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Research article

Comparison of the phenolic contents and epigenetic and genetic variability of wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum* L.)

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ABSTRACT

Background: Epigenetic modifications are key factors modulating the expression of genes involved in the synthesis of phytochemicals. The knowledge of plant epigenetic and genetic variations can contribute to enhance the production of bioactive compounds. These issues have been little explored thus far in *Rorippa nasturtium* var. *aquaticum* L. (watercress), an edible and medicinal plant. The aim of the current study was to determine and compare the phenolic composition and epigenetic and genetic variations between wild and cultivated watercress.

Results: Significant differences were found in the quantitative phenolic composition between wild and cultivated watercress. The eight primer combinations used in the methylation-sensitive amplification polymorphism (MSAP) method revealed different epigenetic status for each watercress type, the cultivated one being the most epigenetically variable. The genetic variability revealed by the *EcoRI*/*MspI* amplification profile and also by eight inter-simple sequence repeat (ISSR) primers was different between the two types of watercress. The results of the Mantel test showed that the correlation between genetic and epigenetic variations has diminished in the cultivated type. Cluster analyses showed that the epigenetic and genetic characterizations clearly discriminated between wild and cultivated watercress.

Conclusions: Relevant chemical, epigenetic, and genetic differences have emerged between wild and cultivated watercress. These differences can contribute to fingerprint and develop quality control tools for the integral and safety use and the commercialization of watercress. The richness of epialleles could support the development of tools to manipulate the watercress epigenome to develop high bioproduct–producing cultivars.

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1. Introduction

Epigenetic modifications play a key role in plant growth, development [1], and stress adaptation [2]. DNA methylation is an important epigenetic mechanism involved in the regulation of gene expression [3]. Several studies showed that abiotic and biotic stress cause heritable alterations in cytosine methylation patterns, which can produce sustained gene expression and new phenotypes, thereby providing plants the ability of rapid adaptation through expression of genes involved in the synthesis of bioproducts [4,5].

Counting with strategies for manipulating plant epigenome would enhance the production of plant bioproducts. However, for achieving this purpose, it is necessary to have knowledge of both the basal epigenetic and genetic variability of plants of interest. Some strategies of the epigenetic manipulation for the novo or enhanced production of bioactive compounds have been developed for some fungi species [6]. Gallusci et al. [5] proposed that the complete characterization of epigenetic variations enables the construction of predictive models of the transmission and stability of this variation, which has application in breeding. Additionally, determining the differential contribution of both genetic and epigenetic variability to the rapid adaptation of

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plants to environmental changes is important to reveal the epigenetic potential for plant breeding application. Studies on this topic have been carried out for *Spartina alterniflora* and *Borrichia frutescens* [4].

Watercress (*Rorippa nasturtium* var. *aquaticum* L., syn.: *Nasturtium officinale* W. T. Ayton) is an aquatic perennial plant of the family Brassicaceae native to Europe [7]. This plant is rich in secondary metabolites [8,9]. These bioproducts, having a wide spectrum of biological activities, convert this edible species into a medicinal plant with potent anticarcinogenic properties [10], among others. All these properties have aided research to improve the accumulation of bioactive phytochemicals of watercress. In this context, Voutsina et al. [11] described the first transcriptome of this plant, and Payne et al. [12] evaluated the gene expression and morphologic variation of commercial watercress, among other studies. However, a lack of knowledge still exists about the genetic and epigenetic variability of natural and cultivated watercress, as well as about the potential of epigenetic manipulation to improve the accumulation of its bioactive compounds.

In Mexico, watercress naturally occurs in springs of high valleys [7], where people consume it as a vegetable. This plant is also cultivated in hydroponic systems in some central regions of the country. The aim of the current study was to determine and compare the phenolic composition and the epigenetic and genetic variations between wild and cultivated watercress.

2. Materials and methods

2.1. Reagents

Ethanol, Folin–Ciocalteu reagent, gallic acid, aluminum chloride, polyvinylpyrrolidone (PVP40), vanillin, HPLC standards, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *EcoRI*, *MspI*, *HpaII*, T4DNA ligase, T4DNA ligase buffer, Taq Polymerase, PCR buffer, dNTPs, and MgCl2 were purchased from Promega (Madison, WI, USA).

2.2. Plant material

Leaves of 20 accessions of wild watercress (named 1 to 20) were collected in springs located in Berros and La Constancia, Durango, Mexico (sampling area between 23° 93′ 19” N, 104° 27′ 23” W and 23° 91’ N, 104° 26’ W; altitude between 1760 and 1800 m), in July 2016. The voucher specimen was deposited at Herbarium CIIDIR (curatorial number 16895). The average annual temperature of the locations was 16°C, the minimum was 4°C, and the maximum was 23°C. Photoperiod was 13 h, and the maximum relative humidity was 87%.

Leaves of 20 individuals of cultivated watercress (named 21 to 40) were obtained from an equal number of different lots, in a local market in July 2016. These samples were hydroponically grown under greenhouse conditions in Queretaro, Mexico (20° 51′ 51” N, 99° 55′ 43” W; 1990 m altitude), where the average annual temperature was 17.4°C, the minimum was 12.5°C, and the maximum was 26.5°C. Photoperiod was 12.5 h, and the maximum relative humidity was 94%.

2.3. Preparation of extracts

Each type of sample was independently prepared and analyzed. Samples were dried (at 40°C) and ground. Three subsamples of each watercress type were formed and separately analyzed. Samples (1 g) were extracted with 10 mL of 80% ethanol (*v*/v) for 12 h. After centrifugation (8000 rpm, 10 min), the supernatant was recovered and the pellet was re-extracted under the same conditions. The two supernatants of the same sample were combined and concentrated to dryness. The dried extract was solved in 80% ethanol (*v*/*v*), at a concentration of 2 mg/mL. Aliquots were used in the determination of phenolic composition.

2.4. Phenolic composition

Total phenolic contents were determined according to Skotti et al. [13]. Phenolic contents were calculated by generating a calibration curve of gallic acid (slope = 0.0913, axis crossing point = −0.0144, r = 0.9963) and expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g DE).

Flavonoid contents were determined following Barriada-Bernal et al. [14]. The flavonoid contents were calculated by generating a calibration curve of quercetin (slope = 0.3261, axis crossing point = 0.0277, r = 0.9957) and expressed as milligrams of quercetin equivalents per gram of dry extract (mg GAE/g DE).

Condensed tannins were determined following Julkunen-Tiitto [15]. The contents were estimated by generating a calibration curve of epicatechin (slope = 4.8739, axis crossing point = 0.2050, r = 0.9983) and expressed as milligrams of epicatechin equivalents per gram of dry extract (mg EE/g DE).

The phenolic profile was determined by HPLC-DAD, in a PerkinElmer Series 200 HPLC system (Shelton, Connecticut, USA), using a PerkinElmer Brownlee Analytical C18 column (4.6 × 250 mm, 5 μm) and diode array detection (DAD) (PerkinElmer Series 200) by the gradient method [16]. Structural information of compounds was obtained by considering the number and λ_{max} of bands and shoulders, as well as the whole shape of the UV spectra according to the UV theory developed for flavonoids and phenolic acids [16] and also by comparing the retention time (RT) and UV spectra with those of the following reference compounds: chlorogenic acid (RT: 29.76 min; λ_{max}: 243sh, 296sh, 326), caffeic acid (RT: 53.13 min; λ_{max}: 239sh, 295sh, 318), *p*-coumaric acid (RT: 37.2 min; λ_{max}: 294sh, 308), quercetin (RT: 45.95 min; λ_{max}: 260, 268sh, 299sh, 370), rutin (quercetin-3-*O*-[rhamnosyl(1–6)glucoside]; RT: 33.74 min; λ_{max}: 255, 264sh, 294sh, 355), and apigenin (RT: 59.60 min, 267, 290sh, 335). The relative abundance of each compound was determined by area measurements, using a standard curve of rutin (slope = 8×10^6, axis crossing point = 42.373, r = 0.9987) for flavonols, and a standard curve of chlorogenic acid (slope = 8×10^6, axis crossing point = 9892.900, r = 0.9985) for phenolic acids. Concentrations were reported as milligrams per gram of dry extract (mg/g DE).

2.5. Epigenetic and genetic analysis

Total DNA of each individual was independently obtained and analyzed. DNA extraction was carried out by grinding the samples in liquid nitrogen and using 2% polyvinylpyrrolidone (PVP 40), according to Bhau et al. [17].

Table 1
MSAP (Methylation-Sensitive Amplification Polymorphism) primers used to assess the epigenetic variability of wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*).

MSAP primers	*EcoRI* Sequence	*HpaII*/*MspI* Sequence
Pre-amplification primers	5′GACTGCGTACCAATTC-3′	5′-ATCATGAGTCCTGCTC GG-3′
Selective amplification primers	5′GACTGCGTACCAATTCAC-3′	5′-ATCATGAGTCCTGCTC GGTCAA-3′
	5′GACTGCGTACCAATTCAG-3′	5′-ATCATGAGTCCTGCTC GGAAT-3′
	5′GACTGCGTACCAATTCAAC-3′	
	5′GACTGCGTACCAATTCAT-3′	
Adapter pair	5′-CTCGTAGACTGCGT ACC-3′/3′-CATCTGACGCA TGGTTAA-5′	5′-GATCATGAGTCCTG CT-3′/3′-AGTACTCAGGA CGAGC-5′

The underlying sequences mean the 2 to 4 selective nucleotides added at the 3′ end of the selective amplification primers.

Detection of epigenetic variation was carried out by the methylation-sensitive amplification polymorphism (MSAP) method according to Lira-Madeiros et al. [18], using both *EcoRI*/*MspI* and *EcoRI*/*HpaII* digestions. The sequences of the primers (selective primers and adapters) used are shown in Table 1.

2.5.1. Digestion

Samples were digested with each enzyme. In the first digestion reaction, 3.5 μL of DNA was combined with 0.5 μL of 5 U *EcoRI*, 0.5 μL of 5 U *HpaII* (the methylation-sensitive restriction enzyme), 0.1 μL of 1× multicore buffer, and 0.1 μL of BSA in a final volume of 20 μL, and the mixture was incubated at 37°C for 3 h. The second digestion reaction was conducted in the same manner, but *MspI* (the methylation-insensitive restriction enzyme) was used instead of *HpaII*.

2.5.2. Ligation

The digested DNA (20 μL) was ligated to adapter using 0.3 μL of 0.9 U T4 DNA ligase, 0.3 μL of 1× T4 DNA ligase buffer, 1 μL of 5 pmol *EcoRI* adapter, and 1 μL of 50 pmol *MspI*/*HpaII* adapter in a final volume of 30 μL for 24 h at room temperature.

2.5.3. Preamplification

Two microliters of ligated DNA, 4 μL of 1× PCR buffer, 0.8 μL of 0.4 mM dNTPs, 1 μL of 25 mM MgCl2, 0.63 μL of 10 μM *EcoRI* primer and 0.52 μL of 10 μM *MspI*/*HpaII* primers, and 0.4 μL of 2 U Taq polymerase in a final volume of 20 μL were combined. The preamplification conditions were 25 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. The preamplified products were combined with 200 μL of water and stored at −20°C.

2.5.4. Selective amplification

Eight combinations resulting from the mixture of each of four *EcoRI* selective primers with each of two *MspI*/*HpaII* selective primers were individually evaluated. The combinations and volumes used are shown in Table 2. An aliquot (5 μL) of preamplified product was mixed with each combination of selective primers according to Table 2, in addition to 1 μL of 25 mM MgCl2, 0.4 μL of 0.2 mM dNTPs, 4 μL of 1× PCR buffer, and 0.4 μL of 2 U Taq polymerase in a final volume of 20 μL. The PCR amplification conditions were 12 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, decreasing the annealing temperature by 0.7°C per cycle, and then 24 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 2 min with a final period of 5 min at 72°C. The selective amplification products were separated by electrophoresis on 5% polyacrylamide gels. The MSAP analyses were carried out at least twice for each sample.

Genetic variability was assessed by considering only the loci produced by *EcoRI* + *MspI* digestions of the MSAP analysis, as the use of *MspI* reveals the variation of the CCGG sites spreading throughout the genomes. Genetic variability also was estimated with five inter-simple sequence repeat (ISSR) primers according to Tarikahya-Hacioğlu [19], with some modifications in melting temperature (Tm) (Table 3). The PCR amplification mixture contained 3 μL of DNA, 1.32 μL of 25 mM MgCl2, 0.25 mM dNTPs, 1× PCR buffer, 2.5 U Taq polymerase, and 6 μL of 10 μM oligonucleotide primer in a final volume of 25 μL. The amplification conditions were 45 cycles of 94°C for 1 min, 50–52.8°C for 45 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 2% agarose gels.

Table 2
Combinations of selective primers used in the Methylation-Sensitive Amplification Polymorphism (MSAP) method.

Combination	*EcoRI* selective primer (10 μM)	Volume (μL)	*MspI*/*HpaII* selective primer (10 μM)	Volume (μL)
1	AG	0.53	TCAA	0.44
2	AG	0.53	AAT	0.45
3	AC	0.53	TCAA	0.44
4	AC	0.53	AAT	0.45
5	AAC	0.52	TCAA	0.44
6	AAC	0.52	AAT	0.45
7	AT	0.55	TCAA	0.44
8	AT	0.55	AAT	0.45

Table 3
ISSRs primers and melting temperature (Tm) used to assess the genetic variability of wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*).

Primers	Sequence	Tm (°C)
UBC809	AGAGAGAGAGAGAGAGG	52.8
UBC810	GAGAGAGAGAGAGAGAT	50.4
UBC816	CACACACACACACACAT	50.4
UBC817	CACACACACACACACAA	50.0
UBC847	CACACACACACACACARC	52.8

2.6. Data analysis

Phytochemical data were subjected to an analysis of variance ($p < 0.05$), and means were separated by the Tukey test with XLSTAT 2017.03.44803. Only consistent amplified products were recorded for each genetic and epigenetic marker. For the MSAP analysis, methylated loci (5′-C5mCGG-3′) were indicated by bands present in the *EcoRI*/*MspI* reaction and absent in the *EcoRI*/*HpaII* reaction for the same locus; fragments in both *EcoRI*/*MspI* and *EcoRI*/*HpaII* profiles indicated nonmethylated loci (5′-CCGG3′), whereas fragments present in the *EcoRI* + *HpaII* reaction but absent in the *EcoRI* + *MspI* were counted as hemimethylated loci, thus representing methylation on external cytosines in the 5′-CCGG-3′ context. Absence of loci in both *MspI* and *HpaII* may be the result of hypermethylation or absence of restriction sites [20]. A comparison of the number of methylated loci between wild and cultivated watercress was made by an analysis of variance ($p < 0.05$) and means were separated by the Tukey test. A binary matrix encoded as 1 (presence) or 0 (absence) of individual loci was constructed. This matrix was submitted to a cluster analysis (Ward's method), using Past 1.43.The matrix was also used to calculate the epigenetic variability within groups through Nei's index, diversity index, and polymorphism (P) using InfoGen/E software. The epigenetic variability between groups was estimated with the coefficient of gene differentiation (Gst) and gene flow (Nm) using Popgene 1.32.

The bands from each type of genetic marker (five ISSR primers and *EcoRI* + *MspI*) were considered as single molecular characters and separately assessed in a binary matrix coded by 1 (presence) or 0 (absence). The matrixes were submitted to a cluster analysis (Ward's method) using Past 1.43. The genetic variability within groups was calculated by the estimation of Nei's index, diversity index, and polymorphism (P), whereas the genetic variability between groups was calculated by the Gst and Nm using Infogen/E and Popgene 1.32 software. The correlation between genetic and epigenetic variability

Table 4
Phenolic and flavonoid content of wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*).

Content	Wild	Cultivated
Total phenolics (mg GAE/g DE)	23.16 ± 0.45^{b}	29.11 ± 1.93^{a}
Flavonoids (mg QE/g DE)	13.18 ± 0.11^{a}	5.01 ± 0.15^{b}
Condensed tannins (mg EE/g DE)	9.07 ± 0.39^{a}	5.95 ± 0.12^{b}

The values represent the mean and standard deviation for three independent samples. Different letters in a same line mean significant differences ($p < 0.05$). GAE: Gallic acid equivalents, QE: Quercetin equivalents, EE: Epicatechin equivalents, DE: Dry extract.

was estimated with the respective distance matrixes by the Mantel test using XLSTAT 2017.03.44803.

3. Results and discussion

3.1. Phenolic variation

Total phenolic content was significantly higher in cultivated than in wild watercress (Table 4), with both watercress types having levels of total phenolics higher than those in broccoli (*Brassica oleracea*), for which Bhagat et al. [21] reported 10.55 mg/g. The levels of total phenolics in the cultivated type were found to be higher than those in the wild type of *Valerianella eriocarpa* (1.39 mg/g and 1.18 mg/g, respectively) [22].

The flavonoid content was significantly higher in wild than in cultivated watercress (Table 4), thereby accounting for 56.90% and only 17.21% of the total phenolics in wild and cultivated watercress, respectively. Both watercress types had higher levels of flavonoids than broccoli (2.85 mg/g) [21]. Flavonoids protect plants against UV-B radiation and herbivores [23], roles that are mainly needed by wild watercress.

Wild watercress also accumulated a significantly higher level of condensed tannins than cultivated watercress (Table 4), both having higher levels than white cabbage (0.50 mg/g) and broccoli (0.41 mg/g) [24].

The HPLC-DAD analysis revealed six major phenolic compounds. The chromatograms and UV spectra of each resolved compound are displayed in Fig. 1. Compounds **1** and **3** were two methylisorhamnetin-3-*O*-glycosides. Compounds **2**, **4**, **5**, and **6** were phenolic acids; **6** was identified as chlorogenic acid because its RT and λ_{max} coincided with those of this compound analyzed as standard. In addition to chlorogenic acid, Aires et al. [9] reported gallic acid, caffeic acid, dicaffeoyltartaric acid, rutin, and isorhamnetin, whereas Boligon et al. [25] reported caffeic acid and rutin for cultivated watercress from Portugal and Brazil, respectively. Furthermore, for wild watercress from Pakistan, Zeb [26] reported 12 phenolic compounds including phenolic acids, the flavone apigenin, and one quercetin-3,7-diglycoside. All the above-mentioned phenolic compositions of watercress were different from each other. These differences may be the result of variations in the methods of analysis used in each study. However, as a species-specific trend has been reported for the phenolic profiles [27,28], which results from genetic differences, thus commanding a defined sequential order in the biosynthesis pathway, which is specific for each plant species [29], the inconsistencies between the reports of the phenolic composition of watercress from different geographical occurrence need a chemotaxonomic revision.

Table 5
Concentration of the phenolic compounds present in two types of watercress (*Rorippa nasturtium* var. *aquaticum*).

Number of compound	Phenolic compound	Wild watercress	Cultivated watercress
		mg/g DE	mg/g DE
1	Methylisorhamnetin-3-*O*-glycoside	3.386 ± 0.208[a]	0.038 ± 0.001[b]
2	Phenolic acid	8.078 ± 0.446[a]	4.690 ± 0.411[b]
3	Methylisorhamnetin-3-*O*-glycoside	4.395 ± 0.105	ND
4	Phenolic acid	1.915 ± 0.181[a]	0.805 ± 0.089[b]
5	Phenolic acid	1.128 ± 0.061[a]	0.761 ± 0.053[b]
6	Chlorogenic acid	4.036 ± 0.161[a]	1.293 ± 0.180[b]

The values represent the mean and standard deviation for three independent samples. Different letters in a same line mean significant differences ($p < 0.05$). DE: Dry extract; ND: Not determined due to co-elution along with **2**.

In the current study, the qualitative phenolic profiles of both watercress types were the same (Fig. 1), as expected for conspecific populations. The concentration of each phenolic compound was the variable, all being higher in the wild type than in the cultivated type (Table 5). The relative concentrations of compounds inside a single profile were also different between the two watercress types, in agreement with the proposal that under variable environmental conditions the main changes occur in the concentration of individual phenolics, keeping the qualitative composition constant [27]. The

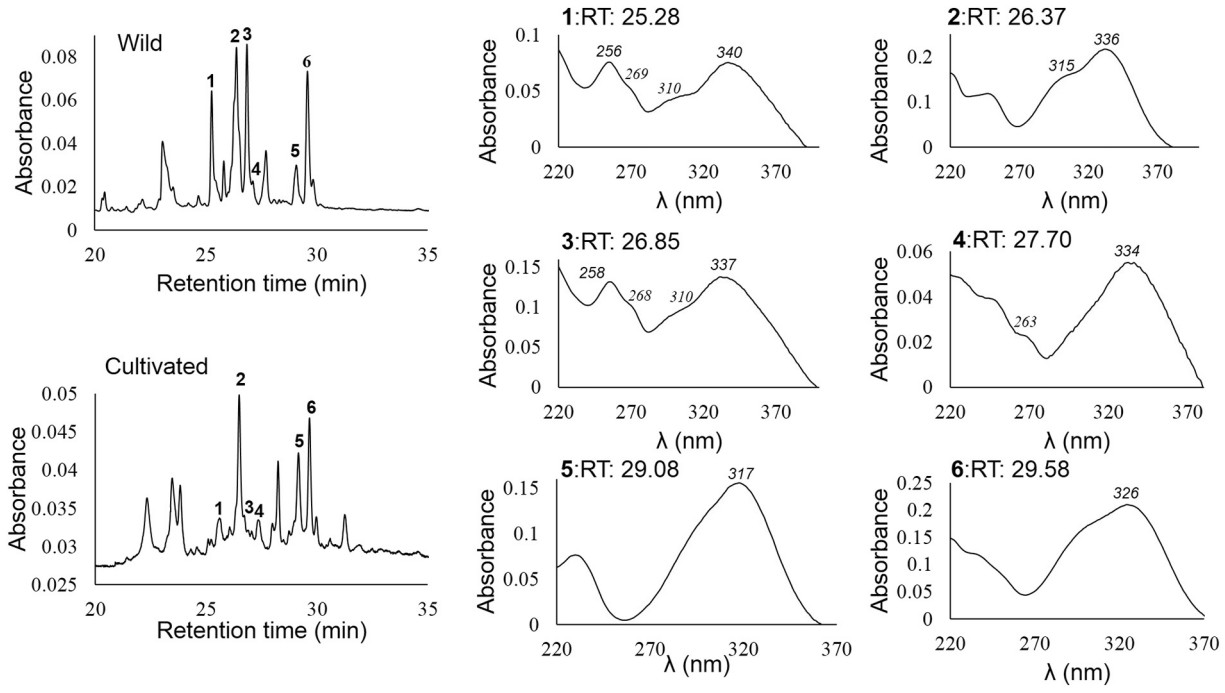

Fig. 1. HPLC chromatograms and UV spectra of the phenolic compounds of wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*). Retention time (min) and λ_{max} (in italics) are indicated.

Table 6
Methylation patterns of wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*) analyzed per *EcoRI* and *MspI*/*HpaII* primer combination.

EcoRI selective primer	*MspI*/*HpaII* selective primer	Methylated loci	Non-methylated loci	Hemi-methylated loci	Loci per Primer combination	Methylation (%)
WILD						
AG	TCAA	17	16	10	43	39.53
AG	AAT	13	11	7	31	41.94
AC	TCAA	23	13	10	46	50.00
AC	AAT	24	20	15	59	40.68
AAC	TCAA	10	20	6	36	27.78
AAC	AAT	11	20	10	41	26.83
AT	TCAA	22	18	13	53	41.51
AT	AAT	9	21	15	45	20.00
TOTAL		129	139	86	354	36.44
CULTIVATED						
AG	TCAA	22	23	24	69	31.88
AG	AAT	12	17	14	43	27.91
AC	TCAA	18	14	12	44	40.91
AC	AAT	16	19	15	50	32.00
AAC	TCAA	13	18	6	37	35.14
AAC	AAT	18	21	11	50	36.00
AT	TCAA	19	15	12	46	41.30
AT	AAT	15	23	17	55	27.27
TOTAL		133	150	111	394	33.76

concentrations of all individual phenolic compounds detected in wild watercress were higher than those reported by Aires et al. [9] for organic-grown watercress from Portugal, which ranged between 2.0 and 200 mg/kg dry weight.

3.2. Methylation patterns

To the best of our knowledge, the natural epigenetic variability of watercress has not been assessed. However, given the important role that epigenetics has on the regulation of the gene expression [5], it is relevant to assess how different growth conditions affect the epigenetic status.

The different *EcoRI*-*MspI*/*HpaII* primer combinations revealed variable levels of methylated loci, nonmethylated loci, and hemimethylated loci between wild and cultivated watercress (Table 6). The levels of methylation found for wild and cultivated watercress were similar to those found for wild and cultivated *B. oleracea* (30–40%), which have been reported as having high levels of methylation [20].

The methylation levels found for both watercress types were higher than those reported for leaves of maize inbred lines (21.44%) [30] and potato [31]; for potato, methylation levels were extremely low (0–3.4%). However, compared to pepper (*Capsicum* sp.) (64.36–67.00%) [32], the methylation levels found for both watercress types were low. All these values suggest the existence of very different profiles of DNA methylation in the plant kingdom.

A total of 354 fragments of wild and 394 fragments of cultivated watercress were amplified by the eight primer combinations used (Table 6). Each primer combination originated between 31 and 69 fragments. For wild watercress, the AC/AAT primer combination produced the highest number of amplified loci, whereas for cultivated watercress, the AG/TCAA primer combination produced the highest number of amplified loci. These differences by themselves indicated epigenetic differences between wild and cultivated watercress. Some of these primer combinations were used by Lira-Medeiros et al. [18] for estimating epigenetic variability of *Laguncularia racemosa* (L.) Gaertn, and they reported that the AAC/AAT primer combination was the most informative, thus producing the highest number of amplified loci (70 of a total of 209); on the contrary, this combination was the least informative in cultivated watercress and the second least informative in wild watercress.

3.3. Epigenetic and genetic variation within populations

The epigenetic and genetic variations within populations are given in Table 7. Estimations of Nei index, diversity index, and polymorphism revealed the cultivated type as the most epigenetically variable. The epigenetic polymorphisms of both watercress types were higher than those found for two varieties of *B. oleracea* (30% and 40%) [20].

The genetic variability within populations evaluated with the *EcoRI*/*MspI* revealed higher levels of variability in cultivated watercress than in wild watercress (Table 7). Genetic polymorphism of both watercress types was lower than those reported for 10 natural populations of *S. alterniflora* (91.67% to 100%) and *B. frutescens* (81.25–100%), two salt marsh perennials [4].

Contrary to the genetic variability revealed by the *EcoRI*/*MspI* marker, ISSR markers showed a higher genetic variability in the wild than in the cultivated watercress (Table 7). These results are in agreement with the proposal of Duchemin et al. [33] that different regions of the genome accumulate changes in a differential manner and suggest that the genetic variability must be estimated with more than one type of marker. The genetic polymorphisms estimated by

Table 7
Parameters evaluating genetic and epigenetic variation within wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*).

Variability measure	Epigenetic variability (*EcoRI*/*HpaII*)		Genetic variability (*EcoRI*/*MspI*)		Genetic variability (ISSR)	
	Wild	Cultivated	Wild	Cultivated	Wild	Cultivated
Nei's index	0.12^a	0.22^b	0.13^a	0.19^b	0.23^a	0.16^b
Diversity index	0.11^a	0.22^b	0.13^a	0.19^b	0.22^a	0.22^a
Polymorphism (%)	42^a	67^b	61^a	71^b	86^a	60^b

Different letters in the same line for each evaluation (epigenetic variability, genetic variability evaluated with *EcoRI*/*MspI*, and genetic variability evaluated with ISSR) mean significant differences ($p < 0.05$).

Table 8
Genetic and epigenetic diversity between wild and cultivated watercress (*Rorippa nasturtium* var. *aquaticum*).

Estadistic	Epigenetic (*EcoRI*/*HpaII*)	Genetic (*EcoRI*/*MspI*)	Genetic (ISSR)
Nm	0.5337	0.6776	2.7023
Gst	0.4837	0.4246	0.1561

Nm = gene flow, Gst = coefficient of gene differentiation.

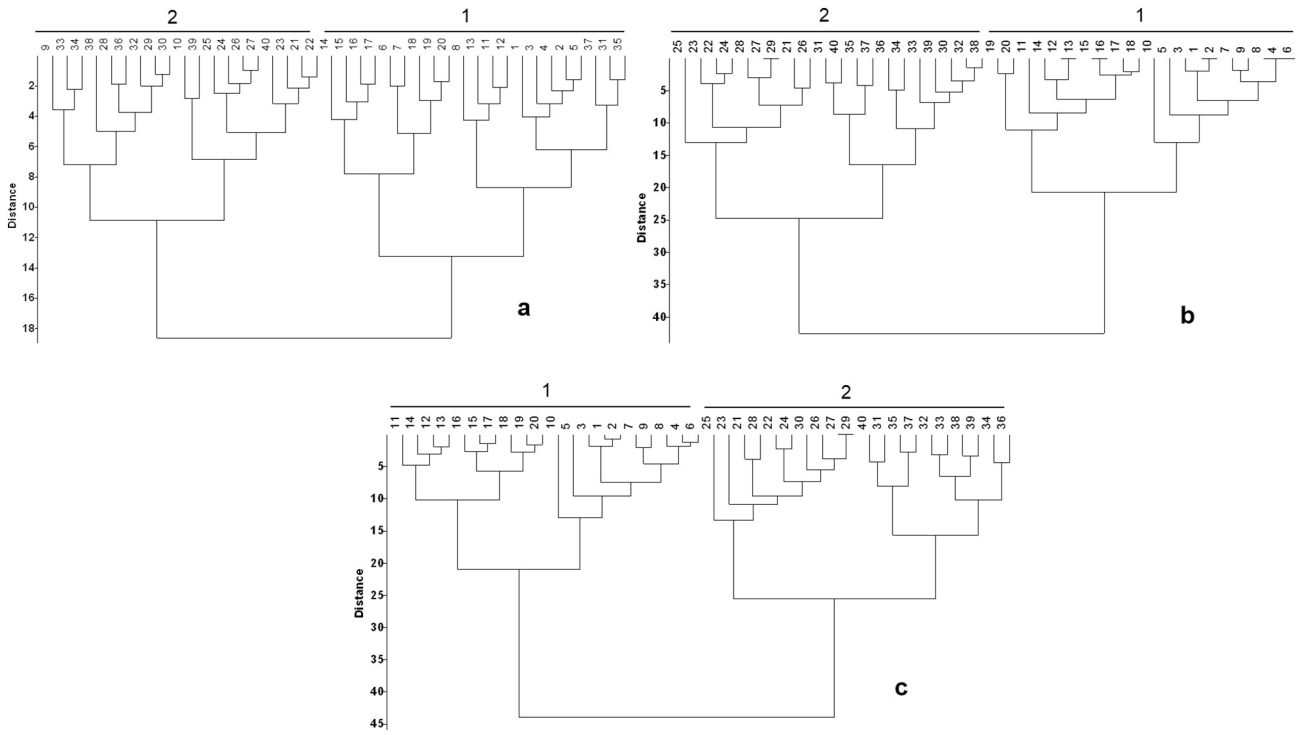

Fig. 2. Results of a cluster analysis comparing **a**: ISSR profiles, **b**: *EcoRI*/*MspI* profiles, and **c**: epigenetic profiles of wild (group 1) and cultivated (group 2) watercress (*Rorippa nasturtium* var. *aquaticum*).

ISSR for both watercress types were lower than those reported for *B. oleracea* subsp. *italica* cv. Green Marvel (85%), calculated with the same type of markers [34].

Cultivated watercress was more epigenetically variable than the wild type, whereas wild watercress was more genetically variable (estimated using ISSR markers) than cultivated watercress (Table 7). The current results are in disagreement with those of Lira-Medeiros et al. [18], who reported a higher epigenetic variability than genetic variability for the wild mangrove *L. racemosa*.

3.4. Epigenetic and genetic variations between populations

Epigenetic and genetic differentiation between wild and cultivated watercress is given in Table 8. The results showed that the epigenetic differentiation between both watercress types (Gst = 0.4837) was higher than the genetic differentiation, estimated by both *EcoRI*/*MspI* (Gst = 0.4246) and ISSR (Gst = 0.1561) markers. The high Nm value (2.7023) revealed by ISSR markers between wild and cultivated watercress indicated that gene flow has promoted important genetic similarities between both types of watercress. Nm values >1 are expected among conspecific population [35]. The gene flow found in the current study was in the range reported for *Alyssum stapfii* (0.1 to 3.0) population by Vaghefi et al. [36].

3.5. Cluster analysis

The variability of both watercress types also is seen in three cluster analysis, each based on the epigenetic profile, *EcoRI*/*MspI* profile, or ISSR profile. With the exception of five of 40 individuals analyzed, the ISSR profiles (Fig. 2a) discriminated between wild and cultivated watercress; however, the *EcoRI*/*MspI* and the epigenetic profiles (Fig. 2b and Fig. 2c) clearly discriminated between wild and cultivated watercress. These results indicate that the accumulation of epigenetic and genetic changes has been of magnitude such that both types of watercress can be clearly differentiated. The epigenetic and genetic results are in agreement with the results of chemical analysis, which

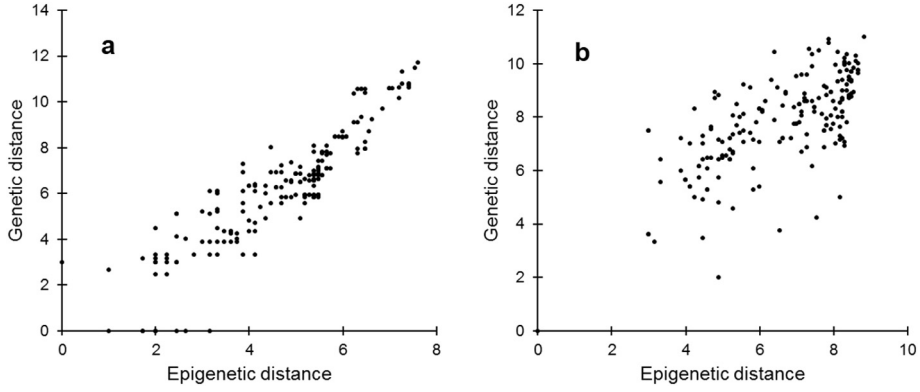

Fig. 3. Mantel test correlation between the genetic variation (evaluated with the *EcoRI*/*MspI* profile) and epigenetic variations for wild ($r = 0.893$, $p < 0.001$) and cultivated ($r = 0.697$, $p < 0.001$) watercress (*Rorippa nasturtium* var. *aquaticum*).

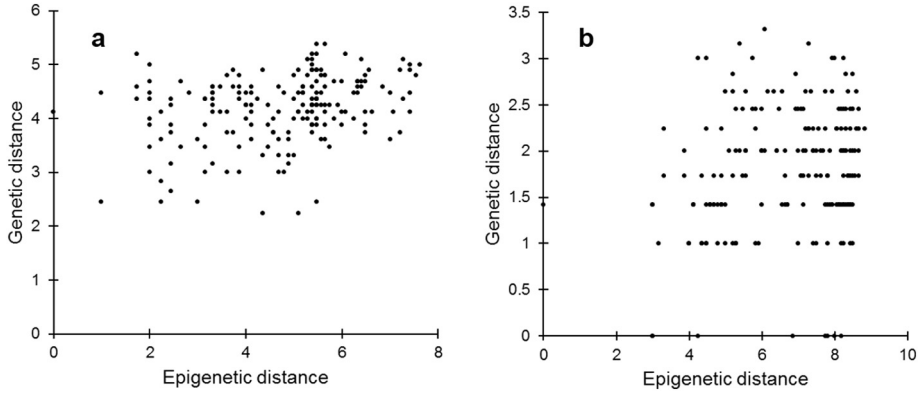

Fig. 4. Mantel test correlation between the genetic variation (evaluated with eight ISSR markers) and epigenetic variation for wild ($r = 0.301$, $p < 0.001$) and cultivated ($r = 0.207$, $p < 0.001$) watercress (*Rorippa nasturtium* var. *aquaticum*).

also provided discrimination between wild and cultivated watercress (Table 4).

The epigenetic and chemical differences found between both types of watercress may be the results of the different environmental conditions under which each one grows, as environmental factors have been reported to alter DNA methylation patterns in plants [2], which in turn alter the expression of genes involved in the synthesis of phytochemicals [5]. In the current study, four of the eight EcoRI selective primers revealed lower levels of methylated loci for wild than for cultivated watercress (Table 6). These lower levels may promote the accumulation of the high levels of flavonoids and tannins found for wild watercress (Table 4 and Table 5), as it has been reported that demethylation enhances the expression levels of genes encoding enzymes of flavonoid biosynthesis [37].

The genetic differences (Table 8) found between wild and cultivated watercress can be the result of the geographical isolation and human management, which generate a current poor ongoing gene flow, thus promoting these differences.

3.6. Relationship between genetic and epigenetic variability

For both types of watercress, a significant correlation between the genetic variation evaluated with the *EcoRI*/*MspI* profile and epigenetic variation was observed (Fig. 3). However, the epigenetic variation of wild watercress ($r = 0.893$) was higher than that of cultivated watercress ($r = 0.697$). Similar results were found when genetic variation was evaluated with ISSR markers (Fig. 4), although the r values were lower ($r = 0.301$ for wild watercress and $r = 0.207$ for cultivated watercress). Foust et al. [4] also found a correlation between genetic and epigenetic variations for wild populations of two salt marsh perennials, obtaining r values between 0.424 and 0.017. The current results indicate an important genetic participation in determining the epigenetic variation of wild watercress. This participation has diminished in the cultivated type. This diminution suggests that the environmental context has a more important participation than the genetic context in determining the epigenetic variation of cultivated watercress.

4. Conclusion

Relevant chemical, epigenetic, and genetic differences have emerged between wild and cultivated watercress in such a way that they can be clearly discriminated by their phenolic composition and their genetic and epigenetic variations. These differences may be the result of the growing conditions and selective processes to which the cultivated type has been subjected by humans. The richness of epialleles could support the development of tools to manipulate the watercress epigenome to develop high bioproduct–producing cultivars. Chemical, epigenetic, and genetic characterization of watercress can contribute to develop quality control tools, fingerprinting, and a kind of passport/ID for the integral and safety use and the commercialization of this edible and medicinal plant.

Declaration of interest

None.

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