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THE COURSES OF NURSERIES CONTROL AND VARIETY INVESTIGATION SYSTEMS FOUNDED ON MOLECULAR MARKERS

Principles of molecular marker systems for breeding and varieties investigation needs were analyzed on model experimental data. The key features of such systems are minimally required number of markers, sets of varieties used as 'localized etalons', and dedicated analytic instruments ready for enhancement. Attention is paid to improve discriminant ability of these systems. UPOV recommendation to use 8 MS markers is considered as not sufficient.

Key words: *molecular markers, microsatellite analysis, variety investigation, identification, winter bread wheat.*

Introduction. Molecular markers are considered to be an indispensable tool of modern genetic research because of their clear manifestation of genetically determined differences in varying environmental conditions and relative simplicity combined with accessibility of the analysis.

Over the past three decades, the total number of the proposed molecular markers reached such a level, which makes it possible to construct high density genetic maps [1, 2]. A large set of markers is being used in fine genetic studies nowadays [3–5], but for the most purposes of the current breeding such a detailed analysis is excessive both in substance and expenditures. Insufficient elaboration of approaches to optimize molecular markers systems of identification and variety control in some way holds back the widespread introduction of the latter into practice of variety investigation. Hence the importance of the problem comes up in the headline of this article.

Material and methods. As a model example there were selected results of our research of 49 lines picked out under the «ear-row» scheme from soft winter wheat varieties breeded by the Plant Breeding and Genetics Institute — National Center of Seed and Cultivar Investigation using 17 microsattellite loci, the part of which was given in the guidelines on the differentiation and identification of wheat varieties and was used in our previous researches [6, 7]. This outlines the range of applications: the model simulates the operation with monomorphic varieties or with the clean lines of composed varieties,

i.e. the final stages of breeding, seed nurseries, dedicated collections and so on. It is assumed that in consequence of breeding these varieties are adapted to the conditions of a certain region, i. e. the genetic basis of such varieties is respectively narrowed compared to the complete gene pool of the culture.

The choice of the microsatellite loci (MS) is dictated by their advantages over other molecular markers that are used in the studies of genetic polymorphism of wheat varieties. Particularly they attract by relative simplicity and accessibility of their analysis, by large number of the mapped loci that uniformly cover the entire genome of *T. aestivum* L., by significant polymorphism, and co-dominant nature of their inheritance [1, 2, 8]. MS analysis attracts also by the opportunity to conduct research of DNA isolated from individual genotypes and by its developed technical basis for the implementation of this method. Also it was foreseen by the research plan to study conditions of application of the approaches for all markers at all, including non-molecular markers.

The list of the selected markers is given in Table 1. It should be noted that some MS-markers can detect additional loci on other chromosomes (they are shown in parentheses), but these additional loci are characterized generally by different size of the alleles which are not investigated in our research.

For the analysis of the data there were used the software tools Excel 2010, FileMaker 12.0, Statistica 6.0.

Results and discussion. Optimizing of the number and composition of the markers. During the research of all 49 wheat lines using 17 MS loci there were revealed 110 alleles with, on the average, 6.5 alleles (Table 1). Theoretically this system is able to identify up to $1{,}925 \times 10^{13}$ alleles uniquely, i. e. about 20 trillion of all the possible combinations of alleles, what is many orders greater than the potential number of all varieties in the world. PIC *(polymorphism information content)* indicates a significant amount of «polymorphic» information in loci.

The represented data indicates that the system of the 17 MS markers would be excessive for the differentiation of a relatively small set of varieties (of about 50). Due to the large amount of diverse information, which should be taken into the account, it is impossible to be bound by purely formal rules of screening of the minimally required set of markers, so it is somewhat empirical process.

Altogether there were formed three sets of markers. First set was composed of loci with the highest number of alleles, further the primary right was given to the index of polymorphism PIC (set 2). All the remaining markers went into the third set. The composition of the three sets of markers is given in Table 2, marked with ordinal numbers from Table 1.

The marker locus number 15 (*Xgwm408*) had to be included in all three sets, the identification without it would not be complete. The same reason was for including the marker locus number 2 (*Xgwm095*) in the composition of set 2 and set 3. The cumulative curves of the number of uniquely identified varieties for all three sets are shown in Figure 1; the curve for set 3, composed of eight markers which detect the least polymorphic loci, was the shallowest one.

Table 1

The quantitative composition of alleles in the studied loci

№	Locus	Cromosome	Alleles	PIC
1	Xgwm186	**5A**	9	0,68
2	Xgwm095	**2A**	5	0,64
3	Xgwm357	**1A**	7	0,76
4	Xgwm18	**1B**(4B)	5	0,74
5	Xgwm190	**5D**	2	0,46
6	Xgwm3	**3D** (2D)	7	0,82
7	Xgwm165/I	**4D** (4A, 4B)	5	0,71
8	Xgwm155	**3A**	10	0,84
9	Xgwm437	**7D**	4	0,55
10	Xgwm389	**3B**	7	0,82
11	Xgwm325	**6D**	11	0,81
12	Xgwm44	**7D**(4A)	6	0,62
13	Xbarc126	**7D**	9	0,74
14	Xwmc405	**7D**(1D, 5B, 5D, 7A)	6	0,61
15	Xgwm408	**5B**	7	0,64
16	Xgwm577	**7B**	5	0,62
17	Taglgap	**1B**	5	0,50

Table 2

The composition of the minimized set of markers

Name	The composition of the selected markers	Alleles/marker	PIC/ marker	A theoretical number of combinations
Set 1	1, 3, 8, 11, 13, **15**	8,8	0,745	436.590
Set 2	**2**, 4, 6, 7, 10, **15**	6,0	0,727	42.875
Set 3	**2**, 5, 9, 12, 14, **15**, 16, 17	5,0	0,580	252.000

Through numerical modeling it was found that the marker system which would be able to identify uniquely at least 99.99 % of varieties must «recognize» the number of allelic combinations of about 2 orders of magnitude greater than the number of the analyzed varieties. The real difference between the theoretical number of combinations given in Table 2 and the number of the identified lines (49) was of 3–4 orders of magnitude. The main reason for this large discrepancy lies in very uneven distribution of alleles in each of the studied loci. Consequently the combinations of the «minor» alleles are very low-probable and within small subsets of varieties (50–100 samples) simply not occur. Thus, for the pairs of loci *Xgwm155/Xgwm325* there were found no lines which combine alleles 129, 135, 137, 139, 141, 143 bp from the first locus (*Xgwm155)* and 115 , 120, 128, 134, 138 bp — from the second one (*Xgwm325)* (Fig. 2). The larger part of these alleles has a frequency under 10 %, respectively the probability of their combinations is below 1 %. It is also likely that the probability of some combinations may deviate from the theoretically expected due to the formation of the co-adapted associations

of alleles of genes or of alleles of loci. The possibility of that arises from the genetic proximity of varieties originated from one breeding center, caused by joint descent from a number of precursor varieties, and also by systematic selection in the same natural conditions, using identical plant breeding criteria. Although the microsatellite loci could not display selectivity, though the data on regional differences [9–12] and the association with a number of breeding traits [13–17] suggest otherwise. This may be due to «canalization» — the selective fixation of only certain combinations of alleles and eliminating of «undesirable» combinations from some eco-niches.

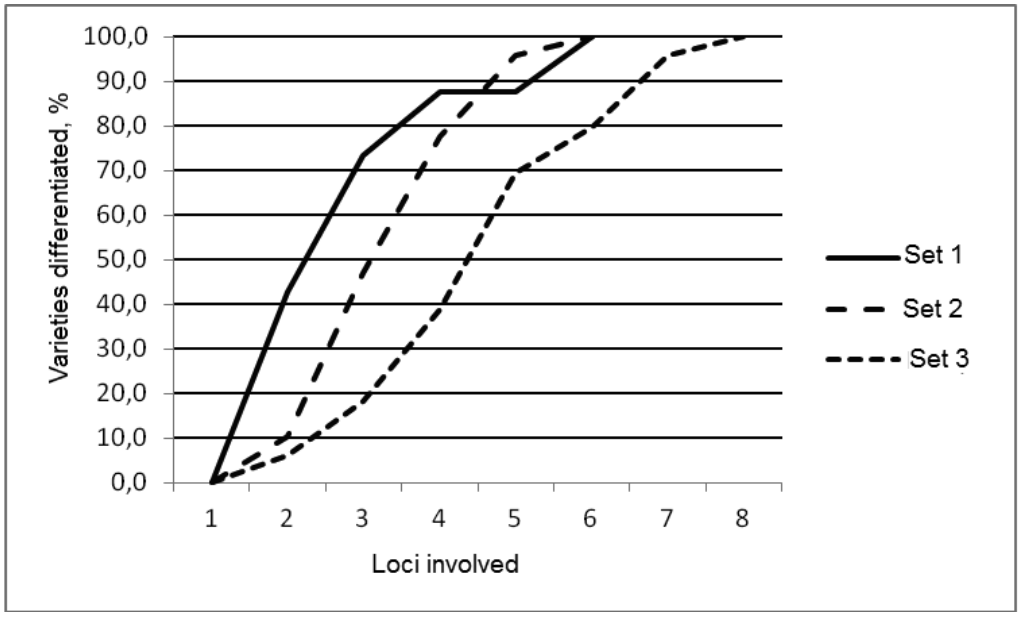


Fig.1. The rise of the separation ability of the marker system at step by step including of markers

Alleles of Xgwm155	*Alleles of Xgwm325*									
	115	120	128	134	138	142	144	146	148	150
129						1				
135							1			
137							1			
139							3	1	1	
141							3		1	
143							2	2		
145	1		3					4	1	1
147	1		2		1		2	1		
149	3	2		1	1		2	3		1
152	1						1	1		

Fig. 2. The distribution of allele combinations studied in 49 lines by two marker loci (*Xgwm155* and *Xgwm325*) in numerical and graphical modes

At first glance, the obtained results (the possibility of reducing the number of markers to 6–8 SSRs) are in good agreement with the recommendation

of UPOV about using 8 microsatellite markers [18]. However, please note that our results were obtained on the lines of the 49 varieties of close breeding origin while the regulations of UPOV should apply to much wider range in both quantitative and qualitative meanings. In the light of the above considerations it appears that the optimal number of markers during the official registration of new varieties should be slightly higher.

Selection of reference varieties. For practical reasons it is handy to have a compact work collection that is «localized», i.e. is consisted of varieties adapted to the certain region and contains in all the necessary alleles required for unique identification. This task was one of the goals of present investigation and as a result practically acceptable solutions were found. It should be noted that the outcome result depended on the action sequence. Out of the total 49 lines two sets were picked out, each containing all 110 alleles that were found in the investigated 17 loci. The first (minimal) set consists of 26 lines, and the second (35 lines) differs from the first one by relatively higher proportion of rare alleles. Formed in the present study the sets will later become the basis of the specialized collections of reference lines. Collections will be implemented into practice of variety investigation, and the composition of them will be improved.

The tools for the results analysis. For set of monomorphic varieties (lines) the key indicators which characterize the degree of difference/similarity between them are the total number of the same / different alleles, and also by which loci pairs of varieties are similar or different. Such information was the foundation at optimizing of both marker sets and at selection of the potential reference varieties. Table 3 shows a fragment of the similarity matrix of the examined lines which is denoted by the number of loci (out of the total their number, 17) which carry the same alleles. (The full matrix even for a such relatively small set of objects is too cumbersome to bring in the article). Each element of the matrix contains the result of generalization of 17 pairs of comparisons. In general, to fill out the entire table it was needed to perform about 40,000 operations of comparison (as matrix is symmetric — 20,000) and summarize them. With the growth of the quantity of analyzed varieties the scope of calculations increases with the rapid pace proportionally to the square of the samples number. To avoid the unproductive «manual» work dedicated tool for Excel environment was designed. It runs the cycle of calculations for each of the varieties automatically, only leaving the formation of the final table to be made «manually». The algorithm of the entire work could be used to develop fully automatic applications.

The tables of similarity (differences) can be subjected to further analysis without even additional preparation. It was verified by conducting the cluster analysis by software package Statistica, ver. 6.0 (Fig. 3).

Another important task in the context of this paper is the identification of the unknown samples by the results of the analysis of a certain set of markers. The general principle is simple — to find the appropriate row in the previously

Table 3

The fragment of the similarity matrix — the number of loci with identical alleles for 17 studied MS markers

Varieties	Hospodynia	Skarbnytsia	Kosovytsia	Antonivka	Zamozhnist'	Blahodarka odes'ka	Misiia odes'ka	Dal'nyts'ka	Yednist'	Kiriia	Liona	Kuial'nyk
Hospodynia	**17**	8	7	5	6	5	7	5	5	9	5	12
Skarbnytsia	8	**17**	7	7	7	7	7	6	4	6	7	9
Kosovytsia	7	7	**17**	8	7	6	7	9	4	6	10	8
Antonivka	5	7	8	**17**	7	6	6	6	5	4	5	6
Zamozhnist'	6	7	7	7	**17**	8	6	7	6	7	8	6
Blahodarka odes'ka	5	7	6	6	8	**17**	7	6	4	6	9	7
Misiia odes'ka	7	7	7	6	6	7	**17**	5	6	7	7	8
Dal'nyts'ka	5	6	9	6	7	6	5	**17**	9	8	7	6
Yednist'	5	4	4	5	6	4	6	9	**17**	4	6	5
Kiriia	9	6	6	4	7	6	7	8	4	**17**	7	9
Liona	5	7	10	5	8	9	7	7	6	7	**17**	7
Kuial'nyk	12	9	8	6	6	7	8	6	5	9	7	**17**

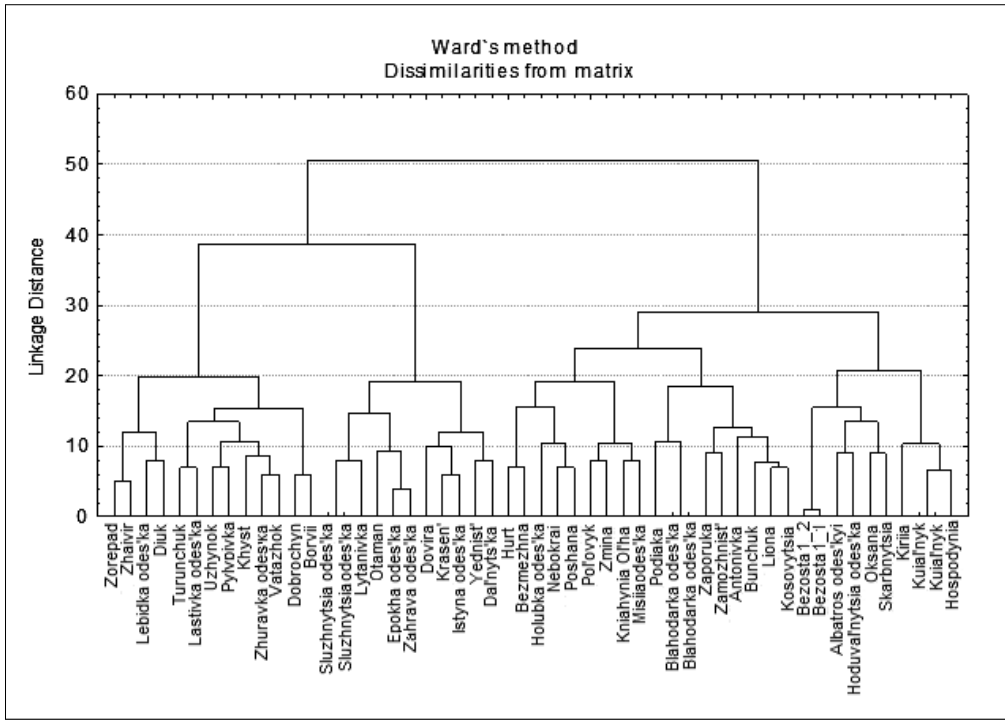


Fig. 3. Cluster analysis of the degree of difference between varieties by 17 MS markers

compiled «reference» table. However, at number of samples as well as markers, it is better to apply here computer technologies. Screenshot of template, which we prepared in Excel, is shown in Figure 4. In present case it is set up to work with 6 markers from set 1. Two different algorithms were prepared for this task. The first one (Dx) requires for identification the complete identity of the allelic composition of the given sample to the certain row of the reference table, and the second (Dbp), which was designed specifically for the analysis of MS markers, permits deviations no more than 1 pair of nucleotides in determining of the size of amplification products no more than in three or four of the six loci. In both cases, the result «0» in the corresponding row of the reference table means the complete coincidence. The procedure returns the catalog number and variety name in a special row of results. Any quantity of varieties which are the closest ones to the given variety under both criteria can be identified after sorting and then the results can be transferred to a separate table for further examination.

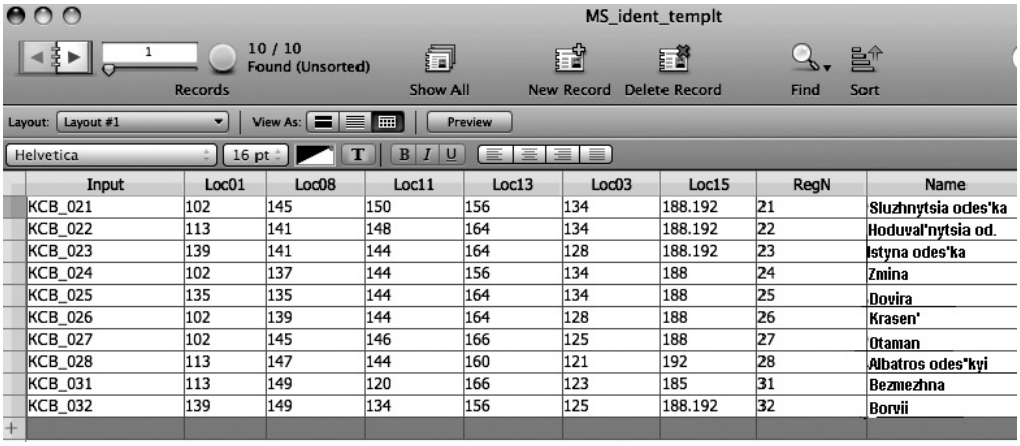
		Xgwm186	Xgwm3	Xgwm325	Barc126	Xgwm357	Xgwm408		**0,27**
		Loc01	Loc08	Loc11	Loc13	Loc03	Loc15		
40	Zhaivir	107	147	146	138	125	188		
RegN	Name	Loc01	Loc08	Loc11	Loc13	Loc03	Loc15	Dbp	Dx
40	Zhaivir	107	147	146	138	125	188	**0,0**	**0**
44	Zorepad	102	145	146	138	125	188	**0,3**	2
4	Antonivka	102	147	144	156	128	188	3,6	4
41	Zhuravka odes'ka	102	152	146	156	125	188	3,7	3
61	Uzhynok	102	145	128	142	128	188,192	3,8	6
56	Pol'ovyk	102	149	144	156	119	188,192	3,9	6
21	Sluzhnytsia od.	102	145	150	156	134	188,192	4,5	6
6	Blahodarka od.	125	143	144	146	116	188,192	4,9	6
24	Zmina	102	137	144	156	134	188	5,3	5
28	Albatros odes'kyi	113	147	144	160	121	192	5,6	5
38	Dobrochyn	102	147	128	156	125	188,192	6,7	4
33	Vatazhok	102	145	128	156	125	188,192	6,8	5
8	Dal'nyts'ka	107	145	148	164	125	188	6,8	3
15	Bunchuk	125	139	144	156	125	188	7,2	4

Fig. 4. The result of identification of tested sample (fully corresponds to variety Zhaivir) in the environment of Excel 2010

For work not with individual samples, but with their sets the similar tool is easy to build in the database management system (DBMS). The results of the identification of number of samples carried out by means of DBMS FileMaker are shown in Figure 5. Here was also implemented the algorithm of identification of samples by the index of Dbp, which was previously tested and tuned up in Excel.

As noted above, the tools were developed so that they could work not only with MS, but also with other molecular, biochemical and even morphological markers. Depending on the situation, it may only be needed some adaptation

of input data through their conversion to an acceptable form. This feature has been successfully tested by us on the example of the published data of the results of electrophoresis of grain store proteins gliadin and glutenin [19, 20], i. e protein markers *Gli* and *Glu*, which at one time were implemented in practice of official identification of USSR varieties [21], and were recommended later by UPOV [22]. It should be noted that these protein markers show disadvantage compared to MS markers by the resolution ability. For example, in research [20] of 75 variety lines 16 individual lines were not clearly identified — despite the fact that the analysis was based on all 10 of the available protein loci. It was impossible to distinguish variety Viktoriia odes'ka from variety Pysanka, each of which consists of 4 lines. The mentioned above is resulted by the fact that the composition of the gluten-forming proteins is under to strong selection pressure during intense screening by the technological quality of grain. That is why, in spite of the existing rich genetic polymorphism in wheat only a limited number of alleles and their combinations are implemented in varieties of PBGI. Thus, from Table 1, shown by the same authors, it could be defined that within the material studied by them 3.7 alleles were fallen on 1 locus on the average, and the mean value of PIC index amounted 0.480. According to these indices *Gli* and *Glu* markers are less powerful comparing even to the least informative MS markers selected from our set 3 (Table 2).



Input	Loc01	Loc08	Loc11	Loc13	Loc03	Loc15	RegN	Name
KCB_021	102	145	150	156	134	188.192	21	Sluzhnytsia odes'ka
KCB_022	113	141	148	164	134	188.192	22	Hoduval'nytsia od.
KCB_023	139	141	144	164	128	188.192	23	Istyna odes'ka
KCB_024	102	137	144	156	134	188	24	Zmina
KCB_025	135	135	144	164	134	188	25	Dovira
KCB_026	102	139	144	164	128	188	26	Krasen'
KCB_027	102	145	146	166	125	188	27	Otaman
KCB_028	113	147	144	160	121	192	28	Albatros odes'kyi
KCB_031	113	149	120	166	123	185	31	Bezmezhna
KCB_032	139	149	134	156	125	188.192	32	Borvii

Fig. 5. The result of identification of 10 tested samples in DBMS of FileMaker 12.0

The approaches we developed on MS markers are easy to adapt to the new markers, if any would be offered. Created within our investigation tools or their algorithms could be integrated to the general computerized systems of the traffic control of seeds in the breeding, seed industry and other institutions.

Conclusions

1. The approaches to optimize the number and composition of molecular markers for variety investigation needs, control of breeding and seed nurseries were developed. There were formed out of 17 PCR markers three mini-

mized sets, which are close by their differential ability, each of which assesses 6–8 loci and is able to distinguish varieties related by the origin of the same breeding centre. The system recommended by UPOV concerning 8 MS markers for wheat may be insufficient in certain situations in spite of hundreds of thousands combinations of alleles theoretically possible for that quantity of mapped loci. A lot of mentioned combinations actually fall out because of a very low probability of their realization and this condition significantly limits the number of varieties that could be uniquely identified.

2. The empirical algorithms of creation of the compact collections of reference lines were developed; and two sets of candidate varieties were created, the minimal and the extended ones consisting of 26 and 35 varieties, respectively, each of which includes all 110 alleles from 17 studied loci.

3. The algorithms and tools for semi-automatic computerized analysis of variety investigation data were created: a) for constructing of a matrix of similarity/difference; b) for individual/group identification of the unknown samples; tools were tested in environments of spreadsheets (Excel) and database management systems (FileMaker).

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ШЛЯХИ ФОРМУВАННЯ СИСТЕМ СОРТОВИВЧЕННЯ ТА КОНТРОЛЮ РОЗСАДНИКІВ ЗА МОЛЕКУЛЯРНИМИ МАРКЕРАМИ

На модельному матеріалі досліджено шляхи конструювання оптимальних систем ідентифікації сортів за молекулярними маркерами для потреб селекції та сортовивчення. Ключовими елементами таких систем є добір мінімально необхідної кількості маркерів, створення «локалізованої» колекції сортів-еталонів, розробка інструментів аналізу даних з можливістю їхнього розширення. Приділяється увага підвищенню роздільної здатності цих систем. Критично аналізуються рекомендації UPOV.

Таблиця — 3. Рисунки — 5. Бібліографія — 22.

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ПУТИ ФОРМИРОВАНИЯ СИСТЕМ СОРТОИЗУЧЕНИЯ И КОНТРОЛЯ РАССАДНИКОВ ПО МОЛЕКУЛЯРНЫМ МАРКЕРАМ

Колесник О. А., Чеботарь С. В., Сиволап Ю. М., Цевма В. Н., Хохлов А. Н., Литвиненко Н. А. Сборник научных трудов СГІ–НЦНС. 2013. Вып. 22 (62).

На модельном материале исследованы пути конструирования оптимальных систем идентификации сортов по молекулярным маркерам для задач селекции и сортоизучения. Ключевыми элементами таких систем являются подбор минимально необходимого количества маркеров, создание «локализованной» коллекции сортов-эталонов, разработка инструментов анализа данных, возможность их расширения. Уделяется внимание повышению разрешающей способности этих систем. Критически анализируются рекомендации UPOV.

Таблицы — 3. Рисунки — 5. Библиография — 22.