The use of trace element analysis for determining the larval host plant of adult Rhagoletis (Diptera: Tephritidae)

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Summary
The limitations of traditional ecological genetic methods have made it difficult to obtain precise and reliable information about gene flow and host preference in Rhagoletis flies, which is of major importance to a proposed mechanism of sympatric speciation via host race formation. A new application of trace element analysis using neutron activation techniques has been developed in order to identify the larval host plant of wild adult insects captured in the field. Statistically significant differences in the concentration of several elements were observed between collections of insects sampled from the fruit of different species of sympatrically occurring host plants.

Résumé
Les méthodes écologiques traditionnelles n'ont pas fourni des données précises et sûres sur la distribution des gènes à travers les populations et le choix d'hôtes chez le Rhagoletis, données qui sont nécessaires pour expliquer le mécanisme proposé de spéciation sympatrique résultant de la création de races d'hôte. On a donc développé une nouvelle application de l'analyse des traces d'éléments en utilisant les techniques d'activation des neutrons, afin d'identifier la plante-hôte larvaire d'insectes sauvages pris dans la nature. On a noté des différences significatives de concentration de plusieurs éléments dans plusieurs groupes d'insectes pris dans différentes espèces de plantes-hôtes sympatiques.

1. INTRODUCTION
The observation of host shifts by frugivorous flies in the genus Rhagoletis from their native North American host plants to introduced apples and cherries represents one of the major examples in support of the process of sympatric speciation via host race formation (7-9, 13). R. pomonella shifted from native Crataegus fruit to introduced apples about 120 years ago (53) and to domestic sour cherry (Prunus cerasus) about 40 years ago (43). It has been proposed that these newly formed populations, in the process of adapting to the particular biochemistry and physiological properties of their host fruit, may develop reproductive isolation while coexisting sympatrically without the influence of any geographic barriers to gene flow or isolation by distance. R. fausta has undergone similar recent host shifts from its native pin cherry (Prunus pensylvanica) to domestic sour cherry, and therefore has also been considered as another possible example of the type of host race formation which, under the right conditions, may lead to sympatric speciation (7, 13). Understanding this process of host race formation and its many ramifications

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is of major importance, not only for efforts aimed at the elucidation of speciation mechanisms, but also for the improvement of our ability to manage a wide variety of harmful and beneficial insects in agricultural systems (16, 20).

One important aspect of this proposed mechanism of speciation is the development of reproductive isolation among the sympatric host races through a divergence in host preference behavior (9, 10). Since mating in these flies occurs on or near the host plant (8, 15, 42, 46), as is typical of many parasitic species (41), a consistent change in host preference results in a simultaneous change in mate preference and a reduction in gene flow between populations which utilize different hosts. Therefore, it is crucial to know if flies which fed on one species or variety of host fruit as larvae consistently return to this host as adults for mating and oviposition. Furthermore, the degree of fidelity of a female insect in the deposition of her eggs preferentially on the host which she herself has fed on as a larva (whether the result of host preference genes or some form of conditioning (26, 43)) is of critical importance in determining if a given intensity of selection acting on the female's progeny will be sufficient to bring about genetic divergence of the populations on the different hosts (10, 17, 31).

If it were possible to determine the species of larval host fruit which had been used by adult Rhagoletis naturally foraging in the field, the question of fidelity of host preference and the consequent gene flow between host races could be addressed directly. It is logistically feasible to sample large numbers of flies from wild populations of Rhagoletis on their host plants in the field (15). Flies may be aspirated while in the act of mating, ovipositing, or while engaged in other behaviors. Recently, there has been an expansion in the application of trace element analysis, utilizing methods of atomic excitation spectroscopy, to a wide variety of problems in the biological sciences (11, 23, 24). In particular, several studies have demonstrated that the geographic source of origin of dispersing animals can be determined by comparing the concentrations of naturally-occurring trace elements in salmon (12), snow geese (28), and a number of insect species including examples in the Homoptera (30), Coleoptera (32, 33, 31, 32), Lepidoptera (5, 34) and Diptera (35). We therefore tested whether the larval host plant of adult Rhagoletis flies at a single sympatric locality could be determined by examining naturally-occurring concentrations of trace elements.

2. METHODS

Trace element concentrations were determined by neutron activation analysis, which involves the production of radioactive isotopes in the sample material by neutron bombardment and the subsequent detection by gamma spectroscopy of decay emissions across a spectrum of energy intensities. Since each element decays by giving off emissions of characteristic intensities, distinct peaks within the spectrum are attributable to the concentrations of particular elements present in the sample. Each fly was individually wrapped in mylar film which did not emit substantial radiation in any of the same peak areas as the fly material. Samples were irradiated for 30 minutes at 290 kW (a flux of 3 x 10^12 n/cm^2/sec). Individual flies were counted upon removal at times of 5-20 minutes and again at 24 hours after irradiation. A Canberra Ge(Li) detector, ND2200 analyser, and NOVA 800 computer were used to accumulate the data and calculate total peak areas of selected gamma radiation peaks (30).

R. pomonella were collected as larvae in infested naturally apple and sour cherry fruit and R. fausta were collected from pin cherry and sour cherry in Door County, Wisconsin during the summer of 1979. These fruit were held in trays over moist vermiculite where the larvae pupated after completing their development within the fruit. The pupae obtained from these collections were stored in moist vermiculite in petri dishes and were chilled for over 3 months, which is necessary to terminate pupal diapause (4), and were then warmed to stimulate adult eclosion in the laboratory. These flies of known larval host origin were held for one to several
weeks in laboratory cages on a standard diet of sucrose, enzymatic yeast hydrolysate, and well water before being frozen in liquid nitrogen for later analysis. At least two weeks prior to neutron activation analysis, all specimens were removed from the liquid nitrogen, permitted to thoroughly dry in open air, and then were weighed.

3. RESULTS

Quantitative analysis of the results indicated that four isotopes (Na24, Mn56, Cl38, and K42) were consistently present in large enough amounts to be detectable against background emission (Fig. 1). Note that some elements emit radiation at several energy intensities due to alternative isotope decay paths. Quantitative analysis was performed on the peaks which exhibited the clearest resolution and the lowest background interference (Mn56, 846.8 KeV; Na24, 1368.5 KeV; Cl38, 1642.0 KeV; K42, 1324.7 KeV). Of these elements, Mn, Na, and Cl were present in dramatically different concentrations in R. pomonella flies which were collected as larvae from different host fruit at a sympatric locality (Table I). Even the small sample of R. fausta also showed a pronounced differentiation of Mn and Na concentrations (Table II).

![Gamma emission spectrum after activation by neutron bombardment for an individual R. pomonella female collected from sour cherry.](image-url)

Figure 1: Gamma emission spectrum after activation by neutron bombardment for an individual R. pomonella female collected from sour cherry.
Table I. Means and standard errors of elemental peak areas per mg for *R.* pomonella flies collected as larvae in the host fruit and analysed as adults after eclosion in laboratory cages and storage in liquid nitrogen. The unit of measurement is the number of radioactive decay counts accumulated under each elemental peak, summed by integration under the peak after subtraction of background emission. Peak areas are standardized for differences among samples in the amount of time which elapsed between removal from the neutron source and counting in the detector to correct for the effects of exponential decay. A non-parametric statistic was used to compare the means because the variances in Mn peak areas were found to be unequal. Since no significant differences were observed between the sexes, males and females were combined in our analysis.

<table>
<thead>
<tr>
<th></th>
<th>Mn$^{56}$</th>
<th>Na$^{24}$</th>
<th>Cl$^{38}$</th>
<th>K$^{42}$</th>
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</thead>
<tbody>
<tr>
<td>Cherry flies</td>
<td>158 ± 8.8</td>
<td>208 ± 13.3</td>
<td>113 ± 4.5</td>
<td>21 ± 3.1</td>
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<tr>
<td>(n = 6)</td>
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<tr>
<td>Apple flies</td>
<td>234 ± 33.1</td>
<td>78 ± 10.8</td>
<td>41 ± 11.7</td>
<td>29 ± 2.3</td>
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<tr>
<td>(n = 7)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
<td>(n = 8)</td>
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<tr>
<td>Mann-Whitney (two-tailed test)</td>
<td>U = 36.0</td>
<td>U = 48.0</td>
<td>U = 48.0</td>
<td>U = 10.0</td>
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<tr>
<td></td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.1 n.s.</td>
</tr>
</tbody>
</table>

Table II. Means and standard errors of elemental peak areas per mg for *B.* fausta flies collected as larvae in the host fruit and analysed as adults after eclosion in laboratory cages and storage in liquid nitrogen with units as in Table I. The means were compared using a parametric t-test because no differences in sample variances were observed.

<table>
<thead>
<tr>
<th></th>
<th>Mn$^{56}$</th>
<th>Na$^{24}$</th>
<th>Cl$^{38}$</th>
<th>K$^{42}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sour cherry flies (n = 3)</td>
<td>103 ± 12.8</td>
<td>68 ± 35.9</td>
<td>73 ± 36.8</td>
<td>30 ± 8.5</td>
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<tr>
<td>Pin cherry flies (n = 3)</td>
<td>233 ± 21.7</td>
<td>296 ± 21.6</td>
<td>148 ± 35.9</td>
<td>35 ± 6.9</td>
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<tr>
<td>t = 5.17, 4df</td>
<td>t = 5.43, 4df</td>
<td>t = 1.45, 4df</td>
<td>t = 0.43, 4df</td>
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<tr>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.25 n.s.</td>
<td>p &lt; 0.7 n.s.</td>
<td></td>
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</table>
4. DISCUSSION

Recent criticism of this proposed example of sympatric speciation has emphasized the importance of documenting host preference behavior (18, 25, 39). Allozyme surveys may be of limited use in resolving this controversy for several reasons. Since these host shifts have occurred in recent historical times, the divergence in gene frequencies may be too small to detect, even if complete reproductive isolation exists between these host races (1). Alternatively, if significant allele frequency differences are observed, it is not possible to positively attribute the differences to either reproductive isolation with genetic drift of neutral loci or natural selection acting on different parts of a freely interbreeding population. Another possible approach would be to directly assay host preference and mate selection behavior in controlled studies in the laboratory or field. However, such experiments are often difficult to conduct under conditions resembling the natural environment of many organisms including Rhagoletis flies. Therefore, these over-controlled experiments may suggest conclusions in discord with the actual behaviors exhibited by the animals when undisturbed in nature and are thus suspect (14, 38).

Another method which should in theory enable us to arrive at a more definitive answer to these questions is the technique of mark-recapture such as used to distinguish the effects of gene flow and spatially heterogeneous selection in Drosophila persimilis (49). Insects of known larval host origin could be marked, released, and then recaptured in the act of mating or oviposition, thus providing a direct estimate of the amount of gene flow between sympatric populations on different host plants. Such necessarily large-scale dispersal studies have already been conducted within a single host race of several economically important Tephritid flies (2, 37). Unfortunately, due to the large local population sizes of these insects and because the number of recaptures tends to be very small, mark-recapture projects would necessarily involve a very substantial investment of time and resources. Furthermore, the validity of such a study could still be limited by problems of toxicity, behavioral changes, and loss of the mark (47, 51).

Ideally, what is needed is some form of naturally-occurring mark which would, in essence, pre-label each wild adult fly with a "chemoprint" such that the identity of its larval host plant could be determined. One method of identifying the larval host plant of adult Heliothis zea utilized a comparison of fatty acid composition (assayed by gas chromatography) of host plant tissue to that in adult insects reared from known hosts (6). The results of this study suggested that such an approach might be feasible. It has also been demonstrated in a study designed to examine other questions that phenols in the diet of the tree locust become bound in the cuticle (3). In addition, scent organ development and pheromone production depend on the ingestion of pyrrolizidine alkaloids which naturally occur at high concentrations in a subset of the larval host plants of Creatonotus moths (44). These findings indicate that larval nutritional differences between different host plant species can influence the chemical properties of adult insect tissue. The large differences in trace element concentrations observed in the present study between the laboratory-reared adult Rhagoletis collected from different larval host plants suggest that this method might provide a means to determine the larval host plant of wild adults in the field with a high degree of certainty.

It remains to be established whether these observed differences associated with use of a particular larval host plant will persist despite possible contamination due to adult feeding in the field (4) as some change in trace element composition over time has been previously reported (27, 33, 31). Research is currently in progress which will investigate a number of additional procedures which are available that may eliminate any possible distortion due to adult feeding (15). First, instead of examining the absolute concentration of each element, the relative amounts of two elements can be compared as a ratio which may exhibit greater stability. This approach was utilized, for example, in the separation of sibling
species within the Anopheles gambiae complex by a method of chemotaxonomy based on comparing differences in the ratio of cuticular paraffins (13). Second, if trace element concentrations are affected by differences in gut contents of the wild-caught and laboratory-reared flies due to different adult food sources in the field and laboratory, this problem may be resolved either by starving all flies or by feeding the wild-caught insects on the laboratory diet for several days before freezing for later analysis. Furthermore, it is known that different parts of the insect’s body differ in trace element concentrations (15, 52). Therefore, it may be possible to obtain more consistent results by analysing only a portion of the body, preferably a heavily sclerotized region of the cuticle, which might be expected to change less in trace element concentration after adult eclosion than softer tissues such as muscle or haemolymph. Lastly, we are currently exploring other techniques of atomic excitation such as x-ray energy spectroscopy. It has been reported that this method is capable of assessing up to 50 different elements in the red turnip beetle (31) although caution should be exercised in order to positively distinguish between activity due to rare elements present at concentrations near the threshold limits of the detection apparatus and background “noise” (32).

It may be considered surprising that the concentrations of these particular elements are subject to influences of the larval environment. Since they are of such crucial importance to a wide variety of metabolic processes, Na, K, Cl, and Mn might be expected to be buffered against change in concentration by homeostatic mechanisms. It is therefore essential for future work to clarify, by a series of controlled cross-rearings, whether the differences observed are due to a passive “imprinting” of the organism by its larval fruit environment, as we assume, rather than due to genetic differences in metabolism between flies collected from the different hosts. The R. fausta samples were obtained from sour cherries which were naturally infested several weeks earlier in the season than the R. pomonella sample as normally occurs (15). At this time the fruit look and taste distinctly different to a human observer and they may also differ in their trace element profile. Without additional data, this confounding factor makes it impossible to determine whether any of the differences in the chemoprints of R. fausta and R. pomonella might be due to genetic differences between the species as opposed to environmental imprinting. Previous studies of insect trace elements suggest a strong environmental influence (27, 35, 51). Similarly, the recently developed method of identifying bacterial strains by mass-spectrometry is significantly influenced by the growth medium employed (21, 22). This is in contrast to the results of another new technique of chemotaxonomy which is based on a comparison of cuticular paraffins in mosquitoes (13) where the results appear to be based on the detection of genetic differences among the organisms.

The use of trace element analysis shows considerable promise for permitting the determination of the larval host plant of wild-caught adult holometabolous insects. Furthermore, some of our specimens used in preliminary trials not reported here were dried in open vials for over a year, yet still gave consistent results. Thus it may not be necessary to freeze samples prior to analysis which would considerably facilitate the application of this technique in a field setting. This result also suggests the possibility that larval host plants might be determined from pinned museum specimens, thus opening up access to a wealth of new ecological information about insect-plant interactions over long periods of time. Such information would greatly enhance our ability to investigate evolutionary questions concerning the interactions of insects and their host plants, as well as improve our understanding of host relations and the status of biotypes in a wide range of harmful and beneficial species (16) including many examples in the Homoptera (36), Coleoptera (48), Diptera (19), Lepidoptera (40), and Hymenoptera (20, 29).
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