Pattern of ecological shifts in the diversification of Hawaiian *Drosophila* inferred from a molecular phylogeny

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Background: The endemic Hawaiian drosophilids, a unique group that are remarkable for their diversity and rapid proliferation, provide a model for analysis of the process of insular speciation. Founder events and accompanying random drift, together with shifts in sexual selection, appear to explain the dramatic divergence in male morphology and mating behaviour among these flies, but these forces do not account for their spectacular ecological diversification into a wide array of breeding niches. Although recognized as contributing to the success of this group, the precise role of adaptive shifts has not been well defined.

Results: To delineate the pattern of ecological diversification in the evolution of Hawaiian *Drosophila*, we generated a molecular phylogeny, using nucleotide sequences from the yolk protein gene *Yp1*, of 42 endemic Hawaiian and 5 continental species. By mapping ecological characters onto this phylogeny, we demonstrate that monophagy is the primitive condition, and that decaying leaves were the initial substrate for oviposition and larval development. Shifts to decaying stems, bark and tree fluxes followed in more derived species. By plotting female reproductive strategies, as reflected in ovarian developmental type, on the molecular tree, we also demonstrate a phylogenetic trend toward increasing fecundity. We find some statistical support for correlations between ecological shifts and shifts in female reproductive strategies.

Conclusions: Because of the short branches at the base of the phylogram, which lead to ecologically diverse lineages, we conclude that much of the adaptive radiation into alternate breeding substrates occurred rapidly, early in the group's evolution in Hawaii. Furthermore, we conclude that this ecological divergence and the correlated changes in ovarian patterns that adapt species to their ecological habitats were contributing factors in the major phyletic branching within the Hawaiian drosophilid fauna.

Background

Speciation on isolated oceanic islands is often rapid and accompanied by a phenomenal degree of adaptive radiation, generating diverse products quite unlike continental forms and posing questions about the mechanisms of insular speciation. More than 35 years ago, Zimmerman [1] drew attention to the remarkable Hawaiian drosophilid fauna, and issued a challenge to geneticists and evolutionists to explain the fact that some 25 % of the world's species in the genus *Drosophila* were endemic to the six major islands of the Hawaiian archipelago. The representation of this group in Hawaii by about a thousand species is clearly disproportionate to the total land area of these volcanic islands (only 16 576 km²), their remoteness and their geological youth (0.4–5.1 – million years [2]).

A multidisciplinary effort to explain the rampant speciation of these drosophilids [3,4] initially focused on the morphology and systematics of the flies [5], their ecology [6], behaviour [7], internal anatomy [8], cytology as revealed by karyotypic analyses [9], and on comparisons of banding patterns in the giant polytene chromosomes [10] — Carson's seminal approach to their phylogenetic analysis. These early studies amply confirmed initial impressions of the diversity and uniqueness of the Hawaiian *Drosophila* and of their value to evolutionary biology; these impressions have been reinforced by the research of more recent decades and by the implementation of molecular approaches [11]. This remarkable group of organisms is now well recognized as a model for the study of the process of insular speciation [12].

Morphologically, these endemic Hawaiian flies are very diverse. Adults range in size from some of the smallest to some of the largest in the genus (wing spans range up to 2 cm), and males display some unusual modifications of the head, mouthparts and legs [5]; these secondary sexual characteristics correlate with remarkably diverse male courtship and mating behaviours [7,13]. Taxonomically, the Hawaiian drosophilids comprise two main genera — *Scaptomyza*, which includes several subgenera, and *Drosophila* (*Idiomyia*, sensu Grimaldi [14]), represented primarily by the subgenus *Drosophila*. The latter genus is the predominant component of the endemic drosophilids, and is divided into two major clusters — the picture-winged flies, which have heavy pigmentation on

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their wings, and the heterogeneous non-picture-winged flies. Within each of these clusters, several species groups have been recognized on the basis of specific male morphological traits, such as modifications of the legs (modified tarsi) or of the proboscis (modified mouthparts) [5]. These groups are further divided into species subgroups, based on even more specific traits, such as a particular set of polytene chromosome inversions [4] or the nature of the male leg modification (split-tarsus, bristle-tarsus and spoon-tarsus subgroups).

Despite this diversity, the endemic Hawaiian drosophilids are cytologically conservative [9,10,15] and molecular divergences are minimal [16–18], confirming the genetic cohesiveness of the group. Their conserved internal anatomy first prompted Throckmorton to suggest that the group derived from one or, at most, two original founders [8]. Because the Hawaiian Islands are so remote from any continental land mass, and inter-island distances are also substantial, founder events are implicated both in the initial colonization of Hawaii and in the sequential spread of drosophilids (and other groups) down the archipelago as new volcanic islands arose and became habitable. Founder events and the accompanying random drift have been inferred to trigger speciation [19,20], and therefore founder effects have been viewed as the major driving force behind insular speciation.

More recently, sexual selection has been identified as another important factor in the speciation of Hawaiian Drosophila, and the interactions between founder events and shifts in sexual selection have been explored [21]. Although the forces of genetic drift and sexual selection may account for the dramatic divergence in male morphology and mating behaviour, they do not account for the ecological divergence which is such a prominent aspect of the adaptive radiation of the Hawaiian Drosophila [6,12]. These saprophagous flies have radiated to use a diverse array of breeding substrates that includes fungi, rotting leaves, flowers, stems, bark and tree fluxes, from some 40 families of endemic Hawaiian plants [6,22,23]. It is unclear whether this spectacular ecological diversification was critical to speciation events or was simply a result of natural selection following speciation, improving adaptation to the respective ecological niches occupied by these flies.

To begin to investigate the role of ecological forces in the diversification of the Hawaiian Drosophila, we have mapped ecological and relevant female reproductive traits on a phylogeny of 5 continental and 42 endemic Hawaiian species, which includes both picture-winged and non-picture-winged flies. This phylogeny was independently reconstructed using nucleotide sequences from a segment of Yp1 [24], one of three genes that encode yolk proteins, and is largely congruent with previous morphological and chromosomal phylogenies [4,15]. Using breeding-substrate data available from field records on the plant family and part of the plant used by 38 of these 42 Hawaiian drosophilid species [6,23], we have used the

parsimony criterion to trace the historical sequence of ecological shifts in the adaptive radiation of this group. The results we report here on the ecological and related reproductive adaptation of these flies [25] suggest that the primary radiation into alternative breeding substrates occurred rapidly, very early in their initial colonization of Hawaii, therefore implicating ecological divergence in the initial phyletic diversification of the group. More recent shifts, such as that to flux breeding, have further contributed to the complex ecological pattern that characterizes the adaptive radiation of the Hawaiian Drosophila.

Results

A molecular phylogeny inferred from a yolk protein gene is congruent with traditional phylogenies

Ovarian maturation and egg development in adult female Drosophila depend upon the expression of many genes. These include the genes that encode the three yolk proteins (also known as vitellogenins [26,27]), which comprise a major portion (40-80 %) of the total egg proteins [28]. The yolk proteins function as storage proteins [29] and also play a role in hormone transport [30]; some regions of these genes may therefore be expected to accumulate non-deleterious changes rapidly, whereas other regions may be quite conserved. The yolk protein genes — Yp1, Yp2 and Yp3 — should therefore be useful for phylogenetic inference over a broad range of divergences.

Using the polymerase chain reaction, we amplified a 1 kb segment of the Yp1 gene — a segment that includes the two introns and 64 % of the coding sequences (278 amino acids) — from the genomic DNA of 42 endemic Hawaiian and four continental Drosophila species [24]. Of the endemic species, 36 were of the well studied picture-winged cluster, with representatives of the five species groups (Primaeva, Adiastola, Planitibia, Glabriapex and Grimshawi), and six species were representative of the non-picture-winged cluster — three species of the Antopocerus group, two species of the modified-mouthparts group and one species of the two-member Anomalipes group. The continental species were members of the Drosophila or Sophophora subgenera and the genus Scaptomyza. The amplified Yp1 gene segments were sequenced, aligned and analyzed with known homologous sequences of D. melanogaster [31]. We observed a high degree of nucleotide conservation in this DNA fragment among the Hawaiian Drosophila species, a reflection of their close genetic affinities.

In order to determine whether these Yp1 sequences were sufficiently informative to infer phylogenetic relationships among the Hawaiian Drosophila, we used three different phylogenetic analyses. The maximum parsimony (MP; [32]) and maximum likelihood (ML; [33]) analyses are character-based, and the neighbour joining analysis (NJ; [34]) is distance-based. The tree topologies that resulted from these analyses were almost identical; the trees derived from the NJ and MP analyses are shown in Figure

1. Our molecular data indicate that all of the Hawaiian *Drosophila* species form a monophyletic group, diverging from the continental species of the subgenus *Drosophila* (*D. virilis*, *D. buzzatii*), whereas the representatives of the subgenus *Sophophora* (*D. birchii*, *D. melanogaster*) resolve as the more basal and primitive group within the genus *Drosophila*. These observations are congruent with phylogenetic hypotheses based on morphological [4,8,35] and

male courtship behavioural data [13], and with sequence analyses (from a smaller number of taxa) of the *Adh* gene [18,36] and of mitochondrial DNA (mtDNA; [37]).

In our *Yp1* gene tree, the placement of the continental species *Scaptomyza adusta* between continental representatives of the subgenus *Drosophila* and the Hawaiian *Drosophila* (nominally also within the subgenus *Drosophila*)
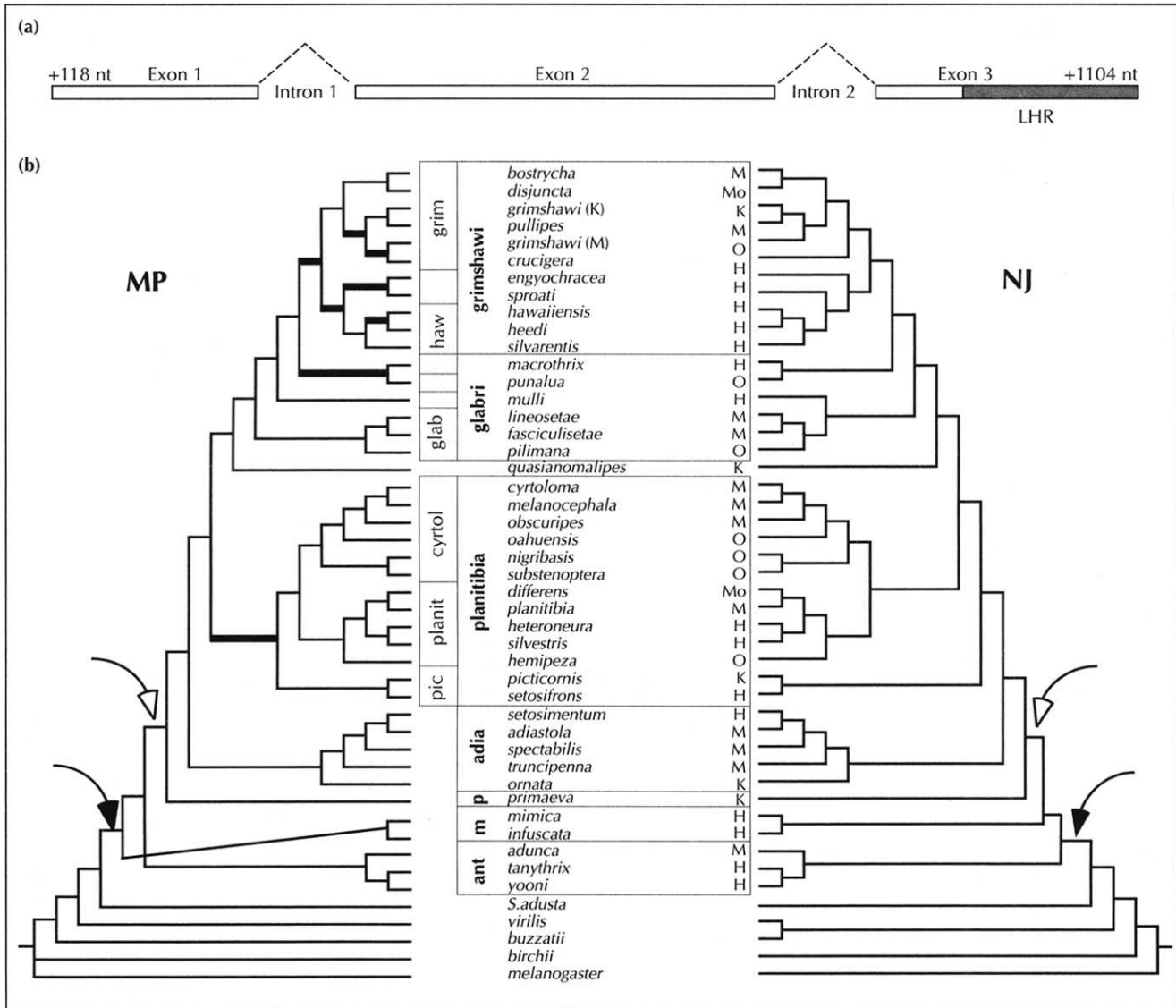

Fig. 1. Phylogenetic analysis of the nucleotide sequences of the *Yp1* gene. **(a)** Organization of the *Yp1* DNA segment that was sequenced, with the nucleotide numbers (nt) that refer to the *D. planitibia* gene [24]. The hormone-binding region (LHR or lipase homologous region [30]) is shown by the shaded block. **(b)** The cladogram to the left (MP) shows one of the 18 equally parsimonious trees (C.I. and R.I. values of 0.594 and 0.701, respectively) generated by the maximum parsimony analysis [32]. The 18 trees differed only in the degree of resolution of the most derived *Grimshawi* group of the picture-wings and in the placement of the *Picticornis* subgroup. The 8 heavier branches (6 in the *Grimshawi*, and one each in the *Glabriapex* and *Planitibia* groups) indicate the branches that collapse in the strict consensus of the 18 equally parsimonious trees. The cladogram to the right (NJ) presents the phylogenetic hypothesis derived using the neighbour joining method of analysis [34]. *D. melanogaster* was used as the outgroup for both analyses in this figure. Filled arrowheads indicate the branching point of the Hawaiian *Drosophila*, and the open arrowheads indicate the branch point for the picture-winged species. Species names are shown between the two cladograms; 46 are members of the genus *Drosophila*, whereas *S. adusta* is a continental member of the genus *Scaptomyza*. The Hawaiian species are boxed according to their morphological classification. Abbreviations for the species groups shown in bold: ant, *Antopocerus*; m, modified-mouthparts; p, *Primaeva*; adia, *Adiastola*; glabri, *Glabriapex*. Abbreviations for the major species subgroups indicated to the left in plain type: pic, *Picticornis*; planit, *Planitibia*; cyrtol, *Cyrtoloma*; glab, *Glabriapex*; haw, *Hawaiiensis*; grim, *Grimshawi*. Letters to the right of species names indicate the island to which each is endemic (listed in order of decreasing geological age): K, Kauai; O, Oahu; M, Maui; Mo, Molokai; H, Hawaii.
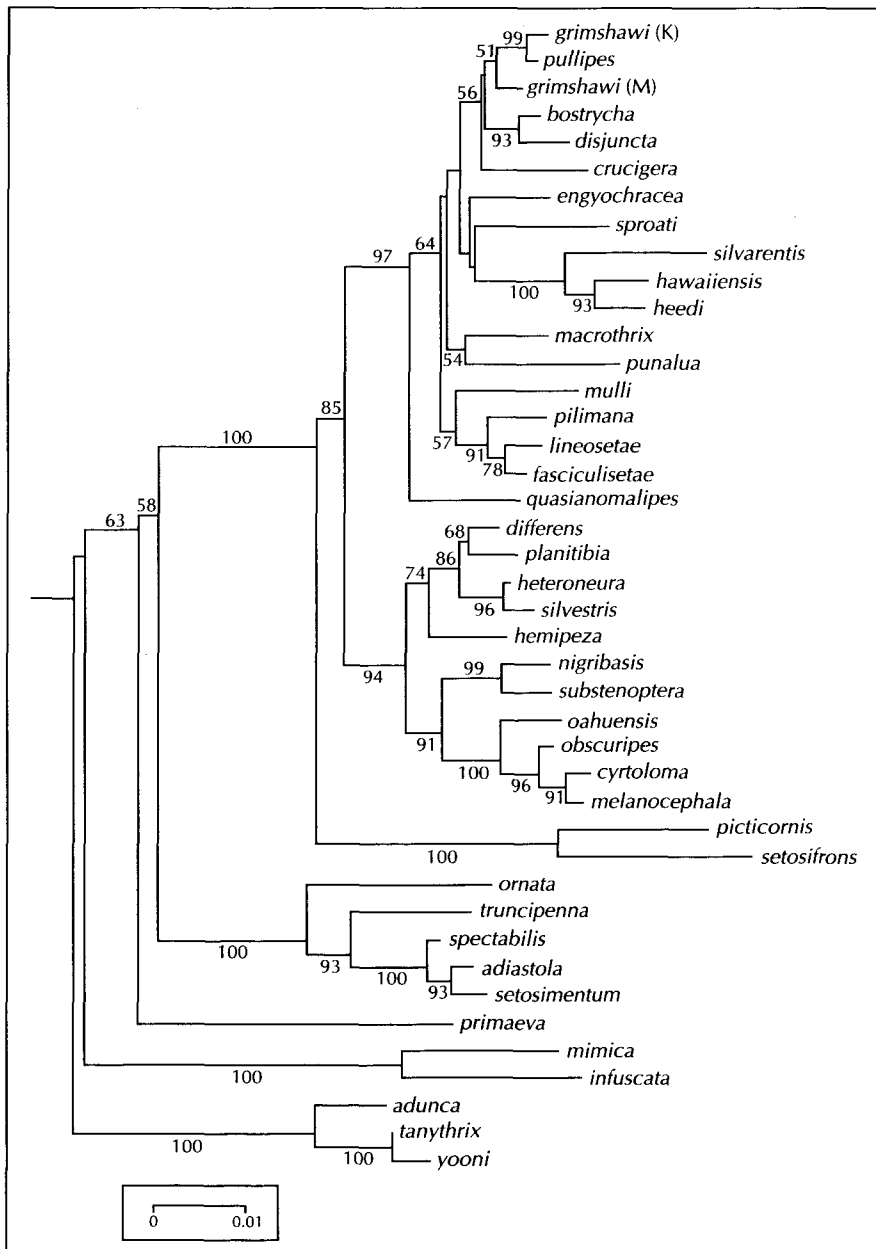
Fig. 2. Phylogram (from NJ analysis) of the Hawaiian *Drosophila* species based on *Yp1* DNA sequences. Branch lengths are drawn to scale, with the scale bar indicating amount of divergence. Numbers on internal branches are bootstrap values from 1000 replications. Bootstrap values below 50 % are not shown. The bootstrap percentage is an indicator of support for the topology determined by that branch. Although the monophyly of species in the *Antopocerus* and the modified-mouthparts clades is strongly supported, the relationship between the groups is not as it varies according to different methods of analysis and strategies of character weighting (data not shown).

Tree tip labels: grimshawi (K), pullipes, grimshawi (M), bostrycha, disjuncta, crucigera, engyochracea, sproati, silvarentis, hawaiiensis, heedi, macrothrix, punalua, mulli, pilimana, lineosetae, fasciculisetae, quasianomalipes, differens, planitibia, heteroneura, silvestris, hemipeza, nigribasis, substenoptera, oahuensis, obscuripes, cyrtoloma, melanocephala, picticornis, setosifrons, ornata, truncipenna, spectabilis, adiastola, setosimentum, primaeva, mimica, infuscata, adunca, tanythrix, yooni

Scale: 0 — 0.01

concurs with the topology of molecular trees based on *Adh* [18,36] and mtDNA [37] sequences. It disagrees, however, with Grimaldi's taxonomic hypothesis based on morphological characters [14], which places the genus *Scaptomyza* at some distance from the Hawaiian *Drosophila* and from continental groups of the subgenus *Drosophila*. Nonetheless, the three independent sets of molecular data are in striking agreement, lending credence to the hypothesis of the origin of the entire Hawaiian drosophilid fauna from a single, rather than two, original introductions to Hawaii [8]. The implication is, therefore, that the genus *Scaptomyza* evolved in Hawaii and then spread to other parts of the world.

The picture-winged flies resolve as a monophyletic group in our *Yp1* phylogeny, with the *Adiastola* clade included as a more primitive picture-winged group; this placement concurs with previous inferences from analyses of

their cytology [4,12,15], morphology [5] and the *Adh* sequence [18]. Such a placement is not congruent, however, with that based on immunological comparisons of a single larval hemolymph protein [38] and that derived from mtDNA sequences [17]; these latter observations place the *Adiastola* clade outside of the picture-winged group.

With the exception of the *Glabriapex* species group, each of the formerly recognized species groups analyzed here — *Antopocerus*, 'modified-mouthparts', *Adiastola*, *Planitibia* and *Grimshawi* — is monophyletic, a result that is congruent with all previous analyses. Although some of the basal nodes of our analysis are not strongly supported (Fig. 2), the low resolution of these nodes is consistent with rapid divergence among these early lineages; this hypothesis is supported by the ecological data (as we discuss below). Not only each species group, but also each

species subgroup analyzed from the picture-wings is monophyletic, in accord with previous interpretations. The only exception is *D. mulli* of the *Orphnopeza* subgroup of the *Grimshawi* species group [4], a controversial species that lacks the wing pigmentation of a typical picture-winged species, and that also has an atypical egg chorion morphology [39].

Unidirectionality of inter-island colonizations
One of the predominant features of the speciation of the Hawaiian *Drosophila* is the central role played by rare inter-island migrants. These are proposed to have been individual gravid females that succeeded in establishing founder populations on new islands [19,20], generally geologically younger islands in the Hawaiian chain. The significance of inter-island migration events is supported by our molecular phylogenetic reconstruction, which demonstrates that the ancestral taxon in each monophyletic group is always found on an older island. Furthermore, the derived taxa show a unidirectional branching pattern from older to younger islands (Kauai to Oahu, then Molokai, Maui and Hawaii) as exemplified by the *Adiastola* and *Planitibia* species groups (Fig. 1).

Back migrations from younger to older islands are not supported among the species analyzed here, except perhaps in the most derived and unresolved *Grimshawi* subgroup. With the analysis of additional species, some back migrations may be found, but these and other molecular data [40] imply that such events are many fewer than suggested by the cytological data [15].

Decaying leaves were the initial breeding substrate in the ecological diversification of Hawaiian *Drosophila*
Ecological divergence refers not only to the variety of endemic Hawaiian plants used by the *Drosophila*, but also to the diversity of substrates from each plant that are used for oviposition and larval development. Table 1 shows that the majority of Hawaiian drosophilid species use a particular decaying part of plants from a single family as their breeding niche (monophagy). A few species can use plants from two to four families (oligophagy), and only a small minority use several families (polyphagy). Of the 114 families of vascular plants represented in Hawaii [41], 40 are known to be used by the endemic drosophilids for breeding. Remarkably, only two of these families, the Araliaceae and Campanulaceae, are the preferred hosts used by 74 % of the 155 species of *Drosophila* analyzed thus far (Table 1). Moreover, from these two families only two genera, *Cheirodendron* (Araliaceae) and *Clermontia* (Campanulaceae), provide the predominant breeding substrates that support the reproduction of 66 % of the *Drosophila* species studied. Once decaying, all parts of these plants — the leaves, stems, bark, flowers, fruits and even aerial roots — are used for oviposition by one drosophilid species or another [6,23].

In an effort to reconstruct the steps in the adaptive radiation of these flies, we have reanalyzed the available ecological data in the context of our molecular phylogeny, as this includes many more Hawaiian species than the available *Adh* or mtDNA phylogenies. Using character reconstruction upon the *Yp1* gene tree, it can

Table 1. Summary of recorded oviposition preferences of endemic Hawaiian *Drosophila* with respect to the use of plant families and specific breeding substrates.

Species group	Ornamented tarsi			*Antopocerus**	Modified mouthparts*	Picture-wings*[†]
	Fork tarsi*	Bristle tarsi*	Spoon tarsi*			
Number of species	11	16	14	11	21	82
% Monophagous	91	94	71	91	71	77
Predominant plant family/genus[‡]						
Family Araliaceae	82	63	79	91	0	37
Genus *Cheirodendron*	82	56	64	91	0	19
Family Campanulaceae	0	0	14	0	52	24
Genus *Clermontia*	0	0	14	0	52	19
Breeding substrates						
Leaves	100	100	100	100	44	6
Stems	0	0	0	0	57	29
Fruits	0	0	0	0	30	7
Flowers	0	0	0	0	13	3
Bark	0	0	0	0	0	35
Flux	0	0	0	0	0	12
Fungi	0	0	0	0	17	2
Others	0	0	0	0	0	6

*Ecological data from Heed [6]. [†]Ecological data from Montgomery [23]. [‡]Percentage of *Drosophila* species from each group using each family and genus. The genus *Cheirodendron* belongs to the Araliaceae family; the genus *Clermontia* belongs to the Campanulaceae family.
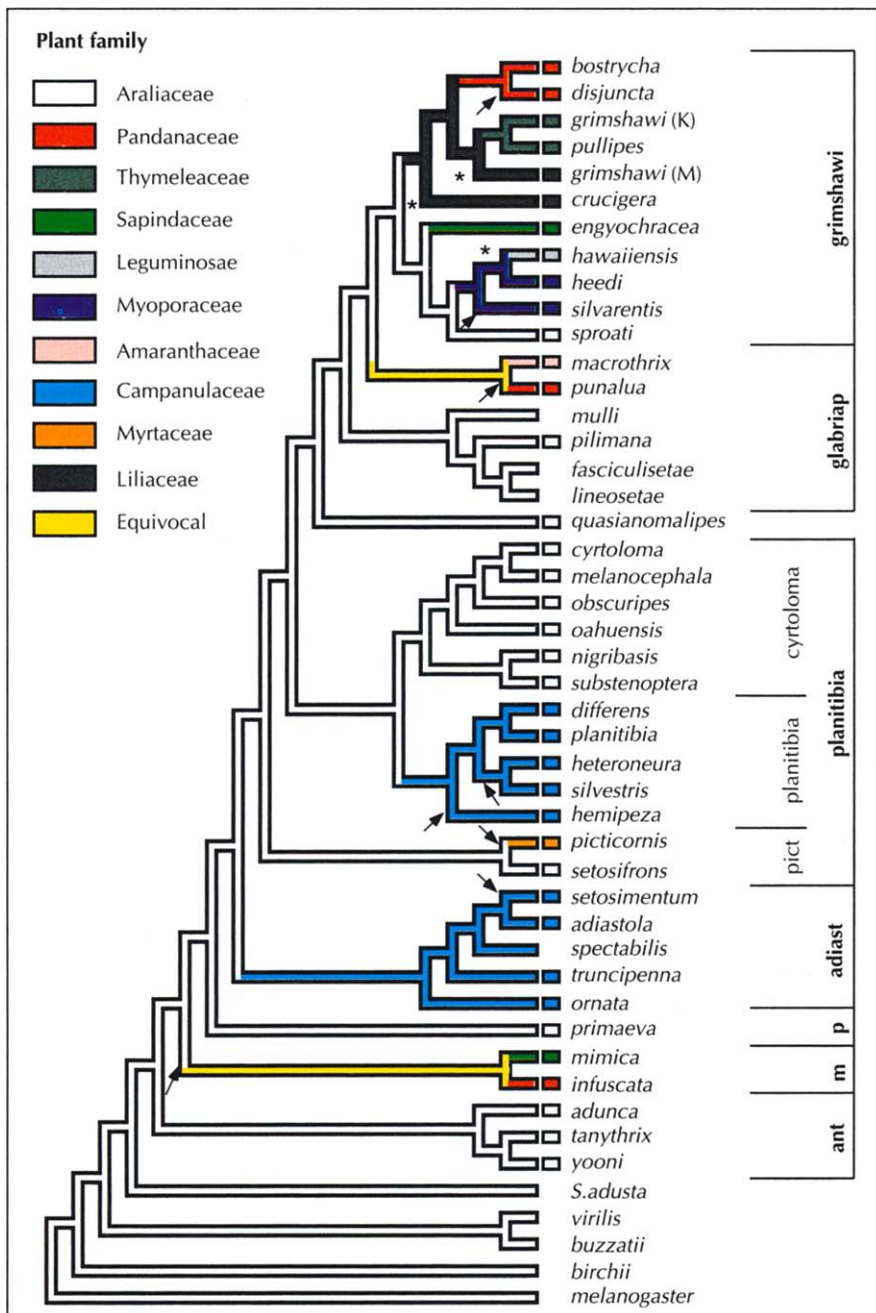
Fig. 3. Ecological character analysis. Using the MacClade program for character reconstruction [49], the plant families used for oviposition by the Hawaiian *Drosophila* species [6,23] were superimposed on the phylogenetic hypothesis generated by the NJ analysis of the *Yp1* DNA sequence data (Fig. 1). Identification of the *Drosophila* species groups shown to the right of the cladogram follows the relationships presented in Figure 1. The majority of the *Drosophila* species analyzed use a single plant family for oviposition (monophagy). A few species (identified by the arrowhead) are oligophagous and use two to four plant families. The rare polyphagous species that use more than four families are identified by an asterisk. For these species, the plant family indicated is that most commonly used. *Drosophila* species for which the breeding substrates are not known lack the small box to the right of the cladogram.

be seen that the most basal Hawaiian clade — the *Antopocerus* species — are monophagous, using plants of the Araliaceae family and decaying leaves as their oviposition substrate (Figs 3,4). Heed's breeding records [6] document the fact that the three ornamented tarsi groups (split-tarsi, bristle-tarsi and spoon-tarsi) are also monophagous leaf miners that primarily use Araliaceae species. Although these groups are not included in our DNA phylogeny, morphological data [8] and molecular data (R. DeSalle, personal communication) resolve them as being among the most primitive Hawaiian *Drosophila*. It might therefore be concluded that the earliest successful *Drosophila* in Hawaiian habitats were monophagous, ovipositing their eggs in the decaying leaves of Araliaceae plants, and that such plants were likely to be of the genus *Cheirodendron*.

Ecological shifts to additional kinds of substrates — decaying stems, fruits, flowers and fungi — appear to have coincided with the evolution of the more derived species of the modified-mouthparts group (Table 1, Figs 3,4). This phase was then followed by the invasion of further novel niches; a shift towards the use of decaying bark appears to have accompanied evolution of the picture-winged species, the most derived group of all, but some picture-wings have further radiated to breed in sap exudates or fluxes.

Divergence in female reproductive strategies and ecological correlates
In Hawaiian drosophilid species, the organization and functioning of the ovaries varies widely among species groups [25]. As shown in Figure 4, the ovarian type maps

relatively cleanly onto the phylogeny, with a phyletic progression from Type II ovaries to Type IIIa, then Type IIIb, and a reversion to Type IIIa in the paraphyletic *Glabriapex* species group. These three ovarian types are distinguished by their number of ovarioles per ovary (few, a moderate number and many, in Types II, IIIa and IIIb, respectively) and by the maximum number of mature eggs per ovariole (one in Types II and IIIa, and several in Type IIIb) [25]. Types IIIa and IIIb are further distinguished by the length of the respiratory filaments relative to the length of the egg (see Fig. 4). Ovarian type is an accurate predictor of female fecundity and reproductive strategy, and is a good phylogenetic character (as demonstrated by Fig. 4). Changes in ovarian structure and functioning must be largely determined at the genetic level, given the complexity of the process of ovarian development which is regulated throughout the life of the fly [26]. The phylogenetic trend in the Hawaiian *Drosophila* is clearly toward increasing fecundity. The question is, what has driven or facilitated this trend?
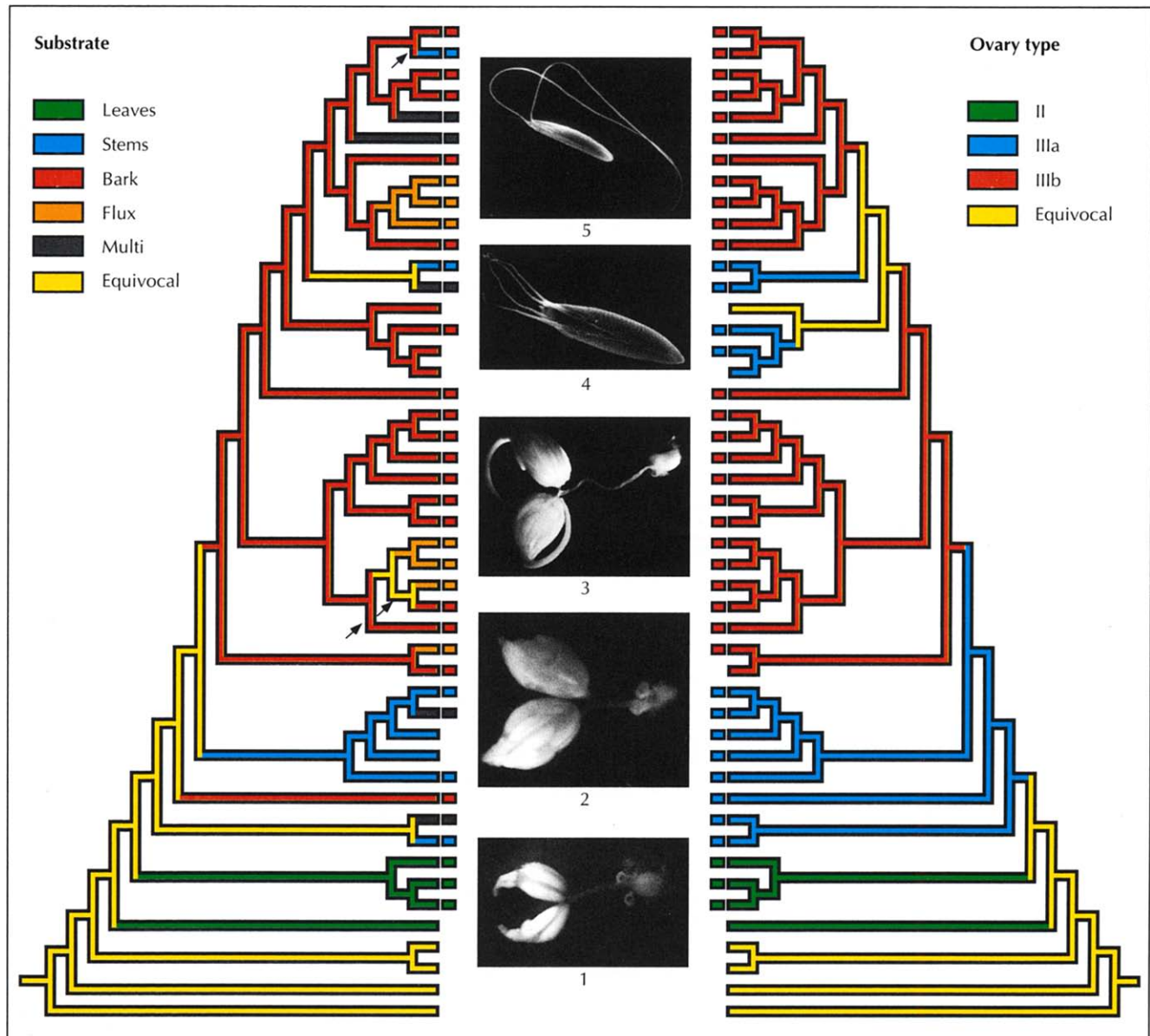

Fig. 4. Association between ovipositional substrate (parsimony reconstruction shown on the tree to the left) and female reproductive strategy (parsimony reconstruction shown on the tree to the right). In addition to the breeding substrates shown in the key to the left, some species, indicated by the arrowheads, are reported to use both decaying bark and stems. Although there is some difficulty in distinguishing between bark and stems (S.L. Montgomery, personal communication), our analysis indicates some association between breeding substrate and ovarian type (see Table 2), supporting a genetic basis to the adaptation of the species to their ovipositional substrates. The ovaries of leaf breeders are exclusively of Type II [25] (photo 1), with few ovarioles (5–15) and a maximum of only one mature egg in some of the ovarioles. Ovaries of stem breeders are typically of Type IIIa (photo 2) with a moderate number of ovarioles and one mature egg per ovariole. The length of the respiratory filaments of eggs from Type IIIa ovaries is less than the length of the egg (see photo 4). Ovaries of bark breeders are generally of Type IIIb (photo 3), with many ovarioles and many mature eggs in each ovariole; respiratory filaments are 2–3 times the length of the egg (see photo 5).

Table 2. Tests of association between ovarian types and breeding substrates.

Character 1 (ovary type)	Character 2 (substrate)	Proportion of gains*	Proportion of losses*	p
Type II	Bark	0/3	0/5	0.638
	Flux	0/2	0/0	0.863
	Stem	0/4	0/1	0.713
	Leaves	1/1	0/1	0.000[†]
Type IIIa	Bark	1/3	2/5	0.149
	Flux	0/2	0/0	0.432
	Stem	3/4	1/1	0.023[†]
	Leaves	0/1	1/1	0.167
Type IIIb	Bark	2/3	3/5	0.104
	Flux	2/2	0/0	0.347
	Stem	1/4	0/1	0.069
	Leaves	0/1	0/1	0.219

*The numerator is the number of gains or losses of a specific substrate falling on branches showing the ovarian type in column 1; the denominator is the total number of gains or losses of that substrate distributed over the whole tree.
[†]A probability value of less than 0.05, as estimated by Maddison's concentrated-changes test [42], is grounds for rejecting the null hypothesis of no association between characters.

Examination of the patterns of substrate specificity reveals a possible answer, although the pattern is complex, particularly within the better sampled picture-winged cluster (Fig. 4). Three patterns can be seen, however. Firstly, ovarian Type II is restricted to flies with the leaf-breeding habit (*Antopocerus* species group); secondly, the transition to ovarian Type IIIa is associated with a shift to the stem breeding habit, as exemplified by the *Adiastola* group; and thirdly, the transition to ovarian Type IIIb is associated with the adoption of the bark-breeding habit in the more derived picture-winged species.

Verification of these proposed associations requires a statistical method that tests the correlation between two multistate characters in the context of the given phylogeny. No such correlation test is currently available for discrete multistate characters, as the algorithms have not yet been devised. We have therefore recoded the three ovarian types and the four distinct substrate categories as an array of binary characters, and then used Maddison's concentrated-changes test [42] to examine the association between pairs of characters. The null hypothesis is that changes are randomly distributed on the cladogram, given the parsimony reconstructions of the evolution of each of the two binary characters (data not shown). The results from analyses of the patterns of character change among 42 Hawaiian taxa are summarized in Table 2. The null hypothesis is rejected ($p < 0.05$) in the two cases where there is statistical support for a concentration of change in the breeding substrate on those branches that have a particular ovarian type. Specifically, there appears to be a significant association between ovarian Type II and leaf-breeding, and between ovarian Type IIIa and stem-breeding. However, the predicted association between ovarian

Type IIIb and bark-breeding is not supported, probably because of the several losses of bark-breeding on the branches of the cladogram that display ovarian Type IIIb. Likewise, the concentration of both of the two gains of flux breeding on branches displaying ovarian Type IIIb was not significantly different from random.

These results from the correlation analyses should be treated as provisional, because the test used is not entirely appropriate for these multistate characters and is a conservative test. The recoding of multistate unordered characters as binary characters can pose problems, as can the ambiguities of parsimony reconstructions of ancestral character states [42]. Furthermore, limitations of the current ecological data and the overlap in substrates in the oligophagous and polyphagous species indicate that the correlation results should be interpreted with caution.

Discussion

Value of the *Yp1* gene for phylogenetic reconstruction
Despite the morphological, behavioural and ecological diversity displayed by the Hawaiian *Drosophila*, members of this group show close genetic affinities. This is supported by the high nucleotide conservation observed in the *Yp1* gene, both in exonic and intronic regions (averaging 91 % and 81 %, respectively, in pairwise species comparisons). Even when all 47 species are considered, approximately 40 % of the nucleotides and 45 % of the amino-acid residues are completely conserved. Unconserved nucleotides are distributed with a regional bias — at a high frequency in the introns and certain coding regions, and at a low frequency in other regions such as the lipase homologous region, which may serve to bind ecdysteroids [30] (Fig. 1). Our sequence data indicate that individual regions of the *Yp1* gene are subject to different selective constraints and evolve at different rates, as will be described more fully elsewhere.

Notwithstanding the general conservation, the yolk protein gene sequences are sufficiently informative for phylogenetic reconstruction. Indeed, the *Yp1* sequences are the most extensive molecular data set currently available for Hawaiian flies, and the phylogenies inferred from these data appear to be robust. The clades indicated by the *Yp1* phylogeny are perfectly congruent with the morphologically defined species groups and subgroups, except that the morphological classification places *D. mulli* within the *Grimshawi* group rather than in the *Glabriapex* group (Fig. 1). Furthermore, the *Yp1* phylogeny shows a higher resolution of the terminal taxa within each species subgroup than non-molecular phylogenies, probably because nucleotide sequences provide more informative characters. Only the most derived *Grimshawi* group is not well resolved; their low phylogenetic signal is the major reason for the multiple trees in the MP analysis. The general consensus between phylogenies based on molecular versus non-molecular characters is reassuring, and validates each of the previously recognized groups and

subgroups of Hawaiian flies as natural groups. Moreover, the molecular *Yp1* phylogeny confirms that the picture-winged group is the most derived group of endemic Hawaiian flies.

The relationship between the more primitive Hawaiian groups is somewhat ambiguous. The MP and NJ trees differ in the branching order of the *Antopocerus* and modified-mouthparts groups, but this node is only poorly supported (Figs 1,2). The branching order suggested by the NJ tree, with the *Antopocerus* group as the more primitive, is congruent with the consensus morphological relationship and with other molecular data (R. DeSalle, personal communication); hence, the NJ tree was chosen as the basis for the character analyses.

Adaptive radiation of the Hawaiian *Drosophila*

The success of Hawaiian drosophilids owes much to the exploitation of a variety of ecological niches and to diversification in female reproductive strategies to adapt to the various breeding niches. A characteristic feature of the ecological relationships of Hawaiian drosophilids is the consistency with which members of a particular species subgroup use the same ovipositional substrate. Indeed, this association is so consistent that the breeding substrate is often used to characterize the subgroup — for example, leaf breeders (*Antopocerus* group), flux breeders (*Hawaiiensis* subgroup) and bark breeders (*Cyrtoloma* subgroup), as shown in Figure 4. The strict specificity in choice of the ovipositional substrate suggests that this behavioural trait has high heritability and is fairly rigidly canalized. Moreover, each adaptation to a particular breeding substrate involves the modification of several morphological attributes of the female reproductive system (from the structure of the ovaries [25] and the eggs [8,39] to that of the ovipositor [8]), as well as modifications to the regulation and coordinated expression of several genes. Clearly, adaptation to a certain substrate has a complex genetic basis, with strong selection acting to preserve the adaptation.

Despite the complex pattern of ecological shifts revealed by the phylogenetic analysis (Figs 3,4), there is some evidence for significant correlations between the breeding substrate and the structure and functioning of the female reproductive system as summarized by the ovarian type (Table 2). It is worth noting that, with some data sets, the concentrated-changes test may lack the power to demonstrate significant correlations between characters even when they do exist [42]; we suspect this to be the case for the putative association between bark-breeding and Type IIIb ovaries. With the future development of better tests that are specifically designed to handle multistate unordered characters, and with the accumulation of more complete ecological and developmental data for the Hawaiian *Drosophila*, these suggested correlations may be more strongly supported.

Our data do, however, appear to indicate that substrate-specific ovarian patterns have evolved to balance the egg mass production against the carrying capacity of the larval niche [25] (Fig. 4, Table 2). For example, decaying leaves can only sustain the growth of a small number of larvae; the leaf-breeding species adapted to this niche accordingly mature and oviposit only a few eggs (ovarian Type II, Fig. 4). Such a strategy would maximize mean offspring fitness. The decaying bark of tree trunks can support the growth of large numbers of larvae, and the bark-breeding species that have adapted to this niche therefore tend to mature and oviposit hundreds of eggs at a time (ovarian Type IIIb; Fig. 4). Furthermore, the rate of larval development correlates with the longevity of the substrate. Bark rots slowly and readily supports the completion of the slow larval development of bark-breeding species; larvae of the leaf-breeding species mature faster, corresponding to the more rapid rate of decay of leaves.

Although not analyzed specifically in this paper, egg morphology [8] — in particular, the ultrastructure of the eggshell or chorion [39] — is also strikingly different among species groups, yet is conserved among members of a particular group and correlates with their breeding substrate [39]. The outer endochorion of the primitive leaf-breeders is rather thin and the egg has only short respiratory filaments (Type II), similar to that of the continental *Drosophila*. The egg of the bark-breeders has a rather thick chorion and the respiratory filaments are very long (Type IIIb; Fig. 4.5); these adaptations provide mechanical support and respiratory exchange for the embryo in the bark substrate. Choriogenesis in *D. melanogaster* depends upon the tightly regulated function of a large number of genes [43]. We predict that the Hawaiian *Drosophila* differ in the regulation of expression of these genes, or that some of the chorion genes are amplified to varying degrees. Such explanations could account for the diverse quantities of chorion proteins produced in different species groups with chorions of very different thicknesses.

From phylogenetic analyses and ecological data on Hawaiian and non-Hawaiian drosophilids, it appears that the original founder to Hawaii was a leaf breeder (Fig. 4, Table 1). This ecological niche of leaf mining is also found in some species from East Asia [44], a region that may have provided the progenitors of the Hawaiian fauna [8]. The lack of continental plants in Hawaiian habitats may have forced a host shift. Indeed, in the initial generations following the original founder event, numerous such shifts may have been underway in parallel, given the availability of open habitats. Such shifts would have led to an early rapid adaptive radiation into the large range of breeding substrates that characterize the Hawaiian drosophilids today. A rapid ecological divergence would suggest that relatively few genetic changes distinguished these ecologically diverse lineages, and would explain the lack of long branches at the base of our phylogram (Fig. 2). In fact, we contend that this initial rapid burst of ecological divergence was one of the primary factors that established the major phyletic branching within the Hawaiian drosophilid fauna. Another factor besides the

ecological divergence and divergence in female repro-
ductive adaptation appears to have been the divergence in
male mating behaviour [7,13] and correlated secondary
sexual characters [5,21], which may well have been
coincident with the female divergence.

Once well adapted to a particular breeding substrate, the
chances of a subsequent host shift may decline rapidly. A
host shift would upset the necessarily complex coordina-
tion between female oviposition preference, ovarian
structure and functioning, structure of the eggshell and
ovipositor, and adaptation of the larvae to particular sec-
ondary plant compounds found in the breeding niche.
The characteristic ecological groupings suggest that the
co-adapted complex of genes that underlie each adapta-
tion must usually be transmitted intact to all the members
of the group that are derived subsequently, and must sur-
vive any genetic 'revolutions' that may accompany specia-
tion events [20]. In most clades, the ecological association
that was established early in the history of the lineage
would therefore be preserved, with the majority of
species displaying exquisite and multifarious behavioural,
anatomical and physiological adaptations to the breeding
substrate. Nonetheless, shifts toward the use of new plant
families and new substrates can sometimes still occur in
later evolutionary stages — as seen in the expansions to
oligophagy and polyphagy (Fig. 3), and in the shift to flux
breeding in the *Hawaiiensis* subgroup of the picture-wings
(Fig. 4). Such ecological shifts in the initial and later
stages of the evolution of Hawaiian drosophilids have
clearly played a major role in their spectacular radiation.

Conclusions

Nucleotide sequences of the *Yp1* gene yield a robust
phylogeny of the Hawaiian *Drosophila* that is generally
congruent with phylogenies derived from other data,
both molecular and non-molecular. Using character
reconstruction on this independent *Yp1* phylogeny, we
have shown that monophagy, or specialization to just one
host plant family, is the primitive and typical condition for
these Hawaiian flies, with oligophagy or polyphagy hav-
ing evolved in only a few derived species. Moreover, we
conclude that one of the initial substrates used by
drosophilids in Hawaii was decaying leaves, and that there
were subsequent shifts to decaying stems, bark and tree
fluxes. Shifts in the female reproductive strategy, as reflec-
ted in the organization and physiological functioning of
the ovaries, have also accompanied the ecological and
phylogenetic diversification of these flies, with a trend
toward increasing fecundity. The data presented here pro-
vide some evidence for significant associations between
particular substrates and particular reproductive strategies,
and suggest that the ecological opportunity opened up by
each shift to a new breeding substrate initiated a burst of
speciation that established each of the major lineages of
Hawaiian flies. Ecological diversification may therefore
be a driving force in insular radiations — of particular
importance in the early stages that follow colonization,

but also significant at later stages in the phylogenetic
process as ecological opportunities become available.

Materials and methods

Sequence determination
Genomic DNA was isolated from adult flies of 46 species (see
Fig. 1) by standard *Drosophila* methods [45]. A 1025 base-pair
DNA fragment of the *Yp1* gene extending from the translation
start point was amplified by the polymerase chain reaction
(PCR) [46] for all species. *Taq* polymerase (Perkin-Elmer
Cetus) was employed for the 30 cycles of amplification (100 μl
reaction volume), conducted in a Coy Model 60 TempCycler
(Coy Laboratory Products). Reaction conditions followed the
supplier's specifications, but were optimized to 4 mM $MgCl_2$.
The universal primers for amplification were V1.2 (sequence
5'-ATGAATCCAATGAGAATGCTTAGCCT-3') and V1.1
(sequence   5'-GGATCCACAAACTCCGCATCGCCGC-
GAG-3'). Amplification products were purified using
GENECLEAN (BIO101). Both strands of each fragment were
sequenced using either Sequenase (USB) or CircumVent (New
England BioLabs) systems. Primers for sequencing were V1.2,
V (5'-GAGCCTAGCTATGTGCCCAGC-3') and VI (5'-
CAGCGAGGAGGACTACA-3') for the 5' to 3' direction,
and V1.1, VII (5'- CATCTTAACAATCCACTTGCC-3')
and VIII (5'-ATGATGGTCACCTCATCCT-3') for the 3' to
5' direction. Sequences are being deposited in the GenBank
database and will be available soon. The authors can be con-
tacted for details of accession numbers.

Sequence alignment and phylogenetic analysis
Nucleotides starting from codon 13 and extending to codon
329 (included in all species) were manipulated initially using
the IBI Pustell program (Eastman Kodak) and the GCG pack-
age (University of Wisconsin), and were then aligned using
Clustal V [47]. Maximum parsimony analysis of unweighted
characters employed a heuristic search with the stepwise addi-
tion option for ten random replications of the PAUP 3.1.1
program [32]. For the ML analysis, the DNAML program in
PHYLIP (Version 3.51c) [33] was used. For the NJ analysis
[34], the complete deletion option of the MEGA program
(Version 1.01) [48] was used. Distances were calculated using
the Jukes-Cantor method, as distance values were less than 0.3
and transition/transversion ratios less than 2 (data not shown).
Bootstrap values from 1000 replications were calculated using
the random-input file option of MEGA [48].

Character reconstruction on the molecular phylogenies
Character analyses for three independent character sets were
performed using MacClade [49], superimposing the various
character states for each trait on the molecular NJ tree (Fig. 1).
The two ecological traits were the plant family and the sub-
strate used for oviposition by each *Drosophila* species [6,23].
The third analysis involved a reproductive trait, namely the
ovarian developmental type [25].

Statistical analyses of character associations
The phylogenetic correlations between ovarian type and
breeding substrate were tested using Maddison's concentrated-
changes test [42], as implemented in MacClade [49]. Each
multistate character was first recoded as a series of binary char-
acters: for example, ovarian Type II versus Types IIIa and IIIb;
use of bark as substrate versus use of other substrates. Using
MacClade [49], each binary character was then optimized on

the molecular cladogram. Character associations in the Hawaiian *Drosophila* clade (42 species) were then tested against the null hypothesis that changes in each character are equally probable on any branch [42]; because of the size of the cladogram and the total number of changes, the probabilities of various numbers of gains and losses of a specific substrate type (character 2) on branches with the selected state of character 1 (ovarian Type) were calculated using simulation of actual changes with 10 000 replications in each case. The probability (*p*) of having as many as the observed numbers of gains and losses of character 2 on the specified branches was then determined.

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