SEGREGATION ANALYSIS AND COMPARISON OF HOP (*HUMULUS LUPULUS* L.) MICROSATELLITE MARKERS VARIABILITY

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Wild hop (*Humulus lupulus* L., *Cannabaceae*) is a dioecious perennial plant, native to the northern hemisphere and its cultural forms are mainly used in brewery. Populations of wild hop are a potential source of new features and therefore valuable for breeders. Further studies of hop populations are informative about the hop evolution as a species. Tested was the mode of inheritance, linkage and possibility of null allele presence at 8 microsatellite loci in 118 plants of one full-sib family and their genetic variability was compared with genetic diversity of 57 plants supposed to be natural population in the locality „Vinařická hora“. We found that one locus does not fit to Mendelian segregation ratio, two are in linkage and in two loci the occurence of null allele is possible. It was also found that 8 genotypes of hybrid progeny do not have expected combination of parental alleles. Heterozygote excess, low number of individual genotypes, small sampling area and population structure assigned highly similar pattern, as a structure of hybrid progeny are indicators that population „Vinařická hora“ has origin in two or only few sexually propagating plants.

Key words: hop, microsatellites, segregation analysis, null allele

INTRODUCTION

Hop (*Humulus lupulus* L.) is a dioecious, perennial climbing species which belongs to *Cannabaceae* family. Wild hops are important source for improvement of present cultivars and they could be helpful for changing of chemical compounds according to requirements of hop growers and processers or be donors of resistance against diseases. Only few studies have compared wild hop and cultivated hop in the sense of relatedness, variability and differences between them (Jakše et al. 2004; Štajner et al. 2008; Murakami et al. 2006). Screening of natural populations is promising way to get demanded genotypes for next breeding process and it is also occasion to study dynamics of populations, their propagation, evolution, phylogeny and structure.

Microsatellites (or SSRs – Simple Sequence Repeats) are usually 1–6 bp long tandem repetitions, dispersed throughout all genome of eukaryotic and prokaryotic organisms. Researchers prefer microsatellites because of their advantages – they are codominant, abundant, hypervariable, specific and highly reproducible. Unfortunately, they have also some disadvantages for proper analysis, which can lead in principle, to enormous genotyping errors. It is silently supposed and only sometimes tested, that microsatellites meet this five basic requirements: no size homo-

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plasy, absence of null alleles, selective neutrality, disomic segregation and an independent assortment according to Mendel´s laws. In our study, we focused on testing of segregation ratios, linkage between loci and occurrence of null allele.

Dakin and Avise (2004) define null allele as any allele that repetitively fail in amplification by PCR. This phenomenon can lead to detection of lower number of alleles, mistyping a heterozygote as a homozygote and underestimation of genetic variability. It can also cause scoring error in a degree of relatedness between tested individuals, omitting true parent in parentage analysis and other genotyping errors.

Our research had two main objectives. The first goal was an optimization and testing of usefulness of chosen microsatellite markers for another analysis and the second was the comparision of variability within one full-sib family of Vital cultivar with hybrid 00/15 and make „pre-test" of supposed natural population of wild hop.

MATERIAL AND METHODS

Plant material

The first tested group was one full-sib family of breeding program realized at the Hop Research Institute Co., Ltd. in Žatec. We evaluated 118 hybrids and their parents. Mother´s component was the new Czech cultivar Vital and father´s component was the hybrid signed 00/15. Progeny has three-row ID number according to evidence of Hop Research Institute Co., Ltd. and we tested numbers 111−129, 131−139, 141−161, 163−193, 208, 210−225 and 228−248, respectively.

We collected the second group, containing only supposed wild hops, by sampling in locality Vinařická hora, in land register of village Třebichovice in the Central Bohemia region. Vinařická hora (50° 11´ northern latitude, 14° 04´ eastern longitude, 358 m above sea level) locality is situated on the shoulder of the tertiary strato-volcano. Population was sampled on 60 m^2 area. Plants were the part of ecological site together with trees and bushes of genera *Prunus, Acer, Ligustrum* and *Crataegus*. Only plants with marks of one year seedlings were sampled. The closest localities with commercially growed hops were situated 12 km (Zlonice), 16 km (Třtice) and 20 km (Veltrusy) from Vinařická hora, respectively. We do not have any information from historical documents whether hop was grown in this locality, so we can suppose that those plants are not so called „run-wild" hop. There were 57 plants (signed VH 1-38, 40-58) completely tested.

Isolation, amplification and analysis of DNA

DNA was extracted from young leaves by DNease Plant Mini Kit (Qiagen) according to manufactor´s recommendation with some modifications. At the beginning of isolation, 100 mg of polyvinylpyrrolidon (PVP) was added. It was also necessary to reisolate some genotypes because of the high content of inhibition substances and to modify the isolation procedure according to Landergott et al. (2006). Variability and segregation ratio was tested at eight microsatellite loci (HlGA3, HlGA4, HlGA9, HlGA29, HlGT5, HlGT9, HlGT10 and HlGT12) with primers published by Jakše et al. (2002). PCR reaction mixture of total volume 12.5 µl contained approximately 20 ng of DNA and 0.5 unit of *Taq* polymerase (Roche). The concentration of other components was following: 1× KCl PCR buffer [10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3] or 1× $(NH_4)_2SO_4$ buffer [75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 0.2 mM dNTP and 0.4 µM each of appropriate primer, 0.4 ug/µl BSA, 2 mM PCR Enhancer [TMA oxalate] (Top-Bio). Final concentration of $MgCl_2$ is showed in Table 1.

We started the optimization procedure with cycling protocol and concentration of $MgCl_2$ according to Jakše et al. (2002). When we were not satisfied with obtained results then we tried to adjust annealing temperature, cycling protocol, concentration of $MgCl_2$ and total number of cycles to gain the best result. Amplifications of SSR markers were carried out in the thermocycler C1000 (Bio-Rad) and DNAengine (Bio-Rad) either with the initiation touchdown protocol (TD-PCR) or only with a standard PCR protocol in the following steps: pre-denaturation at 95°C for 4 min (for both types of PCR), for HlGA3 and HlGA9 primers followed by appropriate number of cycles (N1) at 94°C for 45 sec., then annealing according to initiation annealing temperature (init. Ta) for 30 sec. and elongation at 72°C for 90 sec. then followed (for HlGA3 and HlGA9) or started (for another primers with the exception of HlGA3 and HlGA9) with appropriate number of cycles (N2) of denaturation at 94°C for 45 sec., annealing according to annealing temperature for 30 sec. and elongation at 72°C for 90 sec. The final elongation

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Cycling protocols and concentrations of $MgCl_2$

Locus	TD-PCR			PCR		Buffer	$MgCl_2$ [mM]
	Init. Ta [°C]	Decrement per cycle [°C]	N1	Ta [°C]	N2		
HlGA3	64	1	3	61	25	$(NH_4)_2SO_4$	2.5
HlGA4	–	–	–	56	26	KCl	2
HlGA9	63	0.5	12	57	15	KCl	3
HlGA29				57	26	KCl	1.5
HlGT5				60	29	KCl	1.5
HlGT9	–			55	32	KCl	1.5
HlGT10				57	26	KCl	1.5
HlGT12				56	26	KCl	1.5

Init. Ta – initiation annealing temperature, N1 and N2 – number of cycles, Ta – annealing temperature

was performed at 72°C for 8 min.

The separation of amplified products was performed on vertical electrophoretic cell Sequi-Gene II (Bio-Rad) in 6% *w/v* denaturating (with 8M urea) polyacrylamide gel in 0.5x TBE buffer. Samples were mixed with denaturant solution before separation according to Benbouza et al. (2006) in 1:1 ratio denaturated at 94°C for 5 min., then were put on ice at least for 30 minutes. We used the silver staining procedure according to Benbouza et al. (2006) and when the gel became dry, we documented and digitalized result by scanner.

Identification of sex

Molecular identification of sex as the part of variability testing was performed in all plants. We used a molecular marker by Polley et al. (1997) and we started the protocol according to Patzak et al. (2002), but it was necessary to make some modifications. PCR reaction of total volume 12.5 µl contained 20 ng of DNA and 0.5 unit of *Taq* polymerase (Roche). The concentration of other parts was as follows: 1× KCl PCR buffer [10 mM Tris-HCl, 1,5 mM $MgCl_2$, 50 mM KCl, pH 8.3], 0.2 mM dNTP, 0.2 µM each of pair primers, 0.4 ug/µl BSA and 2 mM PCR Enhancer [TMA oxalate] (Top-Bio). Amplification was performed in the thermocycler C1000 (Bio-Rad). The amplification profile was created by initial pre-denaturation at 94°C for 3 min. followed by 35× cycles of denaturation at 94°C for 30 sec., annealing at 57°C for 60 sec. and elongation at 72°C for 90 sec. with final elongation at 72°C for 10 min.

Statistical analysis

Analysis of independent segregation was performed by the program STATISTICA 8 CZ for Windows via χ^2-test. Linkage analysis was performed by LINKFMEX 2.3 program (developed by R. G. Danzmann). LOD (logarithm of odds) scores and estimation of recombination rates were computed according to sex for each possible pair of loci. LOD score 3.0 was considered as significant (Botstein et al. 1980) for linkage between loci. Description statistic (values of H_E, H_O, PIC) and HWE equilibrium were tested by the Cervus 3.0 (Kalinowski et al. 2007). Program Identity 4.0 (Wagner and Sefc 1999) was used for determination of the number of individual genotypes. Estimation of the effective number of breeders (Nb) for population of locality Vinařická hora was computed by program Nb_HetEx (Zhdanova and Pudovkin 2008) with 1000 bootstrap iterations.

RESULTS

Testing of full-sib progeny with known parents was important for some reasons. We could test the mode of inheritance and segregation of microsatellites

according to Mendel´s laws, possibility of null alele and compare the variability of „population“ created only by two plants with variability of supposed natural population with unknown numbers of reproducing plants.

Segregation analysis

118 hybrids originated from controlled pollination of cultivar Vital and male genotype 00/15 were analysed. We found that eight genotypes (111, 116, 141, 142, 144, 182, 188 and 241) did not have expected allelic combination. This result was confirmed for each of eight genotypes, at least at two loci, except genotype 116 (details in Table 1). In six genotypes, it must have happened to the cross-pollination with another(s) male genotype(s) and both parents of genotype 241 must be different. Genotype 116 was a unique one because it has 3 alleles (two father´s and one mother´s allele) instead of 2 alleles. All of these eight unusual genotypes were excluded from further analyses.

We could check segregation ratio according to Mendelian inheritance and linkage only for 4 of 8 microsatellites because both parents were in loci HlGA3, HlGT9, HlGT10 and HlGT12 homozygotes with the same allele. Table 2 shows result of χ^2 test and further information for every locus. An allelic combination of parents is visible in each row, expected ratio and possible allelic combination of their progeny with their observed numbers in the brackets. As can be seen, only loci HlGA29 and HlGT5 meet requirements for expected segregation with high probability (P>0.05). Locus HlGA9 is nonsignificantly excessed (P>0.55) from independent segregation and locus HlGA4 has significant (P<0.01) segregation distortion.

Null alleles

When loci HlGA29 and HlGT5 were analysed, it came as a surprise that we found high number of unexpected genotypes (in the Table 2 with alleles *b0* and *a0*). Majority of them is different from the eight genotypes mentioned above, with father´s allele, but without any mother´s allele. Afterwards, we performed the statistical analysis of segregation ratio by χ^2 test with expected null allele and this test confirmed our hypothesis about nonsignificant difference of segregation (P>0.05) for both loci.

Linkage analysis

As a part of independent inheritance testing, we used program LINKMFEX ver. 2.3 for testing of linkage between the segregating loci. We tested every possible loci of both parents. Our analysis was more difficult because of mother´s null alleles in the loci HlGA29 and HlGT5. For 56 genotypes, we were not able to distinguish their true allelic combination in both loci, namely to distinguish genotype *aa* from *a0* in locus HlGA29 and genotype *bb* from *b0* in locus HlGT5. We only knew that half of 56 genotypes should have alleles *a0* and half *aa* alleles and the same is valid for locus HlGT5, respectively, only letters are different. That is the reason why we created two testing models to prove possible linkage, called „min. LOD

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Allelic combination of parents and their progeny, expected segregation ratio, results of χ^2 test and genotypes with unusual allelic combination

Locus	Female (4715)	Male (00/15)	Expected ratio	P	Allelic constitution (No. of progeny)				ID number of progeny with unexpected allelic combination							
HlGA3	*aa*	*aa*	none	–	*aa* (115)				111	–	–	–	–	–	188	241
HlGA4	*bd*	*ac*	1:1:1:1	0.006	*ab* (23)	*Ad* (16)	*bc* (30)	*cd* (41)	111	116	141	142	144	182		241
HlGA9	*ab*	*aa*	1:1	0.445	*ab* (51)		*aa* (59)		111	–	–	142	144	182	–	241
HlGA29	*a0*	*ab*	1:1:1:1	0.954	*aa* and *a0* (56)		*ab* (25)	*b0* (29)	–	–	–	–	–	182	–	–
HlGT5	*b0*	*ab*	1:1:1:1	0.954	*bb* and b0 (56)		*ab* (25)	*a0* (29)	–	–	–	–	–	–	–	–
HlGT9	*aa*	*aa*	none	–	*aa* (118)				–	–	–	–	–	–	–	–
HlGT10	*aa*	*aa*	none	–	*aa* (113)				111	–	141	142	144	–	188	–
HlGT12	*aa*	*aa*	none	–	*aa* (118)				–	–	–	–	–	–	–	–

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Values of LOD score for both „min. and max. LOD score“ models

Loci	min. LOD score		max. LOD score	
	♀	♂	♀	♂
HlGA4 vs HlGA9	0.007	–	0.007	–
HlGA4 vs HlGA29	0.197	0.507	0.387	0.507
HlGA4 vs HlGT5	0.387	0.507	0.387	0.507
HlGA9 vs HlGA29	0.387	–	0.961	–
HlGA9 vs HlGT5	0.961	–	0.961	–
HlGA29 vs HlGT5	0.007	**33.113**	**33.113**	**33.113**

score“ and „max. LOD score“. In „min. LOD score“ model are the first 28 genotypes homozygous in allelic combination *aa* in locus HlGA29 and heterozygous in allelic combination *b0* in locus HlGT5. Reversely for the next 28 genotypes, e.g. in locus HlGA29 they are heterozygotes *a0* and homozygotes *bb* in locus HlGT5, which has direct influence on LOD score, mainly from mother´s point of view because the supposed mother´s null alleles in both loci will never meet. In „max. LOD score“ model the first 28 genotypes are homozygous in both loci (*aa* and *bb*) and the further 28 genotypes are heterozygous (*a0* and *b0*). All LOD values are summarized in the Table 3.

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Descriptive statistics of population Vinařická hora and hybrid progeny

Locus	No. of alleles		H_O		H_E	
	VH	Crossing	VH	Crossing	VH	Crossing
HlGA3	2	1	0.632	0.000	0.436	0.000
HlGA4	4	4	0.980	1.000	0.628	0.743
HlGA9	4	2	0.526	0.464	0.424	0.358
HlGA29	4	3	0.351	0.736	0.309	0.628
HlGT5	4	3	0.800	0.736	0.579	0.628
HlGT9	3	1	0.632	0.000	0.597	0.000
HlGT10	2	1	0.175	0.000	0.161	0.000
HlGT12	1	1	0.000	0.000	0.000	0.000
$\bar{x}$	3	2	0.512	0.367	0.391	0.294

VH – population Vinařická hora,
H_O – Heterozygosity observed,
H_E – Heterozygosity expected

Thanks to our models „min. and max. LOD score“ we confirmed with certainty a very tight linkage between loci HlGA29 and HlGT5 in father´s component. As we expected, model „min. and max. LOD score“ has straight influence on the mother´s component only. We have an evidence that if no homozygote *aa* in locus HlGA29 meets with no homozygote *bb* in locus HlGT5 and the same is valid for heterozygotes *a0* in locus HlGA29 and *b0* in locus HlGT5, then the linkage between loci does not exist (LOD = 0.007), of course from female point of view. On the other hand, if every homozygote in both loci and every heterozygote in both loci meets, the linkage between loci is very tight also for mother´s component. Then we also tested the lowest number of genotypes for linkage between loci HlGA29 and HlGT5 of mother´s parent. At the present time, we can only state that for minimal linkage (LOD = 3.232) it is necessary that 22 genotypes of 56 must meet in „right“ allelic combination.

Analysis of variability

Since we also have a natural population of wild hop, we could compare the variability between the population of intended crossing and the supposed natural population. Descriptive statistics are in Table 4 and Table 5 together with test of Hardy-Weinberg equilibrium (HWE) and the estimation of null alleles presence.

The variability of population Vinařická hora is slightly higher in comparision with full-sib progeny, mainly because of higher number of alleles in loci HlGA3, 9, HlGT9 and 10. This result is not so surprising because we know that we have only two parents of the hybrid progeny. The comparision of H_O

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Descriptive statistics, HWE and Null allele tests of population Vinařická hora and hybrid progeny

Locus	PIC		HWE		Null allele	
	VH	Crossing	VH	Crossing	VH	Crossing
HlGA3	0.339	0.000	*	ND	–0.187	ND
HlGA4	0.544	0.691	***	***	–0.239	–0.153
HlGA9	0.379	0.293	ND	*	–0.139	–0.130
HlGA29	0.287	0.555	ND	***	–0.088	–0.066
HlGT5	0.524	0.555	***	***	–0.210	–0.066
HlGT9	0.515	0.000	**NS**	ND	–0.024	ND
HlGT10	0.147	0.000	ND	ND	–0.038	ND
HlGT12	0.000	0.000	ND	ND	ND	ND
$\bar{x}$	0.341	0.261	–	–	–	–

VH – population Vinařická hora, PIC – Polymorphism Information Content, HWE – Hardy-Weinberg equilibrium: NS – not significant, * P<0.05, *** P<0.001, ND – not done, Null allele – estimation of null allele frequency

and H_E values is more interesting. In each locus, values of H_O are always higher than H_E. This result is unusual for the natural population and consequently this un-usual result was confirmed by HWE test because only locus HlGT9 did not show significant excess from the HW equilibrium. We suppose that deviations from HWE at many loci are the indicators of population substructure.

DISCUSSION

Characterization of microsatellites markers

We tested 177 plants by 8 microsatellite markers and we found 28 alleles with an average 3.5 allele per locus. Jakše et al. (2002) analysed these primers in collection of 39 wild and cultivated hops and they found from 3 up to 12 alleles per locus with average 6.6 allele per locus. Another example of high polymophism can be seen in a study of Štajner et al. (2005). They tested 67 plants from all around the world on 25 microsatelite loci and from 5 to 31 alleles were detected for appropriate locus with average 10.6 allele per locus. These results show high degree of polymorphism of hop SSR markers. Our values are much lower but we should realize that tested plants originated in one crossing and in the population which is growing on a small locality and thus variability must be necessarily limited.

During testing of these eight microsatellites we found almost all possible negative appearances. One locus did not segregate according to Mendelian laws, among other pairs of loci is probably very tight linkage, at least for father component, and in the same loci null alleles were detected with high probability.

Segregation analysis

We detected 8 unusual genotypes mentioned in upper part of this article. Genotypes 111, 141, 142, 144, 182 and 188 are probably cross-pollinated plants with another male parent. The Table 2 shows in detail the loci in which they are different against expected allelic combination of parents´ genotypes. It is interesting that genotype 188 was distinguished only with the help of the lowest variable loci. Genotype 241 has in locus HlGA3 combination of alleles different from both parents. We suppose that this mistake could have happened during sampling. Last genotype 116 has 1 mother´s and 2 father´s alleles in locus HlGA4. This unusual pattern can indicate event of unequal crossing-over nearby of this locus and it is well known that an unequal crossing-over often occurs in regions containing tandem repetitions (Kashi and King 2006). Another explanation is possible modified meiosis affecting certain stages of microsporogenesis. This meotic distortion leads to the so called diplogametes (2n gametes). Their occurence is reported for many species, for example potatoes (Carputo et al. 2003), but as far as we know there does not exist any study about diplogametes in hop, so this explanation is only speculative. Our findings also demonstrated an applicability of SSR analysis in hop DNA profiling with the same fidelity at least like any usually used methods.

Only 4 of 8 loci were segregating. The two of these 4 loci, HlGA29 and HlGT5 segregated in agreement with Mendelian inheritance (P>0.05), but only with presumable presence of null allele in mother´s genotype (see below). Locus HlGA9 was nonsignificantly different from expected segregation ratio with lower probability (P>0.55), but in locus HlGA4 segregation distortion with highly significant probability (P<0.01) was two times verified. We found that father´s allele „*c*“ was 1.8 times more represented among offsprings than second father´s allele „*a*“. This result is hard to explain without further analysis. Segregation distor-

tion is usually explained by sex-linked loci, selection or loci occuring in organeles instead of nuclear loci (Selkoe and Toonen 2006). We excluded the possibility of sex-linked locus because the preference for male or female sex was nonsignificant. There might play role a link to an important gene(s) influencing for example the vitality of gametes and consequent selection sweep, but we think that this fact would have a direct ratio effect on the remaining loci. Therefore, the possible explanation is unclear.

Linkage analysis

Linkage between loci could be very useful for some research areas and is applied in mapping studies (Seefelder et al. 2000) or QTL analysis (Čerenak et al. 2006). Unfortunately, our analysis is incomplete because of non-segregating loci. Tight linkage with 0% recombination between loci HlGA29 and HlGT5, at least for male parent, was proven. Irrespective of any possibilities with linkage between loci HlGA29 and HlGT5 for female parent, these loci are suitable for eventual mapping analysis with parents without null allele. In another segregated loci value of LOD score did not reach threshold 3.0 and they could be probably considered as unlinked.

When we take in account our next purpose, i.e. the application of these microsatellite loci also in population genetic studies, that could be a serious problem with misleading statements of these loci. Significant linkage could disrupt assumptions of independent segregation and create correlation between the informativeness provided by these loci (Ardren et al. 1999). The majority of population genetic parameters and models suppose, besides another requirements, unlinked loci. They are important for unbias estimation of effective population size or migration rate (Beerli and Felsenstein 2001), population structure, assigning individuals to population (Pritchard et al. 2000) and gene flow (Hey and Nielsen 2004). So we recommend using only one of these paired loci in population studies.

Null alleles

Null alleles are very common for microsatellites loci, especially when they are developed for one species and tested in another close relative as reported Štajner et al. (2005) where various hop (*Humulus lupulus* L.) plants were tested with *Humulus japonicus* and hemp (*Cannabis sativa* L.) plants. Null alleles can be found on intraspecific level in both plant and animal species. Dakin and Avise (2004) summarize estimated frequencies of null alleles in 233 articles and almost all articles denoted $P<0.4$ and the most usually $P<0.2$. We detected null allele in 2 out of 8 microsatellite loci i.e. in 25% examined loci, but we will not compare this measure of frequency with another studies because we found null allele only in one intended crossing and not in tested population and therefore could be this measure misleading.

Our result for locus HlGA29 is supported by the study of Murakami et al. (2006) because of the same result for hops from american and japanese areas, but without any report in european hops. It is not possible to explain genotypes only with father´s allele by cross-pollination with another female parent, but moreover the number of such plants would be, in hypothetic cross-pollination, very high (29) plus five remaining excluded genotypes comprise together 34. That is 34/118 and this result gives 28.81% possible cross-pollinated plants, althought standard work experience is up to 10% which correspond to our detected unusual genotypes (6.7%).

Null alleles are one of the most common genotyping errors and loci with too high frequencies should be discarted before another analysis (Selkoe and Toonen 2006). We think that the application of locus HlGA29 or HlGT5 could not be a problem if a researcher took into account possible null allele and corrected eventual results approprietly. Our own observation in other tested hops suggests, that frequency of null allele in both loci, at least in european hops, is not too high, but this hypothesis must be supported by additional analysis.

Variability analysis

Our „pre-test“ and comparison of variability showed that the population Vinařická hora is more variable in numbers of alleles in comparison with the hybrid progeny, but it has much lower number of individual genotypes (17 vs. 40). This fact is more highlighted when we take in account that in the full-sib family only 4 loci against 7 polymorphic loci in Vinařická hora population are segregating. Because of unusual finding of „heterozygote excess”, we suggested the hypothesis that population Vinařická hora is subdivided into some closely related families and originates from a few parental plants. Our assumption

is undirectly confirmed by some findings.

We collected only plants with features of one year seedlings. The molecular marker for determining of hop male plants showed that there are at least 11 males. This observed result proved (at least molecularly) a sexually expanding population. We tried to determine the number of the effective breeders by program Nb_HetEx. The number is 2.7 for median bootstrap confidence interval and 3.3 for bootstrap confidence interval 95%, both for 1000 iterations.

Another proof supporting our hypothesis is given by similar pattern for H_O and H_E values and HWE test in the hybrid progeny created by crossing of two plants and relatively small (60 m^2) sampling area.

CONCLUSION

Locus HlGA4 does not fit to expected segrega-tion ratio according to Mendelian inheritance, two loci HlGA29 and HlGT5 are linked together and both of them have probably null allele.

It was found that eight genotypes (111, 116, 141, 142, 144, 182, 188 and 241) of crossing between cultivar Vital and hybrid male parent do not have expected allelic combination probably because of cross-pollination, a mistake during sampling and possible meiotic disorder or unequal crossing-over.

Heterozygote excess together with low variability, low value of the effective number of breeders, small sampling area and high similarity with structure of hybrid progeny indicated that wild population from locality Vinařická hora has origin in two or three sexually propagated plants.

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