Genetic Variation in Resistance Against *Heterobasidion annosum* (Fr.) Bref. in *Picea abies* (L.) Karst. Expressed After Inoculation of Neighboring Stumps

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Abstract

Based on the well-established knowledge that important spread of *H. annosum* is performed through spore infection of stumps and subsequent spread of mycelium to neighboring trees at points of root contact, an inoculation experiment was established in three adjacent fully pedigreed 17-year old Norway spruce field trials. In June 1995, every third row was cut and stump-inoculated with conidia belonging to the two contrasting types, P and S. 5 years later the remaining trees were evaluated by clear-cutting and mapping of rot occurrence. 34 % of the trees were attacked. For each host-tree, the most likely source of infestation (i.e. possible inoculated donor-stump 5 years earlier) was evaluated based on orientation of the rot on the stump surface supplemented with samples of re-isolation of *H. annosum* of the two types. According to these estimates, the tested S-type turned out to be more aggressive towards Norway spruce than the tested P-type. Genetic variation in resistance was most convincingly detected in the most informative trial F175B. The pattern of genetic variation in the two roles as donor and host seems to differ. However, genetic variation was also expressed as general resistance, which is defined as the combined effect of donor stump, living host and across the two tested types of *H. annosum*. No indication of host x pathogen interaction was detected. This evidence combined with the general experience that resistance against root rot pathogens usually is partial and based on the cumulative effects of several genes, suggests that sustainable genetic gains in relative resistance may be obtainable in breeding programs of Norway spruce. The developed experimental set-up represents a useful concept for screening existing genetic trials for field resistance within a time scale of 5–6 years.

Key words: Heterobasidion annosum, *Picea abies*, genetic variation, resistance, stump inoculation.

Introduction

The root rot fungus *Heterobasidion annosum* is the economically most important pathogen of European coniferous forests. Estimated annual losses caused by the disease – excluding windthrow – amount to 790 million Euros (WOODWARD *et al.,* 1998). *H. annosum* is mainly dispersed by basidiospores, which colonize freshly cut stumps and wounds. The vegetative spread of the disease from infected stumps and trees to adjacent trees takes place by means of root contacts and grafts (STENLID and REDFERN, 1998).

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H. annosum has been divided into three intersterility groups (KORHONEN and STENLID, 1998). They have been designated S, P, and F after their main hosts (spruce, pine and fir). These three groups have later been given status as species as *H. parviporum* (Niem. and Korh. = European S type), *H. annosum* (Fr.) *s. stricto* (= European P type), and *H. abietinum* Niem. and Korh. = European F type). S-types are especially adapted to *Picea*, whereas P-types have a broader host spectrum of conifers, amongst those *Pinus* and *Picea*. Up to now, the F-type has not been recorded in Denmark, as its distribution area is restricted to southern and central Europe (KORHONEN *et al.,* 2001).

Reducing the frequency and severity of *H. annosum* root rot in Norway spruce has been the aim of many research projects. One research field is the search for genetic variation in resistance to the fungus. Several types of investigations have been attempted to document genetic variation in resistance against *H. annosum*. These can be categorized in the following groups – partly after DELATOUR *et al.* (1998):

1. Surveys of natural attacks in the field
2. Field resistance trials
3. Inoculation trials
4. Laboratory tests reflecting components of a suspected resistance mechanism

Category 1 has mostly been used to obtain a general idea of differences between species (RÖNNBERG *et al.,* 1999). Recently, a 20-year old cutting-propagated clone-trial in Norway spruce has been mapped for spontaneous attacks (KARLSSON and SWEDJEMARK, 2006).

Category 2 represents situations where hosts to be tested are deliberately placed in a position where they will make contact with plant material colonized by *H. annosum*. The method has been tried in a Danish trial with different tree-species in shelterbelts by ramming infected poles into the soils close to potential hosts (WAGN, 1987). The method was used to demonstrate differences between species, but it may have the potential to detect variation within species too.

Category 3 – inoculation tests – have been used to investigate species, individual and clonal variation by drilling holes through bark into the stem wood and inoculate with mycelium. The method has been used extensively in different designs. Both mature trees with dead heartwood and young plants with only live sapwood as target tissue have been used, with later recording of spread within host. Pronounced tree-to-tree variation

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has been recorded on mature spruces (DELATOUR *et al.*, 1998). SOLHEIM and SKRØPPA (pers. com.) have used the technique on 7-year old juvenile clones and 15-year old parent clones and found a significant parent-offspring regression for lesion length in the bark. SWEDJEMARK (1995) has tailored the method to juvenile plants with only living sapwood and in extensive trials documented clonal variation in *H. annosum* spread in this living tissue. In addition, significant differences in vertical spread of the fungus after inoculation on stumps were found among clones of Norway spruce (SWEDJEMARK and KARLSSON, 2003).

However, the cited inoculation tests do not address possible resistance mechanisms associated with pathogen penetration into roots at the point of root contact between donor and host. Further, no convincing correlations have been documented between the above-mentioned methods or between these methods and actual field tests in the forest.

On this background, the general aim of the reported investigation is:

- To evaluate whether genetic variation occurs in *P. abies* in resistance against *H. annosum* in an experiment reflecting a realistic field plantation situation covering the accumulated penetration from stump inoculation to realized rot in neighboring living hosts and covering the two contrasting *HA*-types at present occurring in Northern Europe.

More specific aims are:

a. To evaluate whether it is possible to detect genetic variation in resistance in the two separate roles as dying donor stump and living neighbor host tree or as "general resistance" (GR) covering the combined effect of both roles.

b. To evaluate whether co-ancestry between donor-stump and living host influences the *H. annosum* penetration from donor to host.

c. To evaluate whether sufficient evidence of useful genetic variation has been obtained to recommend resistance breeding against *H. annosum* in Norway spruce.

d. If c. is reasonably positive, to evaluate whether further development of the field inoculation method is required.

The term "general resistance" is in the present context defined as the combined effect of donor stump, living host and across the two specific strains of *H. annosum* belonging to the S and P type respectively.

The justification for testing whether co-ancestry between donor and host has an effect is based on the cited experience (STENLID and REDFERN, 1998) that the important vegetative spread from infected stumps to adjacent trees occurs through root contacts and grafts. Concerning the latter component, it is well documented in Douglas-fir (COPES, 1970) that genetic variation in graft incompatibility occurs.

Material and Methods

As cited by STENLID and REDFERN (1998) the principal spread of *H. annosum* within stands of 1st generation conifers is performed through basidiospore infection of stumps and subsequent spread of mycelium to neighboring trees at point of root contact.

In the present investigation, this situation was attempted reproduced in three adjacent genetic field trials of Norway spruce (F175A, F175B, and F209). After cutting every third row fresh stumps were inoculated with a suspension of *H. annosum* conidia of either S- or P-types. These stumps are termed "donor stumps." 5 years later the remaining spruces (termed living "hosts") were felled, and the amount of *H. annosum* attack was recorded and most likely neighboring donor-stump was estimated (*Fig. 1*).

By comparing frequencies and the quantitative amount of decay in the host trees and estimating the

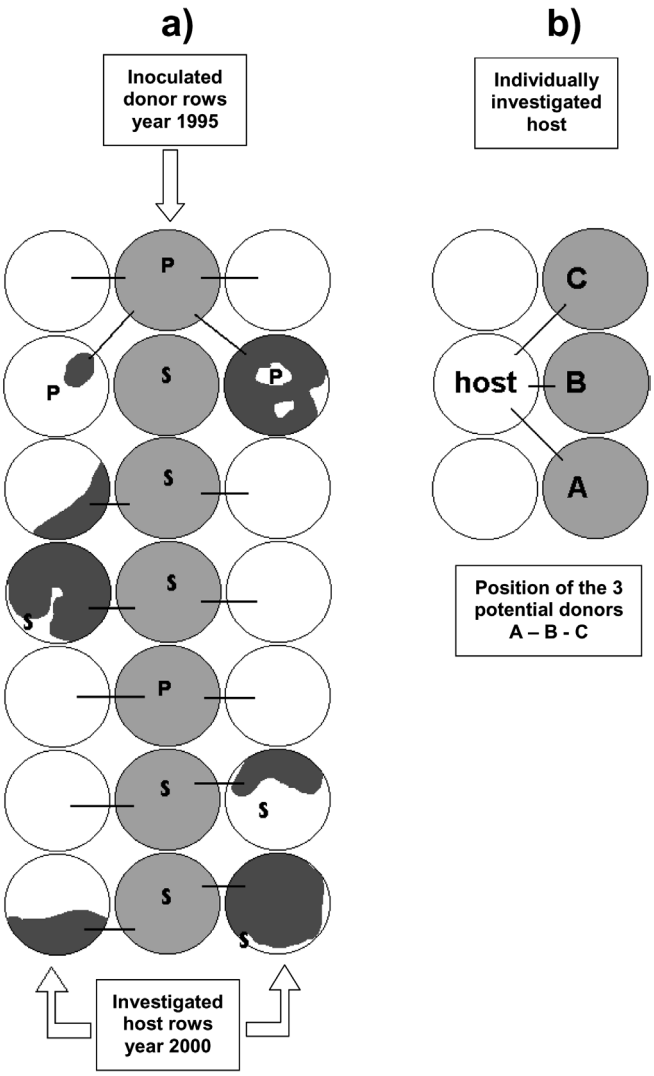

Figure 1. – Donor – Host pairing.

a) General layout of the 1995 stump inoculation rows and neighboring rows of investigated hosts in 2000.

b) Individually investigated host with position (A – B – C) as potential donors.

Legend. Shaded circles: trees Inoculated 1995. Clear circles with or without dark areas: hosts with or without rot on stump surface 5 years later in 2000. P and S: the two strains of *H. annosum* used as inoculum 1995 and reisolated in a sample of 24 attacked hosts 2000. Lines from donor to host: concluding donor – host pairing resulting in presence or absence of attack.

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most likely donor of each attack, it should be possible to evaluate, whether significant variation from the following genetic sources have been expressed:

- *H. annosum* genotypes (S- versus P-strains), i.e. reflecting pathogen aggressiveness.
- *P. abies* genotypes covering three roles: donors, hosts and the accumulated effect "general resistance," i.e. reflecting components of resistance.
- Co-ancestry between donor and host.

Plant material

The experiment utilized three adjacent then 17-year old genetic field trials, F175A, F175B, and F209, growing on fertile soil near the Hørsholm Arboretum in NE Sjælland. The trials represent first-generation Norway spruce on former agricultural land. No selection for resistance against *H. annosum* was performed prior to the reported investigation.

Spacing in all trials were 2 m between rows and 1,5 m within rows. The three adjacent trials were sheltered against the environment by two plant positions with surplus plants from the trials.

F175A and -B were composed of seedlings originating from controlled crosses performed in 1974, sown in 1975 and outplanted 1978. The mating design was two disconnected factorials (F175A shown in *Table 1*, F175B shown in *Table 2*). In each of these factorials, 3 female clones were mated to 11 and 8 males respectively. The designs of these two field trials were independent, although adjacent and in both cases randomized blocks with single-tree plots and 30 replications (blocks). Representation was imbalanced due to fewer than 30 available family-members in approximate 50% of the families. The common Danish reference population (F300 Rye Nørskov) was over-represented by 1–2 plants per replication in all three trials. The imbalance was randomly distributed to the 30 replications, which in each trial was set to a fixed size.

The third trial, F209, was composed of 23 cutting-propagated clones originating from controlled pollinated families and further three cutting-propagated clones selected in two different populations of known origin (provenance). Single-tree plots and 12 replications (blocks) were applied. The scattered mating design is shown in *Table 3*.

Matings between the V-numbered parents (V939-V1369) were performed at the Hørsholm Arboretum. The remaining crosses have been performed in Escherode in Germany, and the offspring represented by cutting propagated clones have been transferred from Escherode to Denmark prior to establishment of the trial. During the development of these trials, repeated recordings of height, DBH, Pilodyn penetration, branch characteristics and needle retention have been accumulated.

Initial tests revealed that in order to obtain sufficient representation of each genetic unit parents rather than individual FS-families were analyzed. In F209, representation of individual cutting-propagated clones was insufficient, and therefore FS-families in which the clones were selected, were attempted as genetic units in the analyses.

Inoculum and inoculation

Inoculum was composed of suspensions of conidia. These spores originated from two reared *H. annosum* isolates in Petri dishes. The isolates were founded from discs with active rot cut from two mature, infected trees after felling. The type of *H. annosum* was determined by confrontation with haploid testers of known type according to the method of KORHONEN (1978). Each spore suspension used for inoculation then contains asexual spores representing the genotype of the mycelium from the trees. In F175A only the S-type was applied, whereas in F175B and in F209, either the S- or P-type *H. annosum* isolates were applied in a randomized design, so their effects could be compared.

Table 1. – F175A mating design. Each FS-family represented by the indicated number of individuals (average 22, 7). W-C: West-Continental origin; Scan: Scandinavian origin.

Parents:	West-Continental origin						Carpathian origin				
Males→ Females ↓	V 6694	V 6731	V 6706	V 6684	V 6680	V 6773	Ås-høj 2	Ås-høj P9	Ås-høj 40	Ås-høj 17	Hasl O 57
V48 (W-C)	30	30	26	20	18	24	30	30	26	26	30
V1175 (W-C)	30		13	16	14	11			12		30
V1369 (Scan)	20	30	20	25	25				17		15

Table 2. – F175B mating design. Each FS-family represented by the indicated number of individuals (average 24, 7). W-C: West-Continental origin; Scan: Scandinavian origin.

Parents:	West-Continental origin				Carpathian origin			
Males→ Females ↓	V 6704	V 6676	V 6675	V 6775	Åshøj 36	Åshøj 22	Åshøj 41	Hasl O 70
V1161 (W-C)			30		19			
V49 (W-C)	30	30	30	30		21	30	
V1367 (Scan)	26	30	30		9	9	27	24

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Table 3. – F209 mating design. Each FS-family is represented by a variable number (1-7) of cutting-propagated clones. A few clones originate from non-pedigreed provenances Istebna and Nagold. The reference provenance Rye Nørskov is represented by seedlings (S6440).

Male parents → Female parents ↓	V939 (Scan)	V1367 (Scan)	V49 (W-C)	SiFi 38 *)	EngFi 13 *)	Prov Istebna	Prov Nagold	Ref Prov Rye
V48 (W-C)	V3783	V3784						
V1367 (Scan)			V3906 V3909 V3910 V3912					
V1369 (Scan)			V3919 V3921 V3922 V3924 V3926 V3927 V3929					
V1368 (Scan)			V3933					
Ga12 (W-C)				V5338 V5339 V5340 V5341 V5344				
GA14 (W-C)					V5342 V5343 V5345 V5346			
Prov Istebna						V3781 V3875		
Prov Nagold							V3780	
Ref Prov Rye								S6440

*) Pollen sampled on *Picea sitchensis* (SiFi) and *Picea engellmannii* (EnFi) in Escherode, Germany. However, the progeny looks like pure *Picea abies.*

Inoculation was performed in 1995 when the trees were 17 year from planting. In early June, every third row of the trials was felled leaving one-meter high stumps. All trees in the remaining rows were adjacent to a stump (*Fig. 1*). Felled trees were removed from the trials. On the 19th of June, inoculations were carried out. The one-meter stumps were cut down to approximate 0.5 m, and the spore suspension was sprayed on the fresh stump surfaces, at least 10,000 spores pr. cm^2. The F175A trial was assigned an additional role, as three protective stump treatments were applied in comparison with non-protected controls. These treatments were Urea in 20% and 30% concentrations and a competing fungus *Phlebiopsis gigantea* (Rotstop®). The stump treatments were applied between cutting down the 1-m stumps to 0.5 m and application of the spore suspensions. For further details of this experiment, see THOMSEN (2003). In the other two experiments F175B and F209 no such protective treatments were applied, i.e. all 0.5-m stumps received the spore-suspensions direct on the fresh stumps.

Six months after inoculation a disc was removed from each stump in F175A to confirm infection and the effect of the applied stump protection. A thin disc was removed from the top of the stump and a two-cm thick disc taken just below was incubated in plastic bags. The presence of *H. annosum* was confirmed by appearance of the conidial stage (*Spiniger meineckellus*) on the surface of the incubated discs. 88% of the untreated stumps were infected and on average 9% of the treated stumps (THOMSEN, 2003).

Scale of the 1995-inoculations

The scale of the experiment is presented in *Table 4*. As the design of the field trials is randomized blocks, neighboring host trees to the inoculated stumps are expected to be a random sample of the represented host genotypes.

Estimation of sources of H. annosum infection (donor – host pairing)

5 years later (June 2000) the stands, now 22 years old, were clear-felled. Thus all remaining trees (potential hosts) neighboring the inoculated rows from 1995 (potential donors) were accessible for a complete mapping of the spread of root rot within and between host trees, all of which have been neighbors to the 1995-inoculated stumps – see *Fig. 1*. This situation was realized in two of the three trials, F175B and F209. As described earlier, in F175A the situation is complicated by the additional protective treatments of the fresh stumps back in 1995 prior to inoculation. This implies that only

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Table 4. – The scale of the 1995 inoculations.

	Number of inoculated stumps		
H. annosum inocula →	S-type	P-type	Total
F175A: *) 25 FS + 1 reference	64	0	64
F175B: 15 FS + 1 reference	76	74	150
F209: 26 Clones + 1 reference	48	48	96
Total	188	122	310

*) Ignoring stumps on which protective treatments were applied, even if the treatment failed.

the unprotected stumps were considered as potential donors in the present genetic investigation. However, this particular trial delivers convincing evidence, that the untreated stumps really were the active agent for the spread of root rot to neighboring host trees (THOMSEN, 2003).

The area and placement of the decay on the newly cut stump surfaces (hosts) was recorded in order to quantify the attack. In addition, the vertical column of discolouration up through the stems was recorded. For each individual clear-felled spruce, it was judged from the map, which of the three nearest neighboring 1995-inoculated stumps was the most probable source (donor) of potential infestation – realized or not – through points of root contacts – see *Fig. 1.* In F175B and in F209 where the two *H. annosum* strains S- and P types were compared as inocula, it was possible in 24 situations to check the identity of the invading strain through re-isolation and tests of mutual somatic compatibility and compare it to the most obvious source judged by placement of rot.

In *Fig. 1*, size and distance between stumps are not to scale, but rot size is drawn approximately relative to stump diameter. Shaded stumps represent stumps inoculated 1995; clear stumps with or without dark areas represent host with or without rot 5 years later in 2000. P and S represent the two *H. annosum* types. As starting point, the closest host neighbor in position B is judged the most likely potential donor. Deviations from this are accepted based on:

i) re-isolation and test of somatic compatibility (P versus S, see *Fig. 1a*),
or
ii) a subjective judgment of orientation of the rot area against position A or C (see *Fig. 1b*).

Co-ancestry

Co-ancestry (FALCONER, 1989) between source of infestation (donor tree) and host tree might take values between 0.5 and 0 according to the definition of this population-genetic parameter: "Co-ancestry of any two individuals is identical with the inbreeding coefficient of their progeny if they were mated." Levels of realized co-ancestries in the investigated populations are 0,125 for HS-pairing and 0,25 for FS-pairing. In the clonal trial F209 pairing between ramets of the same clone did not occur.

Statistical analysis

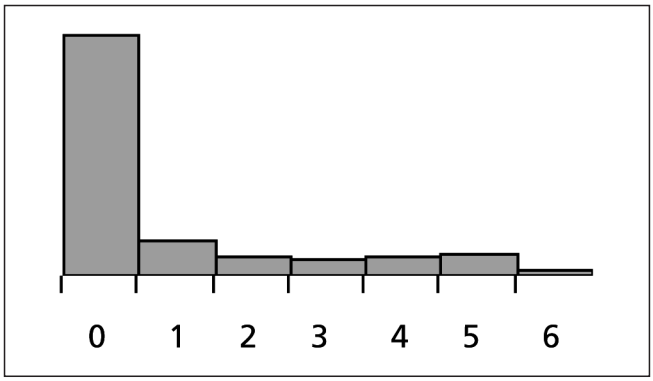
Data set

Each individual host represents an observation. Most important information is then coordinates in the trial (row and number within row), pedigree, degree of attack 2000, DBH, donor (pedigree). In F175B and F209 *Heterobasidion* type of donors were available from the 1995 inoculation (P or S). For a sample of 24 attacked hosts, re-isolation and results of type determination were available.

Distributions

The response variable Y (rot area and rot height) were highly correlated ($r \approx 0.75$, d.f. = 207). For this reason, emphasis was put on analyses of rot area.

Rot areas were heavily skewed with a large proportion (51–74%) of not-attacked hosts and a tail of gradually larger rot areas. Square roots and higher level root transformations were not able to create distributions



Rot area (sq cm)	Rot score
0	0
1-15	1
16-30	2
31-60	3
61-120	4
121-250	5
> 251	6

Figure 2. – Distribution of rot areas to a limited number rot scores of successive thresholds in trial F175B (ordinal variable).

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approaching normality, primarily because of the large proportions of 0-observation, i.e. healthy trees with no rot or stains in the cut surfaces of the stumps.

Subsequently, the concept of nomial and ordinal response variables (SAS, 2000) was applied in parallel for comparison. The term nomial response variable is used when we only distinguish between "rot" and "no rot." The alternative term ordinal response variable is used when a not necessarily continuous variable causes the observable variable to change as it crosses various thresholds (SAS, 2000). *Fig. 2* shows the distribution of "rot scores" and the definition of the score values.

Choice of statistical method

As a consequence of the pronounced skewed distributions of the continuous response variable rot area, both nomial and ordinal maximum likelihood – ML – methods (SAS, 2000) were used on the rot occurrence (nomial) and degree (ordinal rot score). The applied method is logistic regression.

Statistical modeling

Due to limitations of number of observations, an overall model considering all effects is not possible. Instead, individual models with restricted number of factors are formulated from the aims of the investigation.

Effect of HA-type P versus S

The applied model for testing this in trials F175B and F209, where both strains were compared, is

$$Y = Repl + HA\text{-}type + Random\ error \quad (1)$$

Where *Repl* = Replications superimposed on the original smaller blocks due to local environmental conditions and of necessity to obtain stable parameter estimates in successive analyses.

Donor and Host effects tested separately

In an attempt to separate the two effects the following two models were applied

$$Y = Repl + Donor\ (Female\text{-}and\ Male\ Parent) + Random\ error\ (including\ actual\ Host) \quad (2)$$

$$Y = Repl + Host\ (Female\text{-}and\ Male\ Parent) + Random\ error\ (including\ actual\ Donor) \quad (3)$$

assuming hosts and donors were random samples respectively. In F209, donor and host pedigrees refer to FS-families. In the upper pure donor model (2), observations where the reference population acted as hosts were included. In the lower pure host model (3) observations where the reference population acted as donors were included.

Effect of co-ancestry between donor and host

The co ancestry effect has been tested both by its own merit and in combination with the effect of parents:

In F175A: $Y = Repl + Co\text{-}ancestry$ (4)

In F175A: $Y = Repl + Donor\ pedigree + Co\text{-}ancestry$ (5)

In F175B andF209: $Y = Repl + Ha\text{-}type + Co\text{-}ancestry$ (6)

In F175B andF209: $Y = Repl + Ha\text{-}type + Donor\ pedigree + Co\text{-}ancestry$ (7)

Genetic variation in General Resistance (GR) in *Picea abies*

To implement the concept of "general resistance" without regard to the role as donor or host, the effects of individual parents to the identified pairs of donor and host individuals are sought. To obtain this, in trials F175A and F175B a dummy variable for all parents to the actual donor-host pair was introduced. In principle, these variables could then be allocated numerical values 0–4. However, as no selfings were performed in the mating designs (*Table 1* and *2*), in practice for individual parent's only values 0, 1 and 2 were realized. In trial F209 the limited degree of representation restricted us to introduce the full-sib (FS) family in which the clones were selected, i.e. the dummy variables indicated FS-families and not the original parents as in F175A- and B.

Table 5 demonstrates an example of allocation of dummy variables to the dataset with only five parents. The actual models formulated were then:

In F175A inoculated with only one HA-type:

$$Y = Repl + P_1 + \text{------} + P_{14} \quad (8)$$

In F175B inoculated with two HA-types:

$$Y = Repl + HA\text{-}type + P_1 + \text{------} + P_{11} \quad (9)$$

P_i represents parents to the two members of the judged host - donor pair, i.e. 4 parents are represented for each host, namely the two parents to the host itself and the two parents to the donor. Some of these parents may be identical. As F175A and F175B are disconnected, no parents are common in the two experiments. In F209 inoculated by the two HA-types, the model was analog to F175B, but the dummy variables now refer to the 7 FS-families behind the specific donor – host pair.

Table 5. – Demonstration of the method of allocation of dummy variables to 5 parents in trials F175A and F175B.

Donor parents	Host parents	Dummy variables for parents P_1 - P_5					
		P_1	P_2	P_3	P_4	P_5	Total
P_1 X P_2	P_3 X P_5	1	1	1	0	1	4
P_1 X P_2	P_1 X P_4	2	1	0	1	0	4
P_1 X P_2	P_1 X P_2	2	2	0	0	0	4

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Association between statistical tests, parameter-estimates and apparent rot-frequencies in the trials.

In an attempt to demonstrate "statistical significance" to actual frequencies of rot in the field trials, regression between parameter-estimates (model 3) of the genetic units and rot-frequencies was performed.

An alternative approach is to consider the realized rot frequencies as a dose-response situation in "General Resistance", where the degree of representation of each parent in the pedigree of specific donor-host pairs is looked upon as "doses" of individual parents. These values are directly represented in the above-mentioned "dummy" variables in models (8) and (9). In F175B, these doses were represented by values 0–2. Higher doses were not realized, because selfings were not represented in the mating designs.

Results

General level of H. annosum attack

The frequency distribution of trees with rot attack in year 2000 is presented in *Table 6*.

A priori, the best genetic resolution power occurs with attack-frequencies at 50% and with large sample sizes. According to these criteria, trial F175B might be expected to be the most informative and F209 the least informative.

Test of effect of HA-type P versus S

In the first step, the results of model testing is presented for the two alternative response variables, Nomial (–/+ rot) and Ordinal (0–6 scores) scales. The nomial present or absence scale and the semi-quantitative ordinal rot-score scale give comparable results, and this trend applies for all subsequent analyses. For this reason, only the nomial analyses are reported in the following.

Results of these alternative tests according to model (1) in the two trials where both HA-types are compared are shown in *Table 7*, and in *Fig. 3* the actual rot frequencies are illustrated. *Heterobasidion* type is of importance – the S-type is more aggressive than the P-type in the two investigated populations of Norway spruce.

Check of host – donor pairing.

For those 24 hosts, where a double determination of donor identity were available, i.e.

1. HA type-determination of neighboring donor candidates
2. Judgment of most likely neighboring donor candidates based on rot orientation

It was possible to check the correspondence between the two methods. The outcome of this check is shown in *Table 8*. The reasonable correspondence between meth-

Table 6. – Frequencies of attacked trees recorded year 2000, 5 year after inoculation of neighboring stumps during thinning in 1995.

Trial		Occurrence of rot on stump surface year 2000		
		+	–	Total
F175A	Numbers	77	80	157
	%	*49*	*51*	*100*
F175B	Numbers	85	193	278
	%	*31*	*69*	*100*
F209	Numbers	41	117	158
	%	*26*	*74*	*100*

Table 7. – Test of effect of HA-type P versus S according to model (1).

Trial F175B – Including observations on reference population.

Model	Response Nomial (- / + rot)			Response Ordinal (0-6 scores)		
	d.f.	R^2	P-level	d.f.	R^2	P-level
Whole model N = 278	6	0,06	0,0012	6	0,04	0,0005
Components:						
*Replications *)*	5		0,056	5		0,041
HA-type	1		0,0004	1		0,0002

*) 6 replications superimposed on the original 30 blocks.

F209 - Including observations on reference population.

Model	Response Nomial (- / + rot)			Response Ordinal (0-6 scores)		
	d.f.	R^2	P-level	d.f.	R^2	P-level
Whole model N = 156	2	0,07	0,0021	2	0,04	0,0044
Components:						
*Replications *)*	1		0,009	1		0,056
HA-type	1		0,024	1		0,008

*) 2 replications superimposed on the original 12 blocks.

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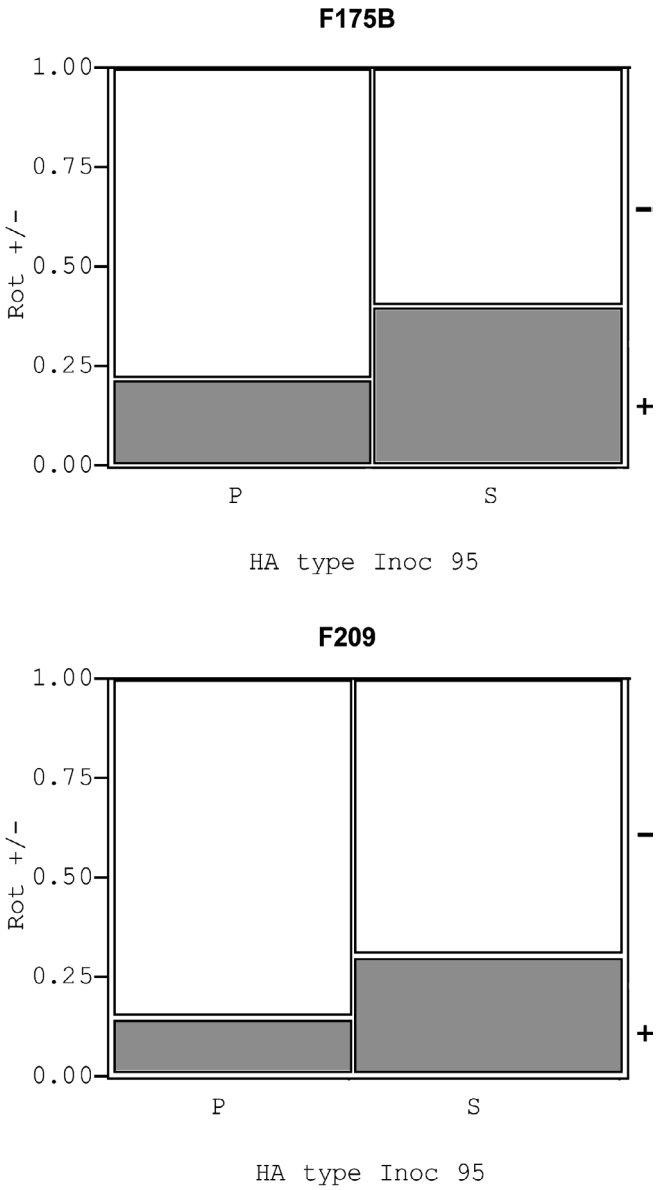

Figure 3. – Year 2000 distribution of frequencies of rot caused by the two *H. annosum* types P and S inoculated 1995 on neighboring stumps in trials F175B and F209.

ods justified the described procedure of accepting the host - donor pairing according to rot orientation alone in the remaining material. Further, based on this result, it was assumed in case of no attack could be interpreted that the nearest tree in position B was the most likely donor of the failed opportunity of attack.

Separate tests of pedigree-effects on resistance of donor or host

Table 9 shows the outcome of the separate test of genetic effects of parents on rot frequencies in hosts in progeny trials F175A and F175B. In the clonal test F209, the genetic effect of the full-sib family in which the progeny clones were sampled is shown.

The genetic effects are separated into the two roles as estimated donor to the host and the host itself. Of the ten tests, three show significant effects: Female parents as donor and male parents as host in F175B, and FS-families as host in F209. Female parents of host had effect at the 10% significance level in F175A.

Tests of pedigree-effects on "General Resistance" (GR)

Table 10 shows the results from the three trials concerning apparent genetic variation in "general resistance" covering the accumulated effect of the two roles, donor and host. Of the five tests, one shows significance close to the 1% level: Male parents in F175B. As above, female parents as host had effect at the 10% significance level in F175A.

The overall picture of *Table 9* and *10* confirms that F175B is the most informative trial.

Table 11 shows the detailed result of the logistic regression model (9) for GR with dummy variables for female and male parents.

Test of effect of co-ancestry between donor and host

The co-ancestry effect has been tested both by its own merit (model 4 and 6) and in combination with the effect of donor pedigree (models 5 and 7), i.e. reflecting effect of co-ancestry when the fungus moves from donor to host through root-to-root contacts or grafts. No traces of

Table 8. – Correspondence between host – donor pairing (i.e. donor determination for each host)) based on i) *Heterobasidion* type determination and ii) orientation of rot area on host stumps in trials F175B and F209. Position A – B – C refers to *fig 1b*). Numbers indicate number of cases among the 24 possible.

i) Heterobasidion type ii) Rot orientation	Position A	Position B	Position C	Total
Position A	1	0	0	0
Position B	2	15	1	18
Position C	0	3	3	5
Total	3	18	3	24

A χ^2 LR-test with 1 d.f. of no correspondence between i) and ii) is rejected at the P=0,01 level.

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effects of co-ancestry were detected in the available material (results not shown).

Association between statistical tests, parameter-estimates and apparent rot-frequencies in the trials.

Fig. 4 shows the regression between parameter estimates of 8 male host parents (model (3) and simple rot-frequencies expressed in the trial F175B. R2 = 0, 90, i.e. 90% of the variance of male host rot percentage is explained by the parameter estimates in model (3).

Based on the idea of "General resistance" covering both roles as donor and host *Fig. 5* shows a dose – response curve of apparent effect on rot frequencies of inclusion of the most resistant male parent in the real-

Table 9. – Overall results from the three trials concerning apparent genetic variation in resistance as dying donor stump or living host tree. In each cell model number (N) and significans level shown.

Effects		F175A	F175B	F209
Donor	Female parents	(2) / NS	(2+HA-type) / **	
Donor	Male parents	(2) / NS	(2+HA-type) / NS	
Donor	FS-families			(2+HA-type) / NS
Host	Female parents	(3) / (*)	(3+HA-type) / NS	
Host	Male parents	(3) / NS	(3+HA-type) / *	
Host	FS-families			(3+HA-type) / *

NS: non-significant 10% < P;
(*) significant at 5% < P < 10%; * significant at 1% < P < 5%; ** significant at 0,1% < P < 1%.

Table 10. – Overall results from the three trials concerning apparent genetic variation in "general resistance" covering the accumulated effect of the two roles, donor and host.

Effects	F175A Model (8)	F175B Model (9)	F209 Model (9)
Female parents	(*)	NS	
Male parents	NS	*	
FS-families			NS

NS non-significant 10% < P; (*) significant at 5% < P < 10%; * significant at 1% < P < 5%.

Table 11. – F175B. Test of pedigree effect on "General Resistance" according to the logistic regression model (9) and the mating scheme in *Table 2.*

Components in Model	N	R^2	d.f.	Wald X^2	Param Estimate	Std Error	X^2- test P-level
Whole Model	186	0,173	15	40,72			0,0004
Replications			5	12,49			0,029
HA-type Inoc			1	8,42			0,004
Female Parents			*2*	*2,624*			*≈0,20*
V1161			1	1,491	+0,67	0,55	0,222
V49			1	1,133	+0,33	0,31	0,287
V1367			0	-	Zeroed	-	-
Male Parents:			*7*	*17,182*			*≈0,011*
V6704			1	7,093	-1,84	0,69	0,008
V6676			1	3,280	-1,17	0,65	0,070
V6675			1	3,658	-1,26	0,66	0,056
V6775			1	0,052	+0,17	0,75	0,819
Åshøj36			1	1,760	-1,05	0,79	0,185
Åshøj22			1	0,782	-0,68	0,77	0,377
Åshøj41			1	0,557	-0,47	0,63	0,456
Haslv Or70			0	-	Zeroed	-	-

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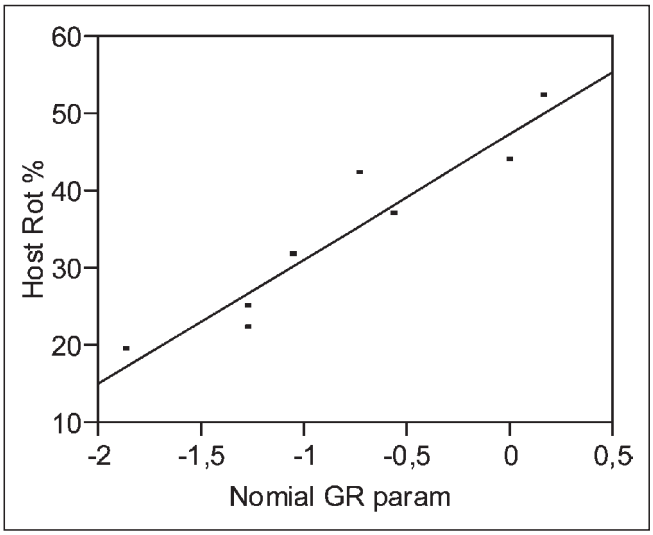

Figure 4. – Regression between host parameter-estimates of General Resistance (GR) of the 8 male parents (model 3 incl. HA-types) and simple average rot-frequencies expressed in trial F175B. $R^2 = 0{,}90$.

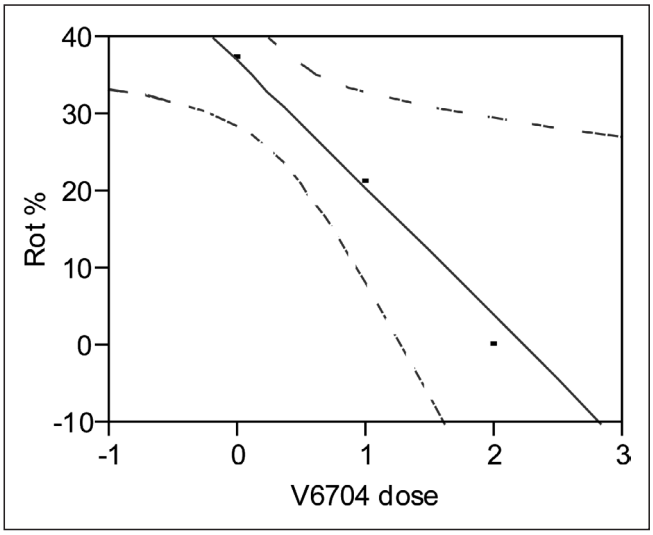

Figure 5. – Based on the General Resistance (GR) concept in model (9) in F175B, the figure shows a dose-response regression of inclusion of most resistant male parent in three successive doses 0, 1 and 2 in the controlled crosses behind the host-donor pair. Weight factors behind the three levels are 131, 52 and 3 respectively, reflecting observations behind. The regression is significant at the 5% level. 95% confidence interval marked.

ized controlled crosses in F175B. Although the confidence level is broad and the number of observations for double representation of the most resistant parent is only 3, linearity might be suggested.

Discussion

Experimental set-up

In relation to the experimental set-up various aspects may be discussed. The stand chosen represented a fairly typical Norway spruce plantation on agricultural land in Denmark. It is important to use unthinned, 1st generation stands in order to avoid the risk of *Heterobasidion annosum* or other root decay fungi such as *Armillaria ostoya* (Romagn.) Herink being present beforehand. Almost no discolorations and no active rot were observed on the inoculated stumps during inoculation and other decay fungi were only found on the host trees in very few cases. Often the discolorations and non-*H. annosum* rot found could be traced to Pilodyn penetrations. It was concluded that all *H. annosum* rot found in host trees originated from the stump inoculation five years earlier.

For the study of transfer from stumps to living trees, high infection success on stumps is essential. It was judged in 1995, that the direct and abundant application of conidia represented a much higher level of inoculum than normal spontaneous fall-out of basidiospores in Denmark. The highest rate of natural infection observed in Denmark was 44% (YDE-ANDERSEN, 1962), and often less than 20% infection success on untreated stumps was achieved during many years of stump treatment trials in the 1970'ies and 1980'ies (M. EGEBJERG PEDERSEN, pers. comm.). In addition, no sources of basidiospores were present in the vicinity, because the nearest forest stand was more than 3 km away. Thus the necessary infection success could best be achieved by artificial inoculation. The evaluation of stump infection in F175A after six months confirmed that a high infection rate had been achieved (and in fact even some of the treated stumps had been infected (THOMSEN, 2003)). The investigation may be close to a 'worst possible' scenario, such as was seen in a Swedish experiment, where almost 88% infection of stumps occurred via natural inoculation with basidiospores (BERGLUND *et al.,* 2005).

The choice of using conidia instead of collecting fresh basidiospores was mainly made for practical reasons, as it is much easier to produce the necessary amounts of spore suspensions from mycelia on artificial media. When using basidiospores the resulting primary mycelia must first undergo plasmogamy to form heterokaryons that may then compete for the stump. In our experiment, the conidia used represented heterokaryons of known S or P types, which had already proven their ability to infect standing trees, as they had been isolated from recently felled, living trees with pronounced decay. The fact that 88% of *H. annosum* inoculated stumps in F175A and 34% percent of neighboring trees became infected demonstrates the aptitude of the used isolates. KUHLMANN and HENDRIX (1964) concluded that infections originating from conidia had less long term survival and growth rate than those caused by basidiospores, but offered no explanations as to why this should be the case. It is our judgment, that it is not possible to draw any conclusions concerning differences in infection ability of the two types of spores.

The time period from inoculation of stumps till felling of neighboring host trees was quite short, i.e. only five years. Thus, it could be argued that a rot free stump surface did not represent a lack of transfer to the host, but only that the rot had not yet reached the above ground part of the tree. However, the amount of stump surfaces with rot and especially the rot height found in many host trees gave convincing evidence that *H. anno-*

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sum had had enough time to cross the distance via growth from the inoculated stumps and through host root systems to the basis of the stem. The growth rate necessary for the horizontal transfer from tree-to-tree centres at stump height was 52 cm pr. year (THOMSEN, 2003). This is in accordance with other studies (BENDZ-HELLGREN *et al.,* 1999; STENLID and REDFERN, 1998). No attempts were made to excavate stumps to check whether there was in fact rot further out in the root systems, as the cost and time needed was prohibitive compared to funds available for the project. However, the main reason for the high annual losses caused by *H. annosum* in European forests is the stem rot, which degrades timber value (WOODWARD *et al.,* 1998). Even if the observed resistance to *H. annosum* only amounts to a slower arrival of the fungus in the stem, this might still be worth pursuing.

The use of one S and one P type isolate may raise the question of whether this is enough to test the resistance and to distinguish between the two types. Resistance against necrotrophs such as root rot pathogens is usually partial and based on the cumulative effects of several genes (LINDHOUT, 2002). Thus, it is likely that any resistance expressed against one isolate of *H. annosum* s.l. would also function against other isolates of the same type and even against both types (now species) of the fungus. Our detection of genetic variation in the most informative trial F175B where both P and S types were inoculated yielded significant genetic effects of HA-types, and on the spruce side both as donor and host as well as in the accumulated effect as "general resistance." This result of our experiment was in accordance with earlier studies showing that S type is more aggressive towards Norway spruce than the P-type (VASILIAUSKAS and STENLID, 1998).

Separate donor and host roles and importance of co-ancestry

In the most informative trial F175B, the pattern of genetic variation seems to differ depending on which role the spruce genotypes act (*Table 9*). As donors, progeny of the three female parents differ. As living hosts, there is no significant difference between the progeny of the same female parents. The opposite pattern occurs for the set of eight male parents. They do not differ in their role as donors, but differ as hosts. This might be interpreted that at least partially different resistance mechanisms are acting in the two roles.

In the dying donor stump, the following stages have to be completed before contact occurs with neighboring potential host roots:

1. The conidia germinate on the fresh-cut stump.
2. The mycelium continue penetration of the newly cut stump into the root system.
3. Infected roots from the dying stump are in contact with neighboring spruces.

Chemistry might influence the first two stages. Stage 3 must be dependant on spacing, in our case the distance between host and donor centres is 2 m if the donor is in the B-position and 2,5 m for donors in the A or C position (*Fig. 1*). Further root architecture of both donor and host may be a factor. Genetics might influence stage 1 and 2, to a lesser degree stage 3.

After contact is established with a potential host, active resistance mechanisms may play a role, again in multiple stages:

4. Has a direct grafting already taken place when the donor was still alive, infection might easily pass from donor to host.
5. If no grafting has occurred, a more active penetration must be performed overcoming an active defence mechanism in the root cambium of the host.
6. Further penetration of host through living roots up to the base of the stem and further up.
7. A second attack from sapwood into the living cambium may kill the host.

If closer co-ancestries than our material represents are analyzed, effects might be detectable. Therefore, our material does not contribute much to this potential important resistance mechanism that may be relevant in clonal forestry. In a Swedish clone trial it has been observed, that after thinning in mono-clone plots, some stumps remained alive apparently because neighboring ramets of the same clone through root graftings kept the stumps supplied with water and nutrition (Bo Karlsson, personal communication).

As our experiment is empirical, and we have not dissected the actual root contacts, we cannot conclude which particular mechanisms have been decisive. However, according to our results the mechanisms seems at least partial different in the dying donor and the living hosts. The most obvious difference is an active defence in the root cambium, which is only expressed in the living host. This mechanism may very well be under genetic control.

General resistance

For the practical forester and breeder it might be relevant to investigate the even more empirical theory that selection for "general resistance" is possible. The concept then ignores the distinction between roles as donors and hosts. Again, the most informative trial F175B delivers evidence that this might be possible to pursue in practice. In *Fig 4.* is shown obtainable genetic gains expressed as decreases in rot frequencies 5 year after unprotected stump exposure to spore inoculation. The situation in *Fig. 5* reflects the effect of crossing a relative resistant parent into a wood productive population not previously selected for HA resistance.

Evidence from other published field trials

KARLSSON and SWEDJEMARK (2003) reported genetic variation in success of stump inoculation with conidia among 50 15-year-old cutting-propagated clones in Norway spruce. In the 60–70 cm high inoculated stumps 98% were infected two months later.

Apparently, only a few clones expressed "resistance" at this level. SWEDJEMARK and KARLSSON (2006) recorded in a 20-year-old clonal test with 50 clones a spontaneous attack frequency of 47% among felled trees coupled with a significant clonal variation.

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Conclusion

Based on the cited Swedish and Danish empirical evidence, it is concluded that genetic variation in resistance in Norway spruce against *Heterobasidion annosum* is expressed under field condition in southern Scandinavia.

Concerning the applied inoculation method, the experimental set-up represents a useful concept for screening existing genetic trials for field resistance within a time scale of 5–6 years. The method might be extended with excavation of actual root contacts.

Concerning genetics of *Heterobasidion* earlier evidence, that the S-type is more aggressive than the P-type in Norway spruce, is confirmed. No trace of non-additive genetic pathogen x host interaction is recorded. This evidence combined with the general experience that resistance against root rot pathogens usually is partial and based on the cumulative effects of several genes, suggests that sustainable genetic gains in relative resistance may be obtainable in breeding programs of Norway spruce.

Concerning resistance mechanisms a hypothesis is put forward, that two partially different mechanisms are acting, one unspecified in the inner heart- and sapwood and another more active in association with the cambium actively defending against penetration from root contact and defending the cambium from infection from already infestated inner heart- and sapwood.

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Microsatellites Reveal Clonal Growth and Genetically Distinct Groups in *Cryptocarya chinensis* in Fragmented Lower Subtropical Forest, China

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Abstract

The lower subtropical monsoon evergreen broadleaved forest in South China (about 22° ~ 24° N) has a high conservation status, because of its uniqueness and high biodiversity. During the last few decades, most of these forests have been destroyed, and the remaining are being degraded by fragmentation. However, genetic information concerning the effects of fragmentation is currently lacking for plant species in these forests. In this study, therefore, eight microsatellites were used to study six *Cryptocaya chinensis* fragmented populations in Guangdong Province South China, and the results revealed a complex pattern of genetic variation within and among *C. chinensis* populations. Firstly, genetic variations demonstrate hitherto undetected clonal growth in *C. chinensis*. Secondly, current population structure of *C. chinensis* reflects an interaction between ancient homogeneous level of genetic variation and contemporary bottleneck via fragmentation. Small populations maintain substantial genetic variation of the initial populations through clonal growth, and do not show genetic depauperation compared to larger populations. Finally, two genetically distinct groups (West and Middle-East groups) are found in this area, connected by highly mixed contact zone.

Key words: clonal growth, *Cryptocarya chinensis*, habitat fragmentation, microsatellite, genetically distinct groups, contact zone, genetic diversity.

Introduction

The fragmentation of continuous habitat into smaller patches is a world-wide phenomenon. Its effects have been discussed and studied comprehensively (HOBBS and YATES, 2003; HONNAY et al., 2005; REED, 2004). Fragmentation leads to restricted gene flow among populations, leading to loss of alleles, increased inbreeding and genetic drift within populations (HAMRICK and GODT, 1996; YOUNG et al., 1996), and eventually influence species long-term persistence (FRANKHAM, 2005). Sometimes, however, fragmented populations may experience normal or even enhanced gene flow and do not suffer from genetic erosion (YOUNG et al., 1996, references therein, WHITE et al., 2002). Clonal growth is another way that may mitigate the adverse effect of habitat frag-

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