

Comparative analysis of genetic diversity in Norway spruce (*Picea abies*) clonal seed orchards and seed stands

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Abstract

Norway spruce, *Picea abies* (L.) Karst. is the most important conifer species in Romania and the most planted tree species in the Carpathian Mountains. Here we compare the genetic diversity of four Norway spruce clonal seed orchards and two seed stands located in the Eastern Carpathians. A set of highly polymorphic nuclear microsatellite markers was used. The analysis of genotypic identity of ramets for each Norway spruce clone in all seed orchards indicated that nearly all sampled ramets (97%) were genetically identical. The genetic diversity in seed orchards ($H_e=0.700$) was slightly smaller compared to the seed stands ($H_e=0.718$). Allelic richness was higher in seed stands (10.874), compared to clonal seed orchards (8.941). The Bayesian analysis indicated a genetic structure with two clusters, one corresponding to the clonal seed orchards and a second one consisting of the two seed stands. Our results provide valuable information for the management of Norway spruce seed orchards in Romania.

Keywords: genetic diversity; microsatellite; Norway spruce; seed orchards; seed stands

Introduction

Seed orchards are the most used method for obtaining forest reproductive materials with superior genetic properties (Funda *et al.*, 2009; Funda and El-Kassaby, 2012). Seed orchards are an important and efficient type of transmission of superior genetic traits to offspring, by creating synthetic varieties (Chaloupková *et al.*, 2019).

Long-term tree improvement implies, among other things, ensuring a balance between expected genetic gain and an appropriate level of genetic diversity, and the most common method of ensuring this is by establishing seed orchards (Tang and Ide, 2001). High genetic diversity in seed orchards may increase resilience and capacity to adapt to changing environments and thus productivity and quality of forest plantations. Genetic diversity among seed orchards crops is significantly influenced by the relatedness of orchard clones, parental fertility variation, and pollen contamination (Geburek, 1997; Ertekin, 2012). At the same time, of great importance is the number of parents involved in cloning the material for the installation of the seed orchard (Kang *et al.*, 2001; Lindgren and Prescher, 2005; Hansen, 2008; Sønstebo *et al.*, 2018).

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In this study, we focused on Norway spruce (*Picea abies* (L.) Karst.), one of the most important forest trees in the boreal and subalpine conifer forests (San-Miguel-Ayanz *et al.*, 2016). Norway spruce occupies approximately 30 million hectare (Jansen *et al.*, 2017; Schiop *et al.*, 2017) and it plays an important role for the society and economy. At present, it is the most common conifer tree species in Romania, occupying approximately 1.488.000 ha and 23.2% of the forest cover (Budeanu *et al.*, 2019). The study of genetic diversity of Norway spruce in Romania is summarized only in a few studies based on allozyme (Curtu *et al.*, 2009; Teodosiu, 2011; Radu *et al.*, 2014) and nuclear microsatellite markers (Mihai *et al.*, 2020) and no genetic analysis was done on seed orchards. In Romania, there have been installed only nine Norway spruce seed orchards that occupy approximately 72.9 ha (Mihai *et al.*, 2019).

Previous studies that aimed at comparing genetic diversity between natural populations and seed orchards have shown a higher genetic diversity in seed orchards than in natural populations (Muona and Harju, 1989; El-Kassaby, 1992; Chaisurisri and El-Kassaby, 1994; Stoehr and El-Kassaby, 1997; Williams *et al.*, 2001) in terms of allelic diversity and heterozygosity. In general, in forest tree species with a high degree of polymorphism, phenotypic selection in the early stages of breeding does not imply a significant reduction in genetic variability, as in *Picea abies* (Bergmann and Ruetz, 1991) or *Picea glauca* (Namroud *et al.*, 2012). However, there are also data that indicate lower genetic diversity in seed orchards compared to natural populations, as an effect of the number of parents selected for cloning (Johnson and Lipow, 2002; Ilinov and Raevsky, 2017), or studies that report a similar genetic diversity (Rungis *et al.*, 2019). Given these differences, we aimed to assess the level of genetic diversity in Norway spruce seed orchards and seed stands in the Eastern Carpathians, a region with a widespread distribution of Norway spruce. The specific objectives were: 1) to assess the genetic identity of ramets for Norway spruce clones used in seed orchards and 2) to compare the genetic diversity in clonal seed orchards and seed stands using highly polymorphic DNA markers.

Materials and Methods

Sampling design

Four Norway spruce clonal seed orchards (Paltinoasa – Cso-P, Bodești – Cso-B, Dalhăuti – Cso-D and Alunis – Cso-A) and two seed stands (Cucureasa – Nat-C, Manastirea Casin – Nat-M) have been sampled (Table 1). The seed orchards are located in the Eastern Carpathian region and were established between 1970 and 1981 with a different number of vegetative copies of plus trees (Cso-P - 33, Cso-B - 33, Cso-D – 81 and Cso-A - 197). Most of the plus trees were selected in natural seed stands distributed across the Eastern Carpathian Mountains, only several plus trees used for Cos-D seed orchard originated from the Southern Carpathian Mountains (Table 1). The sampling was done in 2017 and some of the initial clones were not found in the field. At least one individual per clone was sampled in each seed orchard. Two ramets per clone were sampled randomly for most of the clones in all seed orchards to verify the clonal identity.

Table 1. Geographic location of Norway spruce clonal seed orchards and seed stands

Nr. crt.	Abr.	Population	Region of provenance for the source population *	Number of sampled individuals	Number of unique genotypes	Latitude/ Longitude
1	Cso-P	Paltinoasa	A2	50	27	47.571791/ 25.941412
2	Cso-B	Bodesti	A2,G3	90	54	47.042570/ 26.447690
3	Cso-D	Dalhauti	A2,B2,C1	79	69	45.707435/ 27.007750
4	Cso-A	Alunis	A2, G3	190	154	46.325000/ 27.452275
5	Nat-C	Cucureasa	A2	56	56	47.397383/ 25.045132
6	Nat-M	Manastirea Casin	A2	77	77	46.168066/ 26.678455

*Region of provenance (ecological region) according to The National Catalogue of Approved Basic Material for Production of Forest Reproductive Material (Parnuta *et al.*, 2012)

DNA extraction and PCR amplification

DNA was extracted from buds, cambium or leaves using the CTAB (Doyle and Doyle, 1987) or ATMAB (Dumolin *et al.*, 1995) methods.

A number of 12 nSSR nuclear microsatellites (WS00716.F13, WS0022.N15, WS0073.H08, WS00111.K13 and WS0023.B03 (Rungis *et al.*, 2004) Pa₄₄ and Pa₄₇ (Fluch *et al.*, 2011), EAC1F04 (Scotti *et al.*, 2002), EATC1E03, EATC1B02, EATC2G05 (Scotti *et al.*, 2002), SpAG2 (Pfeiffer *et al.*, 1997) were used. EAC1F04 was excluded from further analysis because of some ambiguities in its interpretation and due to the presence of a large number of null alleles.

The PCR amplifications were performed using a PCR thermal cycler (Corbett), in reaction mixtures (15 µL) containing 5 ng of template DNA, 1x Qiagen Multiplex PCR MasterMix 2x, 2µM for each primer and RNase free water. The PCR cycling conditions were as follows; 10 min at 95 °C followed by 30 cycles of 1 min. at 94 °C, 1:30 min. at a primer-specific annealing temperature (53 °C, 55 °C, 58 °C and 62 °C), 1 min. at 72 °C and a final elongation step of 30 min at 60 °C.

Amplified PCR products were diluted and were then run on a GemoneLab GeXP Genetic Analyser (Beckman Coulter) using Frag-3 method and Size Standard 400.

Data analysis

Microsatellite markers were tested for genotyping errors due to large allele drop-out, scoring of stutter peaks and non-amplified alleles using MICRO-CHECKER 2.2.0.3 (Van Oosterhout *et al.*, 2004). The software indicated the presence of null alleles at very low frequencies (less than 7%) for two markers (WS00716 and WS00023). No evidence of large allele drop-out or scoring of stutter peaks was found.

The software GenAlEx ver. 6.5 (Peakall and Smouse, 2006, 2012) was used to estimate a standard genetic diversity indices: average number of alleles per locus (N_a), effective number of allele (N_e), observed heterozygosity (H_0), expected heterozygosity (H_e), number of private alleles (N_p) and fixation index (F). Principal component analysis ($PCoA$) was performed using the same software. Allelic richness (A_R), a measure that is independent of sample size, was estimated with FSTAT 2.9.3 (Goudet, 2001).

A matrix of pairwise genetic differentiation measures between all populations pairs was computed. For genetic differentiation among spruce populations, pairwise F_{ST} 's were computed using ARLEQUIN 3.5.2.2 (Excoffier and Lischer, 2010). The significance of the F_{ST} statistics was tested by 10000 permutations. The graphical representations of all pairwise F_{ST} were done using an Rfunction (*pairFstMatrix.r*) implemented in ARLEQUIN software. Analysis of Molecular Variance (AMOVA) was performed using the same software.

An Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering was computed with 100 bootstrap replications, based on Nei's (1972) standard genetic distance using the software Populations 1.2.31 (Langella, 1999) and TreeView 1.6.6 (Page, 2003).

The Bayesian clustering method implemented in STRUCTURE software ver. 2.3.3 (Pritchard *et al.*, 2000) was used to genetically assign individuals to clusters. Simulations were run for 50000 steps following a burn-in period of 100000 steps, considering values of K (number of clusters) from one to 7, with 3 replications for each value of K . The analysis was performed using admixture, correlated allele frequencies and no prior information on sampling location. The number of population clusters was estimated using ΔK parameter according to (Evanno *et al.*, 2005) using the STRUCTURE HARVESTER program (Earl and vonHoldt, 2012). The highest value of ΔK statistics was obtained for $K = 2$.

Results and Discussion

Genetic diversity

It was assumed that two ramets originating from the same clone possess identical genotypes. Genotypes of two ramets per clone were compared with each other at three highly polymorphic loci. The multilocus genotypes were identical in nearly 97% of the comparisons. Only in 9 out of the 297 clones (Cso-P - clone 4, Cso-B - clone 163, 169, 265, 267, 272 and 279; Cso-A - clone 270 and 300), the two ramets did not match at two or three loci. This is probably due to the growth of the rootstock to the detriment of scion (Prescher *et al.*, 2007) or because of sampling errors. Only unique genotypes were included for further analyses. Thus, the total sample size was made of 437 individual trees, out of which, 304 and 133, were from seed orchards and seed stands, respectively.

All eleven microsatellite loci showed polymorphism across populations, with the total number of alleles ranging from 33 at the locus WS0023 to four at the locus Pa_47. The mean number of alleles per population was 11.921. Effective number of alleles across all populations was 6.503 ($SD \pm 0.566$), with mean value 5.081 in the seed orchards and 7.945 in natural stands (Table 2).

Table 2. Standard genetic diversity indices

Population		Na	Ne	Ho	He	F	A _R	N _p
Cso-P	Mean	8.455	4.505	0.693	0.674	-0.070	8.331	1
Cso-B	Mean	11.182	5.466	0.683	0.682	-0.036	9.253	6
Cso-D	Mean	10.909	5.071	0.734	0.732	-0.028	8.994	0
Cso-A	Mean	12.091	5.280	0.672	0.710	0.022	9.196	3
Nat-C	Mean	13.001	8.088	0.683	0.712	0.014	10.927	9
Nat-M	Mean	13.364	7.763	0.717	0.725	-0.011	10.820	9
Clonal seed orchards	Mean	10.659	5.081	0.696	0.700	-0.027	8.941	10
Seed stands	Mean	13.182	7.925	0.700	0.718	0.001	10.874	18
Total	Mean	11.921	6.503	0.698	0.709	-0.013	9.901	28
	SE	0.793	0.566	0.022	0.025	0.024	0.021	-

* Na - average number of alleles per locus, Ne - effective number of alleles, Ho - observed heterozygosity, He - expected heterozygosity, F - fixation index, A_R - allelic richness, N_p - number of private alleles; SE - standard error.

One of the roles of seed orchards is to maintain a high level of genetic diversity, which may reflect the genetic diversity of original populations (Ertekin, 2012). Our results show that expected heterozygosity of seed orchards (0.700) is slightly lower than that of natural stands (0.718). This might be because seed orchards are generally derived from a limited number of clones. The mean He in natural stands (0.718) were slightly higher than in seed orchards (0.700), which is consistent with other studies in Norway (Sønstebo *et al.*, 2018) and Latvia (Ruņģis *et al.*, 2019). Furthermore, the mean He was lower than it was previously

reported for Norway spruce core stands in Slovenia (0.935) (Westergren *et al.*, 2018) and Czech Republic (0.780) (Máková *et al.*, 2018).

Allelic richness is one of the most important genetic diversity parameters, particularly when analysing populations of different sample size. This parameter is of importance when elaborating genetic conservation strategies (Foulley and Ollivier, 2006). In our study, larger differences were observed for allelic richness (A_R), which varied between 8.331 and 11.071. Mean A_R over all samples was 10.619. The allelic richness in seed orchards (8.941) was higher than previously reported values for this species using genomic SSRs markers in seed orchards (5.990) (Sønstebo *et al.*, 2018). Although it has been reported that allelic richness increases with increasing number of parents (Sønstebo *et al.*, 2018), allelic richness in Cso-A (9.196), the seed orchard with the highest number of clones, was similar to the other seed orchards. Moreover, Cso-P, which has the lowest number of clones, has the lowest value for A_R compared to the other three seed orchards. Compared to the seed orchard with the highest number of clones (Cso-A), in Cso-P the value of A_R is with 9.4% lower. On the other hand, the highest level of allelic richness was observed in the two natural stands.

The fixation index (F) ranged from -0.070 (Cso-P) to 0.022 (Cso-A). The total number of private alleles (N_p) was 28, out of which 10 alleles in seed orchards and 18 alleles in the natural stands. Overall, the mean values of the genetic diversity parameters were slightly higher in the natural Norway spruce populations compared to the clonal seed orchards.

Genetic differentiation among populations

The genetic divergence among all Norway spruce populations was measured using F_{ST} . Pairwise Wright's F_{ST} showed the lowest genetic differentiation between Nat-C and Nat-M ($F_{ST} = 0.0004$), and the highest genetic differentiation was detected between Cso-D and Nat-C ($F_{ST} = 0.0496$). The genetic differentiation among seed orchards was relatively low (Table 3).

Table 3. Matrix of pairwise Wright's F_{ST}

	Cso-P	Cso-B	Cso-D	Cso-A	Nat-C	Nat-M	
Cso-P	0.0000						
Cso-B	0.0109	0.0000					
Cso-D	0.0173	0.0277	0.0000				
Cso-A	0.0244	0.0147	0.0154	0.0000			
Nat-C	0.0486	0.0467	0.0496	0.0424	0.0000		
Nat-M	0.0439	0.0428	0.0450	0.0366	0.0004	0.0000	

PCoA analysis showed that the first principal coordinate separated clonal seed orchards from the seed stands. The second principal coordinate separated population Cso-D from other clonal seed orchards (Cso-A, Cso-P and Cso-B). This may be a consequence of using plus trees from three region of provenance (C2 – region from the Southern Carpathians, A2 and B2 – region from the Eastern Carpathians) in the clonal seed orchard Cos-D. However, even if plus trees from the same regions of provenance were used for the installation of the Cos-A and Cos-B seed orchards, the observed differences can be due to the number of selected clones (the number of clones in population Cos-A is larger than the number of clones from Cos-B population). A balance between the expected genetic gain and the assumed but reduced loss of genetic diversity is necessary. Also, for advanced generations of seed orchards, the breeding strategy must provide the infusion of new genotypes in the breeding program, in order to avoid the risk of reducing genetic diversity (Funda and El-Kassaby, 2012).

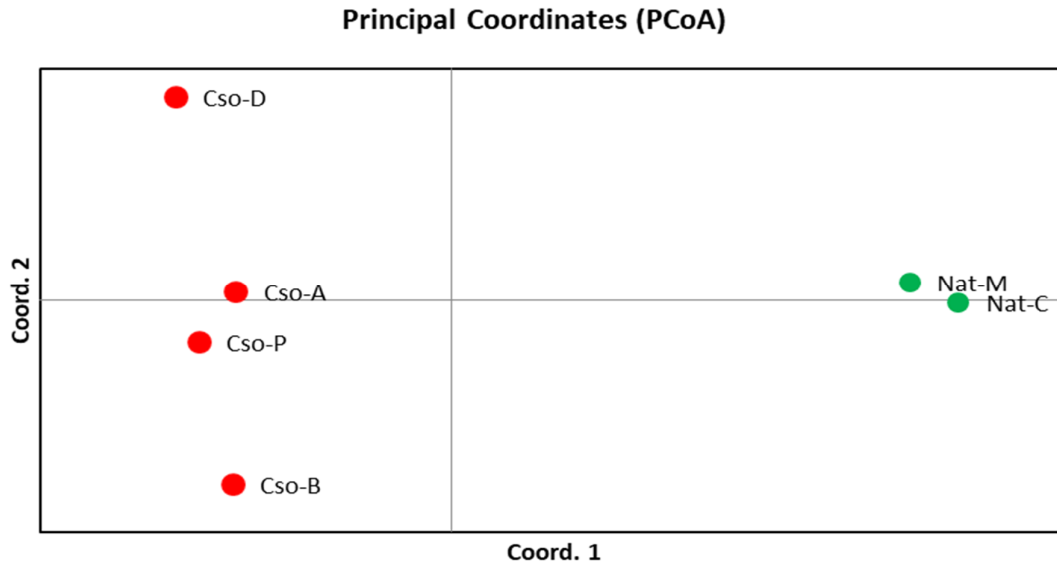


Figure 1. Principal component analysis of six Norway spruce populations. Axes 1 and 2 explain 57.28 % and 19.95 % of the genetic variation detected

Relationships among all six Norway spruce population were further illustrated by a dendrogram, using UPGMA based on Nei's (1972) standard genetic distances (Figure 2 - B). The dendrogram showed a clear separation (bootstrapping value for the pair was 85) between natural Norway spruce stands and clonal seed orchards. Nat-C and Nat-M were grouped together although the geographical distance among them was considerable.

Population genetic structure

Using the Bayesian analysis (Figure 2 - A) we found that the best inferred number of genetic clusters is two ($K = 2$). All seed orchards showed a higher membership in the red genetic cluster. However, there were many admixed individuals and even individual clones with a higher membership value in the second genetic cluster (with green colour). As in the UPGMA dendrogram, the two seed stands are closely related to each other, showing a high membership in the second genetic cluster. This separation is also valid for $K = 3$ (Figure 2 - A).

The four seed orchards consists of vegetative copies of plus trees selected in several seed stands located across the Eastern Carpathian region, including the two stands sampled in this study. This can explain the observation of individual clones with high membership values in the genetic cluster that is specific for the two natural stands. The similarity between the two natural stands might be due to extensive gene flow between Norway spruce forests along the Eastern Carpathians. Little genetic differentiation was found among natural Norway spruce populations across the Romanian Carpathians (Radu *et al.*, 2014).

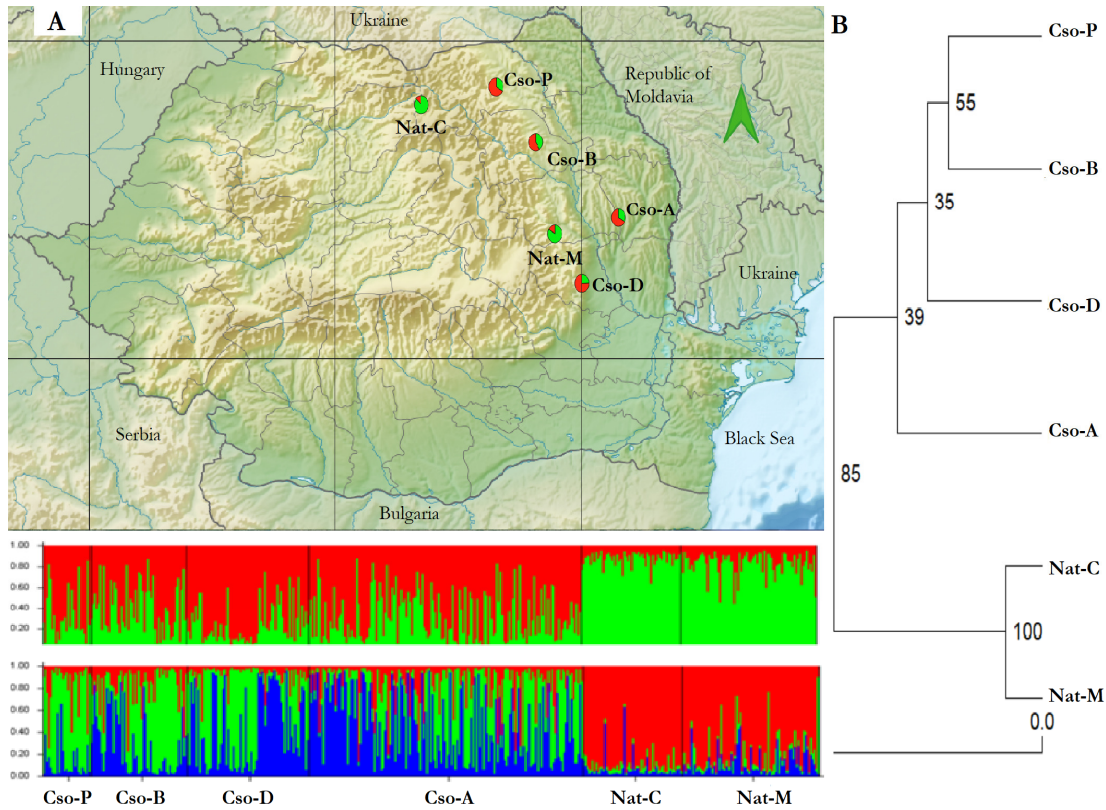


Figure 2. Map of Norway spruce populations. Pie charts represent the average inferred ancestry of individuals for each cluster identified by STRUCTURE for $K=2$. STRUCTURE results for two and three distinct genetic clusters (A). UPGMA dendrogram constructed using Nei's genetic distance (B)

Table 4. Analysis of molecular variance (AMOVA) for Norway spruce populations using 11 microsatellite loci

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	<i>p</i>
Among populations	5	89.477	0.10541 V_a	3.02	0.0224
Among individuals within populations	431	1484.095	0.06322 V_b	1.81	<0.001
Within individuals	437	1449.50	3.31693 V_c	95.16	<0.001
Total	873	3023.072	3.48557	-	-
Among groups (first group seed orchards, second group seed stands)	1	50.963	0.10819 V_a	3.06	<0.001
Among populations within group	4	38.514	0.04700 V_b	1.33	0.0127
Among individuals within populations	431	1484.095	0.06322 V_c	1.79	<0.001
Within individuals	437	1449.500	3.31693 V_d	93.82	0.0615
Total	873	3023.072	3.53535	-	-

* *d.f.*- degrees of freedom; V_a , V_b , V_c , V_d - associate covariance components; *p* - significance level.

Two different AMOVA analyses were conducted. The first analysis included all populations and the second one considered the two different groups established by STRUCTURE (first group for seed orchards and second group for natural stands). Most of the genetic variation between the six Norway spruce populations can be explained by intraindividual variation 95.16 % ($p < 0.001$) (Table 3). When the genetic variance was partitioned into two distinct groups, a small but significant ($p < 0.001$) amount of genetic variation (3.06 % of the total) was the result of differences between groups (Table 4).

Conclusions

The genotypic identity of the putative ramets of the same clone was certified for Norway spruce clonal seed orchards based on highly polymorphic DNA markers. Mismatches were very rare and may be explained by the growth of the rootstock to the detriment of scion or sampling errors. Slightly higher values for genetic diversity parameters were found in seed stands compared to clonal seed orchards. As expected, the degree of genetic admixture was higher in the four clonal seed orchards than in the two studied seed stands. Our molecular analysis provides valuable information for the management of Norway spruce seed orchards in Romania.

Authors' Contributions

Conceptualization - EC, ALC; Data curation - EC, ALC, NS, GM, MT; Formal analysis - EC, MT;; Investigation - EC, ALC, NS, GM, MT; Methodology - EC, MT; Project administration - ALC; Resources - ALC; Software - EC, MT; Supervision – ALC, NS, GM; Validation - NS, GM; Visualization - EC, ALC, NS, GM, MT; Writing - original draft- EC; Writing - review and editing – EC, ALC, NS, GM, MT. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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