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ORIGINAL ARTICLE

Optimum neighborhood seed orchard design

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Abstract Original seed orchard algorithm "Optimum Neighborhood Seed Orchard Design" was developed as local heuristics to facilitate the establishment of advanced-generation seed orchards with complex configurations. The scheme leads to uniform spatial distribution among adjacent genetic entries, promoting panmixia. The resulting scheme is thus suitable to establishing both productive seed orchards as well as clonal archives of threatened species, etc. Further, it can accommodate variable clonal sizes, uneven grids, relatedness, assortative mating. It may supplement existing layouts (clonal rows, etc.). Layouts outperformed existing advanced-generation alternative schemes in most parameters and thus could be recommended to operational forestry and gene conservation management.

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Introduction

Seed orchards are the production vectors linking tree improvement activities with reforestation practices. The main objective of a seed orchard is to produce frequent and abundant high genetic quality seed for the production of genetically improved seedlings for regeneration. The mating pattern within a seed orchard is a major player in determining the level of inbreeding and is mainly influenced by clonal genealogy, size, reproductive phenology, gametic contribution, propensity to selfing, spatial arrangement on orchards' grids, and the extent of extraneous gene flow (El-Kassaby 1995; Burczyk and Prat 1997).

Seed orchard designs have evolved from those focused on randomization for maximizing mating among unrelated clones such as the completely random (Giertych 1975) and permutated neighborhood (Bell and Fletcher 1978), to those dealing with advanced generation orchards for avoiding mating among relatives, promoting assortative mating, and minimizing inbreeding (randomized complete block (White et al. 2007); replicated randomized staggered clonal rows (R^{2}SCR) (El-Kassaby et al. 2014); minimum inbreeding design (MI) (Lstibůrek and El-Kassaby 2010; Lstibůrek et al. 2015)).

The spatial arrangement of clones on the orchard's grid is, by far, among the most important factors affecting not only the mating pattern but also the level of assortative mating which is, sometimes, advocated by breeders for capturing both additive and non-additive genetic variances controlling selected traits. The R^{2}SCR and MI designs focused on the frequency of clonal adjacency and its impact on panmixia. Here, we present a new and simple algorithm that

specifically deals with clonal adjacency and its optimization. The proposed algorithm is based on heuristics, which is expected to effectively allow the discovery of optimum orchard's arrangement through the "trial-and-error" method, even under complex scenarios. The efficiency of the proposed algorithm is demonstrated and compared to those designs developed for managing clonal adjacency; namely, R^2SCR and MI designs. The algorithm was developed in R system (R Development Core Team 2008) and is publicly available (Lstibůrek 2016).

Methods

Optimum neighborhood algorithm (ONA)

We assume an orchard grid with m and n dimensions, it consists of sliding 3×3 rectangular neighborhoods with the target clone located centrally (i.e., every ramet is surrounded by 8 positions). We considered the "optimum neighbourhood" as the arrangement that produces a minimum variance of the number of times (i.e., counts) of adjacency between two different clones. This arrangement is a practical proxy of panmixia as the variance of counts reaches zero when all adjacency counts are equal. It is conceivable that a solution is reached (i.e., minimum variance) where incident adjacency between some clones exist.

Mathematical solution

Let **V** be the square symmetric matrix of sums of adjacency among clones with the dimension N_c (i.e., the number of clones). We optimize the following criterion function, which comprises of two addends:

$$min \rightarrow \frac{1}{\binom{N_c}{2}} \sum_{i=1}^{N_c} \sum_{\substack{j=1 \\ j \neq i}}^{N_c} \left(N_{ij} - \frac{N_g}{\binom{N_c}{2}} \right)^2 + \sum_{i=1}^{N_c} N_{ii} p, \quad (1)$$

where N_{ii} and N_{ij} depict the number of single and different clones' adjacency, respectively, N_g is the total number of neighboring positions existing within the rectangular orchard grid which can be calculated as

$$N_g = m(n-1) + n(m-1) + 2(n-1)(m-1). \quad (2)$$

The first addend in Eq. 1 represents the variance of upper triangle counts in **V** and the second addend represents a penalty (p) implemented to limit same clone adjacency (diagonal counts in **V**). Potential value of p is of higher order compared to the first addend (ex. 10 or 100), which leads to an effective elimination of direct neighborhoods of same genetic entries. Value of the criterion function (later designated as σ^2_{min}) is therefore, in ideal case, equal only to the first addend.

Proposed heuristics to optimize a given orchard design

The first step is to create an empty **V** matrix followed by selecting the first position on the orchard grid (see a graphical representation of the proposed heuristic algorithm in Fig. 6). On the selected position, a genetic entry is randomly drawn out of all available candidate entries (the ramet population of all clones) and the selection of the second and subsequent positions are made after evaluation by the criterion function (Eq. 1). At each step, the criterion function is maintained at minimum after considering all possible candidate entries and this position is assigned and the process is sequentially repeated until the entire orchards grid is fully populated. This process considers all candidate entries (clonal representation) in case of equal or unequal clone size through a candidate entry vector. Therefore, even under different clone sizes, the random selection of candidate entries is proportional to their respective clone size.

Reference level for evaluating the resulting arrangement

The resulting orchard arrangement is compared to a theoretical minimum variance "reference" to validate the effectiveness of the heuristics as well as the impact of the penalty p on the minimization of variance (Appendix).

Presented examples

Seven different scenarios were constructed to assess the efficiency of the proposed orchard layout. Six included balanced clonal size (100, 80, 50, 40, 20 and 10 ramets per every clone), and one unbalanced clonal size (10, 18, and 4 clones with 20, 10 and 5 ramets per clone, respectively). The orchard grid is square and contains 400 positions (i.e., 20×20, in all cases). Optimum solution for each assignment was generated within 30 iterations, and the assigned p value (penalty) was 100.

Comparison to existing designs

The resultant orchard layouts were compared to (R^2SCR) (El-Kassaby et al. 2014) and MI designs (Lstibůrek and El-Kassaby 2010; Lstibůrek et al. 2015). To generalize the comparisons, balanced and unbalanced designs were investigated. The balanced design consisted of 40 clones with 10 ramets each and the unbalanced design consisted of using 10, 18, and 4 clones, each is represented by 20, 10, and 5 ramets, respectively. Additionally, as a reference, a completely randomized layout (RD) was also generated. Results

from the different schemes were evaluated by d_{min} and σ^2_{min}, where d_{min} is a proxy to potential inbreeding (selfing in this case) termed as "minimum distance" estimate:

$$d_{min} = \sum_{l=1}^{N_c} \sum_{i=1}^{N_k} \sum_{j=i+1}^{N_k} \frac{1}{d^2_{il,jl}}, \qquad (3)$$

where d is the distance between the ith and the jth ramet of the lth clone, N_c is the number of clones, and N_k is the number of ramets within a clone. The d_{min} represents the inverse sum of squares of all possible distances between any two ramets of the same clone and across all clones. The closer the distance between any two ramets of the same clone, the higher the value and, conversely, the further the distance, the lower the value (Lstibůrek and El-Kassaby 2010).

Results and discussion

The proposed Optimum Neighborhood Algorithm (ONA) seed orchard layout proved efficient in minimizing the variance of the **V** matrix under both balanced and unbalanced clonal sizes.

Balanced clonal size

The balanced clone size of 40 clones each with 10 ramets is illustrated in Fig. 1 along with the corresponding **V** matrix (Fig. 7). In this scenario, we did not detect any specific geometrical pattern, suggesting efficient distribution of ramets on the orchard's grid. The local separation zone of 3×3 secured 100 % avoidance of having any two ramets of the

Fig. 1 Balanced scenario with 40 clones each with equal clonal size of 10 ramets/clone

Fig. 2 Unbalanced clone size scenario illustrating differently sized group (one clone per each group size is highlighted)

same clone in adjacent positions on the orchards grid. This is a product of the second addend in the criterion function (Eq. 1) and it seems that a penalty of $p = 100$ was sufficient (i.e., all diagonal elements in the **V** matrix are equal to zero). From the above inspection, we highlight excessive optimization space under balanced scenarios with relatively large number of clones, accommodating larger, yet feasible

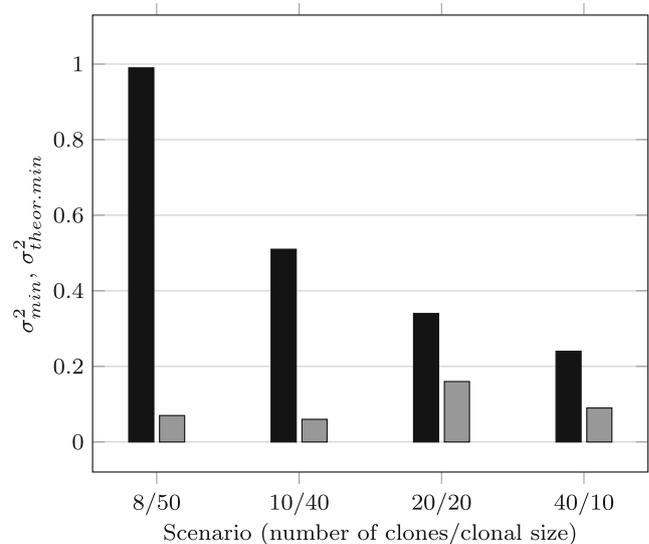**Fig. 3** Comparison of various scenarios (by criterion function value, σ^2_{min}, *black columns*) to a theoretical minimum variance "reference" ($\sigma^2_{theor.min}$, *gray columns*) (see Appendix) for validating the heuristics' effectiveness. σ^2_{min} and $\sigma^2_{theor.min}$ were 3099.67 and 0, respectively, for scenario 4/100; and 12.09 and 0.16 for scenario 5/80 (data not shown)

Table 1 The evaluated criteria values (d_{min} and σ^2_{min}) for the optimum neighbourhood (ONA) with minimum inbreeding (MI), replicated randomized staggered clonal rows (R^{2}SCR), and randomized (RD) schemes

	Balanced clonal size				Unbalanced clonal size			
	ONA	MI	R^{2}SCR	RD	ONA	MI	R^{2}SCR	RD
d_{min}	32.25	21.58	40.34	66.30	60.94	51.39	59.66	98.62
σ^2_{min}	0.24	2.26	1.63	5.04	3.85	14.94	5.54	40.92

The completely random layout was used as a reference value (100 %)

local separation zones. Reducing the clonal number results in a substantial increase of the clonal size (e.g., 4 clones and 100 ramets each) leading to less favorable scenarios (results not shown), where the layout shows repeated patterns and although the σ^2_{min} is minimized, there is no feasible solution to accommodate completely the second addend, i.e. diagonal elements in **V** matrix become larger than zero.

In the above scenarios, σ^2_{min} of the best heuristic solution was close to its theoretical minimum limit (Appendix); however, the presence of the additional constraint, i.e. separation zones, prevented its attainment, particularly under extreme scenarios (small number of clones and large clonal sizes).

Unbalanced clonal sizes

The average number of neighboring positions for the clones is 7.23, 7.58, and 7.70 for the first (20 ramets/clone), second (10 ramets/clone), and third (5 ramets/clone) groups, respectively, indicating the presence of significant dependence of this parameter on clonal size. Resulting layouts

and **V** matrix are illustrated in Figs. 2 and 8, respectively. Similar to the balanced design observation, we conclude the absence of any geometrical specific patterns, suggesting even distribution of ramets within the grid. Additionally, the used local separation function of 3.85 did not result in any clonal adjacency on the orchard grid. While a visible shift exists, the results produced acceptable minimum variance for such exceedingly unbalanced scenario. Furthermore, the strength of the ONA scheme will be highlighted during its comparison with the other advanced designs (R^{2}SCR and MI) as well as the random layout.

Evaluation of resulting schemes by means of reference levels

The evaluation of resultant balanced clonal contributions to the respective $\sigma^2_{theor,min}$ is depicted graphically in Fig. 3, where the value of σ^2_{min} drops with higher N_c and asymptotically progresses towards $\sigma^2_{theor.min}$. For unbalanced clone

Fig. 4 Relative comparison of optimum neighbourhood (ONA) with minimum inbreeding (MI), replicated randomized staggered clonal rows (R^{2}SCR), and randomized (RD) designs using a balanced completely random layout
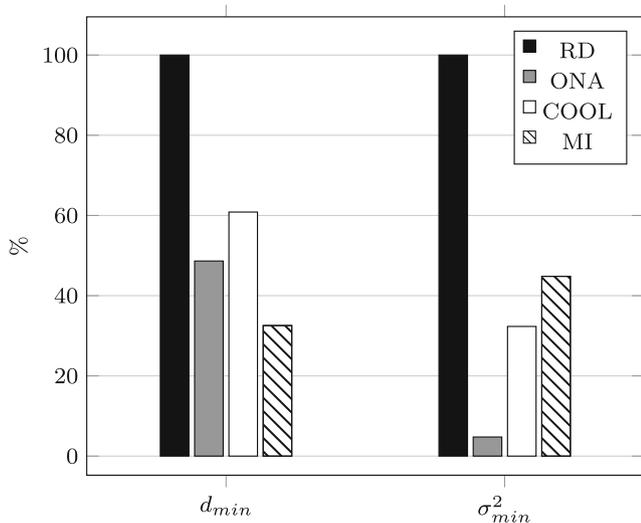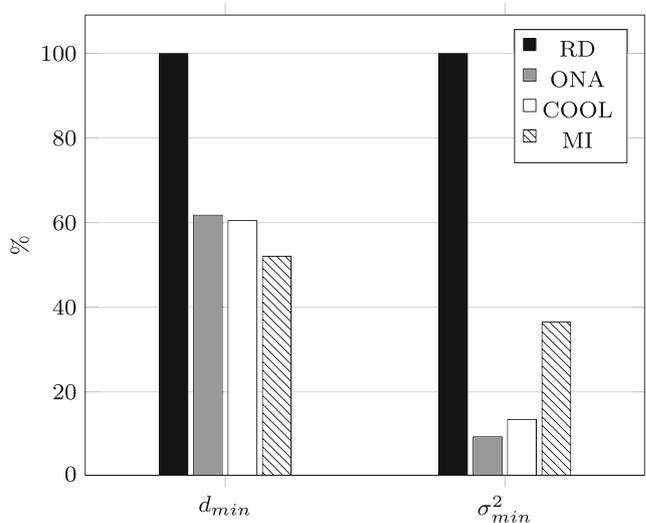

Fig. 5 Relative comparison of optimum neighbourhood (ONA) with minimum inbreeding (MI), replicated randomized staggered clonal rows (R^{2}SCR), and randomized (RD) designs using an unbalanced completely random layout
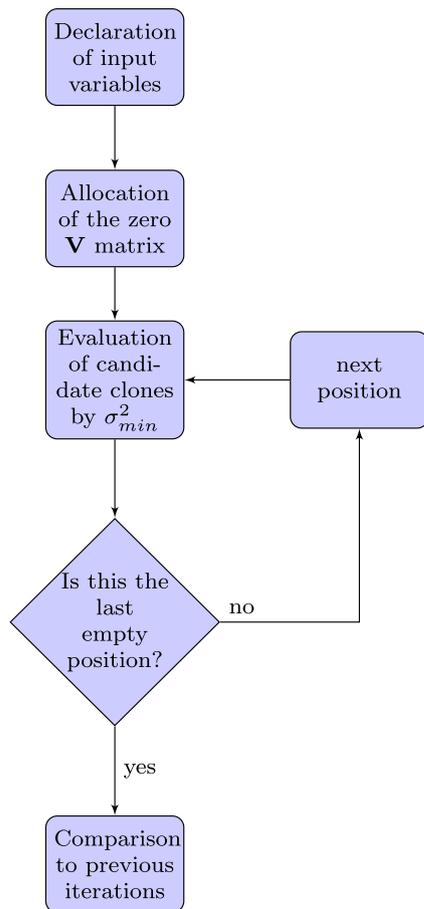
Fig. 6 Schematic description of the ONA's algorithm

sizes, the comparison resulted in $\sigma^2_{min} = 3.85$, while $\sigma^2_{theor.min} = 3.39$ (data not shown in the graph).

Comparison of resulting ONA schemes with other designs by means of d_{min} and σ^2_{min}

The observed differences of d_{min} and σ^2_{min} in R²SCR and MI schemes are relatively lower than that observed for the RD design, indicating these two designs' effectiveness in separating clonal ramets on the seed orchard grid (Table 1). The relative differences became more pronounced for the unbalanced design (Table 1 and Fig. 5). It must be emphasized that the MI outperformed the R²SCR and ONA based on d_{min}. However, it should be noted that the criterion function of MI design is built differently and focuses primarily at minimizing inbreeding.

It is clear that under balanced cases, the ONA outperformed R²SCR in terms of d_{min} and this could be considered as an added benefit to its primary efficiency in minimizing σ^2_{min} (Fig. 4). When considering σ^2_{min} as a key comparison criterion (a proxy of panmixia), the ONA is by far the best layout under the studies' assumptions. It is

worth mentioning that under unbalanced scenarios, the relative differences between the ONA and R²SCR schemes become marginal (Fig. 4 vs. Fig. 5).

The results show that the value of the objective function of the final scheme is closely related to the way of defining close proximity and the type of specific task (the number of clones and space for optimization as the size of the set). It should be noted that smaller clones are placed in the layout center, where the position stretches to the maximum number of direct neighborhoods (8), thereby enhancing their chances to participate in reproduction and thus supporting panmixia (Figs. 6, 7 and 8).

The current study provides a theoretical insight into seed orchard layout optimization. There are two theoretical limits underlying the spatial distribution, one provided by the completely randomized scheme (RD) where all restrictions are relaxed and all genetic entries are treated equally. Such a scenario, when averaged across large number of independent iterations, mimics what could be called a random mating (union of gametes). In a real situation, as shown in Figs. 4 and 5, this approach clearly lacks parameters desirable in optimum seed orchard layout. Clear disadvantage of simple randomization is the lack of separation among the same (ramets of the same clone) or similar (i.e., half-siblings) genetic entries. Second and equally important is the theoretical limit embodied by the MI layout, which is a global optimization protocol, leading to the minimum expected inbreeding (Lstibůrek and El-Kassaby 2010; Lstibůrek et al. 2015), but disregarding panmixia in its nature. The new ONA design is centered somewhere in between RD and MI with its ability to avoid any visible patterns in final scheme (promotion of panmixia) and to control direct neighborhood of the same genetic entries (Figs. 4 and 5). This study also highlights important factors relevant to seed orchards' designs specifically in advanced generations where higher selection differential (smaller number of clones are desired) inevitably leads to increase of common ancestry. As the MI design implies, attention is often directed to intentional selfing avoidance (large weight on separation of ramets of the same clone); however, as the number of clones goes down, this is accompanied by increase in the number of ramets per clone which adds additional levels of complexity. This problem could be further intensified by relatedness among clones or under unequal deployment. This additional complexity has to be dealt when the ONA approach is considered. The penalty value (p) is an integral part of the criterion function and enables us to allocate extra weight to various levels of relationship. When the penalty is given a negative value, it can even promote assortative mating, which is, of course, a rather extreme scenario. Moreover, the criterion function can accommodate various natural constraints of the particular plot—for example, prevailing wind direction, slope etc.

Fig. 7 The balanced scenario **V** matrix (subset of the matrix is presented)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	.	.	Nc
1	0	3	2	2	2	2	1	1	2	1	2	2	2	2	1	2	2	1	1	2	.	.	.
2	3	0	2	2	1	1	2	2	2	2	2	3	2	2	2	2	2	2	2	1	.	.	.
3	2	2	0	2	2	2	2	2	2	1	2	2	1	1	1	2	2	2	1	2	.	.	.
4	2	2	2	0	2	2	2	2	2	1	3	2	2	3	2	2	2	2	2	2	.	.	.
5	2	1	2	2	0	2	2	2	2	2	2	1	2	2	2	2	1	3	2	2	.	.	.
6	2	1	2	2	2	0	1	2	2	3	2	2	2	2	2	2	2	2	2	3	.	.	.
7	1	2	2	2	2	1	0	2	1	2	2	1	2	3	1	1	2	3	2	2	.	.	.
8	1	2	2	2	2	2	2	0	2	2	1	2	2	2	2	2	2	2	2	1	.	.	.
9	2	2	2	2	2	2	1	2	0	3	2	3	2	1	3	2	3	2	2	2	.	.	.
10	1	2	2	1	1	3	2	2	3	0	2	2	2	2	2	2	1	2	2	2	.	.	.
11	2	2	2	3	2	2	2	1	2	2	0	2	1	2	2	3	2	2	2	2	.	.	.
12	2	3	2	2	1	2	1	2	3	2	2	0	3	2	3	2	2	1	2	1	.	.	.
13	2	2	1	2	2	2	2	2	2	2	1	3	0	2	2	2	2	2	2	2	.	.	.
14	2	2	1	3	2	2	3	2	1	2	2	2	2	0	2	2	2	2	2	2	.	.	.
15	1	2	1	2	2	2	1	2	3	2	2	3	2	2	0	2	2	1	2	2	.	.	.
16	2	2	2	2	2	2	1	2	2	3	3	2	2	2	2	0	2	2	2	2	.	.	.
17	2	2	2	2	1	2	2	2	2	1	2	2	2	2	2	2	0	1	2	2	.	.	.
18	1	2	2	2	3	2	3	2	3	2	2	1	2	2	1	2	1	0	2	2	.	.	.
19	1	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	3	.	.	.
20	2	1	2	2	2	3	2	1	2	2	2	1	2	2	2	2	2	2	3	0	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Nc	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

In addition, the ONA can be used in combination with other existing layouts without losing its efficiency. We conducted a case study of ONA connected with predefined MI design of clonal rows (results not shown), where the ramets of the same clone were merged together in clonal-rows without imposing constraints on selfing (El-Kassaby 2003; El-Kassaby et al. 2007; El-Kassaby et al. 2014). This makes biological sense in some species as evidence of low selfing rates in conifers supports this notion (El-Kassaby 1995; O'Connell 2003). Clonal rows were assigned to those clones sharing a common pedigree, and subsequently, distance of these related clones within the layout was maximized by MI. In the next step, ONA layout was literally wrapped around the optimized clonal rows.

The upper triangular matrix concept for defining all possibilities of outcrossing among clones in the seed orchard, which is partially equal to the **V** matrix utilized in the ONA algorithm, has been used before (Nester 1994); however, the **V** matrix has not been used as a design evaluation criteria, but already at the point of the layouts' creation.

Fig. 8 The unbalanced scenario **V** matrix (subset of the matrix is presented)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	0	7	9	8	7	7	9	9	7	8	5	4	4	4	3	3	4	3	3	4	2	3	5	7	4	4	4	7	3	3	2	2
2	7	0	7	7	7	7	8	7	9	9	2	3	4	5	2	4	4	3	3	2	4	3	4	4	3	2	3	4	2	2	2	0
3	9	7	0	8	8	7	7	7	7	8	6	5	3	4	5	3	3	3	3	4	4	4	4	4	4	3	3	2	3	2	2	2
4	8	7	8	0	8	7	8	8	8	10	4	4	3	4	4	4	3	4	3	3	4	6	4	3	3	3	4	3	2	1	1	4
5	7	7	8	8	0	8	9	7	8	8	3	3	6	5	3	4	4	3	4	3	5	4	4	3	2	3	2	2	2	2	2	2
6	7	7	7	7	8	0	6	9	7	7	5	4	4	3	2	4	4	3	4	4	3	3	3	3	3	3	3	1	2	1	1	1
7	9	8	7	8	9	6	0	8	7	6	4	4	2	3	4	4	4	3	3	3	4	4	4	4	3	4	5	3	3	1	1	2
8	9	7	7	8	7	9	8	0	8	8	4	5	4	4	3	3	4	3	5	3	4	5	5	3	3	4	4	4	1	2	2	2
9	7	9	7	8	8	7	7	8	0	8	2	3	5	4	4	4	4	5	3	5	4	4	3	5	4	3	3	2	2	2	2	2
10	8	9	8	10	8	7	6	8	8	0	3	4	3	5	4	4	4	3	4	4	4	4	5	2	5	5	1	3	2	2	2	2
11	5	2	6	4	3	5	4	4	2	3	0	3	2	2	2	2	2	3	1	2	2	4	2	1	1	2	3	1	1	1	1	1
12	4	3	5	4	3	4	4	5	3	4	3	0	2	3	3	1	1	1	2	2	2	2	2	3	2	2	1	1	1	1	1	1
13	4	4	3	3	6	4	2	4	5	3	2	2	0	2	2	3	1	3	3	2	2	2	2	1	3	3	2	0	2	1	1	1
14	4	5	4	4	5	3	3	4	4	5	2	3	2	0	2	3	1	2	2	3	2	3	1	1	3	2	2	1	0	1	1	1
15	3	2	5	4	3	2	4	3	3	4	2	3	2	2	0	3	2	4	2	1	2	2	1	2	1	2	2	2	1	0	1	1
16	3	4	3	4	4	4	4	3	5	4	2	1	2	3	3	0	2	2	1	1	2	2	2	2	2	2	1	2	1	0	1	1
17	4	4	3	4	4	4	4	4	5	4	2	1	3	1	2	2	0	2	3	2	2	1	1	4	3	1	3	2	1	1	0	0
18	3	3	3	3	3	3	3	3	4	4	3	1	3	2	4	2	2	0	2	3	1	3	2	2	3	2	3	2	1	1	1	2
19	3	3	3	4	4	4	3	5	4	3	1	2	1	2	2	1	3	2	0	1	2	2	2	3	2	2	3	1	1	2	1	1
20	4	2	3	3	4	4	3	3	4	4	2	2	3	3	1	1	2	3	1	0	2	3	3	2	2	2	3	3	1	1	2	1
21	2	4	4	3	3	3	3	4	4	4	2	2	3	2	2	2	2	1	2	2	0	2	1	1	2	3	1	2	1	1	2	1
22	3	3	4	4	4	3	4	5	5	4	4	2	2	3	2	2	1	3	2	3	2	0	1	1	2	2	2	2	0	1	1	2
23	5	4	4	6	4	4	4	5	3	4	2	2	1	1	2	1	2	2	3	1	1	1	0	1	1	2	2	2	1	0	0	0
24	7	4	4	4	4	3	4	3	5	4	1	2	2	1	2	2	4	2	2	2	1	1	1	0	3	2	2	2	2	0	0	1
25	4	3	4	3	4	3	4	3	4	5	1	3	2	3	1	2	3	3	3	2	2	2	1	3	0	2	2	2	1	0	1	1
26	4	2	4	3	2	3	3	4	3	2	2	2	2	2	2	2	1	2	2	2	3	2	2	2	2	0	3	1	1	1	1	1
27	4	3	3	3	3	3	4	4	2	5	3	2	3	2	2	3	3	2	3	1	1	2	2	3	2	3	0	2	1	2	1	1
28	7	4	3	4	3	3	5	4	3	5	1	1	2	2	2	1	2	2	3	3	2	2	2	2	2	1	2	0	1	1	1	1
29	3	2	2	3	2	1	3	1	2	1	1	1	1	0	1	1	2	1	1	1	1	1	2	2	1	1	1	1	0	0	0	0
30	3	2	3	2	2	2	3	2	2	3	1	1	2	0	0	1	0	1	1	1	1	1	1	0	0	1	2	1	0	0	1	0
31	2	2	2	1	2	1	1	1	2	2	1	1	1	1	1	1	0	1	1	2	2	1	0	0	1	1	1	0	0	1	0	1
32	2	0	2	4	2	1	2	2	2	2	1	1	1	1	1	1	0	2	1	1	1	0	1	1	1	1	0	0	1	0	1	0

Important information is that ONA is not limited to rectangular shapes. The only input parameters needed for optimization are the coordinates within the plot.

Data archiving statement

In our study, we were developing a mathematical model to optimize spatial allocation of trees in seed orchards. We did not use any real-world data of any species nor any sequencing data.

Appendix

The calculation of $\sigma^2_{theor.min}$ for balanced clonal sizes is based on the assumption that in an ideal clonal deployment, $\mathbf{V}$ contains only 1 or 2 specific values. Minimum and maximum values of $\mathbf{V}$ are denoted as V_{min} and V_{max}, respectively. V_{min} and V_{max} are derived after rounding down and up the expected value $\frac{N_g}{\binom{N_c}{2}}$, respectively. The following system of equation is used to derive the number of repetitions of these values in $\mathbf{V}$:

$$N_g = R_{min}V_{min} + R_{max}V_{max}, \tag{4}$$

$$\binom{N_c}{2} = R_{min} + R_{max}, \tag{5}$$

where R_{max} and R_{min} are the number of repetitions of V_{min} and V_{max} in $\mathbf{V}$, respectively. The last step is to calculate $\sigma^2_{theor.min}$ as

$$\sigma^2_{theor.min} = \frac{\left(\frac{N_g}{\binom{N_c}{2}} - V_{min}\right)^2 R_{min} + \left(\frac{N_g}{\binom{N_c}{2}} - V_{max}\right)^2 R_{max}}{\binom{N_c}{2}}. \tag{6}$$

This reference level does not take into account the fact that in case of unequal clonal sizes the less numerous clones are placed in positions with a larger number of direct neighborhoods. For this reason, for unbalanced clonal sizes, a second option for calculating the $\sigma^2_{theor.min}$ was defined as

$$\sigma^2_{theor.min} = \frac{1}{\binom{N_c}{2}} \sum_{\vee i j \in C} \left(V_{i,j} - \frac{N_g}{\binom{N_c}{2}}\right)^2, \tag{7}$$

where the theoretical ideal number of close neighborhoods V_{ij} is calculated for each clonal pair ij as

$$V_{i,j} = \frac{N_g r_i r_j}{\prod_{i,j} r_i r_j}, \tag{8}$$

where r_i is the number of ramets of the ith clone and r_j is the number of ramets of the jth clone. This calculated minimum variance is merely theoretical, because its calculation is based on real V_{ij} values.

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