Evolution of frugivory across a large radiation of non-seed-dispersing animals

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ABSTRACT

The origin of Angiosperm fleshy fruits, a novel nutritious resource, has led to the evolution of frugivory in a wide range of animals. While the (co)evolutionary dynamics between fruits and seed dispersers has received much attention, the evolution of frugivory in non-seed-dispersing animals remains overlooked. Here, we elucidate the macroevolution of frugivory in a large radiation of an insect, butterflies, by combining macroevolutionary analyses and field experiments. Our comparative analyses spanning 80 million years of evolution across Nymphalidae butterflies, the only family which feed on fermenting fruits, revealed that frugivory has deep origins ~60 million years ago and has evolved convergently from nectar-feeding lineages. Notably, ancestral state estimations indicate that the evolution of frugivory roughly coincides with the evolution of larger fleshy fruit sizes in Angiosperms. Our field experiments across the tropics further showed that frugivorous butterflies from diverse lineages have convergently evolved a strong preference for fermenting over fresh fruit bait, suggesting that the evolution of frugivory is associated with behavioural specialization. Together, our study shows that fleshy fruits likely created a new ecological opportunity that butterflies exploited and required behavioural specialization to exploit this novel resource.

INTRODUCTION

Novel dietary resources have frequently emerged throughout Earth's history, and subsequent diet shifts have altered fundamental aspects of organisms' morphology, behaviour, life history, and physiology. The origin of angiosperm fleshy fruits ~130 million years ago (mya) was one such novel nutritious resource (*1*–*4*). Diverse microbes and animals have subsequently shifted to facultative or obligate frugivory (*5*, *6*). While fleshy fruits likely evolved to facilitate seed dispersal by offering frugivores a nutritious reward (*1*, *6*; for alternative hypotheses, see *4*, *7*), many non-seed-dispersing frugivores (e.g., microbes, insects) have exploited this interaction (*8*, *9*). In fact, these frugivores can engage in direct competition with seed dispersers and negatively affect plant fitness, for instance, by puncturing fruits and introducing microbes that increase fruit spoilage and fermentation, thereby making fruits unattractive to seed dispersers (*8*–*10*). While much emphasis is placed on fruit and seed-disperser (co)evolution, how the availability of fleshy fruits shaped the evolution of non-seed-dispersing frugivores on a large macroevolutionary scale remains an important yet overlooked dimension.

Butterflies provide an interesting system to address questions on the evolution of frugivory in non-seed-dispersing animals. While adult butterflies are generally known to feed on nectar, many species across diverse lineages, especially in the tropics, feed on fermenting fruits (Fig. 1) (*11*–*13*). Dietary specialization in adult butterflies is generally strict – species either feed on nectar/pollen or fermenting fruits (*12*, *14*). In fact, traps baited with fermented fruits are often used to sample frugivorous butterflies across the tropics (e.g. *12*, *13*). While some species utilize supplementary resources, including feeding on rotting carcasses, faeces, tree sap, and imbibing nutrients from moist soil (*11*, *15*), they typically do not specialize exclusively on these diets. Out of seven major butterfly families, frugivory, however, has evolved only in Nymphalidae, which comprises ~6400 species forming 34% of the total butterfly diversity (*13*).

Diet shifts from nectar to fermenting fruits (*14*, *16*) likely have had key impacts on life history evolution. Fermented fruits can contain higher concentrations of amino acids than nectar (*14*). Since the diets of both larval and adult butterflies are generally poor in amino acids, which are vital for somatic maintenance and reproductive investment (*17*), intake of amino acids through frugivory is suggested to be a causal factor explaining why some adult frugivorous butterflies have a longer lifespan, with some living >200 days in the wild (*18*, *19*). Despite the important evolutionary implications, the mode of diet transitions in adult butterflies on a large macroevolutionary scale remains to be uncovered.

Butterflies originated ~100 million years ago (mya), and Nymphalidae are estimated to be ~80 million years (my) old (*20*–*22*). Angiosperm fleshy fruits are estimated to have evolved ~130mya, initially as small and likely not sugar-rich fruits, with size increasing markedly ~70my onwards and undergoing little or no change until the Oligocene (34-23my) (Fig.1) *(1, 3, 4, 7, 25)*. Importantly, fleshy fruit diversity and abundance peaked during the early Cenozoic (66my-current) *(4)*. Thus, the timing of the increase in the size of fleshy fruits and abundance roughly coincides with the early diversification of Nymphalidae butterflies. This raises the possibility that availability of fleshy fruits created an ecological opportunity for the evolution of frugivory in butterflies. Exploiting this opportunity, however, would have required changes across a suite of traits for locating, consuming, and metabolizing fermenting fruits. For locating fermenting fruits, specifically, animals can use fermentation volatiles as

reliable olfactory cues (*24*), facilitated by the fine-tuning of the olfactory system (*25*, *26*). Consistent with this, antennal sensilla of some frugivorous butterflies, but not nectar-feeding butterflies, respond to fermentation volatiles (e.g., alcohols, acetic acid), suggesting that their olfactory systems may be specialized for detecting cues associated with fermenting fruits (*25*, *26*). Overall, fermenting fruits likely provided butterflies with an ecological opportunity, and diet transitions from nectar to fermenting fruits likely involved behavioral specialization to locate this resource.

Here, we carry out macroevolutionary analyses of diet transitions across Nymphalidae butterflies to uncover the timing of the evolution of frugivory in butterflies and whether it coincided with the increase in fleshy fruit size and abundance. We then carry out field experiments across the tropics to test a specific hypothesis that, when given a choice between fresh and fermented fruits, frugivorous butterflies will have a strong preference for fermented fruits, as has been shown for African butterflies (*27*). Support for this hypothesis, would suggest that frugivorous butterflies actively discriminate between these resources and preferentially exploit fermented fruits, and that evolution of frugivory is linked with a higher preference for fermented than fresh fruits. In summary, our study examines how a large radiation of insects exploited a novel resource and whether exploitation of this resource involves similar behavioural specialization.

RESULTS

Frugivory is widespread across Nymphalidae

Our genus-level phylogeny, derived from the largest species-level phylogeny of Nymphalidae butterflies (*22*), comprised 477 unique genera (86% of all currently accepted genera) and included 11 outgroups represented from four butterfly families (Fig. 1B, 2). We used this genus-level phylogeny for comparative analyses. Adult diet is strongly conserved at the genus level, with a few exceptions (e.g., genera such as *Vanessa, Hypolimnas*). Thus, our genus-level analysis captures the broad macroevolutionary patterns needed to formally address questions on the origins and evolutionary dynamics of frugivory.

Based on our diet classification (fermenting fruit vs nectar, which includes pollen-feeding), as many as 47% of Nymphalidae genera (224 genera out of 477) are frugivores. Frugivory is widespread across Nymphalidae, occurring in 10 out of 12 subfamilies, with the proportion within subfamily varying from the entirely frugivorous Charaxinae and Pseudergolinae to 87% of Biblidinae, 63% of Limenitidinae, 52% of Satyrinae, and 23% of Heliconiinae (Fig.1B).

Deep origins and extensive convergence of frugivory across Nymphalidae

Fitting several Markov (*Mk*) models suggested that models accounting for rate heterogeneity across clades overall had a better fit than homogeneous-rate models (Fig. S1, Table S1). This suggests that transitions in diet occurred at different rates across clades or are lineage specific. Maximum likelihood ancestral state estimation using the best-fitting model (the equal-rates hidden-rates model, Table S1) suggested that frugivory has deep origins in Nymphalidae. Two independent and well-supported instances with >72% marginal probability of being a frugivore, indicated that frugivory evolved roughly 60mya (see nodes marked with * in Fig.2). Ancestral states at deeper nodes (>60my, barring the root and immediately following nodes) remain uncertain (Fig. 2), and generally ancestral states at such deep nodes should be

interpreted with caution (*28–30*). Within subfamilies, most exhibited independent instances of transitions between fruit and nectar-feeding across different clades (Fig. 2). This was especially evident in the subfamily Satyrinae, where, in two large clades, nectar-feeding has evolved from fruit-feeding, and *vice versa* (Fig. 2). Better support for the equal rates model further suggested that the transitions between diets were equally likely and labile (Fig S2, Table S1). Ancestral states estimated from 1000 stochastic maps were similar to the maximum likelihood reconstructions (Fig. S3).

Our sensitivity analyses by including a polymorphic state (i.e., 16 genera classified as feeding on both fruits and nectar, Fig. S4), changing a phylogenetic position of an important subfamily (Libytheinae, Fig. S5), and removing outgroups and fixing the ancestral state of Nymphalidae to nectar-feeding (see *Methods*) suggested that the ancestral state estimates are robust and similar to the core analyses (Table S1, Fig.S6-S8).

Field experiments suggest a strong preference for fermented over fresh fruit bait in frugivorous butterflies

We tested the hypothesis that frugivorous butterflies from diverse phylogenetic backgrounds prefer fermented over fresh fruit bait, which would suggest that convergence in frugivory is associated with a strong preference for fermenting fruits. We tested this hypothesis by conducting field experiments in Serra do Japi (Brazil) and Goa (India), representing diverse butterfly lineages by offering butterflies a choice between 3-4 days fermented banana pulp and fresh banana pulp in fruit-bait traps (Fig. 3A, 4A).

In total (across experimental designs and replicates), 21 species from three subfamilies (Biblidinae, Charaxinae, Satyrinae) were captured in Serra do Japi and 10 species from two subfamilies (Biblidinae and Satyrinae) in Goa (Fig.5, Table S2). Together, species captured in the experiments represent ~75 million years of evolutionary history from diverse phylogenetic backgrounds (Fig. S9).

For Design 1, traps were placed serially, and bait types were assigned randomly (Fig. 3A). We found that traps baited with fresh banana pulp had lower species richness ($\beta_{\text{fresh bait}}$= -1, 95% CI = -1.50, -0.510; P = <0.001) and abundance ($\beta_{\text{fresh bait}}$= -1.36, 95% CI = 1.84, -0.880; P = <0.001, Fig. 3B, Table S3) than fermented banana bait in Serra do Japi. The pattern was similar in Goa, with traps baited with fresh banana having lower species richness ($\beta_{\text{fresh bait}}$= -1.11, 95% CI = -1.53, -0.69; P = <0.001) and abundance ($\beta_{\text{fresh bait}}$= -1.58, 95% CI = -1.95, -1.21; P = <0.001, Fig 3B, Table S4). Overall, species richness was 3x higher and abundance was 4x higher in traps baited with fermented than fresh banana bait in Serra do Japi (Fig.3B). The pattern was similar in Goa with fermented banana bait capturing 3x more species and 4.8x more individuals than fresh bait (Fig. 3B). At both locations, some species were captured only by fermented bait, whereas there was no species that was captured only in fresh banana bait (Fig. 3B, 3C).

For Design 2, which was only carried out in Goa, traps were placed in pairs, with pairs containing fresh and fermented bait (Fig.4A). The pattern was similar to Design 1. The traps baited with fermented banana had higher species richness ($\beta_{\text{fresh bait}}$= -1.29, 95% CI= -2.07, -0.50; P = 0.0013; Table S5) and abundance ($\beta_{\text{fresh bait}}$= -1.66, 95% CI = -2.56, -0.76; P = 0.003; Fig.4B, Table S5) than fresh banana bait. Overall, traps baited with fermented banana had 3.6x more

species richness, 5.2x more abundance, and captured several unique species compared to traps baited with fresh bait (Fig. 4B, 4C).

DISCUSSION

The evolution of Angiosperm fleshy fruits ~130mya presented a novel nutritious resource to diverse organisms (*31*). But much of the focus remains on seed dispersers, and the evolution of frugivory in non-seed-dispersing animals remains overlooked. Here, by combining phylogenetic comparative methods and field experiments, we elucidate how the availability of fleshy fruits shaped diet evolution and behavioural specialization in a large radiation of non-seed-dispersing frugivores, the butterflies. We discuss the evolutionary implications that diet shift has had on phenotypic evolution.

Our ancestral state estimates suggest that frugivory has a deep origin in Nymphalidae, with a most confident estimate ~60mya. This timeline roughly coincides with when fleshy fruits started to increase in size between ~70 and ~60mya (Fig 1C) (*1–4*, *7*, *25*). These fleshy fruits early in the evolutionary history were likely dispersed by vertebrate dispersers such as early mammals and non-avian dinosaurs (*4*, *23*). A likely causal connection between an increase in fruit size and the evolution of frugivory in butterflies is that large vertebrate-dispersed fruits tend to be more sugar-rich (*32*) and hence can ferment more, releasing more fermentation volatiles, which butterflies and other frugivores use to locate fruits (*24*, *25*). As of now, we cannot fully reject the possibility of a slightly earlier origin of frugivory, as ancestral states at deeper nodes remain uncertain, which remains a common issue in comparative analyses of discrete traits (*28*, *29*). Future studies using alternative approaches, for example, the threshold model with phenotypic traits that are linked with frugivory (e.g., proboscis morphology) as a liability (*33*, *34*), will be valuable. Data for such phenotypic traits are currently not available for enough clades across Nymphalidae for carrying out large-scale comparative analyses. Finally, the better fit for the equal-rates model likely suggests that the transitions between diets (nectar to fermenting fruit, and *vice versa*) were equally likely and labile. This is surprising, as feeding on fermenting fruits is expected to require adaptive changes across a suite of traits, for instance, olfaction to detect fermentation volatiles (*25*), proboscis morphology to feed on viscous fluids from the fruit surface (*16*, *39*), and physiology to detoxify ethanol from fermenting fruits (*39*). In sum, our macroevolutionary analyses revealed that an increase in the size of the fleshy fruits and their fermentation by yeasts likely opened a new dietary niche and was subsequently exploited by butterflies. Although these patterns are inferential rather than causal, future studies comparing the timing of frugivory in other groups, such as moths and flies, will be important for testing the generality of this pattern.

Our field experiments to quantify the diet preference suggested that frugivorous butterflies across the tropics (i.e. Brazil and India) have a strong active preference for fermenting over fresh fruit bait, and a similar pattern was also demonstrated in Afrotropical butterflies (*37*). This preference for fermenting fruits was general, as reflected by capturing species from diverse subfamilies (Fig.5), which further indicates that the convergence of frugivory is associated with increased preference for fermenting fruits. We posit this is likely an adaptive behavioural response. Fruits (or fallen fermenting fruits) can be seasonally abundant, but their distribution can be spatially clumped (*6*), and diverse animals use the volatiles released during ripening or fermentation to locate this rich resource (*24*, *38*). If fermentation volatiles

reliably indicate the availability of nutrient-rich fruits, selection may favour sensory and behavioural adaptations that enhance detection and exploitation of fermenting fruits (*25*–*27*). Moreover, if fermenting fruits provide greater nutritional benefits than other resources such as nectar (*14*), their preferential use could confer a fitness advantage (see below). The consistent attraction of frugivorous butterflies to fermenting bait over fresh bait across diverse clades suggests that responses to fermented fruit may be broadly conserved among frugivorous butterflies. This behavioural consistency across diverse lineages (Fig. 5) indicates sensitivity to fermentation-related volatiles, which could provide more reliable cues to fermenting fruits than the potentially less predictable chemical profiles of fresh fruits (*39*). However, our field experiments cannot distinguish responses to fermentation volatiles from responses to other chemical or physical changes associated with fermentation. Future studies quantifying volatile profiles of fermenting fruits in the wild and quantifying butterfly responses via electroantennography to diverse fermentation volatiles will be valuable.

Dietary shifts from nectar to fermenting fruits likely provided butterflies with several advantages in addition to being more nutritious, thus translating into fitness benefits. The biomass of the fruits is much higher, and the duration of their availability is much longer in the tropics than in the temperate regions (*6*). This may explain why frugivorous butterfly diversity is higher in the tropics as well, compared to temperate regions. Fermenting fruits, therefore, can be an abundant and seasonally reliable alternative resource to nectar. Butterflies could also use leftover or half-eaten fruits by vertebrates, which could be fermenting. From a nutritional standpoint, fermenting fruits seem to be relatively more nutritious than nectar. Fermenting fruits of *Dipteryx panamensis,* on which Neotropical butterflies were observed feeding, had higher concentrations of sugars and essential and non-essential amino acids compared to nectar (*14*, *37*). Such comparative studies, however, are rather rare. Alongside sugar, ethanol from fermentation can be a source of energy. Ethanol has a higher caloric value than carbohydrates or proteins (7 vs 4 kcal/gm), and animals can use ethanol-derived carbon in resource allocation (*40*). Both invertebrate and vertebrate frugivores are routinely exposed to ethanol concentrations of ~1% v/v in the wild (*41*, *42*). Frugivorous butterflies likely have an efficient alcohol detoxification pathway, as they can readily feed on a diet containing >4% ethanol with only slight negative effects on behavior (*36*, *43*). While nectar can also ferment and contain ethanol (*44*), whether nectar-feeding butterflies can metabolise ethanol and use it as an energy resource is not known. Consuming yeasts from fermenting fruits, as in *D. melanogaster* (*45*), could be an additional protein source.

Better nutritional quality of fermenting fruits may have also had dramatic consequences on life-history evolution. For instance, frugivorous butterflies have longer life spans than nectar feeders (*19*, *46*). In fact, adult frugivorous butterflies comprise some of the longest-living butterflies in the wild (e.g., 293 days in *Euphaedra medon*, 230 days in *Charaxes fulvescens*) (*18*). This life history consequence is similar to extraordinarily long lifespans in *Heliconius* butterflies, some of them living >250 days, owing to their amino-acid-rich pollen diet (*47*, *48*). Experimental evidence also suggests that a diet of fermenting fruits over sugar water can increase fecundity in the frugivorous *Euphaedra* butterflies (*49*), but this advantage was relatively minor in *C. fulvescens* (*50*) or absent in *Bicyclus anynana* (*51*). In addition to life history traits, transitions to frugivory have also been suggested to have shaped a suite of traits including wing patterns (*52*) and proboscis morphology (*16*, *35*), but large-scale comparative studies are lacking. In sum, transition to frugivory has probably had strong consequences on

phenotypic evolution, especially life history evolution, and future studies integrating experimental and macroevolutionary studies will be necessary to test the generality of these patterns.

In summary, by carrying out macroevolutionary analyses and field experiments, we show fleshy fruits likely opened a new ecological opportunity which was exploited by butterflies, and feeding on this resource required behavioural specialization to locate fermenting fruits. The deep and convergent evolution of frugivory will open future research on the ultimate and proximate drivers of frugivory in butterflies, moths, and other frugivorous insects.

METHODS

Diet classification of adult butterflies

We classified diet as a binary trait: fermented fruits versus nectar (which also includes pollen feeding). To classify genus/species as a frugivore, we leveraged studies that carried out bait trapping using fermenting banana, sometimes mixed with sugarcane juice, palm wine, or beer, as a bait. Since adult butterflies generally have a clear diet preference, all species captured in bait traps were classified as frugivores and the rest as nectarivores (but see below). Most frugivorous butterfly lineages occur in the tropics; hence, we searched for studies (including PhD and Master's theses) mainly in Africa and South-East Asia using Google Scholar and used an already existing database which catalogued fruit-feeding butterflies in Atlantic forests (mainly Brazil and adjacent countries) (*53*). We then searched for additional bait trapping studies in Central and South America. In total, we extracted a list of frugivorous species from 18 publications for Africa (1635 records, 61 unique genera, 373 unique species), 22 for South-east Asia (789 records, 87 unique genera, 242 unique species), and 17 from the Neotropics (836 records, 117 unique genera, 397 unique species) for which data were available from the literature (see Fig. S10). It was often the case that multiple studies were carried out in the same locality or skewed towards certain countries (see Fig. S10**).** In such cases, we chose two to three studies as representatives. We checked and updated the taxonomy of species by referring to an online resource:
https://www.nymphalidae.net/Nymphalidae/Classification/All_species.htm

Bait trapping studies on butterflies in the temperate regions are almost non-existent, thus, we checked for papers mentioning records of feeding on fermenting fruits. We also carried out opportunistic bait-trapping in Europe (Croatia and Sweden) using wine mixed with balsamic vinegar and sugar as a bait, acting as a proxy for fermenting fruit, and used this information for diet classification (Table S6).

It should be noted that some species that do not specialise on fermenting fruits, for instance, from families Hesperiidae, Lycaenidae, Nymphalidae species from the Danaine subfamily and other Nymphalidae genera (e.g. *Acraea, Neptis, Pantoporia, Ypthima*, *Telchinia, Precis*) occasionally get attracted to fermented fruits in very few numbers, likely due to moisture (*13*) Further, although we classified genera (as our analyses are at the genus level, *see below*) as frugivores versus nectarivores, Nymphalidae species from some genera (e.g. *Siproeata, Adelpha, Doxocopa, Marpesia, Ariadne*) can occasionally feed on both nectar and fermenting fruits. In such cases, we either relied on our several years of experience in bait trapping butterflies across Africa (OB, FM), India and Malaysia (SH, DH, HB, UK, FM), and the

Neotropics (JPS, AVLF), or consulted with the local experts (see names in Acknowledgements). Our final list of frugivorous genera thus relied on published bait-trapping studies and then confirmed these records based on our experience and by consulting local experts. While our core dataset uses binary diet classification (fermented fruit vs nectar), we carried out additional sensitivity analyses by classifying genera that feed on both fruits and nectar as a polymorphic state (see below).

Phylogenetic comparative analyses

We carried out all analyses in R ver. 4.6.1 (*54*). Base R and *tidyverse* ver. 2.0.0 (*55*) were used for general data processing, *ape* 5.8.1 (*56*) for handling and plotting phylogenies (and associated data) with added functionalities from *phytools* 2.5.2 (*57*, *58*), *ggplot2* 4.0.3 (*59*) for plotting non-phylogenetic figures, and *viridis* 0.6.5 (*60*) for colour palette. Details of R packages used for specific analyses are provided when describing the analysis details. Figure panels were made using Inkscape (https://inkscape.org).

Phylogeny and character coding: We focused on the family Nymphalidae because frugivores are exclusively from this family. We used the most comprehensive phylogeny of Nymphalidae (*22*), comprising ~45% of extant diversity (2869 Nymphalidae + 11 outgroups) from this family. Outgroups comprised other butterfly families. From this species-level phylogeny, we derived a genus-level phylogeny of Nymphalidae comprising 477 unique genera with 11 outgroups. The diet of adult butterflies is generally conserved at the genus level, thus, using the genus-level phylogeny captures the broad macroevolutionary patterns we aim to elucidate in this study. We removed two undescribed genera and two genera (*Brassolis* and *Dynastor*), which have reduced/non-functional proboscis and likely do not feed as adults (*61*), from the phylogeny before comparative analyses.

Fitting Markov (Mk) models and ancestral state estimation: The ancestral state for butterflies is expected to be nectar-feeding (*14*, *16*), and should be the case for Nymphalidae as well, as slightly older non-Nymphalidae butterfly families are nectar feeders. Since fruit-feeding has only evolved in Nymphalidae, we included outgroups classified as nectar-feeders in our comparative analyses, as it provides additional information that the ancestral state for Nymphalidae was nectar-feeding (but see sensitivity analyses).

To estimate ancestral states, we first fitted homogeneous-rate time-continuous *Mk* models to estimate the transition rates using maximum likelihood. Homogeneous-rate *Mk* models assume constant transition rates across time and clades. We fitted two *Mk* models: (1) the equal-rates (ER) model with forward and backward transitions having the same rate, and (2) the all-rates-different (ARD) model, with all transitions occurring at different rates (Fig. S1). Heterogeneity in evolutionary rates, however, is pervasive (*62*), and rates are especially not expected to be constant across 100 million years of butterfly evolutionary history. To account for putative rate heterogeneity across clades, we fitted hidden-rate *Mk* (HRM) models. HRM models do this by including hidden-rate classes to account for 'non-observable' processes that may explain variation in transition rates across clades (*62*, *63*). We fitted the ER and ARD models with two hidden-rate classes (R1 & R2), resulting in the total state-space of four states: nectar-R1, nectar-R2, fruit- R1 and fruit-R2 (Fig. S1). For all HRM models, dual transitions (e.g. nectar-R1 ←→ fruit-R2, *vice versa*) were not allowed (Fig. S1), as such simultaneous transitions without an intermediate state are biologically unlikely (*57*, *58*). All *Mk* models were fitted

using the *corHMM* 2.8 R package (*65*). We used 100 random starts to fit all models, estimated root probability using the 'yang' procedure, and assessed model fits using the AICc score and AIC weights.

We carried out marginal ancestral state estimation using maximum likelihood (*66*) and stochastic character mapping (*67*) using the transition rate estimates of the best-fitting *Mk* model. We generated 1000 stochastic maps, which were then summarized to represent the marginal probability of each state at the nodes. We used *corHMM* for stochastic mapping and functions from *phytools* to summarize and plot ancestral estimates.

Sensitivity analyses: We carried out three sensitivity tests to test the robustness of ancestral estimation: (1) We classified 16 genera as a polymorphic state, as some species are known to feed on both nectar and fruit, or when genera are predominantly nectar-feeding on nectar and only some species feed on fruits (Fig. S4); (2) We changed the position of Libytheinae subfamily such that it is sister to the rest of the Nymphalidae (Fig. S5*)*, as the position of Danaine and Libytheinae with respect to the rest of Nymphalidae differs across studies (*20*, *21*). In the phylogeny we used from Chazot et al. (*22*), Danaine is sister to the rest of the Nymphalidae; (3) We removed outgroups and fixed the root of the Nymphalidae to be nectar-feeding, as the presence or absence can influence ancestral estimation (*29*, *68*).

Quantifying the diet preference of fruit-feeding butterflies in the wild

Field experiments: We carried out field experiments to test whether frugivorous butterflies have an inherent preference for fermented fruits using two experimental designs. Experiments were carried out in Serra do Japi, Brazil (coordinates: -23.2381, -46.9337), and Goa, India (coordinates: 15.2462, 74.2126), thus testing diet preference across diverse lineages. In Serra do Japi, the study site was a forested habitat, and we used the existing road as a transect to place bait traps. In Goa, the habitat was mainly comprised of coconut plantations, with the edges bordered by riparian forested habitat. We used two experimental designs to quantify the diet preference.

For Design 1 (Fig. 3A), which was carried out both in Serra do Japi and Goa, we placed 10 fruit-bait traps (MegaView Science, Pop-up Butterfly Bait Trap, Cone Type, DC0022) serially along the transect, with the distance between each trap of ~50-60 meters. Of 10 traps, five were baited with fresh banana mash and the other five with fermented banana. Fermented bait was made by finely mashing the pulp of ripe bananas (using a kitchen blender) and letting the pulp naturally ferment for four days in Serra do Japi and three days in Goa (as it was much warmer and humid), while fresh bait (blended similarly) was prepared in the morning on the day of the experiment. The type of bait each trap received was randomly shuffled using a random number generator to account for potential spatial effects. The bait was placed in the morning between 8-9 hours and removed between 14.30-16 hours when the traps were checked for butterfly species, sex, and their abundance. All butterflies were released after emptying the bait trap. Each day of the experiment represents one temporal replicate. In Brazil, we carried out nine replicate trials (from 02/10/2024 to 23/10/2024) on every alternate day and eight daily replicate trials (from 26/11/2024 to 06/12/2024) in Goa. Note that, in Brazil, one sampling day was conducted on a different transect located ~600-700m from the main study transect. Because this transect was in close proximity and sampled under the same experimental protocol, we included this data in the main analyses.

For Design 2 (Fig. 4A), which was only carried out in Goa, we used a paired-trap design such that at each location along the transect, we placed two traps 2-3 meters apart, forming a pair, each containing fresh and fermented bait. The rationale here was that butterflies will have to make an active choice to choose one of the baits, which allows ruling out the possibility that the butterfly entered the trap just because it was in the vicinity or flying by (which could happen in Design 1). We placed five such trap pairs with a distance between each pair of 50-60 meters. We carried out four temporal replicates of this experiment (from 10/12/2024 to 14/12/2024), and the remaining experimental procedures are the same as for Design 1.

***Statistical analyses*:** We tested the bait preference of frugivorous butterflies (fresh vs fermented bait) using Generalised Linear Mixed Models separately for both locations. We fitted models with either species richness or abundance as a response variable and bait type as a fixed effect. For Design 1, the date of the experiment and the trap numbers (n=10) were included as two separate random effects with model structure as follows: *Species richness/abundance ~ Bait type + (1|Experiment date) + (1|Trap number)*. Similar to Design 1, for Design 2, the date of the experiment and the trap pair number (n=5) were included as two separate random effects with model structure as follows: *Species richness/abundance ~ Bait type + (1|Experiment date) + (1|Trap pair number)*. We initially fitted models with the Poisson family with a log link, and if there were model convergence issues, we used the negative binomial family with a log link. We checked model fits using the *performance* 0.17.1 (*69*). We fitted models using *glmmTMB* 1.1.14 (*70*, *71*) and *emmeans* 2.0.4 (*72*) to extract marginal means from the fitted models.

AUTHOR CONTRIBUTION

Conceptualization: SH (lead); NW, CWW & AVLF (supporting). **Methodology:** SH (lead), NW, CWW & AVLF (supporting). **Investigation:** SH (lead), DH (supporting). **Data curation:** SH. **Validation:** All authors. **Formal analyses:** SH. **Software:** SH. **Visualization:** SH. **Funding acquisition:** SH. **Writing-original draft:** SH. **Writing-review & editing:** All authors

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COMPETING INTERESTS

The authors declare that they have no competing interests.

DATA AND MATERIAL AVAILABLITY

All raw data, processed data, fitted models, and R scripts required for reproducing analyses can be found on Zenodo: https://doi.org/10.5281/zenodo.22791710

AI USE

Generative AI (ChatGPT, OpenAI) was used to write small sections of R code (highlighted in the R scripts) and occasionally to explore some alternative phrasing in the final versions of the manuscript.

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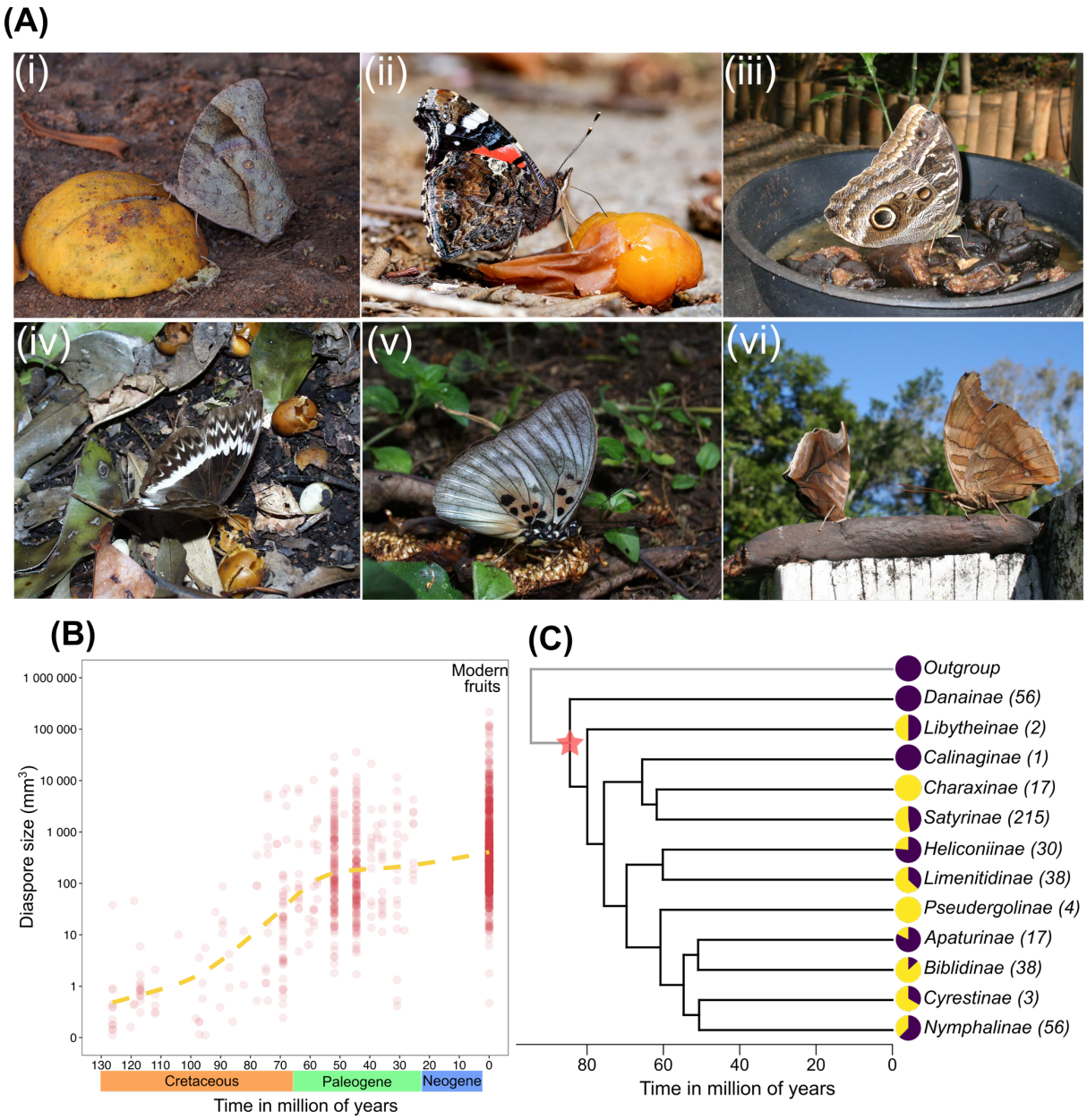

Figure 1: Evolution of fleshy fruits and widespread evolution of frugivory in butterflies. (A) Representative examples of frugivorous butterflies (see image credits at the below). (B) Evolution of Angiosperm diaspore size (or fruit size) based on fossil records from *(4)*, with a loess-smoothed trend line. Fruit sizes at 0 million years indicate the size of modern fruits. (C) Proportion of frugivorous versus nectarivorous genera (yellow = frugivory, violet = nectarivory) across subfamilies of the Nymphalidae butterflies (root indicated by a star), based on our dataset. Numbers in parentheses after each subfamily name indicate the number of genera represented in the dataset/phylogeny. Species names and image credits for panel A are as follows: (i) *Melanitis leda* (©Bias Chakraborty, Wikimedia Commons); (ii) *Vanessa atalanta* (©Charlie Jackson, Wikimedia Commons); (iii) *Caligo illioneus* (©André V.L. Freitas); (iv) *Cymothoe herminia* (©Freerk Molleman); (v) *Euphaedra edwardsi* (©Freerk Molleman); (vi) *Historis odius* (©André V.L. Freitas).

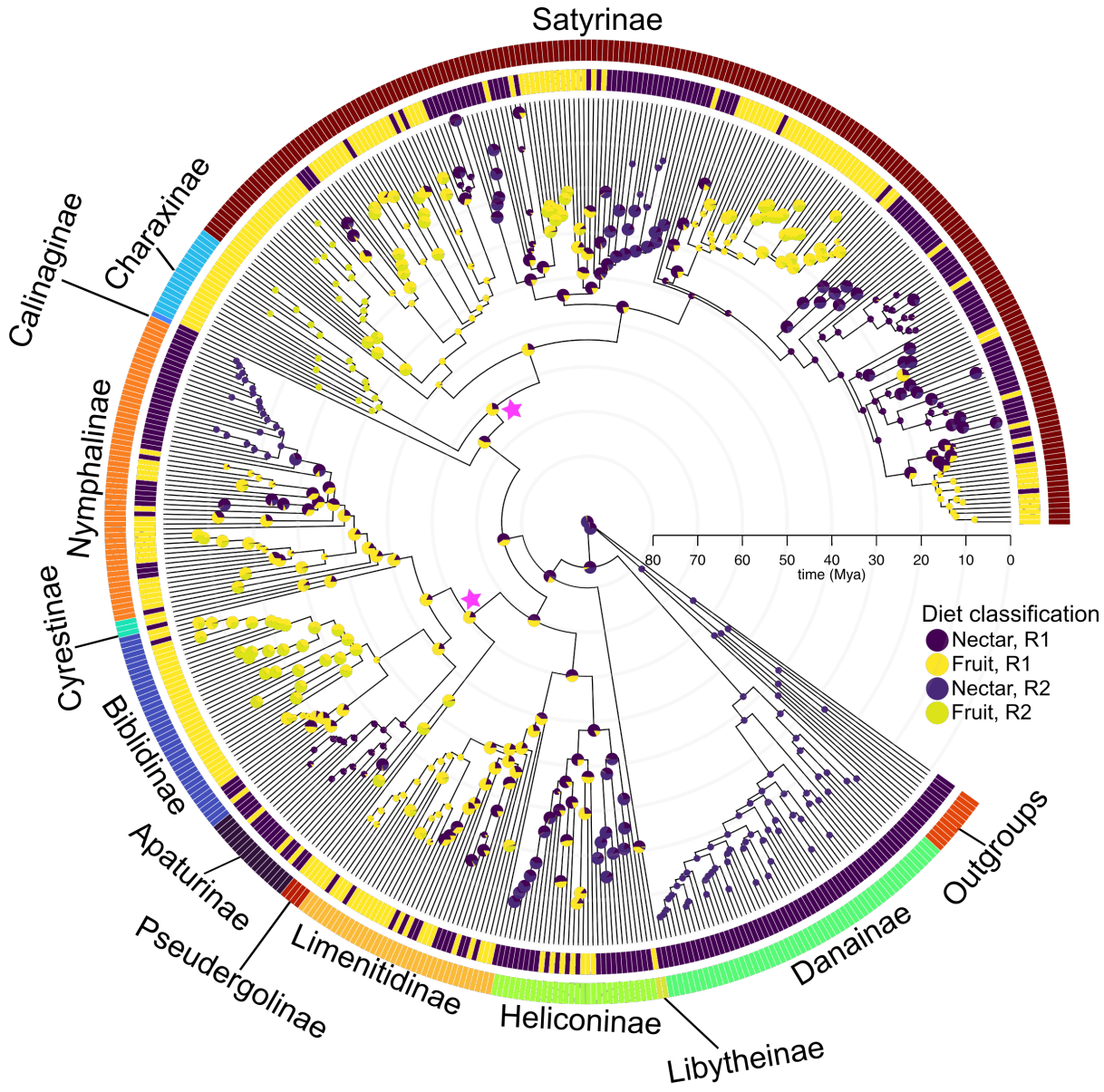

Figure 2: Ancestral state estimation of adult diet transitions. Maximum likelihood ancestral estimation of diet evolution across the Nymphalidae genera using the best-fit *Mk* model (equal-rates hidden-rate model). Outgroups (n=11 tips) are represented by other butterfly families. Diet states with R1 and R2 indicate two hidden-rate classes (see legend on the right). The outer ring of the phylogeny indicates different Nymphalidae subfamilies (colour-coded), while the inner ring indicates adult diet (frugivory vs nectarivory). Two deepest nodes having >72% marginal probability of being frugivorous are marked with 'stars'. Node sizes are scaled such that nodes with >85% marginal probability of being in a certain state will have a smaller size.

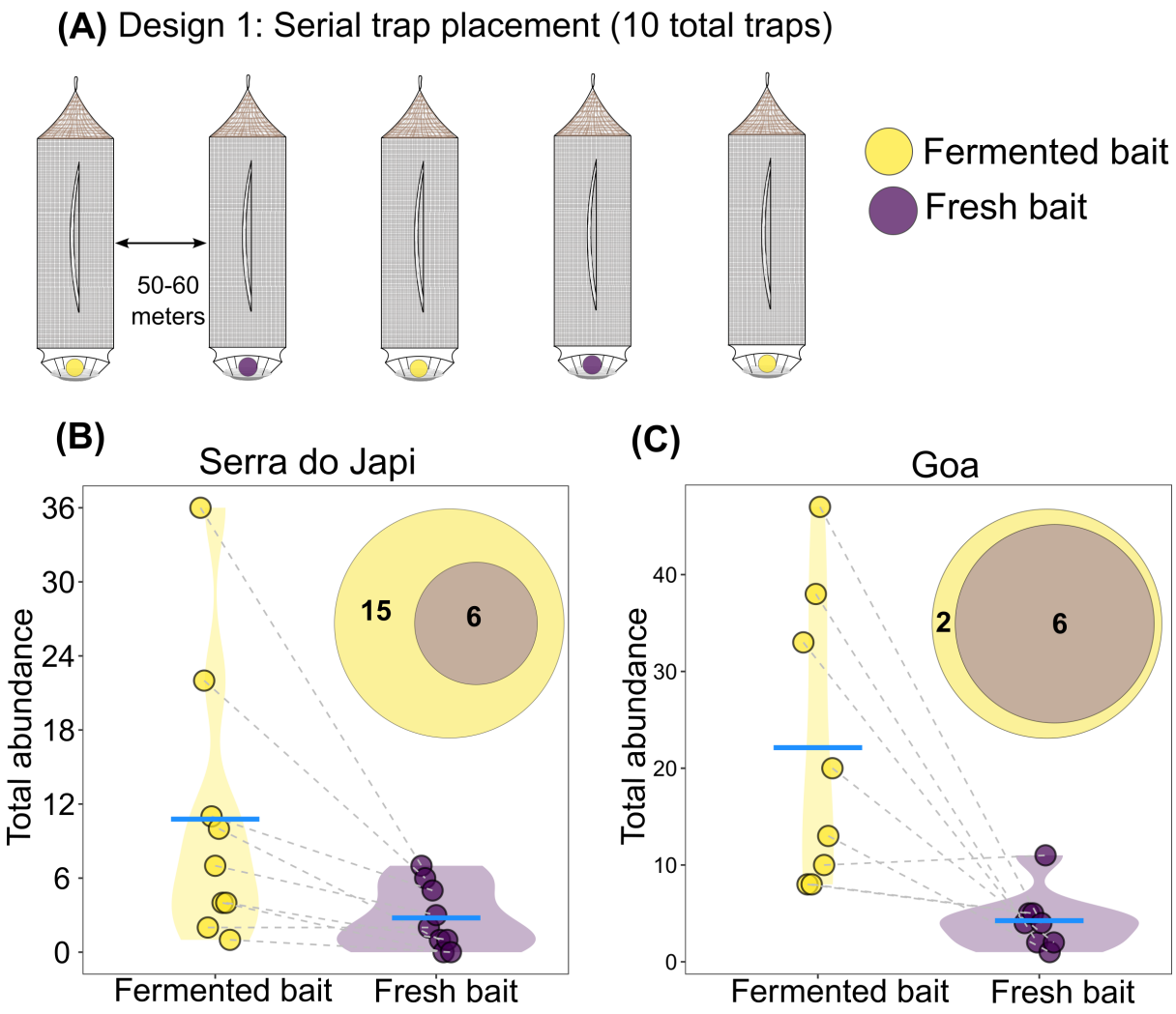

Figure 3: Results of field experiments testing bait preference of frugivorous butterflies in the field. Experimental design 1 (A) was used for testing the bait preference of frugivorous butterflies in Serra do Japi (Brazil) and Goa (India). Ten traps baited with fermented or fresh banana were placed sequentially along the transect (A), and butterfly richness (*Fig. S11*) and total abundance in the traps were recorded in Serra do Japi (B) and Goa (C). Each point in panels B and C represents a temporal replicate, with points from the same sampling day connected by dotted lines. The horizontal blue line for each treatment indicates the mean species abundance. Venn diagrams in the insets of panels B and C show the number of species unique species captured by fermented bait (yellow), by fresh bait (purple), and species common to both bait types. The placement of the fresh-bait circle within the fermented bait circle indicates no unique species were captured in traps containing fresh bait.

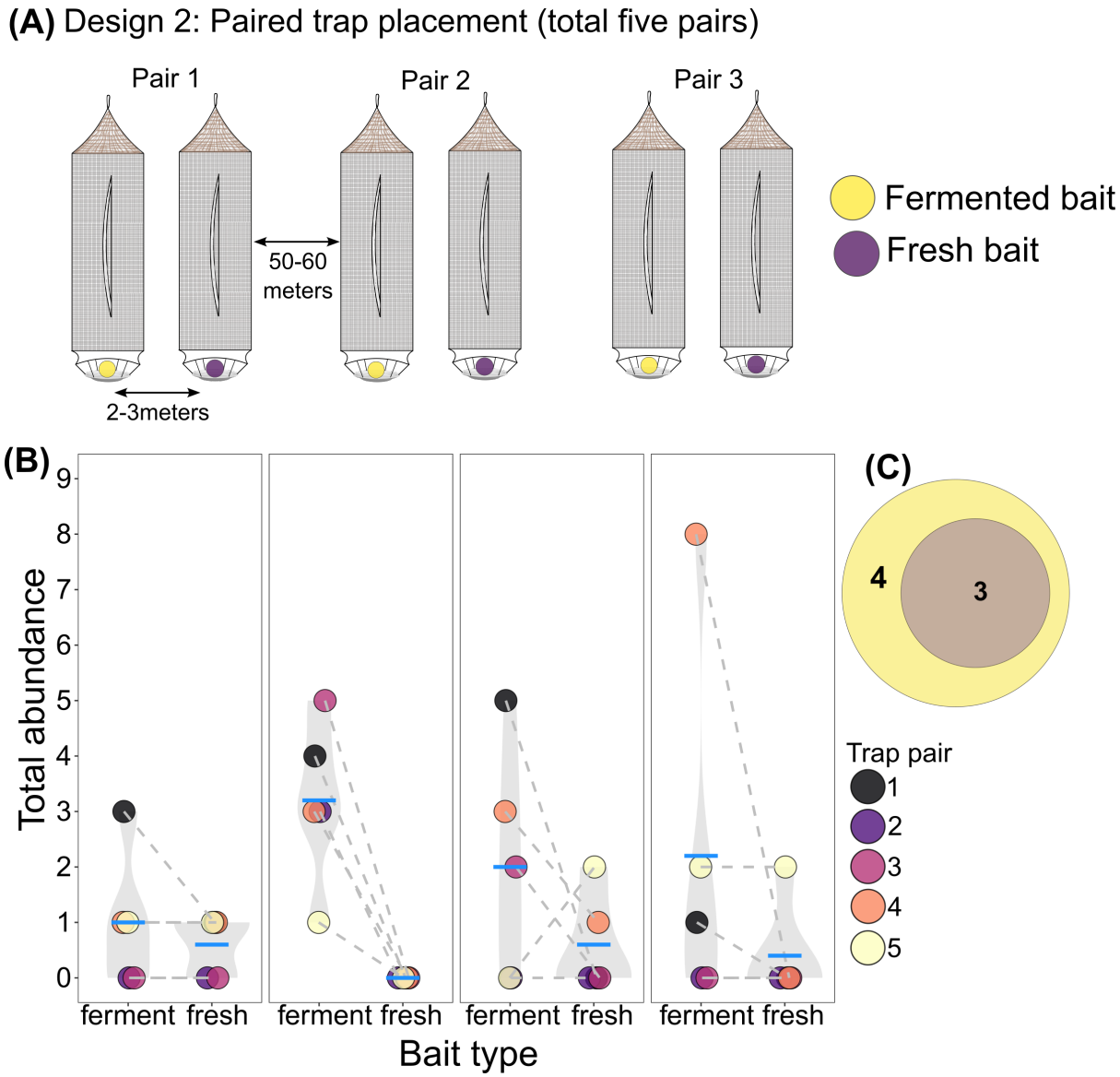

Figure 4: Results of field experiments testing bait preference of frugivorous butterflies in the field. Experimental design (A) used for testing the bait preference of frugivorous butterflies in Goa (India). Five pairs of fruit-bait traps, with one trap containing fermented banana and the other fresh banana bait, were placed along the transect. Butterfly species richness *(Fig.S12)* and total abundance (B) were recorded. Each panel in B represents a temporal replicate. Within each panel, each data point represents a trap pair, and the paired observations from the same sampling day are connected by a dotted line. Trap pairs are highlighted using different colours (*see legend, bottom right*). The horizontal blue line for each treatment indicates the mean abundance for each bait treatment. Venn diagrams in panel C show the number of species unique species captured by fermented bait (yellow), by fresh bait (purple), and species common to both bait types. The placement of the fresh-bait circle within the fermented bait circle indicates no unique species were captured in traps containing fresh bait.

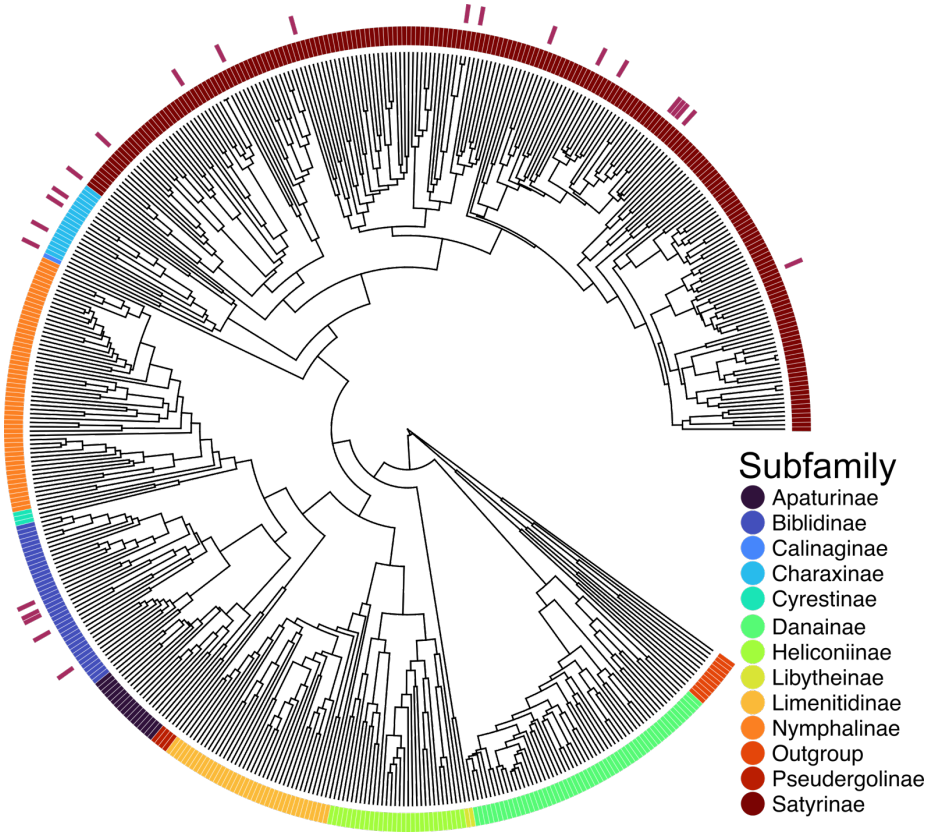

Figure 5: Mapping of unique genera captured in the field experiments onto a genus-level phylogeny of Nymphalidae butterflies. Unique genera from experiments are marked on the outer ring of the phylogeny and the inner ring indicates Nymphalidae subfamilies (color-coded, see legend).