differentiation occurred in the progeny regardless of parental tree habit. For example, most of the offspring were spreading and a few offspring were drooping

Table 3. Genetic diversity indicators of candidate parents and offspring at 14 loci. Na: number of alleles, Ne: effective number of alleles, He: expected heterozygosity.
when an upright female was crossed with a spreading male. Approximately 2% of progenies were weeping when two spreading parents were crossed. However, the proportion of spreading offspring (approximately 10%) was significantly increased if the male or female parent was drooping or weeping.

Parents had similar effects on the leaf shape and fruit size of offspring in the 7 full-sib families. It was rare that these two traits in offspring deviated significantly from those of the parents.

Table 6 shows the results for four traits, including leaf surface, glossiness of skin, fruit calyx, and blooming period. The leaf surface of offspring was affected by the parents; most progenies were similar to the parents, but
a few were differentiated (transgressive inheritance). There were two types of strongly expressed and weakly expressed glossiness of skin in parents. The phenotypes of offspring were mostly within the range of values observed for the parents, but some progenies did not display one of the parents’ phenotypes.

Only two types of blooming periods were observed for the parents and offspring, early and medium, with 80.00% of progeny showing the medium type. Similarly, the fruit calyx trait had only two states: absent and always present. The fruit calyx was absent in 63.85% of progenies, regardless of parental phenotype.

**Discussion**

Genetic diversity is not only an important index used to measure species’ ability to adapt to changing environments but also a key factor affecting plant genetic improvement. Studying the genetic diversity of ornamental crabapple varieties provides a molecular basis for hybridization selection. Hokanson et al. used 8 pairs of SSR primers to analyze the genetic diversity of 142 *Malus* plants, and the average He was 0.623. Kumar et al. used SSR molecular markers to analyze the genetic diversity of wild crabapple populations in the Himalayan region of India, and the He value was 0.506. In this study, the average He of 91 ornamental crabapple varieties was 0.81, which was significantly higher than the previously reported values. On the one hand, the 91 parental varieties...
in this study were from a wide range of sources, and compared with that of wild populations, their genetic background was complex, so the diversity level was higher. On the other hand, the number of markers used in this study was higher than that used in previous studies, and the capillary electrophoresis technology was more accurate. Moreover, the 384 progenies of the 4 families also had a high level of genetic diversity (He = 0.76), but the level was lower than that of the candidate parents (0.81). The main reason was that not all candidate parents provided pollen, and the paternal parents of these progeny came from only 44 varieties.

Paternity analysis has been extensively used in various plant studies, such as paternity testing, pedigree reconstruction, mating system examination and dynamic changes in genetic diversity in generations. Ai et al. used 11 pairs of SSR primers to analyze 286 seeds from a Pinus massoniana seed orchard with 129 candidate paternal clones, and paternity at a 95% and an 80% confidence level was determined for 25 seeds (8.80%) and 107 seeds (37%), respectively. However, in a small candidate parent population of Moringa oleifera with only 60 male parents, 8 pairs of SSR primers assigned fathers for 155 of 288 seeds (53.82%) based on a 95% strict confidence level. By comparing the results of the two studies, it can be seen that the number of candidate parents and polymorphism of the SSR molecular markers used are the main factors that affect the accuracy and efficiency of paternity analysis. Furthermore, the sampling intensity of candidates is another important factor in paternity analysis. In this study, 91 candidate paternal parents were screened through phenological observations. Using 14 pairs of SSR primers at a 95% confidence level, 273 offspring (71.1%) were matched. It was obvious that using capillary electrophoresis to replace polyacrylamide gel electrophoresis was also an important way to improve efficiency and accuracy. Further analysis of the distance between the males and females in the nursery showed that M. 'Red Sentinel' and M. 'Winter Red', the male parents that produced the most offspring, were located far from the female parents, indicating no obvious relationship between pollination success rate and the distance of pollen transmission. The compatibility of male and female gametes might be the key factor affecting the pollination success rate. M. 'Red Sentinel' and M. 'Winter Red' had good compatibility with most varieties when used as male or female parent and are suitable as parents for hybrid experiments.

In this study, the genetic characteristics of traits in parents and offspring were analyzed through reconstructed full-sib families to provide a basis for parental selection in crossbreeding. Flower color is one of the main ornamental traits of crabapple, but there are no reports on the genetics of this trait in ornamental crabapple. According to genetic studies on the flower color of Camellia azalea Wei and Hibiscus coccineus Walter, white flowers are recessive to red flowers. However, Han et al. found that the petal color of F1 offspring derive from the cross between cabbage with yellow petals (♀) and Chinese kale with white petals (♂) was white, and the segregation conformed to a Mendelian ratio of 3:1 in F1 offspring originating from self-pollination of F1 plants, proving that white petals were dominant over yellow petals. In the natural population of ornamental crabapples examined in this study, the flowers were mostly white. If flower color was divided into white and non-white (pink and red), the offspring from the cross between parents with white flowers (♀) and non-white flowers (♂) all had white flowers, indicating that white flowers were dominant over non-white flowers. When both parents had white flowers, only 7.04% of the offspring had non-white flowers, which again indicated that the white flower trait was dominant. Of course, the results need to be confirmed by crossing two parents with red flowers. In addition, the intensity of red was a continuous trait showing quantitative characteristics, and the molecular mechanism underlying its formation needs to be further studied.

The accumulation of anthocyanins causes red leaves in many crops and ornamental plants. Previous studies proved that the inheritance of red or purple leaves followed a monogenic recessive pattern. In contrast, red or purple leaves are controlled by a single dominant gene in birch, copper beech and Brassica juncea. Huang et al. found that leaf color was determined by a single locus and that the purple leaf phenotype was recessive to the green leaf phenotype by selfing Kalanchoe garambiensis (purple leaves) and K. garambiensis G. (green leaves). Our study showed that 97.53% of the offspring resulting from crosses between parents with green leaves also produced green leaves. The green leaf trait was observed among 79.59% of offspring from red (♀) × green (♂) crosses. This finding suggests that green leaves were dominant over red or purple leaves. However, the segregation ratio of green and red leaves in offspring deviated from that expected under Mendelian inheritance, which might be related to the quantity of offspring.

Previous studies on apples found that fruit color was composed of background and surface color. Red was dominant over yellow and controlled by the single gene R. However, some scholars believe that the inheritance of skin color is regulated by major genes as well as polygenes. Although red is dominant, the intensity of red is affected by polygenes. It is a qualitative trait with a quantitatively inherited character. In our study, the progenies with red fruits accounted for 80% of the progenies obtained from red (♀) × green (♂) crosses, and the segregation ratio of red and yellow was close to 1:1 in some full-sib families. We speculated that red peel was also dominant in crabapple.

Tree habit is a major ornamental trait controlled by both major genes and polygenes. It has been proven that the weeping trait is regulated by single recessive genes in peach and Canadian redbud. There were not only major genes but also some polygenes involved in controlling the weeping trait in Prunus mume. In our data, upright and spreading were the main tree habits in offspring, and progenies rarely exhibited drooping and weeping. A few drooping and weeping progenies appeared in the crosses of upright (♀) × spreading (♂) and spreading (♀) × spreading (♂), respectively. The proportion of drooping and weeping in offspring was significantly increased if a parent was drooping or weeping. The results showed that weeping and drooping were recessive. However, the segregation ratio of two full-sib families' offspring deviated from that expected under Mendelian inheritance, and we speculated that the production of weeping branches in crabapple may be similar to that in P. mume. Dougherty et al. revealed four genomic regions, W1, W2, W3 and W4, that were significantly associated with weeping by performing genetic mapping in the F1 generation of a cross between Malus 'Cheal's Weeping' and Malus 'Evereste', and W was the major locus, which further supported the inference of this study.
We should select a parent with drooping or weeping branches for crossbreeding if we want to obtain more offspring with weeping branches.

The blooming period is an important trait in ornamental plants. It is valuable because earlier or later blooming periods will extend the viewing period of the species. Shen et al. found that the blooming period of progeny from the cross between Plumbago auriculata and P. auriculata f. alba was much earlier than that of the parents, which showed obvious heterosis. Furthermore, the authors proved that the blooming period was controlled by two pairs of major genes with additive dominance, with dominant effects predominating. In our study, the parents had early or medium blooming periods, and most progenies showed medium blooming periods. However, three progenies resulting from the cross between two medium-blooming parents showed early blooming. This result indicated the possibility of crossbreeding ornamental crabapple during the blooming period, although there were no later flowering plants among the existing hybrid combinations.

Conclusion
At present, the method used to breed novel varieties of ornamental crabapple mainly involves open-pollination offspring. This approach is inefficient and depends on the abundance of male parents, as well as the physical distance between the parents. Over time, efficiency will gradually decline and be difficult to sustain. By paternity analysis, we found that ‘Red Sentinel’ and ‘Winter Red’ were suitable as parents for hybrid experiments. The green leaves and white flowers were dominant traits, and they might be a dominant qualitative trait in crabapple. The weeping trait was rare and recessive compared with the upright and spreading traits. Interestingly, some progeny had an earlier blooming period than their parents, which indicated the possibility of changing the blooming period by cross-breeding. According to our results, we identified hybrid combinations with a high success rate, plentiful progeny variation and an increased possibility of producing ornamental varieties for artificial hybridization, which will improve the efficiency of new variety breeding.

Data availability
Development SSR primer pairs have been deposited to GenBank, accession numbers are ON402244, ON402245 and ON402246.

Received: 22 January 2022; Accepted: 10 August 2022
Published online: 18 August 2022

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Acknowledgements
We thank the editor and anonymous reviewers for their efforts and insightful comments that allowed us to improve our manuscript.

Author contributions
H.R. and B.H. contributed equally to this work. H.R. and B.H. performed the experiments and analyzed the data. X.H. and K.W. participated in some experiments. H.R. drafted the manuscript. L.X. and M.X. conceived and designed the experiments. W.Z. and F.Y. provided materials. L.X. reviewed the manuscript. All authors read and approved the final manuscript.

Funding
This study was supported by the Key Research and Development Program of Modern Agriculture in Jiangsu Province (BE2019389).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-18352-z.

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