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Cross-Species Transferability of Specific SSR Markers from *Carex curvula* (Cyperaceae) to Other *Carex* Species

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Abstract: Microsatellites are codominant markers that, due to their high polymorphism, are a common choice for detecting genetic variability in various organisms, including fungi, plants, and animals. However, the process of developing these markers is both costly and time-consuming. As a result, the cross-species amplification has become a more rapid and more affordable alternative in biological studies. The objective of this study was to assess the applicability of 13 SSR markers, originally designed for *Carex curvula*, in other 14 species belonging to different sections of the genus. All the markers were successfully transferred with a mean of 90.76%, and 100% transferability was reached in two species (*C. baldensis* and *C. rupestris*). The lowest transferability was registered in the G165 marker, which did not produce amplification in six species. Together, the microsatellites amplified a total of 183 alleles, ranging from 10 to 19 alleles per locus, with an average of 14.07. The mean number of different alleles ranged from 0.846 to a maximum of 2.077 per locus. No significant departures from the Hardy–Weinberg equilibrium were detected in polymorphic loci. The transferability of the 13 SSR markers proved highly successful in various *Carex* species, across different clades and sections of the genus.

Keywords: *Carex* clades; genetic polymorphism; microsatellites; phylogeny; phylogeography

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1. Introduction

Microsatellite markers, also known as simple sequence repeats (SSRs), are intensively used in genetic studies for several distinctive features:, as follows: each marker is locus-specific and capable of revealing multiple alleles, they are inherited as codominant markers, they demonstrate a high level of reproducibility, and they offer broad genomic coverage [1,2]. Owing to the substantial variability between the number of tandem repeats present in individual loci, SSRs are recognized as one of the most polymorphic types of genetic markers [3].

Currently, microsatellites are the markers of choice in evolutionary biology studies for the assessment of genetic diversity among species, the determination of population structures, phylogenetic reconstructions, phylogeographic patterns, genetic mapping, evolutionary analyses, and molecular breeding [4,5].

When genomic data for specific species are unavailable, the use of microsatellites in genetic studies encounters a major bottleneck. This challenge primarily involves considerable time, research efforts, and financial resources for the development of customized primers to amplify distinct SSR loci. Therefore, overall, the information on SSR markers for less economically important species is still limited. A different approach to address this issue involves using the cross-amplification potential of microsatellite markers. This method allows for the exploration of genetic variability in various species, utilizing the conserved DNA sequences adjacent to these SSRs [6,7]. The efficacy of this method relies heavily on the degree of nucleotide similarity in the SSRs' flanking regions across various species. Consequently, it is anticipated that phylogenetically related taxa will exhibit a greater likelihood of success in achieving amplification due to their closer genetic relationships and more similar genomic structures [8,9]. Heterologous amplification has been extensively applied across a broad spectrum of plant species, at intergeneric (e.g., [10–14]) and infrageneric levels (e.g., [15–17]). As expected, the greatest success in terms of using cross-amplification was found when applying this approach at the infrageneric level (success rate of about 60% [8]).

Carex L. (Cyperaceae), with ca. 2061 accepted species, is the fourth most diverse angiosperm genus [18,19]. This genus is present all over the world, except in Antarctica [20,21]. *Carex* exhibits greater species diversity in the Northern Hemisphere, in temperate and boreal biomes [22]. In contrast, its diversity significantly diminishes in tropical regions, where its presence is largely confined to montane ecosystems [23].

The tremendous diversity of the genus has rendered the classification and taxonomic treatment of *Carex* species particularly challenging. Traditionally, the *Carex* genus has been divided into four subgenera, as follows: subg. *Psyllophora* (Degl.) Peterm.; subg. *Vignea* (P. Beauv. ex T. Lestib.) Peterm.; subg. *Vigneastra* (Tuck.) Kük.; and subg. *Carex* [24,25]. However, recent molecular studies have demonstrated that this treatment does not accurately reflect the phylogenetic relationships between taxa. Furthermore, the genera *Cymophyllus* Mack., *Kobresia* Willd., *Schoenoxiphium* Nees, and *Uncinia* Pers. have been reevaluated and included in *Carex* [21,26]. The most recent studies [18,26] divided the *Carex* genus into six subgenera (subg. *Siderosticta* Waterway, subg. *Psyllophorae* (Degl.) Peterm., subg. *Uncinia* (Pers.) Peterm., subg. *Vignea* (P. Beauv. ex T. Lestib.) Heer, subg. *Euthyceras* Peterm., and subg. *Carex*), which are further subdivided into 62 sections and groups.

Despite its enormous species diversity, only a few microsatellite markers have been specifically developed in the *Carex* genus. Initially, microsatellite markers studies targeted only few species (e.g., *Carex scabrifolia*, *C. moorcroftii*, *C. helodes*, *C. angustisquama*, *C. kobomugi*, *C. macrocephala*, *C. pumila* [27–34]), but more recently, 42 and 17 markers, respectively, were tested for 106 *Carex* accessions [33,35]. Almost all taxa included in these studies belong to the *Carex* or *Vignea* subgenera. There have been even fewer attempts to assess the transferability of microsatellite loci within the genus. Consequently, in a previous study, we developed and characterized a set of 13 polymorphic SSR markers in the alpine sedge, *Carex curvula* All. (*C. c.* subsp. *curvula* and *C. c.* subsp. *rosae* Gilomen), and we tested their applicability in species phylogeography and subspecies delimitation [36].

In the present study, we explored the transferability of these genomic SSR markers, which were specifically developed for *C. curvula*, to 15 populations belonging to 14 species of *Carex*. We specifically targeted species within the *Euthyceras–Psyllophorae* Clade [18]. To the best of our knowledge, only one study has been conducted on a single species from this clade, namely, *Carex onoei* [35]. This particular clade is intricate, encompassing species that were formerly placed within the obsolete genera, *Schoenoxiphium* and *Kobresia*.

2. Materials and Methods

- Plant sample collection and DNA extraction

Young, healthy, green leaves from two randomly chosen individuals per species/population were sampled from 15 locations across the Carpathians, Alps, Pyrenees, and

Svalbard (Table 1). After collection, the plant material was stored in tubes filled with silica gel, at room temperature, until DNA extraction.

Table 1. The Carex species used in the study (Clade/Section are in accordance with [18]).

Species	Clade/ Section	Country	Range	Massif	Longitude (E)	Latitude (N)
Carex pauciflora Lightf.	*Pauciflora*	Romania	Carpathians	Vlădeasa	22.69	46.44
Carex pyrenaica Wahlenb.	*Pauciflora*	Romania	Carpathians	Făgăraș	24.74	45.6
Carex parallela (Laest.) Sommerf.	*Physoglochin*	Norway	Svalbard	Ny-Friesland, Flatøyrdalen	15.99	79.28
Carex dioica L.	*Physoglochin*	Slovakia	Carpathians	Low Tatras	19.35	48.94
Carex baldensis L.	*Curvula*	Italy	Alps	Garda Mountains	10.36	45.61
Carex maritima Gunnerus	*Disticha*	Norway	Svalbard	Ny-Friesland, Wijdefjorden, Ringhorndalen	15.99	79.28
Carex pulicaris L.	*Psyllophorae*	France	Alps	Plateau Matheysin	5.77	44.99
Carex nardina Fr.	*Capitata*	Norway	Svalbard	Ny-Friesland, Flatøyrdalen	16.02	79.28
Carex microglochin Wahlenb.	*Capitata*	France	Alps	Vanoise Massif	7.1	45.37
Carex rupestris All.	*Capitata*	Romania	Carpathians	Bucegi	25.46	45.41
Carex dacica Heuff.	*Phacocystis*	Romania	Carpathians	Făgăraș	24.62	45.59
Carex nigra (L.) Reichard	*Phacocystis*	Slovakia	Carpathians	High Tatras	20.1	49.16
Carex simpliciuscula Wahlenb.	*Kobresia 2*	Romania	Carpathians	Bucegi	25.46	45.41
Carex myosuroides Vill. (pop. 1)	*Kobresia 1*	Spain	Pyrenees	Maladeta Massif	0.68	42.59
Carex myosuroides Vill. (pop. 2)	*Kobresia 1*	Andorra	Eastern Pyrenees	Aston Massif	1.7	42.58

The total genomic DNA was extracted from 13 to 15 mg of silica gel-dried leaf tissue, using TissueLyser II (Qiagen, Hilden, Germany) and the innuPREP DNA Mini Kit (Analytik Jena AG, Jena, Germany), in accordance with the manufacturer's protocol; however, the final elution took place in 50 µL in order to increase the DNA concentration. DNA quality was estimated using a 1% agarose gel stained with ethidium bromide, and the concentration was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

The nomenclature of the species listed in Table 1 adheres to the taxonomy provided in the *Plants of the World Online (POWO)* database [19].

- Biological validation

The amplification of the 13 SSR markers previously developed in *C. curvula* was tested on DNA extracted from 15 populations/14 species of *Carex*. According to [18], nine of the studied species are included in the *Euthyceras–Psyllophorae* Clade (subgenus *Psyllophorae*: *C. baldensis*, *C. pulicaris*; subgenus *Euthyceras*: *C. simpliciuscula*, *C. myosuroides*, *C. microglochin*, *C. nardina*, *C. rupestris*, *C. pauciflora*, *C. pyrenaica*), three species in subgenus

Vignea (*C. parallela*, *C. dioica*, *C. maritima*), and two are included in the subgenus *Carex* (*C. nigra*, *C. dacica*).

PCR protocol was followed in accordance with the one described by [36], as summarized below:

(i) PCR cycling conditions. Three min at 94 °C, 35 cycles for 1 min at 94 °C, 1 min at 55 °C (except VG139 and VG100, at an annealing temperature of 57 °C) and 2 min at 72 °C, followed by 7 min at 72 °C. PCR cycling was performed in a Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany).

(ii) PCR reaction conditions in a total volume of 10 µL. Here, 0.4 U KAPA Taq DNA Polymerase (Kapa Biosystems, Wilmington, MA, USA), 1× KAPA Taq Buffer A ($MgCl_2$ 1.5 mM included), 0.2 µM of each primer (the forward primer was fluorescently labelled with 6FAM: VG139, VG152, VG100, VG168, G110, VG203; NED: VG153, VG174, VG131, VG119; or HEX: VG175, VG108, G165), 0.25 mM of each dNTP (Thermo Fisher Scientific), 0.1 mg/mL BSA (Ambion, now part of Thermo Fisher Scientific), and 1.5 µL of DNA template (diluted four times) were used.

More details about the primers (including their sequences), the purification of the PCR products, and the subsequent separation that occurred through capillary electrophoresis can be found in [36].

- Microsatellite data analysis

GeneMapper v.4.0 software (Applied Biosystems, now part of Thermo Fisher Scientific) was used for scoring the alleles.

The reliability of the experiment was assessed via a comparison of two duplicates from different populations. Only the unambiguous and repeatable peaks between duplicates were scored.

The number of alleles, number of private alleles, percentage of polymorphic markers, observed (Ho) and expected heterozygosity (He), and departure from the Hardy–Weinberg equilibrium (HWE) were determined using GenAlEx 6.5 [37,38].

3. Results

The SSR markers developed for *C. curvula* showed great success in cross transferability in the analysed species. The amplification bands were scored to reveal polymorphism based on the presence or absence of alleles, and variations in allele size, at the same locus. The size range of these reproducible bands is presented in Table 2.

All the 13 SSR primers were successfully transferred with a mean percentage of 90.76. Of the 13 primer pairs, only five primer pairs produced amplification in all 15 populations, namely, VG174, G110, VG119, VG108, and VG153, indicating 100% transferability. Moreover, 93.33% transferability was recorded for VG139, VG168, and VG131. A lower percentage of transferability was exhibited by VG152, VG100, VG203 (86.66%), and VG175 (80%), and the lowest transferability (only 60%) was registered in G165.

One hundred percent transferability for all 13 SSR primers was recorded in two species (*C. baldensis* and *C. rupestris*). This was followed by 92.3% transferability in eight species (*C. pyrenaica*, *C. microglochin*, *C. nardina*, *C. parallela*, *C. maritima*, *C. pulicaris*, *C. dacica*, and *C. simpliciuscula*), and 84.61% in five populations (*C. pauciflora*, *C. dioica*, *C. nigra*, and the two populations of *C. myosuroides*).

A total of 183 alleles were identified at these 13 amplified loci, ranging from 10 (G165) to 19 (VG168) alleles per locus, with an average of 14.07 (Table 2).

Table 2. Cross-species amplification: size range of reproductible bands and number of alleles for 13 microsatellite loci developed for *Carex curvula* across 15 populations of *Carex*. IA = inconsistent amplification; NA = non-existent amplification.

Species/Population	**VG139**	**VG175**	**VG152**	**VG100**	**VG174**	**G165**	**VG168**	**G110**	**VG119**	**VG203**	**VG108**	**VG131**	**VG153**
Carex pauciflora	126	261	141	73	165	NA	203	99	113	**NA**	90	120	143
Carex pyrenaica	118	173–178	141	73	166	NA	188–197	90	107–122	259	109	118	171
Carex nardina	118	185	159	79	151	NA	166	97	110	194	105	124	145
Carex parallela	142	171	133	75	167	220	177	94–97	105–110	206–216	193	NA	138–166
Carex baldensis	142–148	149	134–160	90–97	146	146	157	86	109–122	196–223	94–100	119	131–136
Carex maritima	139	242	158–184	80	164	NA	156–170	97	105	248–257	224–259	119	137–138
Carex pulicaris	143	203	143	86	163	92	NA	87	61	193	100–103	117–118	143
Carex microglochin	147	NA	139	79–80	158	168	150	87	100	182	87	130	145
Carex rupestris	149–151	271–274	132–139	73–88	160–170	165–178	168–174	86–92	93–109	208	98	119–122	149–152
Carex dacica	137–175	NA	151	83–92	160	147–161	161–165	87–96	107–114	382–401	91–96	113	132–134
Carex nigra	NA	NA	152	77–93	157–161	165–171	145–193	89–92	107	368–398	96–113	117	134
Carex dioica	118–142	203	138	87	164–167	NA	175–179	86–98	128–130	NA	198–199	122–125	145
Carex simpliciuscula	142	393	141	92	167	NA	161	111	107–109	207	107	120	140
Carex myosuroides (1)	151	103–134	IA	IA	169	185	153	93	103	242	90–98	124	140
Carex myosuroides (2)	136–153	103	IA	IA	159	185	153	99	103	242–244	98–102	124	140
No. of alleles	17	13	13	12	14	10	19	12	13	17	18	11	14

All the 13 SSR markers that were successfully transferred from *C. curvula* were polymorphic across the 15 evaluated *Carex* populations (Table 2). However, none of the populations showed polymorphism for all the loci (100% polymorphism), and the average percent of polymorphism was 27.69% (Table 3). *C. rupestris* was noted as the most polymorphic species, with only two invariable loci (84.6%), followed by *C. dacica* (69.23%) and *C. baldensis* (53.85%). *C. pauciflora* and *C. nardina* were the only two species monomorphic for all the 13 loci (0.00% polymorphism). Nevertheless, low values of polymorphism were noted for *C. microglochin*, *C. nigra*, and *C. simpliciuscula* (7.69%), followed by *C. myosuroides* (population 1) and *C. pulicaris*, with a value of 23.08% for the polymorphic loci (Table 3).

Table 3. Average number of different alleles (NA); average number of private alleles (NAP); observed heterozygosity (Ho); expected heterozygosity (He); and percentage of polymorphic loci (%P) for 13 microsatellite loci developed for *Carex curvula* across 15 populations of *Carex* sp.

Species/Population	NA	NAP	He	Ho	%P
Carex pauciflora	0.846	0.308	0.000	0.000	0.00
Carex pyrenaica	1.231	0.692	0.115	0.192	23.08
Carex nardina	0.923	0.462	0.000	0.000	0.00
Carex parallela	1.231	0.846	0.154	0.308	30.77
Carex baldensis	1.538	1.077	0.250	0.462	53.85
Carex maritima	1.308	0.846	0.192	0.308	38.46
Carex pulicaris	1.077	0.692	0.067	0.115	15.38
Carex microglochin	1.000	0.615	0.038	0.000	7.69
Carex rupestris	2.077	1.385	0.404	0.308	84.62
Carex dacica	1.846	1.385	0.317	0.385	69.23
Carex nigra	1.462	0.846	0.192	0.269	38.46
Carex dioica	0.846	0.692	0.038	0.077	7.69
Carex simpliciuscula	1.000	0.385	0.029	0.038	7.69
Carex myosuroides (1)	1.000	0.154	0.077	0.077	15.38
Carex myosuroides (2)	1.077	0.385	0.096	0.077	23.08
Mean	**1.231**	**0.718**	**0.131**	**0.174**	**27.69**

The mean number of different alleles ranged from 0.846 in the case of *C. pauciflora* and *C. dioica*, to a maximum of 2.077 per locus in the case of *C. rupestris* (Table 3). The number of private alleles (defined as the number of alleles unique to a single population) ranged from 0.154 in the case of *C. myosuroides* (population 1), to 1.385 for *C. rupestris* and *C. dacica* (Table 3). Expected heterozygosity ranged from 0.000 (for monomorphic species *C. pauciflora* and *C. nardina*) to 0.404 (*C. rupestris*), with an average of 0.131 across all *Carex* populations (Table 3). Observed heterozygosity ranged from 0.000 (for species *C. pauciflora*, *C. microglochin*, and *C. nardina*) to 0.462 (*C. baldensis*), with a mean of 0.174 across all *Carex* populations (Table 3). The departure from the Hardy–Weinberg equilibrium was noted as non-significant for all polymorphic loci.

4. Discussion

- Interspecific transferability potential

Our research findings once again emphasize the value of the cross-species transferability of microsatellites, highlighting its role as a cost-effective method for the generation of genetic markers across various species within the same genus. A set of 13 SSR markers, previously developed for *C. curvula*, were evaluated for polymorphism in 15 different populations/14 species of *Carex*. The cross-amplification experiment was successful, and a total of 183 alleles were detected, with a range of 10 to 19, and an average of 14.07 alleles per locus. The total number of alleles and mean per locus, as revealed by these SSR markers, were higher than the values exhibited by *C. curvula*, 137 alleles and a mean of 10.53 alleles per locus respectively [36]. Furthermore, our values were relatively high, even compared with previous studies amplifying other SSR loci in different *Carex* species, such as the study by [35], which found a total of 173 alleles produced by 17 pairs of primers and an average of

10.18 for each locus. Similarly, the study by [33], reported 178 alleles in 79 *Carex* accessions, with an average of 4.3 alleles per microsatellite, generated by 42 SSR primer pairs.

The transferability rate varied from 60% to 100%, the lowest transferability being registered in G165, which did not produce amplification in six species. One hundred percent amplification for all 13 SSR primers was recorded in *C. baldensis*; this species is very closely related to *C. curvula*, and it belongs to the same clade (*Curvula* Clade). However, 100% amplification was also registered for *C. rupestris*, which is part of another clade, the *Pauciflora* Clade. Moreover, a high percentage of amplification (92.3%) was detected for species belonging to different clades and sections of the *Carex* genus, as follows: *Capitata* Clade, *Kobresia* Clade 2, *Disticha* Clade, Section *Physoglochin*, and Section *Psyllophorae*.

The percentage of polymorphism exhibited by the 15 *Carex* populations ranged from 0.00% to 84.6%, with an average value of 27.69% (Table 3). These values might be considered to be very low in comparison with the values noted for *C. curvula* (mean value = 87.97%). However, the present polymorphism results should be treated with caution, given that sample size in each population was lower than that of the *C. curvula* populations (five individuals) mentioned in [36]. This would suggest that, for an exhaustive assessment of genetic variation in natural populations, increasing the number of sampled individuals per locality would offer a better resolution of the genetic structure of these species.

Nevertheless, we tested how the sample size actually influenced the polymorphism rates in the case of these 13 SSR markers, by including additional individuals (data not shown) of *C. pauciflora* (up to a total of four individuals) and *C. myosuroides* (five for each of the two populations) in the analysis. In the case of *C. pauciflora*, the polymorphism value increased slightly, from 0.00% to 7.69%, whereas in *C. myosuroides* (both populations), it remained unchanged (15.38% and 33.85%) despite the addition of three more individuals.

- Inter-populational discriminative potential

Carex myosuroides was represented in the present study by two populations sampled from different massifs in the Pyrenees, specifically to test how informative these SSR markers might be in the case of phylogeographic studies on other *Carex* species besides *C. curvula*. Both populations showed inconsistent amplification for the same SSRs (VG152 and VG100) and displayed identical alleles for VG119, VG131, G165, VG153, and VG168 markers (Table 2). Again, both populations were recorded as being among the poorest in terms of number of private alleles. However, the population from the Aston Massif proved to contain more unique alleles, with a value of 0.385, compared with 0.154 for the other population (from the Maladeta Massif). Their values of Ho, He, and percentage of polymorphic loci were very similar (Table 3). The number of alleles per population ranged from 0 to 2, depending on the marker, and it reached a maximum of three alleles for both populations. These results remained unchanged when analysing five individuals per population. The number of alleles displayed by both populations of *C. myosuroides* in the case of these 13 SSR markers was lower compared with several other studies reporting SSR markers in the *Carex* genus. For example, an analysis of 14 microsatellite loci in six populations of *C. kobomugi* revealed two to eight alleles per marker [27], and another analysis of nine microsatellite loci in four populations of *Carex scabrifolia* reported two to seven alleles [28], whereas an analysis of 30 SSR markers in three populations of *C. pumila* discovered four to twelve alleles [34]. The low number of alleles and the high level of monomorphism were also reported by [32], for three populations of *C. angustisquama*, analysed using 20 SSR markers, resulting an average of one to five alleles per marker.

Nevertheless, the most important finding was that these SSR markers were able to successfully delineate different populations of the same species.

5. Conclusions

In this study, we have revealed the successful cross-species amplification of 13 SSR loci, specifically developed for *Carex curvula*, in 14 other *Carex* species. Moreover, to the best of our knowledge, this study represents the first report focused on the identification of

SSR markers suitable for genetic variation assessments in *Carex* species from the *Euthyceras-Psyllophorae* Clade.

All 13 primers were successfully transferred, with a mean percentage of 90.76, though only five primer pairs produced amplification in all 14 species andachieved 100% transferability. In addition, all the primers generated polymorphic alleles. In conclusion, our successful cross-amplification results extend the potential usefulness of these loci to other related taxa.

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