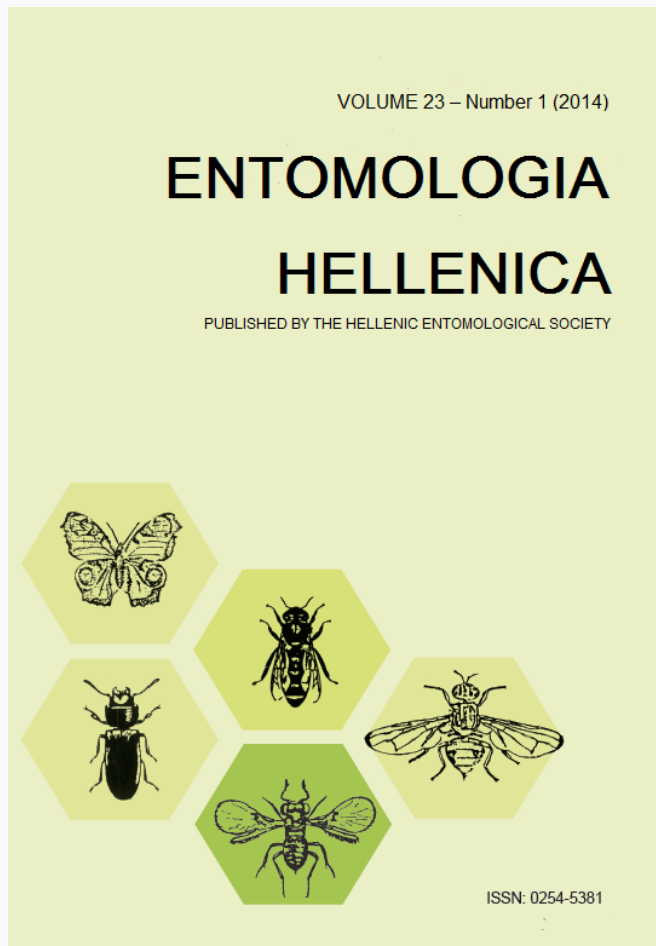


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Αναπαραγωγική ασυμβατότητα μεταξύ γενετικά διαφοροποιημένων πληθυσμών *Tetranychus urticae* από διαφορετικά φυτά-ξενιστές

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Reproductive incompatibility between genetically differentiated populations of *Tetranychus urticae* from different host plants

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ABSTRACT

The study of host plant adaptation in arthropods, and especially agricultural pests, presents great interest, as it relates to patterns of population isolation and differentiation, with effects ranging from gene flow restriction to speciation. Prompted by our earlier isoenzyme studies that revealed genetic differentiation of *Tetranychus urticae* (Koch) collected on citrus, compared to other host plants, we investigated crossing compatibility between *T. urticae* collected from citrus (lemon) trees (ELCI) and *T. urticae* collected from the weed *Mercurialis annua* (ELMA), in the same citrus grove. Crossing compatibility in haplodiploid species where unfertilised eggs develop into males, like *T. urticae*, is assessed based on both the number and the sex ratio of the offspring. When ELMA females were crossed with ELCI males, fecundity was not affected, however the sex ratio was significantly biased towards males (16.6 % females, compared to 66% in the control cross ELMAxELMA). In the reciprocal crosses (ELCIxELMA), fecundity was lower by 30% and the proportion of female offspring was reduced to 52% from 67 %, compared to the control. The fecundity of the F1 hybrid females was significantly reduced and the eggs they laid were less viable, compared to the non-hybrids, further reducing the reproductive potential of inter-strain crosses. Combined with previous data, these results suggest the existence of a citrus-associated *T. urticae* host race.

KEY WORDS: host plant, reproductive incompatibility, *Tetranychus urticae*, two-spotted spider mite.

Introduction

The two-spotted spider mite *Tetranychus urticae* (Koch) is one of the most important agricultural pests globally, infesting a wide range of plant families including economically important crops, ranging from field and greenhouse vegetables and ornamentals to fruit trees and vines. It has been recorded on over 900 plant species, in 478 genera, across 124 families (Bolland et al. 1998). However, such extreme polyphagy at the species level may be harbouring

several specialised, genetically differentiated populations adapted on different hosts, as is often the case with generalist feeders (Fox and Morrow 1981, Thompson 1994). Indeed, at the population level some degree of specialisation has been found in *T. urticae*, as evidenced by the observation of genetically differentiated populations associated with particular host species (reviewed in Magalhães et al. 2007).

Host plant adaptation relates to patterns of population differentiation with effects ranging from gene flow restriction to

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reproductive isolation and speciation. The formation of host races, i.e. of populations partly reproductively isolated due to adaptation on different hosts (Diehl and Bush 1984), is an intermediate stage in this process and has in fact often been implicated to support the plausibility of sympatric speciation (Drès and Mallet 2002). Host race formation, which reduces variation at the population level and increases it at the species level, is not uncommon in the Acari, given their low dispersal ability, strong association with their hosts, host-associated mating, high reproductive rates and short generation times (Magalhães et al. 2007).

The potential for host adaptation has been investigated in selection experiments. The evidence shows that *T. urticae* populations adapt very rapidly to initially unfavourable hosts, in only a few generations (Gould 1979, Fry 1990, Agrawal 2000, Magalhães et al. 2007), although not much evidence exists that artificial selection on different hosts results in reproductive isolation (Fry 1999). In natural populations of *T. urticae*, some studies have revealed host race formation, some have failed to and some show mixed results. In two studies involving *T. urticae* sampled in open fields in Greece and greenhouses in France, Tsagkarakou et al. (1997, 1999) found no plant-associated population genetic differentiation. Gotoh et al. (1993) used allozyme electrophoretic analysis on two strains of the spider mite, one from cucumber and one from tomato, to show that the two strains represent distinct host races. Moreover, mites from the two host races showed host preference for their plant of origin, while mate preference to females of the same host race was also reported: these conditions can maintain the genetic isolation between the two strains. In a preliminary population genetics study based on AFLPs, Weeks et al. (2000) yielded evidence for genetic differentiation between populations collected in the same small area, on two

different plants, also suggesting host race formation.

Navajas et al. (2000) report that populations of *T. urticae* from the rose bay *Nerium oleander* (L.) are clearly genetically differentiated from samples collected on other plants. However, this pattern was only detected in the western Mediterranean area, while in the eastern regions of the basin there was no such evidence. Further investigation of the differentiated populations, showed partial to complete reproductive incompatibility with a strain from another host plant (bean), reflecting what could be a process toward speciation. In an allozyme polymorphism study involving 27 *T. urticae* populations across southern Greece, Tsagkarakou et al. (1998) found that even though genetic differentiation was generally correlated to geographic distance, samples collected from citrus trees at different locations grouped closely together, separately from samples from all other host plants, even those from the same field. More recently, Aguilar-Fenollosa et al. (2011) found evidence for host-associated genetic differentiation between *T. urticae* from clementine trees and from the weed *Festuca arundinacea* (Schreb), along with reduced fitness of the former population when reared on the latter plant. A larger-scale study, based on COI sequence polymorphism and involving *T. urticae* populations from several countries and a variety of host plants, showed that most mites collected on citrus fell in the same clade but did not form a monophyletic group, not providing strong support for the existence of a 'citrus mite race' (Navajas et al. 1998).

It is possible that the detection of host race differentiation is prone to temporal effects. This appears to be the case in the related species *T. kaznawai* (Kishida), where the levels of population differentiation, estimated with microsatellite markers, varied depending on sampling season, reflecting

gene flow fluctuations (Nishimura et al. 2005). If this observation can be generalised, temporal variation should be taken into account in experimental design, as well as data analysis and interpretation. Therefore, population differentiation is likely to be transient and operate at a local level. Also, different patterns emerge from the application of different markers, possibly because they operate at different time scales (Navajas et al. 1998). Another hypothesis that can be formulated is that *T. urticae* may be more likely to form host races on plants that - like citrus and rose bay - produce increased levels of toxic chemicals, exerting high selection pressure on the herbivore, and are long-lived, providing more opportunity for a stable association (Magalhães et al. 2007).

The study of the host plant-associated population structure of a pest like *T. urticae* has important implications for pesticide resistance management, and knowledge on gene flow between populations from different host plants should be incorporated in predictions about the fate of resistance genes. Resistance is more likely to develop within an isolated population, where it is less susceptible to “dilution” by wild alleles, but will have less potential to spread to other populations, due to limited gene flow. In this context, we set out to further explore the nature of the population differentiation observed in our earlier studies. The aim was to investigate whether the evidence for genetic differentiation of the *T. urticae* populations from citrus (Tsagkarakou et al. 1998) is associated with any degree of reproductive incompatibility with other populations. For this purpose, we used a scheme of reciprocal crosses between a field population of *T. urticae* collected on lemon trees in a citrus grove in the Peloponnese (one of those included in the study by Tsagkarakou et al. 1998), along with another from annual weeds in the same field.

Materials and Methods

Mites

Two hundred individuals of both sexes were collected from 15 lemon trees, and 200 individuals from 5 *M. annua* plants, in the same field in the area of Elaionas, in North Peloponnese, one of the main citrus growing areas in Greece. The mites were used to initiate two laboratory colonies, ELCI and ELMA, from the lemon trees and from *M. annua*, respectively.

The colonies were maintained on bean leaves, for 5 generations before the start of the experiment, in a controlled-environment chamber, at 25°C, 16:8 h L:D and 60-70 % humidity. Each collection was maintained at >1000 individuals, in order to avoid bottlenecks and maintain genetic variation.

Several females from each field collection were preserved in 95% ethanol, for DNA analysis. A few males were also preserved in 70% ethanol for morphological identification. Species identification was based on both morphological and molecular data. The examination of different morphological characters, mainly the shape of the terminal knob of the aedeagus of *T. urticae*, was used for the identification of the species (Pritchard and Baker 1955). In addition, 10 females from each collection were subjected to species identification by a PCR-RFLP diagnostic test. This test is based on the ITS sequence differences between morphologically close tetranychid species (Hurtado et al. 2008).

Crossings

Reciprocal crosses were performed between females and males from ELMA and ELCI. Crosses between individuals of the same strain served as controls. Virgin females at the teleiochrysalis stage were isolated and individually placed in leaf-disc ‘arenas’ together with the respective males, in order to ensure controlled mating. Each arena consisting of a small pinto-bean leaf disc (~ 4 cm diameter), placed on a sponge and surrounded by a cotton-wool barrier,

maintained wet by keeping the sponge in a tray with water.

The unmated female in the arena was left with two young virgin adult males (1-2 days old) from the same or a different strain for 48 h. The virgin males were produced as a cohort by groups of unmated females isolated as teleiochrysalis. Males were then removed from the arena and females were allowed to lay eggs for 5 days. The fecundity (number of eggs laid), egg mortality (proportion of unhatched eggs) and progeny sex ratio (proportion of females) were recorded for each cross. Given that in the arrhenotokous species, like *T. urticae*, females arise from fertilised eggs it is important to record the sex ratio of the progeny when studying reproductive compatibility.

Reproductive incompatibility is most commonly expressed in the haplodiploid *T. urticae* as low viability of, particularly the unfertilised (male), eggs laid by F1 females (De Boer 1985). We therefore isolated one to five F1 unmated female offspring from each of the above crosses (except, of course, the ones that had produced only males), set each of them on a fresh leaf disc and recorded the number of eggs she laid in 10 days, as well as the proportion of those that hatched. Females that did not lay any eggs within this time were regarded as sterile. All crosses were performed at 25°C.

Statistical analysis

Data were analysed with one-way analysis of variance (ANOVA) and means were compared using the Tukey-HSD test. To normalise the data, log transformation was used for the number eggs laid per female and arcsin-transformed values were used in analysis of proportions (sex ratio and mortality).

Results

Survival of progeny

The results of the crossing experiment are shown in Table 1. ELCI females produced

less eggs in the 5-day period allowed for oviposition when they were crossed to males from the ELMA strain (29.6 eggs) rather than their own (45 eggs) and this difference was statistically significant. ELMA females did not show the same pattern, as fecundity was not different in inter- or intra-strain crosses (35.9 and 38.9 eggs, respectively). Average egg mortality ranged from 0.8 to 12.1% but the differences between inter- and intra-strain crosses were not significant.

Sex ratio in *T. urticae* is female biased a pattern also found in the control crosses (Table 1). The proportion of females in the F1 progeny was significantly lower in both inter-strain crosses, compared to the respective intra-strain ones, although this difference was much higher between the ELMAxELCI and the ELMAxELMA crosses than between ELCIxELMA and ELCIxELCI. Females from the ELMA strain produced a progeny consisting of 16.6 % females, when mated to ELCI males, compared to the 65.6% female progeny of ELMA females mated to males of the same strain. Females from the ELCI strain produced a 52.4% female progeny when mated to ELMA males, compared to 67% when mated to ELCI males - a lesser difference but still statistically significant.

The fecundity of unmated female F1 offspring from all types of crosses was also assessed and it was found that many hybrid females did not produce any eggs and when they did these were fewer and were less viable, compared to those laid by non-hybrid females (Table 2). The proportion of sterile F1 females from the ELMAxELCI cross was 25% and from the ELCIxELMA cross it was 41%, compared to 5.3 and 2.8% for the respective intra-strain crosses. The number of eggs laid in 10 days by ELMAxELCI hybrid F1 females was 26% lower compared to ELMAxELMA non-hybrids. This difference was even higher for ELCIxELMA hybrid females, which produced 54% less eggs than the daughters of the ELCI intra-strain crosses. The average mortality among

the eggs of the ELMAxELCI and ELCIxELMA hybrid F1 females was much higher (67 and 79%, respectively) compared to non-hybrids (17 and 29%, respectively).

Overall, all parameters considered point to the same direction: the crosses between the two strains produce less offspring than those between mites of the same strain. In order to quantify this effect, and by taking into account fecundity, egg mortality and sex ratio (Table 1), we can calculate how many F1 females were on average produced per P

(parental) female; if the fecundity and egg viability of the unmated F1 female progeny (Table 2) is added to the equation we can project the total reduction in reproductive potential suffered by females mated to males from the other strain rather than their own (Table 3). This reduction is remarkably high and very similar between ELMA and ELCI females: 95.1 and 95.3%, respectively: a dramatic effect in only two generations, albeit loosely estimated.

TABLE 1. Fecundity, egg mortality and sex ratio of intra- and inter-strain crosses.

cross		N	fecundity ± SE	Tukey HSD test #	% egg mortality ± SE	sex ratio: % ♀ offspring ± SE	Tukey HSD test #
♀	x ♂						
ELMA	x ELCI	18	35.9 ± 3.1	ab	9.7 ± 4.0	16.6 ± 4.6	a
ELMA	x ELMA	23	38.9 ± 2.6	ab	0.8 ± 0.6	65.6 ± 4.5	bc
ELCI	x ELMA	20	29.6 ± 2.2	a	4.8 ± 2.3	52.4 ± 3.4	b
ELCI	x ELCI	25	45.0 ± 3.2	b	12.1 ± 4.4	67.0 ± 2.7	c
one-way ANOVA			P = 0.025		P = 0.06		P < 0.001

cross: description of the crossing scheme, referring to the strain (ELMA or ELCI) of the female (♀) and the male (♂) parent; N: number of females crossed per crossing scheme; fecundity: the average number of eggs laid per female in 5 days; % egg mortality: the percentage of eggs that did not hatch and were thus considered unviable; sex ratio: the percentage of female offspring produced on average from each cross; SE: standard error; #: means followed by different letters are significantly different according to the Tukey HSD test (P < 0.05).

TABLE 2. Average fecundity of unmated F1 females and associated egg mortality.

parents		N	% sterile	unmated F1 fecundity * ± SE	Tukey HSD test #	% egg mortality ± SE	Tukey HSD test #
♀	x ♂						
ELMA	x ELCI	28	25.0	40.71 ± 5.6	a	67.43 ± 5.3	a
ELMA	x ELMA	38	5.3	54.92 ± 6.6	b	17.18 ± 6.9	b
ELCI	x ELMA	78	41.0	28.73 ± 5.7	c	78.94 ± 8.6	a
ELCI	x ELCI	72	2.8	62.25 ± 3.4	d	29.26 ± 7.5	b
one-way ANOVA				P < 0.0001		P < 0.0001	

parents: description of the parents of the unmated females, referring to the strain (ELMA or ELCI) of the mother (♀) and father (♂); N: number of unmated F1 females tested; % sterile: percentage of unmated females that had laid no eggs after 10 days unmated F1 fecundity: average number of eggs laid by unmated females in 10 days; *: only fecund females considered

(sterile females excluded); % egg mortality: the percentage of eggs that did not hatch and were thus considered unviable; SE: standard error; #: means followed by different letters are significantly different according to the Tukey HSD test ($P < 0.05$).

TABLE 3. Projected reproductive potential of inter- compared to intra-strain crosses.

cross			total F1 ♀ / P ♀	♀ offspring reduction	total F1 fecundity	total fecundity reduction
♀	x	♂				
ELMA	x	ELCI	5.4	78.7%	53.5	95.1%
ELMA	x	ELMA	25.3		1090.4	
ELCI	x	ELMA	14.8	44.3%	52.7	95.4%
ELCI	x	ELCI	26.5		1134.3	

cross: description of the crossing scheme, referring to the strain (ELMA or ELCI) of the female (♀) and the male (♂) parent; total F1 ♀ / P ♀: the average number of female offspring (F1 ♀) produced per mother (P ♀) for each crossing scheme; ♀ offspring reduction: the average percent reduction of female offspring per mother, when she was mated to a male of the other rather than her own strain; total F1 fecundity: the average estimated number of F2 eggs (grandchildren) produced per parental female; total fecundity reduction: the average percent reduction of F2 offspring (grandchildren) per parental female, when she was mated to a male of the other rather than her own strain.

Discussion

In order to understand the population differentiation identified in a previous study (Tsagkarakou et al. 1998), we investigated the reproductive barriers between *T. urticae* from lemon trees and from the weed *M. annua*, by considering different parameters to assess the success of reciprocal crosses. It is clearly emerging from our data that the reproductive potential of both *T. urticae* strains is greatly reduced when these strains are intercrossed. This effect was demonstrated mainly as reduced fecundity in hybrid crosses involving citrus-collected (ELCI) females and male-biased sex ratio in crosses involving weed-collected (ELMA) females. Both these effects are known expressions of reproductive incompatibility in arrhenotokous spider mites: embryos either die or develop into males. In addition, reproductive

incompatibility is most commonly expressed in the haplodiploid *T. urticae* as low viability of eggs laid by F1 females, particularly the unfertilised (male) eggs (De Boer 1985). We therefore used this parameter as an indication of reproductive intra-strain fertility. In both crosses, the female hybrids displayed reduced fecundity and an increase percentage of egg mortality, compared to F1 females produced from control crosses.

Similar results have been obtained in a study by Gotoh et al. (1993), where the tomato- and cucumber-associated *T. urticae* strains, besides the genetic differentiation in terms of allozyme polymorphism, also showed reduced inter-strain fertility, demonstrated as reduced proportion of females among the progeny of inter-strain crosses and higher mortality of hybrid eggs. Osakabe and Komazaki (1996), also evidenced reproductive barriers between

Panonychus citri spider mites from citrus and *Osmanthus* hosts, which were differentiated by allozyme analysis, as well as each developing at a lower rate on plants other than their host of origin. Combined with these findings, the reproductive incompatibility evidenced in our crossing experiments between populations associated with different hosts, provides an indication of host race formation processes.

Reproductive isolation could be the long-term outcome of population differentiation, as a by-product of independent evolution of strains that remain isolated for any reason, including specialisation on a particular host plant. However, other factors may operate as reproductive barriers, most characteristically the effects of endosymbiotic reproductive parasites like *Wolbachia* or *Cardinium*, that have been shown to infect a wide range of arthropods, including *T. urticae*. In such cases, reproductive incompatibility may be the direct consequence of infection with a symbiont and may in turn lead to population differentiation and perhaps eventually speciation (Wade 2001).

Endosymbiotic bacteria with variable effects on reproductive incompatibility are known to occur in *T. urticae* (Vala et al. 2000, 2002; Perrot-Minot et al. 2000, Gotoh et al. 2007a, 2007b). Preliminary PCR tests for the presence of the endosymbiont *Cardinium* (primers from Weeks et al. 2003) produced different results for the two strains used in the present study: mites collected on *M. annua* did carry the bacterium, while those collected on citrus did not. This result could form the basis for further investigation of the potential implication of this reproductive parasite in the incompatibility observed.

In order to better understand the nature, the origin and the effects of the reproductive incompatibility revealed by our study, it is important to investigate the role of endosymbionts and perform further

experiments assessing, for example, mate choice and potential pre-zygotic barriers in inter-strain mating or performance on different hosts and associated fitness costs. The investigation of host plant associations will greatly benefit from the recent advances on *T. urticae* genome enabling comparative transcriptome studies (Grbić et al. 2011).

If the incompatibility between strains can be explained by the action of endosymbionts, the question remains open on the role of the plant host. There are, however, cases where symbionts are known to be playing a role in herbivore-plant interactions, reflecting the high complexity of interspecific associations. For example, Tsuchida et al. (2004) report that an endosymbiont of *Acyrtosiphon pisum* infecting populations of the aphid on white clover at a much higher rate than those on vetch, also appears to be increasing the fitness of the aphid on white clover (but not on vetch), providing evidence for a possible role of symbionts in host-plant specialisation. In this context, it could even be hypothesised that host plant chemistry, besides driving population differentiation, may be affecting the composition of the symbiotic flora of the spider mites in our study, which in turn affects the outcome of inter-strain crosses.

The findings so far, however, strongly indicate that citrus- and weed-associated *T. urticae* show a highly reduced inter-strain reproductive potential which, in the light of the previous population genetic findings (Tsagkarakou et al. 1998) suggest that *T. urticae* from lemon trees forms distinct host race.

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Αναπαραγωγική ασυμβατότητα μεταξύ γενετικά διαφοροποιημένων πληθυσμών *Tetranychus urticae* από διαφορετικά φυτά-ξενιστές

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ΠΕΡΙΛΗΨΗ

Η μελέτη της προσαρμογής των εχθρών των καλλιεργούμενων φυτών στα φυτά ξενιστές τους παρουσιάζει μεγάλο ενδιαφέρον, καθώς σχετίζεται με την απομόνωση και γενετική διαφοροποίηση των πληθυσμών, με αποτελέσματα που ποικίλλουν από τον περιορισμό της γονιδιακής ροής μέχρι και το σχηματισμό νέων ειδών. Με έναυσμα προηγούμενες μελέτες μας με ισοένζυμα, που κατέδειξαν γενετική διαφοροποίηση μεταξύ πληθυσμών *Tetranychus urticae* (Koch) από εσπεριδοειδή σε σχέση με άλλους ξενιστές, μελετήσαμε την συμβατότητα των διασταυρώσεων μεταξύ *T. urticae* που συλλέχθηκε σε λεμονιές (ELCI) και *T. urticae* που συλλέχθηκε από το φυτό *Mercurialis annua* (ELMA) στο ίδιο χωράφι. Η συμβατότητα των διασταυρώσεων όταν πρόκειται για απλοδιπλοειδές είδος, όπου τα αρσενικά προέρχονται από αγονιμοποίητα ωά, μελετάται λαμβάνοντας υπόψη τον αριθμό των απογόνων αλλά και την αναλογία φύλου μεταξύ τους. Η γονιμότητα των θηλυκών ELMA δεν επηρεάστηκε όταν διασταυρώθηκαν με αρσενικά ELCI, όμως η αναλογία θηλυκών : αρσενικών ήταν σημαντικά μειωμένη (16,6% θηλυκά, σε σύγκριση με 66% στην διασταύρωση αναφοράς ELMA x ELMA). Όταν τα θηλυκά ELCI διασταυρώθηκαν με αρσενικά ELMA, η γονιμότητα ήταν κατά 30% μειωμένη και το ποσοστό των θηλυκών απογόνων μειώθηκε από 67% σε 52%, σε σχέση με τη διασταύρωσης αναφοράς (ELCI x ELCI). Τα F1 θηλυκά που προέκυψαν από διασταυρώσεις θηλυκών και αρσενικών από διαφορετικούς πληθυσμούς γέννησαν λιγότερα ωά, που είχαν μεγαλύτερη θνησιμότητα, σε σχέση με τα F1 θηλυκά προερχόμενα από ενδο πληθυσμιακές διασταυρώσεις, μειώνοντας επιπλέον το δυναμικό αναπαραγωγής μεταξύ των πληθυσμών.