Paternal melanisation defines wing spot area of male *Drosophila nepalensis*: supporting evidence through genetic crosses.

Seema Ramniwas
Chandigarh University
Divya Singh (singh.genetics@gmail.com)
Chandigarh University https://orcid.org/0000-0002-5075-3866

Research Article

Keywords: Drosophila nepalensis, wing melanisation, genetic cross, genetic polymorphism, phenotypic plasticity

Posted Date: November 15th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1020671/v1

Abstract

Males of *Drosophila nepalensis* show dimorphism in wing melanisation but how they evolve and coordinate during evolution is unknown. Heterogeneity in environment helps individuals to adapt accordingly either through genetic polymorphism or through phenotypic plasticity. In this study, we tried to untangle the genetic architecture underlying differences in wing melanisation in males because in nature the frequency of spotted and spotless males is different. We investigated the variation in wing spot formation in males of *D. nepalensis* via genetic cross. We found wing spot formation on wings in male is directly correlated to female body melanization. We report that the wing spot of *D. nepalensis* show very high plasticity and correlated with female body melanization instead of male body melanzation. Only at 21°C temperature we found darker and complete light females, dark female progeny always produced male with spotted wing whereas lighter female produced male without wing spot. The degree of wing melanisation in males of *D. nepalensis* was assessed to check plasticity patterns. We investigated that increased wing spot area (WSA) is negatively correlated with higher temperature. Finally, we find wing spot is highly correlated in reciprocal progeny due to linkage or pleiotropy which could help in evolution.

Introduction

The key purpose of ecology is to understand the evolution of adaptations because the only alternative for organisms (which are not capable to move fast in changing climatic envelopes) to escape extinction is adaptation (AA and CM, 2011) (Bell and Gonzalez, 2011). Phenotypic plasticity in terms of gene expression is one of the main molecular mechanisms of adaptation (Pigliucci, Murren and Schlichting, 2006) and presently when anthropogenic changes is responsible for species extinction, research is prioritizing investigations to find out mechanisms which allow the organisms to cope with changing environment.

Drosophila melanogaster is abundant on Indian subtropics and it possess melanisation variability (Parkash, Rajpurohit and Ramniwas, 2008) (Parkash, Sharma and Kalra, 2008). Different methods were performed to measure pigmentation in *D. melanogaster* like measuring width of abdominal stripe; measuring intensity of thoracic trident and examining each fly's level of melanisation at specific point (Robertson, Briscoe and Louw, 1977) (Michael E.N. Majerus, 1998) (Pool and Aquadro, 2007) (Wittkopp et al., 2011) but, for *Drosophila* species i.e. *Drosophila nepalensis* the inheritance of wing spot dimorphism remains largely unknown to assess the impact of climatic selection.

There are traits like sexual dimorphism, abdominal melanisation and wing melanisation which have independently developed through evolutionary succession (Kopp et al., 2003). Genesis of wing melanisation have long been the subject of speculation and has significantly contributed in adaptation of various species of butterflies via mate choice, providing defense against predators through mimicry and through thermoregulation (Watt, 1968) (Roland, 1982) (Kingsolver, 1987) (Wiernasz, 1989) (Ellers and Boggs, 2002) (Ellers and Boggs, 2003). The *Drosophila* species shows variations in melanisation (either

in body or in wing) and hence in association with abiotic factors there is existence of different phenotypes (dark, light body pigmentation or spotted and spotless wings) in nature. It is observed that appearance of spot on wing of *D. nepalensis* is restricted in particular area and this wing spot area (WSA) is limited to males only. The genetic basis of WSA is not clear yet. Through Figure 1, we showed the bibliographic map analysis of the selected keyword, i.e., 'wing spot area' in the web of science database. A total of 282 studies reported in the previous 22 years, and all of them have been selected for bibliographic analysis. Among the total reported 1830 terms, the most relevant 74 terms have met the threshold with minimum occurrences of 5. Also, proximately 100 % most relevant terms (i.e., 74) terms have been selected for analysis. It has been found most of the studies are in relation to Pest, wing size, sex, evolution, development, host, frequency, age, yeast, courtship, variation and habitat of USA in *Drosophila suzukii* or *buterflies*. But a gap of studies has been observed for different *Drosophila species* for investigations of wing melanisation, wing spot size, sex, different structures, evolution, plasticity, inheritance of wing melanisation and habitat in different island etc. (Figure 2).

The characteristics shown by an individual are basically ancestral inheritance where transmission of qualities occurs generation to generation from ancestors or parents to progeny. To study parental inheritance and genetic basis of WSA in males of *D. nepalensis* we used different body color females (Dark and light) for genetic crosses in present study and simultaneously we checked change in any dimension of male WSA, especially in F_1 and F_2 progeny. We examined effect of growth temperature on WSA to check developmental plasticity. In nature there exists sexual dimorphism for melanisation in *D. nepalensis* (wing spot in males and abdominal melanisation in females). Hence, *D. nepalensis* is suitable model to check (i) paternal inheritance of WSA (ii) plasticity of WSA across different temperatures (iii) frequency variation of spotted and spotless males across genetic crosses. Finally, we compared our observations on WSA from genetic crosses with field data because wing spot dimorphism in *D. nepalensis* could be directed by natural selection with constant changes in climate.

Material And Methods

Culturing

We collected ~100-150 individuals of *D. nepalensis* in pre-winter (Oct - Nov) and winter (Dec-Jan) from nature i.e. from highland localities (Kasauli and Shimla) of Western Himalayas in India. We used the bait trap and net sweeping method to collect flies from fruit markets and godowns. We kept the wild caught flies at 21 ºC based on the T_{ave} data of pre-winter and winter season of highland localities and we obtained the climatic data record from IITM (Indian Institute of Tropical Meteorology; www.tropmet.res.in). We prepared minimum 50 isofemale (IF) lines to assess repeatability of different traits for G_1 and G_2 generations. From each isofemale lines, we took 25-30 pairs of flies to lay eggs at 21 ºC in eight replicates to study the plasticity of WSA across different temperatures and kept two replicates at each temperature i.e. at 17, 21 and 25 ºC.

Dark and light Isofemale lines

Abdominal melanisation variation is visible in females of *D. nepalensis* while males of this species are totally black. Vice-versa, wing melanisation (spotted and spotless wings) is visible in males only and not visible in females. We observed that collections done in winter months, showed totally darker flies whereas, a few lighter flies were obtained along with darker flies from collections done in pre-winter months. From the 50 established IF lines at 21°C, we checked true breeding IF lines from the successive progeny and up to 11 generations homozygosity of each IF line was checked for dark and light phenotypes. Dark, intermediate and light phenotypes were observed in many IF lines in 1:2:1 ratio. We checked the genetic basis of melanisation through F_1 and F_2 by crossing dark and light individuals of homozygous IF lines (The lines which were showing 1:2:1 ratio) and found that in F_1 the light allele was dominant at 25°C while dark allele was dominant at 17°C. Hence, we labeled all the phenotypes as DD (dark), DL (intermediate/heterozygote) and LL (Light).

Color dimorphism and its Genetic basis

In dark and light IF lines 6th and 7th abdominal segments of female D. nepalensis are either dark or light and correspond phenotypically to dark and light IF lines while males lack variation in melanisation for abdominal segments as they are totally black but males of dark IF lines progeny are always with spotted wings while males of light IF lines progeny are always with spotless wings. To assess genetic basis of divergence in melanisation for abdominal pigmentation and for wing melanisation we set up the genetic crosses with individuals of true breeding Dark IF lines and Light IF lines. Single male and single female of each phenotype (DD and LL) were allowed for single mating and in the F_1 progeny 100 female flies were scored for abdominal melanisation and to obtain F_2 progeny 50 males and 50 females from F_1 were selected randomly and 10 pairs were used in five replicates.

Mendelian crosses to determine genetic basis

We collected virgin males (♂) and virgin females (♀) from homozygous true-breeding dark (DD) and light (LL) IF lines of *D. nepalensis*. We further made two crosses to determine genetic basis of WSA. We allowed single-pair mating in 10 replicates for each cross. In cross I, DD ♀ and spotless ♂ and in cross II, LL ♀ and spotted ♂ were crossed to check their F_1 and F_2 progeny.

Calculation of Wing spot area (WSA; mm^2)

WSA (mm^2) in males across different growth temperatures (17, 21 and 25 ºC to get the winter, pre-winter and average temperature) was measured visually using stereo zoom microscope (www.olympus.com) at 3 × magnifications. Wing of each male fly (grown at different temperatures) was mounted on slide and was analyzed with Biowizard image software (www.dewinterindia.com). % WSA per fly was calculated with high significance (correlation value; r = 0.99).

Assessment of phenotypic plasticity

We measured the plastic effects on melanisation (abdominal pigmentation or melanisation in females and wing melanisation in males) due to developmental growth temperatures (17, 21 and 25°C) in

parental IF lines as well as in F_1 progeny of crosses (Cross I and II). After the egg laying in 8 vials from 30 pairs of each parental IF lines and Crosses (I and II) for 8 hours at 21 ºC, two replicate vials having 60-80 eggs were maintained at 3 growth temperatures (17, 21 and 25°C). Each 6 day old fly of the progeny from each vial were analyzed for melanisation (abdominal in females and wings in males) by 2 methods:

a) Visual scoring using stereo-zoom microscope where body melanisation of the female abdomen was scored from dorsal view and values from 0 (no melanisation) to 10 (complete melanisation) were given for each abdominal segments.

b) Analysis using Biowizard software on basis of video camera and captured image where each fly's abdomen minus viscera was mounted on a slide.

Percent melanisation = (Σ observed weighted melanisation scores of abdominal segments per fly)/ (Σ relative size of each abdominal segment × 10 per fly) × 100

Statistical analysis

We use the χ^2 test for all crosses to compare expected phenotypes with observed phenotypes. Mean values were used for representation of data. In F_1 to check dominance effect for melanisation (abdominal melanisation in females and wing melanisation in males) total melanisation value of parents was subtracted from F_1 values. Statistica 5.0 (Statsoft Inc., Tulsa, Oklahoma, USA) was used for analysis. We used VOSviewer for bibliographic analysis.

Results

Genetic analysis of Wing melanisation in males

Inheritance of male wing melanisation of *Drosophila nepalensis* is shown via genetic cross in Figure 3. Darker female progeny when crossed with spotted males always produces spotted wing in males (Figure 3A) while the lighter female progeny when crossed with spotless males always produces spotless wings in males (Figure 3B). In crosses, whenever darker mother is crossed with spotless males then the F_1 progeny is always with spotted wings but size of spot decreases while the F_2 progeny segregates into 3 spotted: 1 spotless ratio of males (Figure 4, Cross I) and whenever the lighter female is crossed with spotted males then the F_1 progeny is always spotted with very small spot area and very less intensity of wing melanization (~ negligible) and the F_2 progeny simultaneously segregates into all spotless males ((Figure 4, Cross II).

Plasticity of wing spot area in males

Spotted males have shown increase (~3.67 fold) in the spot area across growth temperatures (17 to 25 °C) in *D. nepalensis* (Table 2). At 21°C we found spotted wing males and spotless wing males and this is because lighter female always produced male without wing spot whereas darker female produced male with spotted wings. We found no plastic effect in *Drosophila nepalensis* male body color but wing spot

area is highly plastic. Spotted male have shown higher plasticity at all growth temperatures (Table 2, Figure 5). So there is not any correlation between body color plasticity and wing spot melanization plasticity in *Drosophila nepalensis* male.

Wing spot Genetic basis

Total number of male individuals in progeny of crosses (I and II) along with their replicates are shown in Table 3. We observed two types of males in progeny of both the crosses based on size of WSA. In cross I, we observed all spotted males with small WSA in F_1 generation while in F_2 generation all males segregated into spotted and spotless in 3:1 ratio (Figure 4, Table 3). In cross II, we observed all spotless males with very less intensity of WSA in F_1 but in F_2 we observed all spotless males without any WSA (Figure 4, Table 3). The spotted and spotless males of cross I and II are due to genotype of mother irrespective of genotype of father (Figure 4). Thus, the wing dimorphism (wings with and without WSA) in *D. nepalensis* is due to maternal inheritance.

Discussion

Present work on inheritance of WSA is interesting in several respects (a) quite high plasticity for WSA in *D. nepalensis* (b) Increase in number of males with WSA at lower temperature and increase in number of males without WSA at higher temperature. Our result confirms that number of *D. nepalensis* male, eclosing in nature differs significantly in accordance to wing melanisation (Table 1). Thus, we can say thermal variation is playing a significant role for natural selection as presence of intense WSA in males at lower temperature increases the mating propensity. Our analysis of the genetics of male wing spot inheritance is one of the first attempts to genetically dissect a difference within a species.

Brisson and co-workers found body melanism correlation with habitat in *D. polymorpha* of Brazil and gave evidence for presence of dark and light morph in open and forested environments respectively (Brisson *et al.*, 2005). However, in the present studies, habitat selection of wing spot males was not investigated but our results shows that male wing spot is correlated with female body color i.e. if females are darker in color; males are always with wing spot and if females light in color then males are without spot on wings (Figure 1).

Our result (Table 1) explains the variation in frequency of total males (spotted and spotless) in field in varying seasons (pre winter and winter). Winter season select spotted flies because wing spot formation on wings in male is directly correlated to female body melanization and females in winter (lower temperature) are always dark. A phenotypic manifestation of wing melanisation in *D. nepalensis* has two interesting features: (i) WSA in nature as well as in laboratory is influenced by temperature (ii) dominance of spotted male over spotless male.

We are not aware of how wing melanism varies in different insects. We are aware of two parallel cases: (a) In *D. polymorpha* of Brazilian population, lack of thermal plastic effects (Brisson *et al.*, 2005); and (b) For populations of two-spot ladybird, lack of geographical variation in morphs (non-melanic being the

dominant allele) (Majerus, 1994); (BRAKEFIELD, 1985). The occurrence of such genetic strategies across insect taxa should have adaptive significance. The dominance of spotted male over spotless male might help *D. nepalensis* to adjust environmental conditions.

For *D. nepalensis,* occurrence of wing color polymorphism and its inheritance has not been reported previously. It is worth consideration to check fitness of an individual when phenotype of organism is in accordance with behavior. The common trait of insect taxa is melanism and it has mixed evolutionary effects. Ecological significance of melanism in cold adapted temperate insect taxa including cosmopolitan and temperate endemic drosophilids favors thermal melanism (Michael E.N. Majerus, 1998); (Sabath, Richmond and Torrella, 1973). *D. nepalensis* have adapted to climatic stresses by modifying the frequencies of wing spot males through assortative matings. It may be concluded that seasonal changes can help to maintain frequency of spotted and spotless males in *D. nepalensis*.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: The datasets used and/or analysed during the current study are included in this article and its supplementary information files available from the corresponding author on reasonable request.

Competing interests: Authors declare no competing interests.

Funding: SR is grateful to the Department of Science and Technology (DST), New Delhi for the financial support through the SP/YO/775/2018G project and DS is also grateful to the Department of Science and Technology (DST) for the financial support through SP/YO/2019/1165(G).

Author's contributions: SR contributed to the study conception and design. Material preparation, data collection and analysis were performed by SR and DS. Data analysis and draft preparation level was performed by SR and DS. The first draft of the manuscript was written by DS. Both authors read and approved the final manuscript.

Acknowledgements: Authors are grateful to Chandigarh University. SR and DS both are grateful to the Department of Science and Technology (DST), New Delhi for the financial support through the SP/YO/775/2018G and SP/YO/2019/1165(G) project respectively.

References

AA, H. and CM, S. (2011) 'Climate change and evolutionary adaptation', *Nature*, 470, p. 479.

Bell, G. and Gonzalez, A. (2011) 'Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration', *Science*, 332(6035), pp. 1327–1330. doi: 10.1126/science.1203105.

BRAKEFIELD, P. M. (1985) 'Polymorphic Müllerian mimicry and interactions with thermal melanism in ladybirds and a soldier beetle: a hypothesis', *Biological Journal of the Linnean Society*, 26(3), pp. 243–267. doi: 10.1111/j.1095-8312.1985.tb01635.x.

Brisson, J. A. *et al.* (2005) 'Abdominal pigmentation variation in Drosophila polymorpha: Geographic variation in the trait, and underlying phylogeography', *Evolution*, 59(5), pp. 1046–1059. doi: 10.1111/j.0014-3820.2005.tb01043.x.

Ellers, J. and Boggs, C. L. (2002) 'The evolution of wing color in Colias butterflies: Heritability, sex linkage, and population divergence', *Evolution*, 56(4), pp. 836–840. doi: 10.1111/j.0014-3820.2002.tb01394.x.

Ellers, J. and Boggs, C. L. (2003) 'The evolution of wing color: Male mate choice opposes adaptive wing color divergence in Colias butterflies', *Evolution*, 57(5), pp. 1100–1106. doi: 10.1111/j.0014-3820.2003.tb00319.x.

Kingsolver, J. G. (1987) 'Evolution and Coadaptation of Thermoregulatory Behavior and Wing Pigmentation Pattern in Pierid Butterflies', *Evolution*, 41(3), p. 472. doi: 10.2307/2409250.

Kopp, A. *et al.* (2003) 'Quantitative trait loci responsible for variation in sexually dimorphic traits in Drosophila melanogaster', *Genetics*, 163(2), pp. 771–787. doi: 10.1093/genetics/163.2.771.

Majerus, M. E. N. (1994) 'Ladybirds.', *Bulletin of Entomological Research*, p. 367.

Michael E.N. Majerus (1998) 'Melanism: Evolution in action', *Cambridge University Press*, 72(1).

Parkash, R., Rajpurohit, S. and Ramniwas, S. (2008) 'Changes in body melanisation and desiccation resistance in highland vs. lowland populations of D. melanogaster', *Journal of Insect Physiology*, 54(6), pp. 1050–1056. doi: 10.1016/j.jinsphys.2008.04.008.

Parkash, R., Sharma, V. and Kalra, B. (2008) 'Climatic adaptations of body melanisation in Drosophila melanogaster from Western Himalayas', *Fly*, 2(3), pp. 111–117. doi: 10.4161/fly.6351.

Pigliucci, M., Murren, C. J. and Schlichting, C. D. (2006) 'Phenotypic plasticity and evolution by genetic assimilation', *Journal of Experimental Biology*, 209(12), pp. 2362–2367. doi: 10.1242/jeb.02070.

Pool, J. E. and Aquadro, C. F. (2007) 'The genetic basis of adaptive pigmentation variation in Drosophila melanogaster', *Molecular Ecology*, 16(14), pp. 2844–2851. doi: 10.1111/j.1365-294X.2007.03324.x.

Robertson, A., Briscoe, D. A. and Louw, J. H. (1977) 'Variation in abdomen pigmentation in Drosophila melanogaster females', *Genetica*, 47(1), pp. 73–76. doi: 10.1007/BF00122443.

Roland, J. (1982) ‘Melanism and diel activity of alpine Colias (Lepidoptera: Pieridae)’, *Oecologia*, 53(2), pp. 214–221. doi: 10.1007/BF00545666.

Sabath, M. D., Richmond, R. C. and Torrella, R. M. (1973) ‘Temperature-Mediated Seasonal Color Changes in Drosophila putrida’, *American Midland Naturalist*, 90(2), p. 509. doi: 10.2307/2424482.

Watt, W. B. (1968) ‘Adaptive Significance of Pigment Polymorphisms in Colias Butterflies. I. Variation of Melanin Pigment in Relation to Thermoregulation’, *Evolution*, 22(3), p. 437. doi: 10.2307/2406873.

Wiernasz, D. C. (1989) ‘Female Choice and Sexual Selection of Male Wing Melanin Pattern in Pieris occidentalis (Lepidoptera)’, *Evolution*, 43(8), p. 1672. doi: 10.2307/2409383.

Wittkopp, P. J. *et al.* (2011) ‘Local adaptation for body color in Drosophila americana’, *Heredity*, 106(4), pp. 592–602. doi: 10.1038/hdy.2010.90.

Tables

Table 1: Frequency of Total males (spotted and spotless) in field in varying seasons (pre winter and winter)

Seasons	**Pre-winter**		**Winter**	
	Spotted male	**Spotless male**	**Spotted male**	**Spotless male**
No. of individuals	158	52	352	21
Frequency	75.23%	24.76%	94.36%	5.63%

Table 2: Comparison of wing spot area (mm^2) in *D. nepalensis* when grown on 17, 21 and 25 °C.

Type of males	**17 °C**	**21 °C**	**25 °C**	**t- test (17 vs 25 °C)**	**t- test (21 vs 25 °C)**
Spotted	0.56 mm^2	0.48 mm^2	0.15 mm^2	***	***
Spotless	0.0	0.0	0.0	0.0	0.0
t - test	***	***	***		

*** $p < 0.001$

Table 3: Number of male individuals scored in F_1 and F_2 progenies of parental lines and different crosses.

	Replicates		Total Males (n)	Spotted Males	Freq. %	Spotless Males	Freq. %	Mendelian Ratio	χ^2 test
Parental lines									
DD ♀ x S ♂	F_1		175	175	100	0	0	—	—
LL♀ X SL	F_1		139	0	0	139	100	—	—
Crosses									
DD ♀ x SL ♂	F_1		115	115	100				
	F_2	1	105	79	75.24	26	24.76	3.04 : 1	ns
		2	110	83	75.45	27	24.55	3.07 : 1	ns
		3	113	85	75.22	28	24.78	3.03 : 1	ns
		4	106	79	74.53	27	25.47	2.92 : 1	ns
		5	95	71	74.74	24	25.26	2.96 : 1	ns
LL ♀ x S ♂	F_1		100	0	0	100	100	—	—
	F_2	1	106			106	100	—	—
		2	113			113	100	—	—
		3	116			116	100	—	—
		4	95			95	100	—	—
		5	115			115	100	—	—

DD ♀ = Homozygous Dark Female, LL ♀ = Homozygous Light Female, S ♂ = Spotted male, SL ♂ = Spotless male, ns = nonsignificant

Figures

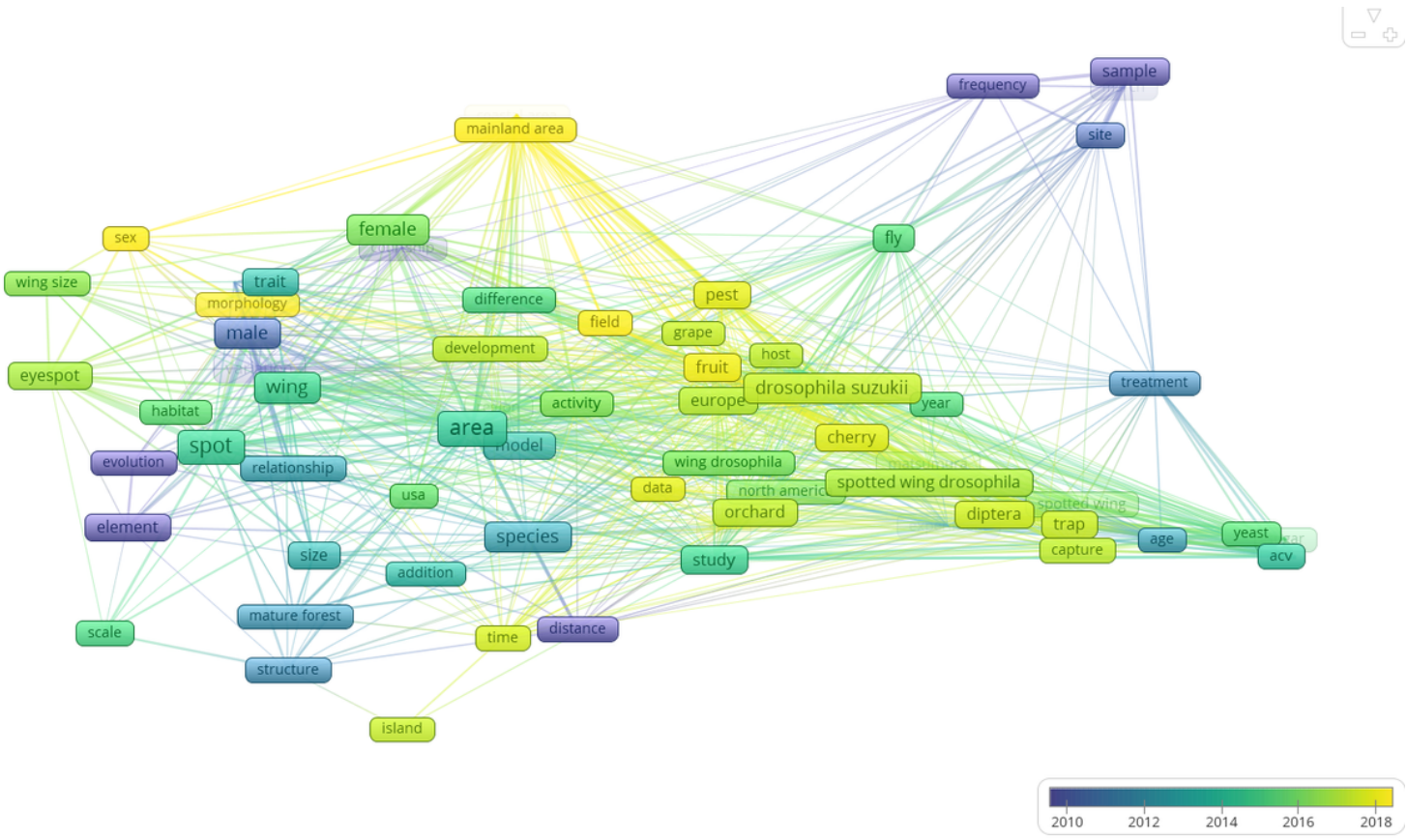

Figure 1

Detailed VOS-Viewer bibliographic analysis for the keyword ‘wing spot area’ (Database source: www.webofknowledge.com) (colors of nodes represent the clusters)

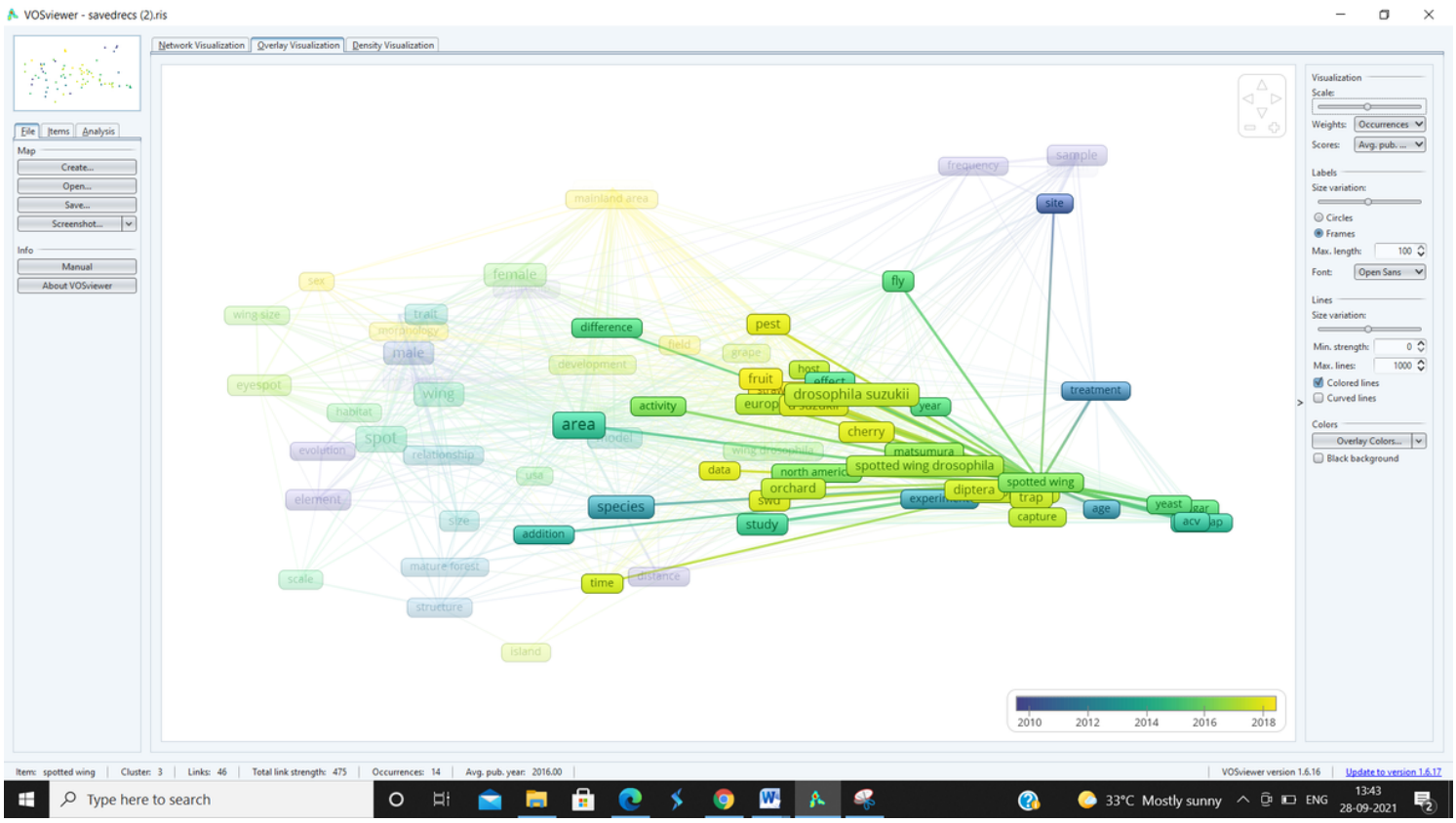

Figure 2

Bibliographic gap analysis for wing spot area from year 2010 to 2018. (Database source: www.webofknowledge.com) (colors of nodes represent the clusters).

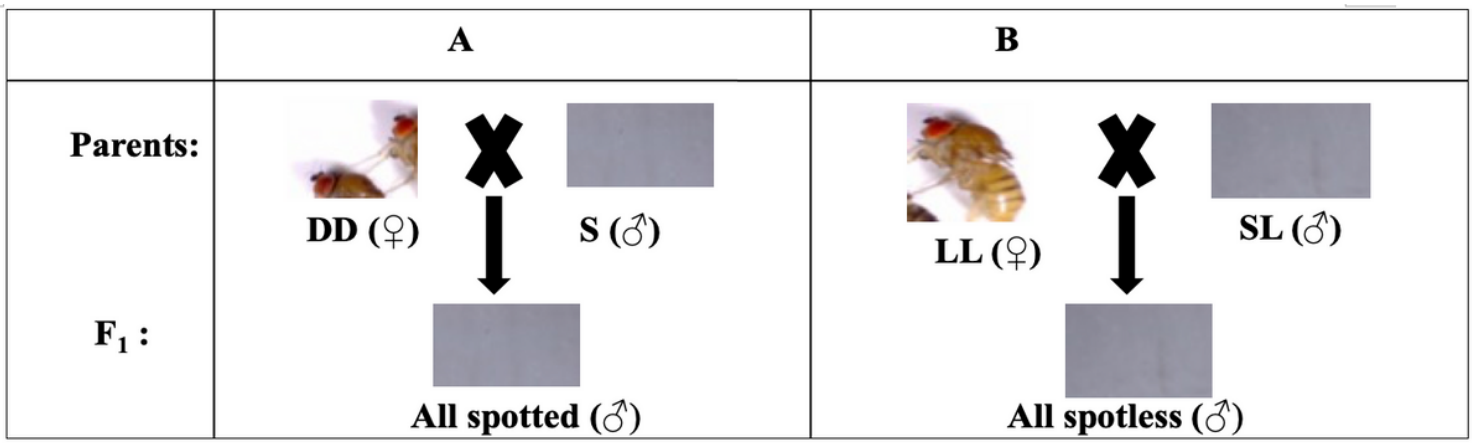

Figure 3

Genetic analysis of wing spot area (WSA) from parental Drosophila nepalensis lines. Homozygous darker females crossed with spotted males (A) and the homozygous lighter females crossed with spotless males (B) to get progeny.

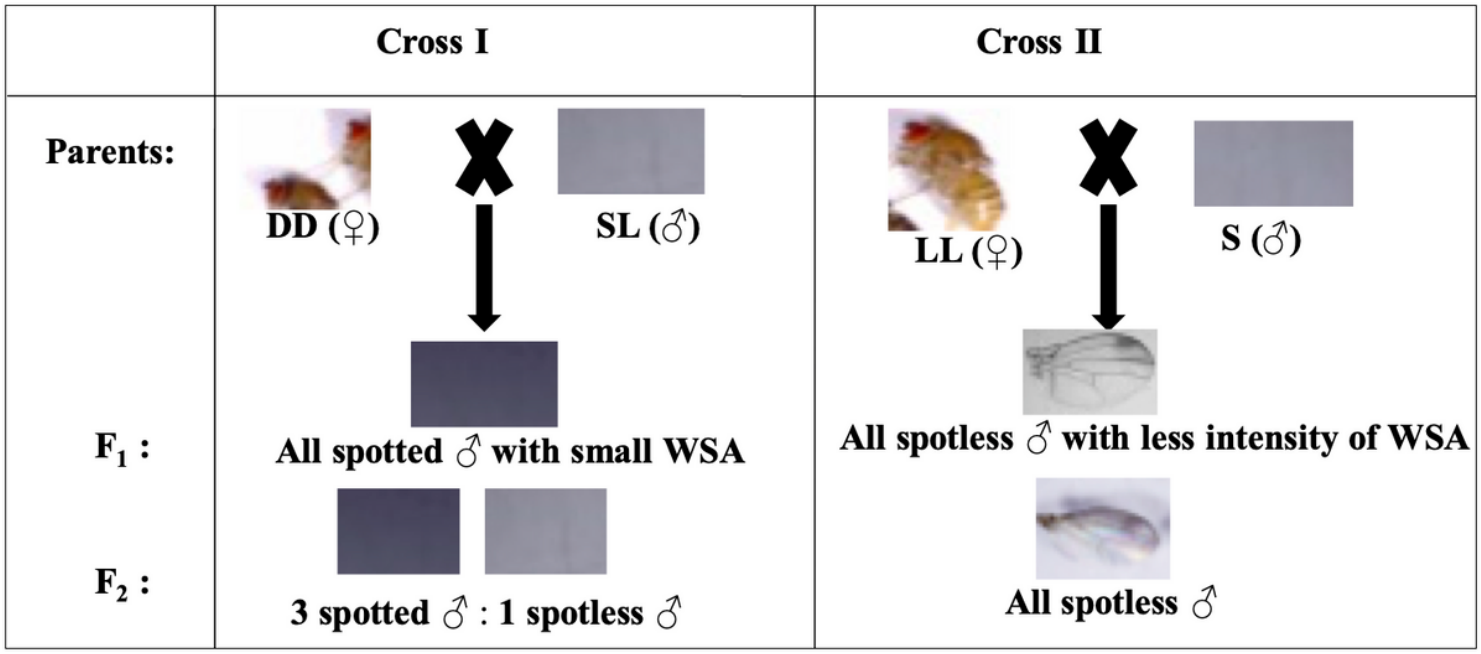

Figure 4

Inheritance of wing spot area (WSA) in males of Drosophila nepalensis checked via F1 and F2 progeny of Cross I (darker mother and spotless male) and Cross II (lighter mother and spotted male).

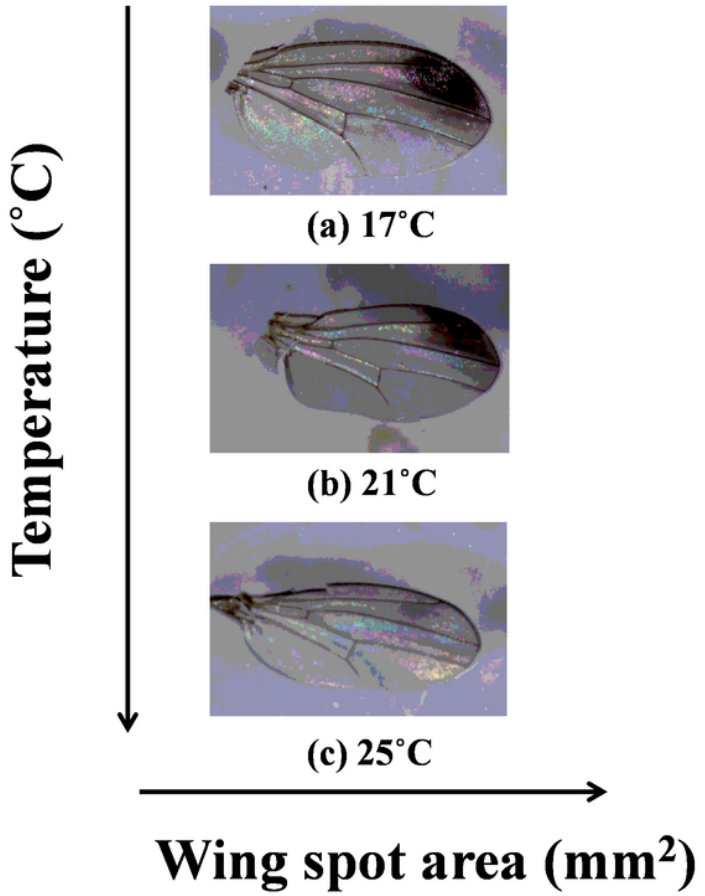

Figure 5

Wings of Drosophila nepalensis males across various growth temperatures (a = 17˚C; b = 21˚C; c = 25˚C) showing developmental plasticity for wing spot area (WSA).