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RAPD Markers and Genetic Information Entropy in Environmental Monitoring: A Case Study with Wild Mushrooms

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Abstract

Mushrooms have a remarkable scientific value due to their nutritional, medicinal properties and industrial applications in enzyme production, so that effort in the maintenance of native wild mushroom varieties is increasing. The present study focuses on the use of Random Amplified Polymorphic DNA (RAPD) markers for biodiversity measure of wild mushroom species of the Northwest mountainous region of Greece. Data mining of similarity matrices from RAPD analysis was used to extract measurable entropy parameters for mushroom biodiversity monitoring based on Shannon's information entropy. Shannon information index provides an easy assessment of the entropy of the genetic information of the germplasm per mushroom species while the total equitability index (E_H) = 0.871 offers an overall estimation of the genetic variation evenness of all species in the population of the studied mushrooms. Application of RAPDs with parallel entropy analysis is an easily applicable and low-cost valuable technology in environmental monitoring, using genetic information of wild mushroom species as an indicator that can lead to future actions in biodiversity maintenance and germplasm protection. The provided methodology can serve as a pilot procedure enriched with other environmental factors to monitor and protect wild mushroom communities native to the Greek countryside or in any part of the world and provide comparable results about biodiversity from different regions using common entropy indices.

Keywords

Random Amplified Polymorphic DNA, Shannon's Index, Entropy, Biodiversity, Dendrogram

1. Introduction

Mushrooms have a great scientific value due to their nutritional, medicinal properties and industrial applications in enzyme production. Their protein content is less than in animals but more compared to plants, while they are low in fat and high in fiber (Pérez-Montes et al., 2021). The use of medicinal mushrooms in Western countries has increased in the last decades, while in Asian countries, there is a long tradition for therapeutic applications (Agarwal & Fulgoni, 2020; Rauf et al., 2023). Singh et al. (2022) comment that mushrooms provide primary metabolites for energy, while secondary metabolites offer medicinal properties for human wellness that will play a significant role in fighting infectious diseases. Mushrooms' metabolites are used as immunomodulatory and anticancer agents. Reports show that they exhibit antitumor and antimicrobial activity and are used in immunotherapy through immunonutrition (Wong et al., 2020; Zhao et al., 2020). They are environmentally friendly contributing to bioremediation conferring specific activities in biodegradation, bioremediation and biosorption (Ganguly et al., 2023; Hultberg et al., 2020; Li et al., 2022) as also in biotechnological applications for energy production with biofuels (Shan et al., 2023; Diamantopoulou & Papanikolaou, 2023).

Effort in the maintenance of native wild mushroom varieties is increasing, as they can have a significant environmental impact and increased commercial value (food, pharmaceutical, etc.). Newer technologies, following the progress in molecular genetics, have been employed to certify and conserve native wild mushrooms (Li et al., 2018; Geng et al., 2022; Wijayawardene et al., 2023). Genome analysis methods can ensure the consumer and the producer for uniquely valuable products with a label of origin avoiding the risk of losing important species of mushrooms, native to rural areas. Due to the lack of substantial genetic sequence information in the field of wild mushroom genetics, the present study focuses on the use of Random Amplified Polymorphic DNA (RAPD) markers for biodiversity measure of wild mushroom species of the Northwest mountainous region of Greece. The RAPD technology is based on the amplification of DNA with short nucleotide sequences of random order, usually 10 bp long. RAPD markers bind to genomic DNA randomly, resulting in the amplification of DNA pieces with unknown sequences. RAPD marker analysis dates to the nineties, where it was reported that "*These polymorphisms*, *simply detected as DNA segments which amplify from one parent but not the other*, *are inherited in a Mendelian fashion and can be used to construct genetic maps in a variety of species*. *We suggest that these polymorphisms be called RAPD markers*, *after Random Amplified Polymorphic DNA*" (Williams et al., 1990; Welsh & McClelland, 1990). Hadrys et al. (1992) comment that "M*olecular genetic markers have been developed into powerful tools to analyze genetic relationships and genetic diversity and the RAPD technology may be used in molecular ecology to analyze mixed genome samples with* (*i*) *suitability for work on anonymous genomes*, (*ii*) *applicability to problems where only limited quantities of DNA are available*, (*iii*) *efficiency and low expense*". RAPD markers serve to distinguish, character-

ize and differentiate fungal populations at species level and have proven to be a rapid and reliable tool in the genetic diversity. Seven RAPD primers were used to assess the diversity within and among twelve populations of three mushroom species *Ganoderma lucidum*, *Leucoagaricus* sp. and *Lentinus* sp. from different sampling sites in central India (Dwivedi et al., 2018). Similarly, genomic discrimination of eleven commercial mushrooms and establishing phylogenetic relationships using 10 RAPD markers has also been attempted (Agarwal et al., 2013). Most of RAPD studies are towards cultivar classification and breeding selection in certain species. RAPD technology was applied to differentiate 11 commercial strains of *A. auricula* and five commercial strains of *A. polytricha* and one white-fruitbody mutant strain, and characterize their genetic diversity. Results showed that all the strains tested could be differentiated by pooled RAPD data, and even one individual primer (S10) could also discriminate all tested strains (Yan et al., 2004). Using PEG-mediated protoplast fusion, a total of nine *pfle* somatic hybrids were developed between *Pleurotus florida* and *Lentinula edodes* and were assessed for genetic closeness, stability and variance compared to their first parental strains using nine RAPD molecular markers (Sarkar et al., 2022).

Information entropy based on mathematical formulas for entropy (Shannon, 1948) has been employed to analyze data from similarity matrices of RAPD genetic markers. Genetic Information Entropy has been extensively reviewed by various authors where the versatility of entropy analytics is presented (Sherwin, 2010; Sherwin & Prat i Fornells, 2019). Applications range from the study of genetic entropy evaluating allele distribution using genetic markers for variety classification and selection in the same species to the study of diversity within and among populations of distant genera in natural environments (Hao et al., 2023; Dwivedi et al., 2018; Zeb et al., 2023; Patel et al., 2020). The present study aims at the application of RAPDs as a valuable technology in environmental monitoring using genetic entropy of wild mushroom species that can lead to future actions in biodiversity maintenance and germplasm protection.

2. Materials and Methods

2.1. Sample Collection

Ten species of wild mushrooms populations, collected from the North-West mountainous region of Greece, were studied at the Genetic Identification Laboratory of The Hellenic Agricultural Organization-DIMITRA. The sampled species were: *Amanita caesaria*, *Boletus aereus*, *Craterellus cornucopiodes*, *Macrolepiota procera*, *Agaricus urinascens*, *Cantharellus cibarius*, *Hygrophorus marzuolus*, *Pleurotus ostreatus*, *Ganoderma lucidum* and *Morchella esculenta*.

2.2. DNA Extraction

DNA extraction was carried out in duplicates using 100 mg of lyophilized mushrooms from each species using the NucleoSpin® Plant II extraction system (Macherey-Nagel) according to manufacturer's instructions. The protocol in-

volves first lysis of the cells, purification with organic solvents and extraction of DNA via column chromatography provided in the company's kit. Quantification of the extracted genetic material was performed by fluorometry on the fluorescent detector Gene-4 (DNA-Technology, Research & Production), suitable for the quantification of genetic material.

2.3. PCR Conditions

Isolated DNA was analyzed for genetic similarity among samples with PCR using six RAPD primers (ten bases long, decamers) with nucleotide sequence listed in Table 1. PCRs were performed in a final volume of 25 μl using KAPA2G Robust PCR Kit (Kapa Biosystems) according to protocol of the kit manufacturer. The reaction mixtures were subjected to the following thermal cycling parameters in a GeneAmp PCR system 9700 (Applied Biosystems): 1 cycle of 3 min at 94˚C (denaturation), followed by 45 cycles of 1 min at 94˚C, 1 min at 35˚C and 1 min at 72˚C.

2.4. Gel Electrophoresis and Data Analysis

PCR products of each sample were assayed by electrophoresis on a 2% agarose gel containing GelRed fluorescence dye in 1X TAE buffer at 90 V for 1 hour and photographed in a BIO-PRINT ultraviolet photographic chamber (Vilber-Lurmart). Photos of the RAPD profiles obtained from the electrophoresis of the genetic material were uploaded in PyElph 1.4 bioinformatics software for the grouping of mushrooms and the construction of a genetic similarity dendrogram (Pavel & Vasile, 2012).

2.5. Genetic Information Entropy

Information entropy resulted from RAPDs analysis was estimated by Shannon's information index:

$$H = -\sum p_i \ln p_i$$

where Σ: sum; ln: natural log; p_i: value refers to the number of unique RAPD bands per species i. The Shannon Equitability Index was calculated using the formula:

Table 1. Nucleotide sequences of RAPD primers used in the genetic analysis of wild mushroom species.

RAPD Primers	Sequence
RAPD1	CAGGCCCTTC
RAPD2	TGCCGAGCTG
RAPD3	AGTCAGCCAC
RAPD4	AATCGGGCTG
RAPD5	AGGGGTCTTG
RAPD6	GTGATCGCAG

$$E_H = H/\ln S$$

where H: the Shannon information index; S: the total number of unique bands for all studied species.

3. Results and Discussion

The overall electrophoresis results of PCR products from ten mushroom species using 6 RAPDs primers are presented in Figure 1.

Electrophoresis photos of PCR products for each one of the six RAPD primers and the ten mushroom species were combined and analyzed simultaneously. Analysis using PyElph 1.4 for the grouping of mushrooms and the construction of a genetic similarity dendrogram is presented in Figure 2.

Based on the genetic information provided from the RAPD analysis, it is noticed that the ten mushroom species are clustered in two major groups with significant genetic distance variation among members of each group as in the case of *Craterellus cornucopiodes* and *Morchella esculenta* in the one group and the *Cantharellus cibarius* in the other group. It has to be strongly stated that the presented RAPD analysis and the corresponding dendrogram are not intended for identification or taxonomic classification of the studied fungi, as they are species of different fungal orders already defined from their collection. The dendrogram is used as a graphic representation for comprehensible visual inspection of genetic similarities among studied fungi for entropic analysis. It was formed using the Neighbor Joining algorithm (Saitou & Nei, 1987) that allows unequal rates of evolutionary progress, so that branch lengths are proportional to amount of genetic variation. Additionally, the observed differences in genetic distances are not interpreted at this stage as an evolutionary advantage or disadvantage but indicate the genetic variability among the studied mushrooms. Monitoring the genetic profile of the wild mushrooms in conjunction with their population size can help in maintenance of natural mushrooms resources. Moreover, indication of high genetic variability can lead to selection from certain populations of wild mushrooms already known for their high nutritional value such as *Pleurotus* sp. and *Amanita* sp. as a source of new genetic material for improvement of existing commercial mushrooms.

Shannon Genetic Diversity Index

Beyond commercial utilization of wild mushroom germplasm based on RAPD markers, the certain technology could be used in the design of strategies for monitoring and assessment of ecosystems biodiversity from the unpredicted effect of various environmental factors such as climate change and anthropogenic interference. For this reason, matrix data that were provided from the software PyElph 1.4, were further analyzed based on Shannon information index for entropy to extract quantifiable parameters about the genetic information content of studied mushrooms (Table 2 and Figure 3), as also were tested for statistical significance (Table 3).

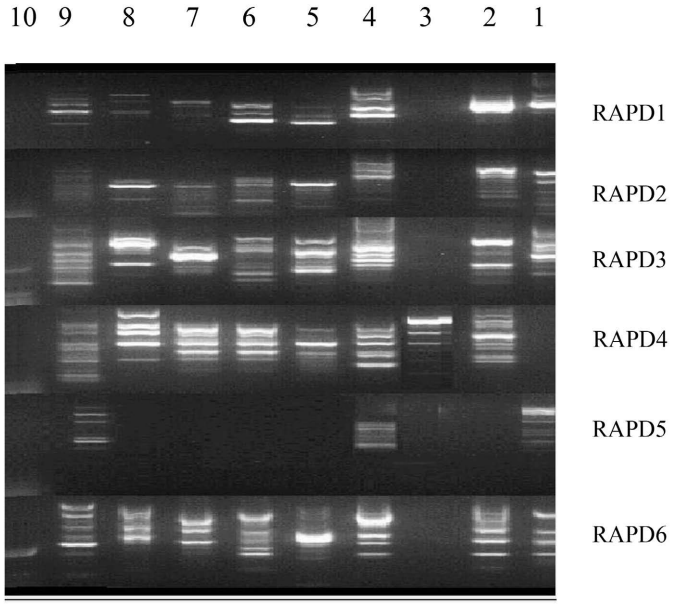

Figure 1. Electrophoresis of PCR products using 6 RAPDs primers and genomic DNA of ten wild mushrooms from North West Greece: 1) *Amanita caesaria*, 2) *Boletus aereus*, 3) *Craterellus cornucopiodes*, 4) *Macrolepiota procera*, 5) *Agaricus urinascens*, 6) *Cantharellus cibarius*, 7) *Hygrophorus marzuolus*, 8) *Pleurotus ostreatus*, 9) *Ganoderma lucidum*, 10) *Morchella esculenta.*

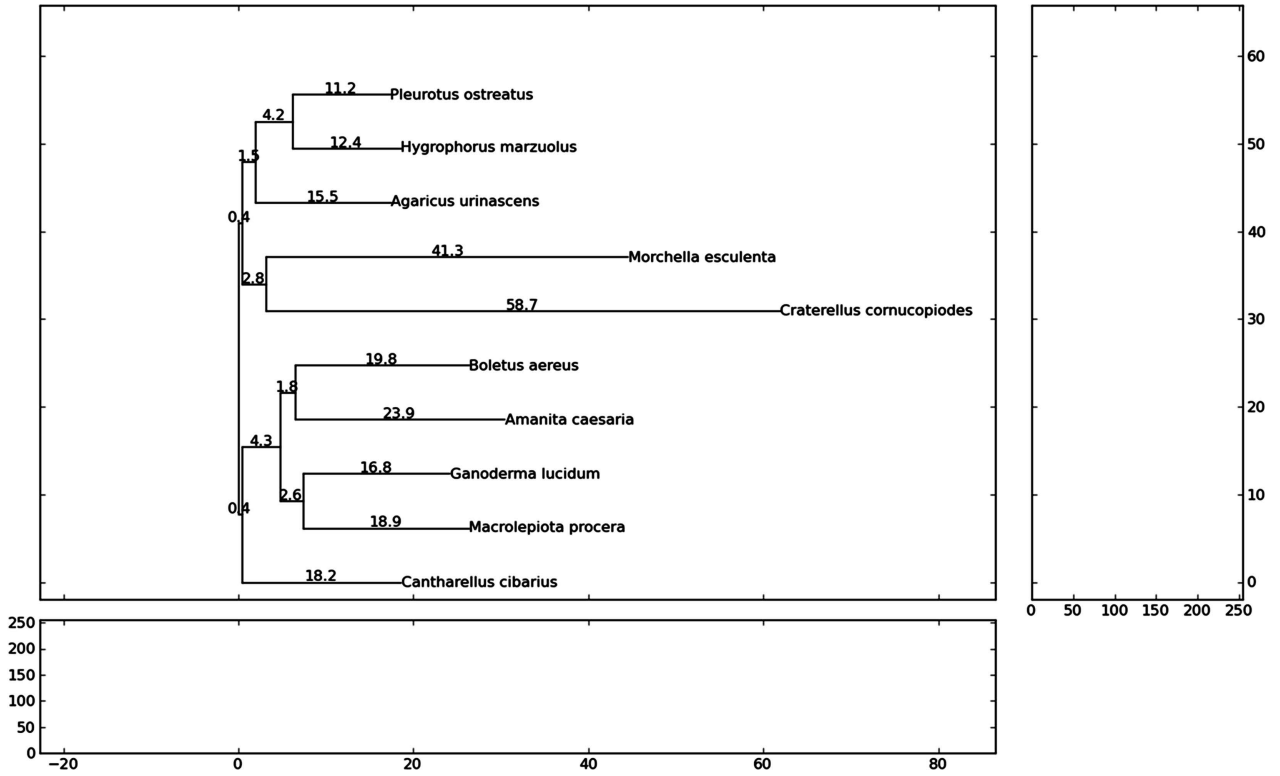

Figure 2. Genetic similarity dendrogram using PyElph 1.4 software and Neighbor Joining algorithm for analysis of electrophoresis results of PCR products using 6 RAPDs primers and genomic DNA of ten wild mushrooms from North West Greece: 1) *Amanita caesaria*, 2) *Boletus aereus*, *3*) *Craterellus cornucopiodes*, *4*) *Macrolepiota procera*, 5) *Agaricus urinascens*, 6) *Cantharellus cibarius*, 7) *Hygrophorus marzuolus*, 8) *Pleurotus ostreatus*, 9) *Ganoderma lucidum*, 10) *Morchella esculenta.*

Table 2. Shannon genetic information index (H) per mushroom population and total equitability index (E_H).

Mushroom Species	H
Amanita caesaria	0.367
Boletus aereus	0.367
Craterellus cornucopiodes	0.186
Macrolepiota procera	0.315
Agaricus urinascens	0.365
Cantharellus cibarius	0.343
Hygrophorus marzuolus	0.367
Pleurotus ostreatus	0.367
Ganoderma lucidum	0.236
Morchella esculenta	0.365
Shannon Total Population Equitability Index (E_H)	**0.871**

Table 3. Anova and pair wise comparisons based on the similarity matrix produced by RAPD marker analysis of ten wild mushroom species.

ANOVA: Single Factor								a = 0.05
DESCRIPTION								
Group	*Count*	*Sum*	*Mean*	*Variance*	*SS*	*Std Err*	*Lower*	*Upper*
Amanita caesaria	43	17	0.395	0.245	10.279	0.072	0.254	0.537
Boletus aereus	43	15	0.349	0.233	9.767	0.072	0.208	0.490
Craterellus cornucopiodes	43	3	0.070	0.066	2.791	0.072	−0.071	0.211
Macrolepiota procera	43	25	0.581	0.249	10.465	0.072	0.440	0.723
Agaricus urinascens	43	18	0.419	0.249	10.465	0.072	0.277	0.560
Cantharellus cibarius	43	22	0.512	0.256	10.744	0.072	0.370	0.653
Hygrophorus marzuolus	43	17	0.395	0.245	10.279	0.072	0.254	0.537
Pleurotus ostreatus	43	17	0.395	0.245	10.279	0.072	0.254	0.537
Ganoderma lucidum	43	31	0.721	0.206	8.651	0.072	0.580	0.862
Morchella esculenta	43	14	0.3261	0.225	9.442	0.072	0.184	0.467
ANOVA								
Sources	*SS*	*df*	*MS*	*F*	*p-value*	*Eta-sq*	*RMSSE*	*Omega Sq*
Between Groups	11.323	9	1.258	5.672	2E−07	0.108	0.363	0.089
Within Groups	93.163	420	0.222					
Total	104.486	429	0.244					
Q TEST								
Group 1	*Group 2*	*Mean*	*Std Err*	*q-stat*	*Lower*	*Upper*	*p-value*	*Mean-crit*
A. caesaria	*B. aereus*	0.047	0.072	0.648	−0.277	0.370	1.000	0.323

Continued

A caesaria	*C. cornucopiodes*	0.326	0.072	4.533	0.002	0.649	**0.046**	0.323
A caesaria,	*M. procera*	0.186	0.072	2.590	−0.137	0.509	0.715	0.323
A caesaria	*A. urinascens*	0.023	0.072	0.324	−0.300	0.346	1.000	0.323
A caesaria	*C. cibarius*	0.116	0.072	1.619	−0.207	0.439	0.980	0.323
A caesaria	*H. marzuolus*	0.000	0.072	0.000	−0.323	0.323	1.000	0.323
A caesaria	*P. ostreatus*	0.000	0.072	0.000	−0.323	0.323	1.000	0.323
A caesaria	*G. lucidum*	0.326	0.072	4.533	0.002	0.649	0.046	0.323
A caesaria	*M. esculenta*	0.070	0.072	0.971	−0.253	0.393	1.000	0.323
B. aereus	*C. cornucopiodes*	0.279	0.072	3.886	−0.044	0.602	0.159	0.323
B. aereus	*M. procera*	0.233	0.072	3.238	−0.091	0.556	0.398	0.323
B. aereus	*A. urinascens*	0.070	0.072	0.971	−0.253	0.393	1.000	0.323
B. aereus	*C. cibarius*	0.163	0.072	2.267	−0.160	0.486	0.846	0.323
B. aereus	*H. marzuolus*	0.047	0.072	0.648	−0.277	0.370	1.000	0.323
B. aereus	*P. ostreatus*	0.047	0.072	0.648	−0.277	0.370	1.000	0.323
B. aereus	*G. lucidum*	0.372	0.072	5.181	0.0490	0.695	0.010	0.323
B. aereus	*M. esculenta*	0.023	0.072	0.324	−0.300	0.346	1.000	0.323
C. cornucopiodes	*M. procera*	0.512	0.072	7.123	0.189	0.835	**0.000**	0.323
C. cornucopiodes	*A. urinascens*	0.349	0.072	4.857	0.026	0.672	**0.023**	0.323
C. cornucopiodes	*C. cibarius*	0.442	0.072	6.152	0.119	0.765	**0.001**	0.323
C. cornucopiodes	*H. marzuolus*	0.326	0.072	4.533	0.002	0.649	**0.046**	0.323
C. cornucopiodes	*P. ostreatus*	0.326	0.072	4.533	0.002	0.649	**0.046**	0.323
C. cornucopiodes	*G. lucidum*	0.651	0.072	9.066	0.328	0.974	**0.000**	0.323
C. cornucopiodes	*M. esculenta*	0.256	0.072	3.562	−0.067	0.579	**0.262**	0.323
M. procera	*A. urinascens*	0.163	0.072	2.267	−0.160	0.486	0.846	0.323
M. procera	*C. cibarius*	0.070	0.072	0.971	−0.253	0.393	1.000	0.323
M. procera	*H. marzuolus*	0.186	0.072	2.590	−0.137	0.509	0.715	0.323
M. procera	*P. ostreatus*	0.186	0.072	2.590	−0.137	0.509	0.715	0.323
M. procera	*G. lucidum*	0.140	0.072	1.943	−0.184	0.463	0.935	0.323
M. procera	*M. esculenta*	0.256	0.072	3.562	−0.067	0.579	0.262	0.323
A. urinascens	*C. cibarius*	0.093	0.072	1.295	−0.230	0.416	0.996	0.323
A. urinascens	*H. marzuolus*	0.023	0.072	0.324	−0.300	0.346	1.000	0.323
A. urinascens	*P. ostreatus*	0.023	0.072	0.324	−0.300	0.346	1.000	0.323
A. urinascens	*G. lucidum*	0.302	0.072	4.209	−0.021	0.625	0.089	0.323
A. urinascens	*M. esculenta*	0.093	0.072	1.295	−0.230	0.416	0.996	0.323
C. cibarius	*H. marzuolus*	0.116	0.072	1.619	−0.207	0.439	0.980	0.323

Continued

C. cibarius	*P. ostreatus*	0.116	0.072	1.619	−0.207	0.439	0.980	0.323
C. cibarius	*G. lucidum*	0.209	0.072	2.914	−0.114	0.532	0.557	0.323
C. cibarius	*M. esculenta*	0.186	0.072	2.590	−0.137	0.509	0.715	0.323
H. marzuolus	*P. ostreatus*	0.000	0.072	0.000	−0.323	0.323	1.000	0.323
H. marzuolus	*G. lucidum*	0.326	0.072	4.533	0.002	0.649	0.046	0.323
H. marzuolus	*M. esculenta*	0.070	0.072	0.971	−0.253	0.393	1.000	0.323
P. ostreatus	*G. lucidum*	0.326	0.072	4.533	0.002	0.649	0.046	0.323
P. ostreatus	*M. esculenta*	0.070	0.072	0.971	−0.253	0.393	1.000	0.323
G. lucidum	*M. esculenta*	0.395	0.072	5.505	0.072	0.718	0.005	0.323

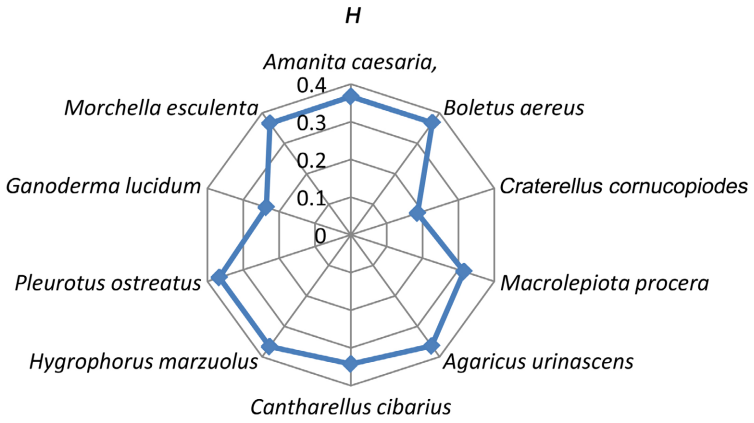

Figure 3. Radar presentation of Shannon genetic information index (*H*) per mushroom population.

Shannon information index provides an easy assessment on the entropy of the genetic information of the germplasm per mushroom species while the total equitability index (E_H) = 0.871 offers an overall estimation of the genetic variation evenness of all species in the population of the studied mushrooms. The higher the value of E_H approaching to 1, the most balanced the observed genetic information among the studied species. The importance of Shannon index is that can reflect genomic drift and if an ecosystem biodiversity will tend to decline. Resampling in time can provide a trend of Shannon index as an indicator for future strategies for environmental protection. In this case, the wild mushrooms germplasm entropy calculated from the RAPDs, could serve as a representation of the overall condition of the ecosystem and it can also be related with other abiotic factors. It has to be noticed that while the Shannon equitability index offers an easily quantifiable measure on the total genetic entropy, information of all the examined mushrooms may mask genetic entropy of individual species. This is observed mainly in the case of *Craterellus cornucopiodes* where there is differentiation from the rest of the mushrooms as it is noticed from the dendrogram distances and the Shannon index. This is also verified from the ANOVA analysis

of the similarity matrix (Table 3) where *p*-value 2E−07 < 0.05 indicates significant differences in the genetic information, as also from the pairwise significance comparison where *Craterellus cornucopiodes* differs from all the rest of the studied mushrooms apart from *Morchella esculenta*.

While RAPDs have been extensively used in species and genera taxonomic classifications, they have also been subjected to criticism as their PCR replications are not consistent. This is a problem that it is observed when specific RAPD markers are used for taxonomic issues, especially in different labs. In the current study, this limitation is not an issue as the analysis is based on measurable data of the total number of the used RAPD markers with statistical estimation about the entropic character of genomes and not targeting specific bands with certain RAPD markers. Additionally, as the described procedure is of statistical nature, increasing the number of markers will reduce even more variations that are caused by experimental conditions.

4. Conclusion

In the present study, the application of RAPDs with parallel entropy analysis was examined as an easily applicable and low-cost valuable technology in environmental monitoring, using genetic information of wild mushroom species as an indicator that can lead to future actions in biodiversity maintenance and germplasm protection. The provided methodology can serve as a pilot procedure enriched with other environmental factors to monitor and protect wild mushroom communities native to the Greek countryside or in any part of the world and provide comparable results about biodiversity from different regions using common entropy indices.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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