ARE MOLECULAR MARKERS STRENGTHENING PLANT VARIETY REGISTRATION AND PROTECTION?

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Abstract

In vegetatively propagated ornamentals, new varieties can be obtained from i.) conventional breeding through crossing and selection or ii.) spontaneous or induced variant types of existing (initial) varieties. Indeed, variant types enlarge the assortment to be produced without the need to alter production systems. However, they inherit the breeding effort needed to create a completely new and profitable variety. Under the rules defined by the UPOV convention of 1991 this new variant type should be considered as an “Essentially Derived Variety” (EDV) from the initial variety (IV). Therefore, the breeder of the initial variety might want to claim at least part of the profits acquired by the breeder of the EDV under the form of a royalty. To detect either EDV or fraud, criteria based on plant morphology are insufficient or even not applicable. Here, some case studies are presented to illustrate the possibilities and the limitations of the use of molecular fingerprints in disputes on fraud and essential derivation of ornamental plants. These examples are put in contrast with genetic analysis work that has been performed for the study of phylogenetic relationships, characterization of hybrids and breeders’ gene pools. From this framework, it is discussed what benefits molecular markers could provide to plant variety registration and protection of Plant Breeders’ Rights.

1. Introduction: What is the framework to apply molecular markers

For ornamentals, the evaluation of new candidates and the granting of Plant Breeders’ Rights are hampered by two facts: i.) virtually every new attractive plant can be a valuable introduction (at least for a short term) giving rise to an infinite list of ornamental species for which in most cases no evaluation guidelines are available, and ii.) for some very popular ornamentals (e.g. roses, chrysanthemums or geraniums), the large number of new applicant varieties make it hardly attainable to compare a new breed with the existing set of registered varieties. For clonally propagated ornamentals, in which case uniformity and stability of a variety is only influenced by eventual somaclonal variation, testing authorities are studying the possibility to apply molecular markers for the assessment of Distinctness, Uniformity and Stability criteria for new varieties, and for the management of reference collections.

In addition, breeders and propagators of ornamental plants need methods that can quickly and efficiently trace infringements of Plant Breeders’ Rights. Morphological characteristics used for the identification of varieties have been shown to be relatively unreliable due to the influence of environmental conditions. Moreover, fraud prevention needs much faster techniques e.g. also applicable on cut flowers sold on a market. Here too, molecular marker techniques could be very helpful.

Spontaneous or induced variant types of existing varieties are a very popular source of new introductions. Indeed, one extra attractive feature expressed in an important
variety offers the growers the benefit that a differentiated assortment can be produced without any single change in the plant production system. In many instances, such variant plant types differ from the original variety from which they have been derived in the expression of an only limited number of genes. Heritable changes in coding sequences by mutation, but also non-heritable changes by transposons and epigenetic effects can be the cause. Even when true mutations are at the basis, this does not imply that all tissue layers of a plant are involved e.g. in some flower color modifications and chimerical banding patterns in leaves. Variant types can occur spontaneously (e.g. bud sports) but can also be generated with that purpose by \textit{in vitro} culture (somaclonal variation), use of mutagens, irradiation and genetic transformation. Variants obtained in this way carry with them the breeding effort needed to create a completely new variety that is attractive and adapted to the most profitable plant production technique. Therefore, the breeder of the initial variety (IV) might want to claim at least part of the profits under the form of a royalty on what is considered as an Essentially Derived Variety (EDV).

By the UPOV-convention of 1991, the concepts for essential derivation and dependency have been defined. Essential derivation is a term being used to define a fact. For example if a new variety meets the criteria of containing "virtually" the entire genotype of a variety from which it was derived and therefore "retains the expression of the essential characteristics of the IV", the new variety is deemed to be Essentially Derived from the IV. This is a fact and has no relationship to whether or not the IV is a protected variety (has Plant Breeders’ Rights). In contrast, Dependency may or may not result from being Essentially Derived. Dependency is a legal consequence of developing an EDV from a protected variety. No Dependency exists for an EDV developed from a non-protected variety. If no Dependency exists, there is no need for the developer/owner of the EDV in question to seek permission to commercialize from the owner of the IV from which it was predominantly developed. However, if Dependency exists, then the developer of the EDV must seek permission from the owner of the IV prior to the commercialization of the new variety.

The criteria and procedures used for DUS-testing and variety registration are often not appropriate to detect EDVs because there will always exist important morphological characteristics that differentiate an EDV from the IV. A test based on the genetic relatedness between IV and EDV should be more appropriate, as even if both varieties have a completely different flower color, for example, they still would share most of their genome.

The burden of proof for dependency of the EDV has to be given by the breeder of the IV. Official variety testing agencies are not authorized to inform the breeder of an IV when they suspect a potential EDV. A publicly available database containing the molecular marker profiles of registered and new candidate varieties could be the feasible system. Because EDV assessment needs that the genetic conformity between EDV and IV to be proven, this is a different concept than distinctness evaluation and both concepts should not be merged. Taking into account that EDV contribute to a larger assortment, it is in nobodies’ interest to ban all EDV and exclude them from being registered.

2. Some case-studies on the use of molecular data

Many recent papers have focused on the application of molecular markers as a tool for cultivar identification in ornamental plants (e.g. Barcaccia \textit{et al.}, 1999; Han \textit{et al.}, 1999; Loh \textit{et al.}, 1999; Torres \textit{et al.}, 1993; Wolff \textit{et al.}, 1995); it was also the subject of recent scientific congresses (e.g. ISHS, 2000) and is discussed in extenso in the UPOV working group Biochemical and Molecular Techniques (UPOV-BMT). Our purpose here is to highlight some practical aspects of the ultimate purpose of these kinds of studies: application of molecular techniques for plant variety registration and reinforcement of Plant Breeders’ Rights protection in disputes on fraud and essential derivation in ornamental plants. Case studies on pot azalea (\textit{R. simsii} hybrids), roses, ornamental crab apple (\textit{Malus}) and \textit{Phalaenopsis} orchids are used to illustrate the possibilities and the
limitations of genetic analysis and marker fingerprinting techniques for these purposes. For further details we refer to the research articles mentioned.

2.1. Phylogenetic analysis on *Rhododendron*

This study was initiated to unravel the origin of the different azalea hybrids. Different wild species are reported in literature to have contributed to the construction of the present pot azalea assortment. Typical examples of the hybrids were compared with diverse material from the supposed ancestors (De Riek et al., 2000). Eighteen natural and semi-wild populations of *Tsutsusi* species seed obtained from China, were tested with AFLP; in addition, at least one individual genotype from each of these populations and single reference genotypes of other *Tsutsusi* species were used for *matK* sequencing (protocol according to Kurashige et al., 1998).

Within the different *Rhododendron* accessions studied, three distinct groups and some outlying species could be distinguished using 179 AFLP markers. The first group contains species belonging to the *Tsutsusi* section of the *Tsutsusi* subgenus. The second group solely holds *R. simsii* populations clustered with its subspecies *mesembrinum*. The third group consists of the in China freely proliferating cultivated species *R. x pulchrum* and *R. mucronatum*, the different groups of cultivated azalea hybrids and the majority of the supposed ancestors for cultivated azaleas. The remaining species (*R. racemosum, R. yunnanense* and *R. mariesii*) are clearly separated based on AFLP data. *R. racemosum* and *R. yunnanense* are lepidote (scaly) species that belong to the subgenus *Rhododendron*, and *R. mariesii* belongs to the *Brachycalyx* section of the *Tsutsusi* subgenus. Phylogenetic analysis data obtained from *matK* sequencing confirmed this structure (J. Dendauw, DvP, unpublished results).

As expected from the parentage of the cultivated hybrids, a closer clustering of *R. x pulchrum* and *R. mucronatum* with Hirado and pot azaleas was observed; Kurume azaleas were more distant. *R. x pulchrum* is regarded to be a hybrid between *R. indicum* and *R. mucronatum*. Both species are also related to pot azaleas. *R. mucronatum* has been cultivated at least for 300 years in Japan and China. The original wild form, native to Japan, is var. *ripense* with rose pink flowers but widely cultivated together with var. *mucronatum*, an albino form. Hirado and pot azaleas share at least one important common ancestor, *R. scabrum*, originating from some small southern Japanese islands. *R. scabrum* germplasm was introduced to *R. simsii* hybrids by ‘Phoeniceum’, one of the early crossing parents with supposed *R. simsii* plants from Sanghai. ‘Phoeniceum’ is accepted to be a cross between *R. mucronatum* and *R. scabrum*. Kurume azaleas are related to *R. kiusianum* and *R. kaempferi*, species native to Japan but with a different geographic origin; Satsuki azaleas are related to *R. indicum*.

2.2. Breeder’s gene pool of *R. simsii* hybrids

For this AFLP study (De Riek et al., 1999), a total of 75 azalea genotypes were chosen from three subgroups distinguished within the evergreen azaleas: pot azaleas (*R. simsii* hybrids; 55 genotypes), Hirado azaleas (*R. scabrum* hybrids; 4 genotypes) and Kurume azaleas (hybrids of *R. kiusianum* var. *kiusianum* and var. *sataenise* and *R. kaempferi*; 7 genotypes). Also 9 individual genotypes of different *Tsutsusi* species were included.

Principal co-ordinate analysis was used to produce a two dimensional ordination for the breeders’ gene pool based on AFLP data. The current pot azalea assortment was clearly separated from the wild *Rhododendron* species. The latter clustered together with the Kurume azaleas. Hirado azaleas were grouped together with *R. scabrum*. Both Kurume and Hirado azaleas were grouped with their most important ancestor. Within the *R. simsii* hybrids two subgroups may be distinguished. The first group contains the archetype of a Belgian pot azalea, - globular shape, dark green leaves, early flowering and carmine red double flowers -, although it has never been created so far in all its details as
a single genotype. The second subgroup is a looser group of cultivars, intermediate to the other groups. They can be generally typified as flowering late and having single or half double flowers. A lot of them are older cultivars. Some are cultivars that originate from intermediate crosses: ‘Cheops’, a new pyramidal azalea created from *R. noriakianum*, ‘Lara’ and ‘Mistral’, crosses between Hirado azaleas and *R. simsii* hybrids and ‘Directeur Van Slycken’, a cross between Kurume azalea and a *R. simsii* hybrid.

2.3. ‘Hellmut Vogel’ and its bud sport series

De Riek *et al.* (1997) analyzed the “Hellmut Vogel” (*R. simsii* hybrid) bud sport series (more than 20 bud sports reported over 30 years of propagation). ‘Hellmut Vogel’ is a not protected variety that has become the most important commercial pot azalea; some of its bud sports have even acquired Plant Breeders’ Rights, e.g. ‘Sima’ and ‘Aquarell’. All of the commercial bud sports have a changed flower colour ranging from carmine red, red, pink white or banding pattern (edge, dots, stripes). For some of them growth is retarded or leaf shape is altered (Heursel, 1999). This series provides at least 60% of the azaleas produced in Belgium.

The 6 AFLP primer combinations used in this experiment yielded a total of approximately 500 molecular markers. Within this set, no polymorphisms between the initial variety and its different bud sports could be observed. The exact mechanism of azalea bud sporting is still unraveled, however, a comparison with already better-studied flower variants of e.g. Petunia indicates that only a limited number of genes are affected by bud sporting (De Schepper, 2001). In fact these results are not surprising if we take into account that using a random fingerprint technique as AFLP it is very unlikely to pinpoint the probably few loci responsible of the phenotypical changes detected.

Although dependency is not valid for ‘Hellmut Vogel’ being a free variety, this bud sport series offers a good example of essential derivation. Since some of the bud sports themselves have been protected, under the latest UPOV convention-act, a quite contradictory situation might arise when finders of new bud sports want to protect these. However, since no dependency can exist to a protected variety that is an EDV already, the finder of a new bud sport would have to seek permission from the owner of ‘Hellmut Vogel’, but this is a free variety. As a consequence, every finder of a new bud sport of a free variety can receive plant variety protection but cannot broaden this protection to other EDVs (as long as they embody essentially all the inherited characteristics of the IV). Until now, there was a kind of courtesy between azalea breeders that the finder of a bud sport informs the breeder of the original variety. Also, quite often, in license contracts for protected varieties, the breeder imposes the grower to return all possible (valuable) new bud sports that he identifies.

2.4. *Rosa* gene pool study

An AFLP study on the genetic relationship of roses was conducted on 88 plants (Leus *et al.*, 2000). The plants were chosen between species, varieties and cultivars of different breeders. The plant species sampled belonged to the sections: *Caninae, Cinnamomeae, Gallicanae, Pimpenellifoliae* and *Synstylae*. The breeding history of some of the plants is clear and the relationships towards other roses are known. The plants used differed in ploidy range from 2n=2x to 2n=6x. Most of the cultivars were 2n=4x. A considerable degree of genetic variation was observed in this gene pool. According to the AFLP data, a clear division could be made between the species group (together with some closely related varieties) and the cultivars. The only species occurring in the cultivar group was *R. chinensis minima*. Next to *R. chinensis minima, R. multiflora* appeared to be more related to the cultivars than other species. Both *R. chinensis* and *R. multiflora* are supposed to be the species with the largest influence during more recent breeding of modern roses (19th century; De Vries & Dubois, 1996). Clear relationships between subspecies were revealed. With exception of the *Caninae* section also a clustering of the
sections could be observed. Known relationships between plants were evident, e.g. *R. x pteragonis* and *R. hugonis*, being one of the parents of the first, were clustered together. Within the group of the cultivars a rough division could be made between true hybrid tea roses and floribunda types. Also, some known relationships (not all ancestors are known) can be mentioned. ‘Ravel’, ‘Rossini’, ‘Pavarotti’, ‘Vivaldi’ and ‘Timeless’ are all cut roses of the same breeder that clustered together. A very close relationship was found between ‘Pailine’ and its sport. From the cross ‘Melflor’ x ‘Melglory’ the cultivars ‘Professor Boesman’ and ‘Melrose’ were selected, these were grouped.

2.5. Presumable case of fraud in roses

The owner of the registered variety ‘Pailine’, which used to be an important yellowish red cut rose, suspected the variety ‘Lena’ to be essentially derived or to be identical and used AFLP fingerprints to strengthen his case (De Riek *et al.*, 1997). ‘Pailine’ is a seedling selected from a pair cross between a salmon-pink *floribunda* type with the appearance of a hybrid tea (‘Kardinal’; seed parent), and a scarlet-red hybrid tea rose typically used as cut rose (‘Lorena’; pollen parent). ‘Lena’ shows more yellowish flowers, but is further phenotypically identical to ‘Pailine’. The breeder of “Pailine” had also found a bud sport with similar flower colours to ‘Lena’. ‘Lena’ has also been registered. According to the PBR-application file, ‘Lena’ was derived from a pair cross between an orange-pink *grandiflora* type of garden rose but showing a type adapted for cut roses (‘Montezuma’; seed parent) and a garnet-red *floribunda* with bushy growth and small flowers (‘Garnette’; pollen parent). The nature of the putative parents of ‘Lena’ supported already the suspicion formulated by the breeder.

Identical fingerprints were obtained when the AFLP marker profiles for ‘Pailine’, ‘Lena’ and the known bud sport of ‘Pailine’ were compared. This indicated already a high degree of similarity between the tested plants. Including the parents of both cultivars, as indicated on the PBR application forms, made the evidence indisputable. For the original variety ‘Pailine’ no AFLP markers could be observed that did not appear in the parents. However, 16 of the approximately 350 peaks detected in ‘Lena’ by the 4 AFLP primer combinations used, could not be traced back to its putative parents. As ‘Pailine’ and ‘Lena’ did not reveal any polymorphism, all peaks of ‘Lena’ could be traced back to the parents of ‘Pailine’. This was also evident from the genetic similarities: ‘Pailine’ and ‘Lena’ are clustered very closely together; ‘Lena’ is more close to the parents of ‘Pailine’ than to its putative parents. For this case, the conclusion is beyond doubt: ‘Lena’ is at least a bud sport from ‘Pailine’, the parents that were entered for registration are false.

2.6. Different micropropagation lines of *Phalaenopsis*

In this complex case, a commercial micropropagation lab producing *Phalaenopsis* in vitro cuttings for different growers was accused by another breeder of producing his registered variety without a license (De Riek *et al.*, 1997). The breeder of the registered variety supported his charge by mentioning that a genetic analysis with molecular markers (not further specified), could not detect any difference between both varieties. The micropropagation lab claimed that they independently selected the specific clone from a seed lot of hybrids they bought almost 15 years ago from the previous breeder, taken over afterwards by their accuser. This initial variety was propagated in the traditional way by in vitro sowing. Therefore, the parents used to produce the hybrid presumably had a high degree of kinship in order to obtain a uniform offspring. By the general introduction of micropropagation techniques for multiplication of *Phalaenopsis*, the little genetic variation still available in traditional seed propagated hybrids was fixed in different clonal lines. These clonally propagated plants completely replaced the old varieties. The micropropagation lab wanted to collect support to their thesis that, in *Phalaenopsis*, there are very few genetic differences in phenotypically very similar hybrids. With that purpose, two clones *L* and *J* were compared by means of AFLP. The first is the attacked clone; the
second is a full sib, selected at the same time by the micropropagation lab. L and J are only different from each other in minor flower details and micropropagation rate. As a reference, ‘Mercatus’ and ‘Jupiter’, that are phenotypically completely different Phalaenopsis hybrids (from each other and from L and J), were included. L or J was never protected.

The fingerprints generated with 3 AFLP primer combinations did not reveal any polymorphic band between L and J. The two other clones ‘Mercatus’ and ‘Jupiter’ were clearly different. If the variety protection granted to the breeding company should have been issued under the UPOV 1991 convention, L and J should probably be considered as identical or at least essentially derived from their cultivar. However, as the origin of the initial seed stock was quite unclear and probably free or at least open to buy by several growers/micropropagators, both parties decided to close the case. Also, due to change in propagation technique, the variety that originally obtained variety protection cannot be reconstituted. This example demonstrates the difficulties one might run into when (somaclonal) variants obtained from original varieties are also receiving variety protection. Growers then might try to broaden this protection towards previously free varieties or to variants obtained by others. However, in such a case one should recall the definitions of essential derivation and dependency. Before a claim can be based on the EDV concept, both the initial variety and the putative EDV must be registered. This includes that they are distinct, using DUS-criteria. Secondly, there must be an intention for essential derivation: an on purpose violation of the copyrights on the initial variety. In all cases, dependency by essential derivation will call back to the first variety obtained in a conventional way, by e.g. crossbreeding, and never to a previous already derived variety (protected or not).

2.7. Authenticity control for ornamental trees

Within the hardy stock nursery a large amount of different species and cultivars are produced and commercialized. Only for a few woody ornamental species exists a labeling system of certified plant material. However, all produced plant material within the EU should fulfil a minimal EU-quality level, for which correct identification and nomenclatures of the cultivar are important criteria. To be able to guarantee authenticity of the produced plants, it is important to have objective control mechanisms. Until now all the identification systems are based only on morphological descriptions of the plants. Especially in wintertime, when most woody plants are soled, identification can sometimes cause problems. For the purpose of authenticity control ornamental crab apples were compared by use of AFLP (Van Huylenbroeck et al., 2001) and microsatellite markers (E. Coart, DvP, unpublished results). Using 12 microsatellite markers, developed for eating apples and covering more or less homogeneously the apple genome, crab apple varieties could be positioned against wild apple accessions and eating apples. All crab apple cultivars could uniquely be identified with this set of markers. Although only 12 microsatellite loci were assayed, because of the highly polymorphic nature of this kind of marker, more than 200 different alleles could be scored in the total dataset.

3. Discussion

The above studies on different hybrids (different azalea hybrids and wild species), gene pools (R. simsii hybrids, Rosa and Malus) using different genetic analysis techniques (matK sequencing, AFLP and microsatellites) clearly proof the potential of such techniques to reveal genetic relationships. This can be at different levels: relatedness of species (phylogeny), origin of interspecific hybrids and use of wild genetic resources, pedigrees in genetically narrow breeding material and mutants of a same initial genotype. Each of the genetic analysis techniques mentioned have their own field of application: e.g. matK sequencing is very unlikely to reveal differences beyond the level of species or hybrid types, microsatellites and to a lower extent AFLP, very powerful in discriminating
among pedigrees, are to be interpreted carefully at higher taxonomic levels (AFLP might amplify completely different loci that result in similar fragment sizes; microsatellite repeat lengths might be similar but evolutionary completely independent). Although there is a substantial correlation (taxonomy before the advent of molecular techniques was based on it), genetic relatedness or differentiation is not right away to be converted into terms of similarity or distinction at the level of phenotypes of cultivars. Differences in use for horticulture or ornamental value can be qualitatively or quantitatively inherited; an estimate on genetic conformity will certainly reflect this in a different way. Moreover, for DUS criteria, most often ready observable and valuable (in terms of application of the plants) characteristics are included. The genetic basis of all these might be very diverse. Also, introduction of certain monogenic traits (e.g. alleles for resistance that are scarce) can take quite some screening and breeding effort, worth to be awarded by granting of PBR although the differences at the genetic level might be minimal. All such considerations hamper at the moment a complete chance over from conventional morphological description of varieties to another system solely based on molecular data. However, due to the benefits of molecular markers, their gradual introduction can be advantageous.

It is a generally aspired principle that for any type of application (identification, DUS assessment or EDV testing), molecular markers should be able to produce consistent, reproducible results across different laboratories and at an acceptable cost. However, the levels of consistency required and reproducibility depend on the purpose of application. On the one hand, for the assessment of distinctness, molecular markers must at least produce consistent differences between varieties in a particular set of conditions (i.e. the laboratory recognized by the testing authority). It might not necessarily require the reproducibility of the same band patterns across different laboratories. On the other hand, absolute reproducibility of molecular band patterns across different laboratories is required for the most effective management of reference collections and composition of databases of registered varieties.

Because of the cost and the accessibility of molecular marker techniques, there can be a tendency to use only the smallest set of markers that can do the job. Microsatellites are then the markers of choice because they are simple in use (PCR-based), locus specific, reproducible among laboratories and highly polymorphic. As indicated by the apple study and also shown by Van Hoof (IdQ BV, the Netherlands) with 4 primer pairs on a set of 250 rose varieties, the distinguishing capacity of microsatellites can already be very high also if only few primer sets are used. Breeders and growers will continue to use morphological characteristics as the most practical system for rapid cultivar identification. Therefore, any new plant variety registration system will incorporate both approaches. The ultimate goal for vegetatively propagated plant material could be a system where PBR are granted on the basis of a morphological description from the breeder and the assessment of a limited set of molecular markers. However, what kind of system is most feasible will only appear after a transitional period were both systems are applied on new applicants and registered varieties. Regardless of what criteria (and what respective weights) the decision on the threshold level for distinctness is based on, the combination of both conventional testing data and molecular markers is useful basic information that indicates the discrimination power of molecular techniques. The comparison between these data and differences observed between close pairs of protected varieties will produce some guidance on the possible impacts on the strength of protection from the introduction of molecular techniques.

Testing of EDV requires a different dimension because it is needed to prove general genetic conformity within certain thresholds. A limited set of highly polymorphic markers, very well adapted for general distinction purposes, only refers to a small number of genetic loci. For a dishonest breeder having the accession to a molecular marker facility, it is only a small task to select for some different alleles for a few loci by e.g. multiple backcrossing. Moreover, because of their highly polymorphic nature, microsatellites spontaneously can have relatively high rates of mutations that can be
selected with a unfair purpose. Therefore, for the assessment of genetic conformity for testing of essential derivation, multiple loci must be taken into account. AFLP markers are one of the most powerful tools to detect similarities in the genome of related cultivars. They reveal detailed information about a large set of loci even with a limited number of fingerprinting reactions. AFLP is a PCR-based marker technique for which no sequence knowledge is needed in advance. It shares with RFLP its focus to mutations in restriction sites and its reproducibility. Moreover, once the technique has been established, it can be extended to a broad range of species in little time. Consequently, AFLP has quite some advantages if one wants to assess quickly a putative case of fraud or essential derivation. This is certainly the case for vegetatively propagated plants, self-pollinated species and hybrids where little genetic variation within a variety is to be expected.

Genetic conformity is most often accessed by definition of a “minimum distance” for essential derivation. However, one of the concerns of the introduction of molecular techniques is an erosion of the “minimum distance” in a manner that might weaken the value of protection. Therefore, one option is to set a threshold of molecular distance corresponding to “minimum distance” observed between known pairs of essentially derived varieties and non-essentially derived varieties. However, it should be noted that, since most molecular markers are not directly linked to traditional characteristics, direct correspondence between traditional characteristics and molecular marker distance couldn’t be expected. Moreover, essentially derivation can also be evaluated by considering if there was an intention to approach the initial variety closely. This can only be revealed by evidence from the breeders’ logbooks. In this view, molecular data can only function as a strong indication and a necessary requisite to ask the competing breeder to open his files.

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