doi:10.14720/aas.2023.119.1.2787 Original research article / izvirni znanstveni članek

Preliminary assessment of genetic diversity between *Glebionis coronaria* and *G. discolor* (Asteraceae) by AFLP markers

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Received July 25, 2022; accepted February 21, 2023.
Delo je prispelo 25. julija 2022, sprejeto 21. februarja 2023

Preliminary assessment of genetic diversity between *Glebionis coronaria* and *G. discolor* (Asteraceae) by AFLP markers

Abstract: *Glebionis coronaria* is a valuable and medicinal herb native of Mediterranean region. Recently, *G. coronaria* var. *discolor* has been elevated to the rank of species as *G. discolor* (d'Urv.) Cano based on morphological characteristics, distinguishing it from *G. coronaria* var. *coronaria* (=*G. coronaria*). To investigate the genetic basis of this diversity, AFLP markers were applied to genotypes of *G. discolor* and *G. coronaria* sampled in three different Mediterranean regions (Italy, Spain, and Portugal). Our results showed that among 1347 fragments identified with five primer combinations 99.55 % were polymorphic. The genetic distance and the Shannon Index values suggested that the two species can be genetically distinguished, but further studies are needed to confirm this hypothesis.

Key words: biodiversity; Compositae; *Chrysanthemum*; daisy; garland chrysanthemum; taxonomy

Preliminarno ovrednotenje genetske raznolikosti med vrstama *Glebionis coronaria* and *G. discolor* (Asteraceae) z AFLP markerji

Izvleček: Vrsta *Glebionis coronaria* je cenjeno zdravilno zelišče, samoniklo na območju Mediterana. V zadjem času je bila različica *G. coronaria* var. *discolor* dvignjena na rang vrste kot vrsta *G. discolor* (d'Urv.) Cano na osnovi morfoloških lastnosti, po katerih se razlikuje od vrste *G. coronaria* var. *coronaria* (=*G. coronaria*). Za preučitev genetske osnove te raznolikosti so bili uporabljeni AFLP markerji za analizo genotipov vrst *G. discolor* in *G. coronaria* vzorčenih na različnih območjih Mediterana (Italija, Španija in Portugalska). Rezultati so pokazali, da je bilo med 1347 fragmenti, identificiranih s kombinacijami petih primerjev 99,55 % polimorfnih. Genetska razdalja in vrednosti Shannonovega indeksa nakazujejo, da sta vrsti genetsko ločeni, a so potrebne nadaljne raziskave za potrditev te hipoteze.

Ključne besede: biodiversiteta; Compositae; *Chrysanthemum*; ivanjščica; užitna ivanjščica; taksonomija

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1 INTRODUCTION

Asteraceae is one of the worlds' richest and the most diverse plant families in biological and ecological terms (Cano et al., 2020). This angiosperm family comprises 1,739 genera including 36,033 species (Hassler, 2021), taxonomically continuously updated. Several of them have various uses around the world, due to their chemical composition for medicinal and food purposes (Mezhoud et al., 2012; Saoud et al., 2019; Pace et al., 2020; Nkuimi Wandjou et al., 2020; Singh et al., 2020; Bhat et al., 2021; Sicari et al., 2021). Among all, *Glebionis coronaria* (L.) Cass. ex Spach (synonym: *Chrysanthemum coronarium* L.) is an annual plant, frequent in ruderal vegetation, in field margins, road verges, and urban wastelands, widely distributed in the Mediterranean basin, Western Africa and Asia (da Silva et al., 2005). In the last few years there has been a growing interest in this species due to its biological activities, such as insecticidal, antifungal, cancer prevention, antioxidant and anti-inflammatory (Yildirim et al., 2018; Khareba et al., 2021). Historically, based on the different colour and morphology of the flowers, d'Urville (1822) distinguished two varieties of this species: *Chrysanthemum coronarium* var. *concolor* d'Urv. (= *G. coronaria* var. *coronaria*) and *Chrysanthemum coronarium* var. *discolor* d'Urv. (= *G. coronaria* var. *discolor* (d'Urv.) Turland). Furthermore, a recent study demonstrated that *G. coronaria* var. *discolor* can be separated and elevated to the rank of species, based on the disposition of the intercostal glands, the size of the disc cypsela wings and bioclimatological traits, as *Glebionis discolor* (d'Urv.) Cano, Musarella, Cano-Ortiz, Piñar Fuentes, Spamp. et Pinto Gomes (Cano et al., 2017). These authors documented that *G. coronaria* has totally yellow ray florets and intercostal glands aligned, while *G. discolor* has white ray florets on a yellow base and intercostal glands arranged randomly. Another feature highlighted by Cano et al. (2017) concerns the distribution of these two species in the Mediterranean basin: indeed, *G. coronaria* is distributed mainly throughout the thermo-Mediterranean bioclimatic belt, while *G. discolor* is spread also in the meso-Mediterranean one. Few other authors phenotypically recognize and/or report *G. discolor*, such as Cueto et al. (2018), Bartolucci et al. (2018), Portal to the Flora of Italy (2022) and POWO (2022). Among them, the last three sources endorse that *G. discolor* is a doubtful taxon or is a synonym of *G. coronaria*, contrary to Cano et al. (2017). In this contest, DNA-based markers (also named molecular markers) represent a powerful tool to fingerprint unequivocally the identity of these species. Indeed, in the last decades, they have been successfully used for investigation of interspecific and intraspecific genetic variability in various plants (Carputo et al., 2013; Villano et al., 2014, 2022, 2023). Among the *plethora* of molecular markers available, the best choice for *Glebionis* spp. can be represented by AFLP (Amplified Fragment Length Polymorphism) markers, due to the absence of a reference genome. Their main advantage is the employment of a standard protocol in combination with different restriction endonucleases to achieve optimal fingerprints without prior knowledge of the organism's genome sequence. These markers have been pioneered by Vos et al. (1995) and have been used in various species, such as *Dioscorea* spp. (Rivera-Jiménez et al., 2011) and *Gynerium sagittatum* (Aubl.) P.Beauv. (Rivera-Jiménez et al., 2008). This paper aims to analyse the genetic variability between *G. coronaria* and *G. discolor* using combinations of AFLP markers.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL AND DNA EXTRACTION

Three samples of *G. coronaria* and three of *G. discolor* (Figure 1) were collected in Italy, Spain, and Portugal in three biological replicates and stored at the herbarium of the Mediterranean University of Reggio Calabria (REGGIO) (acronym follows Thiers, 2023), as below detailed according to the original labels:

Italy:

1) *G. coronaria* - SIC Fiumara di Melito P.S. (Reggio Calabria). 03/05/2018. Collectors: C.M.Musarella & G.Spampinato.

2) *G. discolor* - Spiaggia di Palizzi Marina, Palizzi (Reggio Calabria). 14/05/2018. Collectors: C.M.Musarella & G.Spampinato.

Spain:

1) *G. coronaria* - Near Urbanización Salobreña (Granada). Alt. 13 m asl. 30S0448140/4064980. 05/05/2018. Collectors: E.Cano, A.Cano-Ortiz & J.C.Piñar Fuentes.

2) *G. discolor* - Near Hotel Soto, Andujar (Jaén). Alt. 220 m asl. 30S0405061/4209759. 06/05/2018. Collectors: E.Cano & A.Cano-Ortiz.

Portugal:

1) *G. coronaria* - Faro. 20/05/2018. Collector: R. Quinto Canas

2) *G. discolor* - Tavira, Pedras del Rey. 15/06/2018. Collector: R. Quinto Canas.

The samples were processed using the DNeasy Plant Mini Kit (Qiagen) previously described by Tengel et al. (2001). The quantity and quality of the isolated gDNA were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA).

Figure 1: Capitula of (left) *Glebionis coronaria* (L.) Cass. ex Spach (Pentidattilo, Reggio Calabria, Italy – April 18, 2022) and (right) *G. discolor* (d'Urv.) Cano, Musarella, Cano-Ortiz, Piñar Fuentes, Spamp. et Pinto Gomes (Sevilla, Spain – March 28, 2013). Ph. C.M.Musarella

2.2 AFLP ANALYSIS

The analysis was performed using the method described by Vos et al. (1995) and the commercially available AFLP kit and protocol (Gibco-BRL AFLP analysis System I, Life Technologies, Gaithersburg, MD), which employs EcoRI and MseI as restriction enzymes. For selective amplification, five combinations of primers were used (E-AGG + M-CAG; E-AGC + M-CAC; E-AGG + M-CTT; E-AGC + M-CAC; E-ACT + M-CAG) with the E primer labelled with FAM or HEX. Amplicons were separated with the ABI PRISM® 3130 DNA Analyzer system (Life Technologies, Carlsbad, California, USA). Size calibration was performed with the molecular weight ladder GenScan® 500 ROXTM Size Standard (Life Technologies, Carlsbad, California, USA). AFLP fragments were detected and scored using Peak Scanner® software (Applied Biosystems, Foster) and combined into a binary matrix. Three technical and three biological replicates were considered.

2.3 DATA AND PHYLOGENETIC ANALYSIS

The statistical software Genalex 6.5 (Peakall & Smouse, 2006) was used for data analysis. The input file was created considering each band as one diallelic locus (1 means presence of band, 0 means absence of band). Per each species were calculated the observed number of alleles (Na), the number of total bands and number of bands unique, the effective number of alleles (Ne), the Shannon's information index (I) and the percentage of polymorphic loci (P %). To visualize interspecies and individuals' relationships, a principal coordinates analysis (PCoA) was performed (Nei & Li, 1979).

3 RESULTS AND DISCUSSION

Five selective AFLP primer combinations generated a total of 1347 fragments, distributed between 50 and 500 bp, of which 1341 (99.55 %) were polymorphic (Table 1). The combinations E-AGG/M-CAG and E-AGG/M-CTT were the most informative ones with 248 and 274 polymorphic bands, respectively (Table 1). Two-hundred seventy-two species-specific bands were identified in *G. discolor* and 224 in *G. coronaria* (Table 1).

The genetic variation was measured within the two species considering the number of effective and different alleles (named Ne and Na, respectively), the Shannon's information index (I), the percentage of polymorphic loci (P %) and the number of species-specific bands, called private bands (Table 2). Among all, the Shannon's indexes in *G. coronaria* and *G. discolor* species were 0.369 and 0.353, respectively. The Na values were 1.398 in *G. coronaria* and 1.341 in *G. discolor* while the Ne values were 1.414 in *G. coronaria* and 1.398 in *G. discolor*. The percentage of polymorphic loci showed that the most

Table 1: Results of AFLP analyses used to differentiate *Glebionis coronaria* (L.) Cass. ex Spach and *G. discolor* (d'Urv.) Cano, Musarella, Cano-Ortiz, Piñar Fuentes, Spamp. et Pinto Gomes genotypes

AFLP combinations	Total bands	Polymorphic bands, n°	Polymorphic bands, %	Specie-specific bands, n°	
				G. coronaria	*G. discolor*
E-AGG/M-CAG	249	248	99.60	61	53
E-AGC/M-CAC	310	308	99.35	21	45
E-AGG/M-CTT	275	274	99.64	51	54
E-AGC/M-CAC	327	325	99.39	30	74
E-ACT/M-CAG	186	186	100.00	61	46

Table 2: Statistical analysis of *Glebionis coronaria* (L.) Cass. ex Spach and *G. discolor* (d'Urv.) Cano, Musarella, Cano-Ortiz, Piñar Fuentes, Spamp. et Pinto Gomes. Na = No. of different alleles; Ne = No. of effective alleles; I = Shannon's information index; P % = percentage of polymorphic loci; No. Private Bands = No. of bands unique to a single species

Species	Na	Ne	I	P %	No. Private Bands
G. coronaria	1.398	1.414	0.369	67.12%	359
G. discolor	1.341	1.398	0.353	63.90%	315

Table 3: Nei's Original Measures of genetic distance among samples of *Glebionis coronaria* (L.) Cass. ex Spach and *G. discolor* (d'Urv.) Cano, Musarella, Cano-Ortiz, Piñar Fuentes, Spamp. et Pinto Gomes from: P = Portugal; S = Spain; I = Italy. The highest values are reported in bold

	G. discolor P	*G. discolor* I	*G. coronaria* I	*G. coronaria* S	*G. discolor* S	*G. coronaria* P
G. discolor P	0					
G. discolor I	0.574	0				
G. coronaria I	0.580	0.599	0			
G. coronaria S	**0.642**	0.527	0.556	0		
G. discolor S	0.595	0.546	**0.642**	0.583	0	
G. coronaria P	0.415	0.514	0.576	0.483	0.522	0

polymorphic species was *G. coronaria,* with 67.12 % of polymorphic loci and 359 private bands.

To investigate the genetic distance of the analysed species, the Nei's value was calculated. Our results showed that the highest variation was found between *G. coronaria* from Spain and *G. discolor* from Portugal (0.642), and between *G. coronaria* from Italy and *G. discolor* from Spain (0.642) (Table 3).

In order to obtain further information on the grouping of the two species, we carried out PCoA, using AFLP band pattern as raw data. The PCoA (Figure 2) clearly reflected the relationships among and between the genotypes analysed. The first and second component could explain 22.6 % and 20.7 % of the variation, respectively. *G. discolor* from Portugal have been classified apart from *G. coronaria* genotypes along the two axes. Furthermore, the first axis separated two *G. coronaria* genotypes (Portugal and Italy) from two *G. discolor* ones (Spain and Italy).

The obtained results showed that the *G. coronaria* and *G. discolor* can be distinguished using these markers, but further studies with a higher number of molecular markers are needed to confirm it. The genetic difference between these species has been always investigated using phenotypic attributes; only Ata et al. (2017) investigated the relationships of 12 species belonging to Asteraceae, including *G. coronaria* and *G. discolor*, using ITS sequence barcoding. They affirm that the analysed species could be distinctly separated on the genetic basis. This claim is in line with our results. Indeed, the high number of polymorphic bands, the Shannon index and Nei's gene diversity values suggest that the two groups of genotypes are sharing only part of the analysed fragments, and so can be considered as distinct genotypes. The level of polymorphism obtained here in terms of percentage of polymorphic bands with AFLP markers and the genetic diversity expressed as Nei's gene diversity as well as Shannon's information index values is higher than that

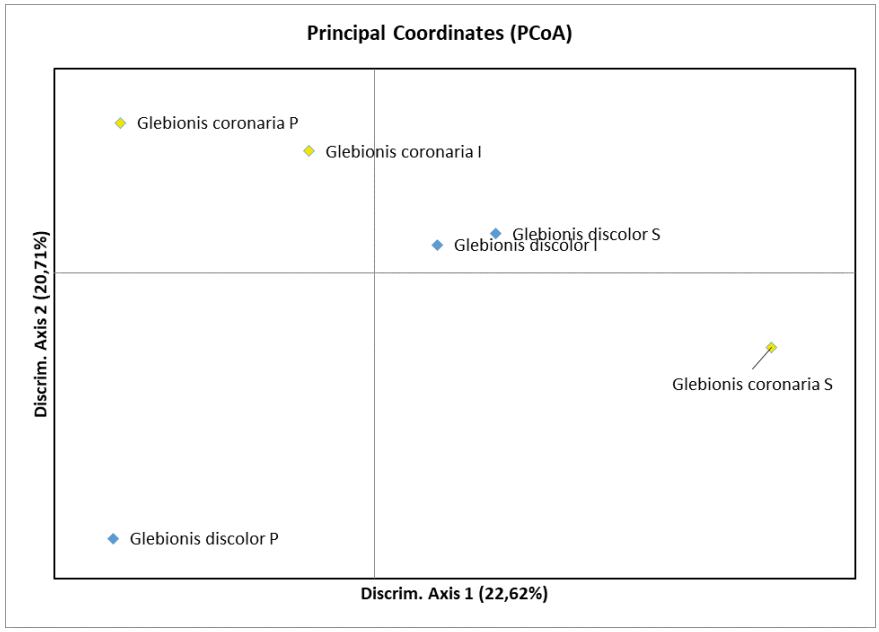

Figure 2: Principal coordinates analysis (PCoA) of three samples of *Glebionis coronaria* (L.) Cass. ex Spach and three of *G. discolor* (d'Urv.) Cano, Musarella, Cano-Ortiz, Piñar Fuentes, Spamp. et Pinto Gomes based on AFLP data (S = Spain; I = Italy; P = Portugal)

reported for other Asteraceae (Nguyen et al., 2013; Kropf et al., 2017; Wu et al., 2019), confirming the effectiveness of the markers used to distinguish the population studied. Furthermore, the PCoA analysis clearly separated *G. coronaria* apart from *G. discolor* along both axes. The different distribution of *G. coronaria* from Portugal and Italy vs genotypes from Spain and of *G. discolor* from Spain and Italy vs genotypes from Portugal could be related to the area of origin.

4 CONCLUSIONS

In the present study, the genetic diversity of two *Glebionis* species has been investigated through an AFLP analysis. The genotypes considered came from *G. coronaria* and *G. discolor* samples collected in three different countries (Spain, Portugal and Italy). Our results, together with the phenotypical studies conducted by Cano and collaborators (2017), allowed the separation of *G. discolor* from *G. coronaria*, confirming that the best way to distinguish some individuals is the combo of molecular markers and phenotypic attributes. We contemplate that this study is showing partial results. Indeed we know that the number of samples used is not fully representative of the *Glebionis* germplasm. However, these preliminary results confirmed the potential resolving power of AFLP analysis for a genome lacking species and could be considered as a starting point for future researches in a larger collection of genotypes.

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