46th World Congress of Vine and Wine
OIV International Organisation of Vine and Wine Intergovernmental Organisation
Available on iVES Conference Series vine & wine

DOI : https://doi.org/10.58233/pL5i2F7v

Rară Neagră 2.0: prospecting, improving and safeguarding the biodiversity in an eastern european heritage grape variety

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Abstract. The *Rară Neagră 2.0* project aims to restore and safeguard the intra-varietal diversity of the ancient Eastern European grape variety *Rară Neagră* through polyclonal selection and the establishment of a certified genetic conservatory. A total of 159 accessions were collected from 30 old vineyard plots in Romania, the Republic of Moldova, and Ukraine, representing plantings aged from 25 to over 100 years. Morphological characterization was carried out in 2024 based on eight key traits related to vine vigour, productivity, bunch and berry structure, and epidermal features. Statistical analyses (PCA, hierarchical clustering, Wilcoxon rank-sum tests) revealed significant intra-varietal variability, allowing the identification of two stable morphological groups with differentiated agronomic and oenological potential. Sanitary testing was performed using the ELISA method on dormant cane samples. Approximately 16.4% of accessions tested positive for one or more major grapevine viruses, mainly GLRaV-1 and GLRaV-3, while only one accession showed the presence of ArMV/GFLV and none were infected with TBRV. All virus-positive vines were excluded from the final selection. The results support the implementation of a polyclonal selection strategy based on both phenotypic diversity and sanitary integrity. The selected 133 accessions will form the foundation for a high-quality mother vineyard, contributing to genetic conservation, sustainable viticulture, and the long-term valorization of this emblematic local variety. Additionally, the identification of a stable parthenocarpic seedless mutant and several atypical pigmentation biotypes provides new insights into the genetic complexity of *Rară Neagră* and highlights the importance of conserving its full phenotypic spectrum.

1. Introduction

The Rară Neagră grape variety (also known by its synonyms *Babească Neagră* in Romania and *Sereksia Ciornaia* in Ukraine) is part of the historical ampelographic landscape of Eastern Europe and is believed to have originated in the broader Carpatho-Dniester region with historical roots in vineyards located in Romania, Republic of Moldova, and southern Ukraine [1]. Valued for its freshness, aromatic elegance, and naturally high acidity in winemaking, the variety currently faces major challenges related to the loss of genetic diversity, the unclear clonal origin of existing plantings, and the lack of certified virus-free material. In recent decades, climatic pressures, vineyard modernization, and the trend toward planting uniform, commercial clones have contributed to the genetic erosion of the variety, as old vines have been replaced by international varieties. In this context, conserving intra-varietal diversity has become a strategic priority [2] - not only for protecting local viticultural heritage, but also for ensuring the future adaptability of the variety to evolving agroclimatic conditions and market demands [3].

Large-scale analyses of grapevine cultivars reveal the complexity of their domestication history and underline the need for structured conservation [4].

The Rară Neagră 2.0 project was launched with the goal of identifying, characterizing, and preserving valuable biotypes of this variety through a modern polyclonal selection approach, supported by morphological, sanitary, and agro-oenological analyses. The current phase of the research focused on the morphological characterization and sanitary status of 159 accessions collected from 30 old vineyards across three countries, laying the groundwork for the establishment of a genetic conservatory and the selection of a certified propagation block.

The aim of this study was to support the certification of the varietal identity of Rară Neagră in old vineyards across its traditional growing area, to visually assess the observable phenotypic diversity and general plant health status, and to perform a preliminary selection of biotypes that show no visible symtoms of disease, particularly viral infections. Additionally, the study provides key methodological components needed to complete the polyclonal selection process, including sanitary testing, comparative morphological descriptors, and the proposal of a conservatory plot dedicated to the collection, evaluation, and propagation of the selected material.

2. Materials and Methods

2.1. Origin of Plant Material, Field Selection and Sampling of Accessions

This study was based on 159 accessions of the Rară Neagră variety, collected during 2023-2024 from old vineyards aged between 25 and over 100 years. The prospection covered the traditional growing area of the variety, across three countries: the Republic of Moldova, Romania, and Ukraine. A total of 30 vineyard plots were included, distributed as follows:

Republic of Moldova: 24 plots, grouped into three geographical areas:

- Lower Dniester Area - PGI Ștefan Vodă (13 plots, 77 accessions);
- PGI Codru (2 plots, 12 accessions);.
- PGI Valul lui Traian (9 plots, 47 accessions).

Romania: 4 plots from the PGI Dealurile Vrancei, totaling 20 accessions;

Ukraine: 2 plots from Southern Bessarabia (Ukraine), totaling 3 accessions.

In each plot, a detailed visual inspection was carried out to:

- confirm varietal identity based on ampelographic traits;
- observe morphological diversity within the vineyard;
- identify and tag apparently healthy vines, with no visible signs of viral infections or physiological disorders.

2.2. Morphological Characterization Criteria and Observation Periods

The traits used for field observation and scoring followed the guidelines of the OIV Descriptor List [5]. Morphological observations were conducted during 2024 at two key phenological stages:

- bunch closure stage (first half of July);
- full ripening stage (second half of September).

Each accession was evaluated using the following 8 morphological criteria, based on a 1- 3 scoring scale:

1. Vine vigour; 2. Productivity; 3. Fertility (of buds); 4. Bunch shape; 5. Bunch compactness; 6. Berry shape; 7. Skin color; 8. Presence of bloom on berry skin.

The eight traits evaluated are commonly used in ampelographic studies to capture key aspects of intra-varietal diversity [6].

2.3. Sanitary Testing (Viruses)

The sanitary tests for the detection of the five major grapevine viruses (GLRaV-1, GLRaV-2, GLRaV-3, ArMV/GFLV and TBRV) were performed by ELISA method, which remains a cost-effective and widely applied approach for sanitary selection in viticulture [7]. The samples were collected in the form of three wood fragments consisting of a merithalus framed by two buds, taken from the base of three branches per vine. The analyses were performed at the IFV laboratory, a specialized French center for grapevine virus diagnostics[1].

2.4 Genetic varietal identification

To confirm the varietal identity of accessions with atypical pigmentation traits, molecular analyses were performed using the PCR-based microsatellite (SSR) method[2].

Genetic analysis was performed on plant material consisting of 5 young leaves collected from each atypical pigmentation accession.

The resulting genetic profiles were compared with the reference genotype of Rară Neagră (syn. Babească Neagră) held by the French national grape collection of INRAE Vassal-Montpellier[3], confirming their identity as belonging to the same variety.

2.5 Statistical Analysis

The data analysis included: (1) Hierarchical Cluster Analysis (HCA) based on Gower distance and Ward.D2 linkage to group accessions according to morphological similarity; (2) Radar plots to compare the average morphological profiles across clusters, supplemented with Wilcoxon rank-sum tests to assess trait-wise statistical significance ($p < 0.05$ considered significant); (3) Principal Component Analysis (PCA) to visualize the main axes of phenotypic variation and trait contribution; and frequency distribution analysis of ELISA results to summarize the phytosanitary status of accessions. All statistical analyses were performed using R version 4.4.3.

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3. Results and Discussion

3.1. Sanitary Testing Outcomes

All 159 accessions were tested for major grapevine viruses. GLRaV-1 and GLRaV-3 were most frequent (22 positive cases), while ArMV/GFLV (Court Noué complex) was detected in only one accession. No infection with TBRV was observed. In total, 26 accessions (16.4%) were excluded due to virus presence, ensuring the selection of a high sanitary quality pool for propagation.

Table 1. ELISA test results for major grapevine viruses in 159 Rară Neagră accessions.

Virus tested	Accessions tested positive	Accessions tested negative
Court Noué (ArMV + GFLV)	1	158
Tomato Black Ring Virus (TBRV)	0	159
GLRaV-1 and GLRaV-3	22	137
GLRaV-2	3	156
GLRaV-4 like	1	158
Legend: ArMV = Arabis Mosaic Virus; GFLV = Grapevine Fanleaf Virus; TBRV = Tomato Black Ring Virus; GLRaV = Grapevine Leafroll-associated Virus.		

3.2. Morphological Variation

To assess the intra-varietal phenotypic variability of the Rară Neagră grape variety, a Hierarchical Cluster Analysis (HCA) was performed based on eight morphological traits. The Gower distance metric was used to measure dissimilarity, as it is well-suited for data containing ordinal categorical variables. Clustering was carried out using the Ward.D2 linkage method, which minimizes within-cluster variance. After testing several numbers of clusters (k = 2 to 6), the optimal partitioning was determined to be k = 6, based on the highest average silhouette score (0.285).

The resulting dendrogram (Figure 2) divided the accessions into six distinct clusters (Cluster 1-6), each comprising samples with similar morphological profiles. The cluster sizes varied, from as few as 4 samples in Cluster 6 to 45 samples in Cluster 2.

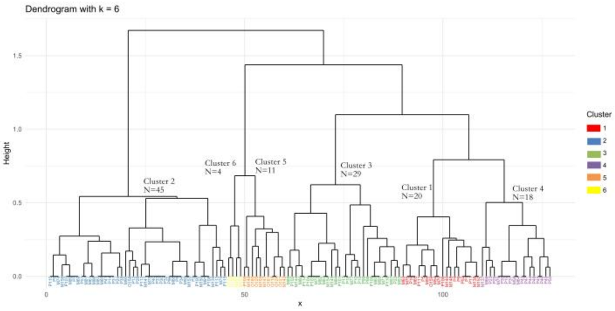

Figure 1. Cluster analysis of the 127 virus-free biotypes of Rara Neagra.

Most of the samples in Cluster 4 originated from the Ștefan Vodă (P) region, suggesting that this cluster may represent a distinctive phenotypic profile characteristic of this specific terroir. Cluster 6 contained only four samples - two from Moldova (M) and two from outside the country (O) - potentially indicating rare or divergent biotypes. The samples from outside Moldova were predominantly grouped in Cluster 5, forming a relatively distinct exogenous group.

In contrast, the larger clusters - Clusters 1, 2, and 3 were primarily composed of mixed samples from both Moldova (M) and Ștefan Vodă (P), indicating a certain degree of phenotypic overlap or convergence between vineyards in these two regions.

Smaller clusters may represent rare or underutilized biotypes with potential value for preserving genetic diversity, while larger clusters are more likely to reflect dominant phenotypic expressions that are stable and widespread in current viticultural practice.

To explore phenotypic differentiation within the Rară Neagră grapevine population, a Kruskal - Wallis test was performed to evaluate the overall variation of eight key morphological traits across the six defined clusters. Traits showing statistically significant differences ($p < 0.05$ - "*", $p < 0.01$ - "**", $p < 0.001$ - "***") were annotated directly on the radar plot (Figure 2). Overall, six out of the eight traits displayed significant variation among clusters, with "Productivity", "Vigour" and "Fertility of buds" being the most strongly differentiated ($p < 0.001$). In contrast, "Presence of bloom on berry skin" and "Berry shape" exhibited comparatively weaker significance ($p < 0.05$).

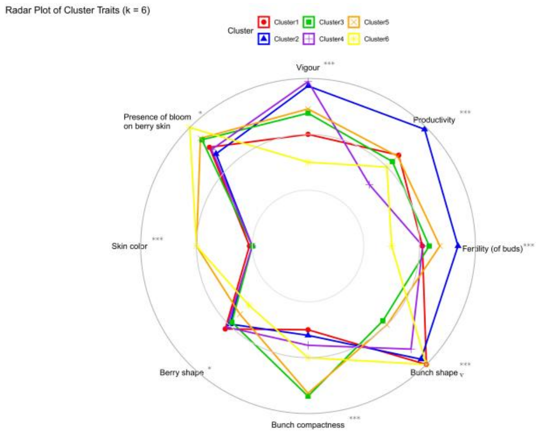

Figure 2. Radar plot for morphological characteristics of Rara Neagra biotype based on the cluster analysis.

To further clarify which specific cluster pairs contributed to these differences, pairwise comparisons were performed using the Wilcoxon rank-sum test. The results were visualized as a significance heatmap (Figure 3), where different colors represent significance levels and non-significant results are marked with "×".

Figure 3. Heatmap of Pairwise Trait Differences Between Clusters (Wilcoxon Rank-Sum Test).

Overall, the Wilcoxon results aligned well with the trends observed in the radar plot, confirming the elevated performance of Cluster 2 for several traits, and the relatively lower expression levels observed in Cluster 6. Notably, structural differences in bunch-related traits were also evident between Cluster 4 and Cluster 5.

The results revealed that “Vigour” and “Bunch shape” showed highly significant differences across multiple cluster pairs, highlighting their importance in distinguishing biotype groups. Notably, although “Presence of bloom on berry skin” and “Berry shape” showed lower overall significance in the Kruskal-Wallis test, they still exhibited statistically significant pairwise differences (e.g., Cluster 6 vs. Clusters 1, 2, and 4 for Berry shape; Cluster 2 vs. Clusters 3, 5, and 6 for berry skin bloom). This suggests that these traits may carry localized phenotypic divergences that were not fully captured in the global analysis. Overall, the heatmap provides additional support for the complex and heterogeneous morphological variability within the Rară Neagră grapevine population.

To further explore phenotypic variation among biotypes, a principal component analysis (PCA, figure 4) was conducted, and a biplot was generated to visualize the structural relationships among samples and the directions of major traits.

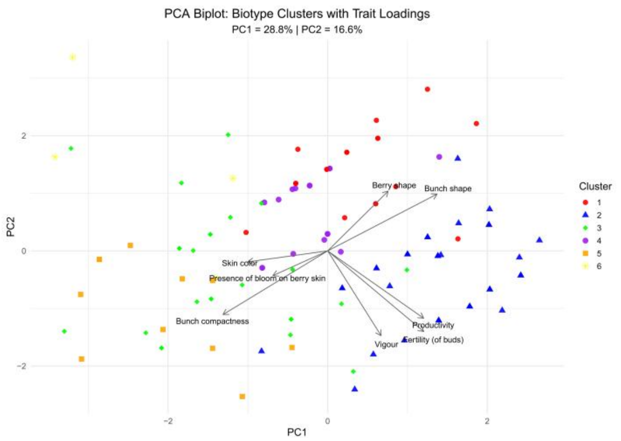

Figure 4. PCA analysis of Rara Neagra biotype based on cluster analysis.

The first two principal components explained 45.4% of the total variance (PC1=28.8%, PC2=16.6%). Although the explained variance is relatively low - likely due to the balanced contribution of multiple variables and heterogeneous sources of sample variation - it still reflects the dominant trends in trait distribution.

In the PCA biplot (Figure 4), Cluster 2 (blue triangles) is distinctly positioned in the positive direction of traits such as “Productivity” and “Fertility (of buds)”, indicating that biotypes in this cluster are characterized by superior performance in growth and reproductive capacity. Cluster 1 (red circles) aligns with the positive axes of “Berry shape” and “Bunch shape”, suggesting that this group may possess distinctive structural features. On the other hand, Cluster 5 (orange squares) is located in the positive direction of “Skin color”, “Presence of bloom on berry skin”, and “Bunch compactness,” highlighting the importance of external appearance traits in defining this cluster. These spatial patterns are consistent with prior results from the radar plot and Wilcoxon pairwise tests, supporting the notion that phenotypic divergence within the Rară Neagră population is driven by both structural and visual traits across different clusters.

A comparative analysis between virus-free accessions and those detected positive for the virus is shown in Fig. 5.

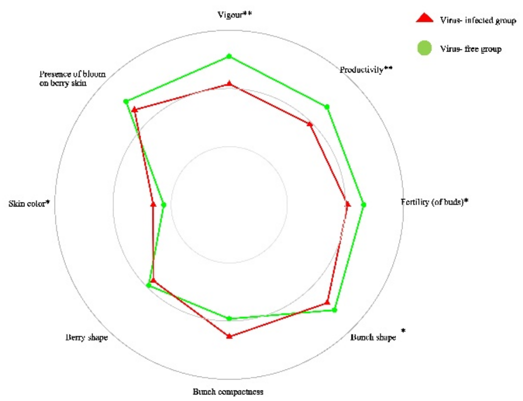

Figure 5. Radar plot for morphological characteristics of Rara Neagra biotype between virus-free and virus-infected groups.

This radar plot illustrates the average performance of eight key morphological traits between the virus-free group (green) and the virus-infected group (red). To evaluate whether these traits differ significantly between the two groups, a non-parametric Wilcoxon rank-sum test was applied. This test is well-suited for comparisons between two independent groups, particularly when sample sizes are unbalanced and normality assumptions are not met - as is the case in this study (Virus-free: N=127, Virus-Infected: N=26).

The results indicate that the healthy group exhibited higher trait scores across several characteristics, with significant differences observed for “Vigour”, “Productivity”, and “Fertility of buds” ($p < 0.01$ or $p < 0.05$), suggesting that viral infection may suppress both vegetative vigor and reproductive potential of grapevines. Additionally, “Skin color”also showed a statistically significant difference between groups (p <

0.05), implying that this visual trait may also be affected by viral presence.

Other traits such as "Presence of bloom on berry skin", "Berry shape", "Bunch shape" and "Bunch compactness" did not reach statistical significance, but visible trends in the radar plot suggest that the infected group generally scored lower on traits related to appearance. Although these trends were not significant, they may still reflect subtle phenotypic disturbances caused by viral infection.

3.3. Atypical Pigmentation Biotypes

During the morphological assessment of the 159 *Rară Neagră* accessions, a small number of vines were identified with unusual pigmentation characteristics. Traditionally, *Rară Neagră* is associated with an intense dark coloration, ranging from deep blue to black. However, in seven distinct accessions originating from different regions (PGI Codru, PGI Valul lui Traian, and PGI Dealurile Moldovei-Romania), noticeable deviations from this standard were observed.

These biotypes exhibited yellowish green, pale pink or violet hues, and in some cases, four bicolored berry skin, with no other visible anomalies. The trait remained stable throughout the growing season and across phenological stages, suggesting a potential genetic basis rather than transient environmental effects.

Although numerically limited, the presence of such atypical pigmentation patterns contributes to expanding the known phenotypic spectrum of the variety. It also raises intriguing questions regarding ancestral lineages, somatic mutations, or localized selection. These accessions were photo-documented and the main morphological characteristics are shown in Table 2 and are proposed for inclusion in the future genetic conservatory, as part of the strategy to safeguard the full extent of intra-varietal diversity.

Table 2. Morfological characteristics of Atypical Pigmentation Biotypes Rară Neagră.

Accession Code	Age of the plot	Skin Color Description	Berry Shape	Bunch Compactness	Notable Feature
RN-PIG-13	41	Yellowish green, no anthocyanin traces	Round	Moderate to compact	White-berried mutant, stable phenotype
RN-PIG-25	55	Light red to rose, uniform pigmentation	Round	Moderate	Stable pigmentation, uniform berry shape
RN-PIG-117	38	Pale pink to translucent yellow, rusty hue	Round to slightly oval	Moderately loose	Slight color variability, regular bunch
RN-PIG-122	32	Orange-pink, subtle variation	Round to slightly oval	Loose	Lax cluster, soft epidermis

To rule out varietal misidentification, all atypical pigmentation accessions were genotyped using the PCR-based microsatellite (SSR) method, which confirmed their identity as Rară Neagră (syn Babească Neagră).

3.4. Identification of a Parthenocarpic Seedless Mutant in the Rară Neagră Variety

During the morphological prospecting activities, a consistent phenotype was observed in several vineyard plots located exclusively in the Republic of Moldova (including PGI Codru, PGI Stefan Voda and PGI Valul lui Traian). This phenotype exhibited parthenocarpic fruit development, resulting in seedless grape bunches.

The mutant is characterized by very loose clusters of medium to large size, bearing small berries that developed without fertilization and lacked seeds, with slightly variable berry shapes.

All accessions corresponding to this mutant were tested using the ELISA method and were found to be free from all five major viruses monitored in the study (GLRaV-1, GLRaV-2, GLRaV-3, ArMV/GFLV, TBRV), supporting the hypothesis that this is a genetically inherited trait rather than a symptom of viral infection.

Field observations conducted at multiple phenological stages - from early fruit formation to full ripeness - confirmed the stability of this trait throughout the vegetative cycle (Figure 6 a-c). The presence of this phenotype in multiple regions and the persistent expression of the trait suggest a fixed genetic mutation, rather than a temporary effect caused by environmental stress. Such a phenotypic mutation, although rare, is already known for other grape varieties, for example: Termarina (mutation of Sciacerello); Cape Currant (mutation of Muscat à petits grains rouges); Corinto bianco (mutation of Pedro Ximenez)[4].

Parthenocarpy in grapevine may result from altered hormonal signaling or floral organ development. [8]**.**

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[4] Collection INRAE de Vassal-Montpellier and https://www.vivc.de/

(a) Early-stage development – loosely formed bunch, unfertilized flowers

(b) Veraison – berry enlargement without seed formation

(c) Full ripening – final bunch aspect with small, seedless berries

Figure 6. a-c. Developmental stages of the parthenocarpic seedless mutant identified in the Rară Neagră variety

The presence of this mutant in a significant number of plots surveyed during this project also illustrates a certain lack of selection of the plant material used, with such non-productive vines logically having to be discarded when selecting cuttings. This fact is also highlighted by certain recurrent varietal mixes within plots, particularly when these are geographically close (Merlot, Cabernet-Sauvignon, Pinot, etc.).

From an applied perspective, this mutant presents significant interest for genetic studies on parthenocarpy and may serve as a valuable source of germplasm for the development of seedless grape cultivars adapted to the local terroir. Moreover, it illustrates the intra-varietal complexity present within the Rară Neagră variety and underscores the importance of conserving such biological diversity.

4. General Conclusions

The study conducted within the Rară Neagră 2.0 project aimed to identify, characterize, and perform polyclonal selection of the most valuable biotypes of the indigenous Rară Neagră variety, with the purpose of conserving its biodiversity and establishing a healthy and diverse planting material base.

To meet these objectives, each accessions will be grafted separately onto a certified rootstock from a sanitary-controlled source, then planted with respect for clonal identity in a genetic repository.

Through the prospection of 30 vineyard plots across the Republic of Moldova, Romania, and Ukraine, a total of 159 accessions from old vineyards were collected, representing the initial genetic pool of the selection program. Morphological characterization was carried out in 2024, based on 8 key traits observed at two phenological stages. Statistical analyses (PCA, clustering, radar charts) revealed a high level of intra-varietal variability and allowed the structuring of accessions into stable morphological groups with differentiated agronomic and oenological potential.

Sanitary testing (ELISA) showed a moderate incidence of GLRaV-1 and GLRaV-3, while other viruses were rarely detected. All infected accessions were excluded from the final selection.

The identification of atypical pigmentation biotypes, displaying stable variations in berry skin coloration (greenish-white, pale pink, orange-pink, light red to pink), reflects the intra-varietal complexity of Rară Neagră and supports the inclusion of such forms in conservation strategies aimed at preserving the full genetic spectrum of this heritage variety.

The observation and documentation of a parthenocarpic seedless mutant of the Rară Neagră variety, identified in multiple vineyard plots across the Republic of Moldova, highlights the presence of unexplored genetic mechanisms within the variety and opens new avenues for research on parthenocarpy and the development of seedless cultivars.

Based on these findings, the study supports the implementation of a polyclonal selection strategy that fully utilizes the existing diversity while ensuring the conservation of the genetic resources of the variety. The resulting conservatory will form the basis for the certification of a high-quality mother vineyard for graftwood, strengthening regional viticultural heritage and contributing to the long-term sustainability of the sector.

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