REVIEW ARTICLE

Drosophila pallidosa- whether a separate species or a light form of *D. ananassae*

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Keywords. *D. ananassae*; *D. pallidosa*; sibling species; light and dark forms.

Running title: *D. pallidosa* and *D. ananassae*- separate species or light and dark forms

Abstract

Drosophila pallidosa is a species belonging to the *D. ananassae* complex, in which a total of ten species are included. Earlier it was known as light form of *D. ananassae* but later it was described as a new species and sibling with *D. ananassae*. Both these terms, light form and sibling species were used by Futch. Therefore, it is very clear that this sibling species pair having certain similarities as well as dissimilarities, which makes the taxonomic status of *D. pallidosa* confusing. While keeping this fact in mind, we have tried to put both the views to understand the actual status of this sibling species pair in this review article. Considering similarities and dissimilarities, we suggest that *D. pallidosa* does not have the full status of a species, rather it is in the process of speciation, *statu-nascendi*. Our suggestion is strengthened by the fact that male genitalia are identical in both the cases and they lack postmating reproductive isolation because hybrids between them are normal and fully fertile.

Introduction

Drosophila pallidosa is a species belonging to the *D. ananassae* complex, in which a total of ten species are included (Tobari 1993). Earlier it was known as light form of *D.ananassae* (Futch 1966) but later it was described as a new species (Bock and Wheeler 1972; Futch 1973) simply on the basis of sexual isolation and variations in the sex-comb tooth numbers while both the species possess identical male genitalia which are very important taxonomic character. Thus, these two different statements given by Futch himself have goaded us the most.

Hence, because of these two contradictory statements, always an intriguing question about the status of *D. pallidosa* comes in our mind that it is a separate species or the light form of *D. ananassae* which is in the process of speciation. From the literature, it is very clear that this sibling species pair having a number of similarities and dissimilarities, which make their status confusing and mysterious for us. While keeping this fact in mind, in this review article we tried to put both the views to understand the actual status of this sibling species pair. Therefore, we have divided the whole article under two headings: evidence in favour of light form, dealing with similarities and evidence in favour of separate species, dealing with dissimilarities.

Evidence in favour of light form

Certain similarities which show that the position of these two species is not yet clear, are mentioned below:

Male genitalia

Singh (2016) has discussed about myriad of sibling species of genus *Drosophila* in his review article but not a single pair of sibling species was found which have identical male genitalia

except *D. ananassae* and *D. pallidosa* (Bock and Wheeler 1972). The occurrence of identical male genitalia is very rare and matchless condition which makes this sibling species pair very unique because it is well known fact that male genitalia are very important taxonomic trait for the classification. Even though, Hsu (1949) defined many of the species groups of genus *Drosophila*, on the basis of comparative study of periphallic organs in the family Drosophilidae. However, no obvious morphological difference in male genitalia of *D. aldrichi* and *D. wheeleri* was found, suggesting that their status as valid species is questionable (Villela 1983), whereas, they produce sterile males (Patterson and Alexander 1952), reflecting the presence of postmating isolation but *D. ananassae* and *D. pallidosa* show the presence of identical male genitalia as well as lack of postmating isolation, making the status of these sibling species very mysterious.

Hybrid sterility

Species maintain their integrity through reproductive isolating mechanisms, which restrict the intermingling of genomes from different species (Mayr 1942). Due to these reproductive barriers, species in vicinity are unable to mate (premating) and if they do so, they are unable to produce fertile and viable progeny (postmating). Even the species in incipient stage of speciation shows asymmetrical type of hybrid sterility in males (Haldane's rule 1922).

But contrary to the above facts, this species pair is unique because they are separated only on the basis of strong sexual isolation but there is no postmating isolation between them (Futch 1973). Therefore, when mated they produce fully normal and fertile hybrids. However, for complete separation or divergence, species should need to achieve complete isolation (premating and postmating) which would cause the complete cessation of gene flow because postmating reproductive barriers might be really important in completing reproductive isolation between two species. In addition to this, interspecific hybrids of the

two species show the same level of FA as parents. Therefore hybrids are as stable as parents (Vishalakshi and Singh 2009), whereas, it is evident that divergence is directly related to developmental instability. However, Futch (1973) reported that these two species cross in nature, which suggests substantial gene flow between these species. Thus, we can say that these species have not diverged completely and are still at incipient stage of speciation. Thus, we can depict that these species are very unique and not diverged completely yet, because species in incipient stage of speciation even shows asymmetrical sterility of males, as level of divergence increases degree of sterility also increases. As Banerjee and Singh (2016) reported sterility in hybrid daughters in distantly related species but in this species pair even asymmetrical male sterility has not been found.

Interpulse interval (IPI)

Interpulse interval, a parameter of the courtship song of *Drosophila*, has been shown to play an important role in female preference among closely related species (Bannet-Clark and Ewing 1969). A number of genetical studies indicate that the differences in interpulse intervals between closely related *Drosophila* species are typically controlled by multiple genes (Tomaru and Oguma 1994). Yamada *et al.* (2002) have identified identical IPI and IPF between *D. ananassae* and *D. pallidosa*. However, it is known that IPI has a tendency to differentiate during speciation, particularly between sympatric species of *Drosophila* (Ewing and Bennet-Clark 1968; Neems *et al.* 1997). Therefore, *D. ananassae* and *D. pallidosa* which have identical value in interpulse interval represent a very rare and unique case, though they are sympatric species. Yamada *et al.* (2002) found that the other parameter of song like pulse length (PL), cycle per pulse (CPP) and burst length (BL) show species specificity. Thus, occurrence of identical interpulse interval (IPI) indicates that still divergence is needed between these two species to achieve dichotomy of IPI like crucial song parameter, which are

very important from the speciation point because IPI has shown to play an important role in female preference (Bennet-Clark and Ewing 1969).

Parthenogenesis

Parthenogenesis strains of numerous species have been reported in genus *Drosophila*. Futch (1972) found that in collection from different geographical localities, few flies from both the species, *D. ananassae* and *D. pallidosa* have parthenogenetic ability. He screened lots of geographical stocks from Mexico, Hawaii, Palmytra Island, Fiiji, Cook Islands and Papua New Guinea populations for the occurrence of parthenogenesis and found that only *D. pallidosa* from the Western Samoa population, had parthenogenetic capacity. Few flies of the two species, *D. ananassae* and *D. pallidosa* were found to have the *Parth* gene in the Samoa Islands. This may indicate that the *Parth* gene has been derived from their ancestral species and conserved in both the populations during the course of speciation. Alternately, the presence *Parth* gene in both the species, may also suggest the occurrence of substantial gene flow between them. Also, from the results of Matsuda and Tobari (1999, 2004), it is evident that interspecific hybrid females also produced impaternate progeny efficiently like the parthenogenetic strains of both the parental species. Thus, based on these findings, it is clear that *Parth* genes of both the species are thought to be homologous or don't show incompatibility. Therefore, it is likely that genetic basis for parthenogenesis is identical or not diversed so much between these two sibling species.

Molecular phylogeny

Comparative genetic and molecular research in these two sibling species is very essential for understanding the demographic history of each species as well as for resolving the evolutionary forces acting on molecular sequences. By keeping this fact in mind, Matsuda *et al.* (2009) constructed the phylogeny of *D. ananassae* species subgroup by employing

molecular approach to understand the evolution in the *ananassae* species subgroup. They examined phylogenetic relationship in the *ananassae* subgroup based on the mitochondrial COI and Y-chromosomal *K/2* loci because mitochondrial and Y-chromosomal sequences don't recombine and have smaller effective populations size than autosomal genes, causing more frequent coalescence and thus potential for the resolution of phylogeny to the greater extent between the closely related species. Matsuda *et al.* (2009) found common sequences in *D. ananassae* and *D. pallidosa* at the COI locus as well as no evidence of differences either within *D. ananassae* and *D. pallidosa* or between *D. ananassae* and *D. pallidosa* at *K/2* locus. Evidence for the lack of interspecific and geographic dichotomy at the *K/2* locus suggests that the divergence between these species pair is a very recent event. It may also be possible that due to lack of differentiation at *K/2* locus of Y-chromosome, postmating isolation is absent in these species pair because it is known that *K/2* locus of Y-chromosome play very important role in reproductive isolation. Therefore, analysis of Y-chromosomal and mitochondrial haplotypes suggests that these species pair represent a recent evolutionary radiation and may experience substantial gene flow.

Metaphase karyotype

Metaphase karyotypes of both the species are same (Futch 1966). Both are having two pairs of large V shaped metacentric autosomes; a pair of small V shaped autosomes; a pair of medium size V shaped metacentric sex chromosomes in females as well as J shaped Y chromosome in place of X chromosome in male. Therefore, existence of identical metaphase karyotypes between these two sibling species in spite of being separate species indicates that they are in early stage of speciation.

Allozyme

Johnson *et al.*(1966) found that all form of Est are equally present in both the species, indicating that if divergence has taken place between these sibling species then it is only in the narrow sense whereas in the broad sense they are very much similar. In addition to this, Johnson *et al.* (1966) did not find any hybrid band for Est C and APH. Thus, occurrence of no hybrid bands indicates genetic homology of Est C and APH between these two sibling species.

Evidence in favour of separate species

Sexual isolation

As it is known that sexual isolation is a potential cause of speciation, strong sexual isolation has been observed between these sibling spices (*D. ananassae* and *D. pallidosa*). In fact, the basis of their separation as two individual species is sexual isolation and differences in the number of sex-comb teeth (Bock and Wheeler 1972). Prior to the findings of Bock and Wheeler (1972), Futch (1966) also reported sexual isolation between two forms (light and dark forms) of *D. ananassae*. Further, Futch (1973) also found sexual isolation between these two species after discovery of Bock and Wheeler (1972) as two separate species. Vishalakshi and Singh (2006) tested sexual isolation between *D.ananassae* and *D. pallidosa* and they found strong ethological isolation between them, which is not affected by different experimental conditions. Whereas Doi *et al.* (2001) mapped some loci on distinct positions near the *Delta* locus on the middle of the left arm of the second chromosome that controls female discrimination in each species. As it is well known that mate discrimination is the only known mechanism that prevents gene flow between them, these loci may have played very important role in the evolution of reproductive isolation, and ultimately, in the speciation process between these two species. Sawamura *et al.* (2008) analysed genetic basis of female discrimination behaviour by using isogenic females from interspecific mosaic

genome lines that carry homozygous recombinant chromosomes and found that not only left arm of chromosome 2(2L) had significant effect on the willingness of females to mate with *D. ananassae* males but also the left arm of chromosome X (XL) and right arm of chromosome 3 (3R) had significant effects on the willingness of females to mate with *D. pallidosa* males.

Thus, it is clear that this species pair has only one genetic barrier that is sexual isolation because no post mating isolation is present between these species, which demonstrates the occurrence of incomplete reproductive isolation between them. Therefore, the occurrence of incomplete isolation between these sibling species may indicate that a process of speciation, of splitting of a single species into two or several derived ones, is under way.

Cuticular hydrocarbon

Despite being closely related, hydrocarbon compositions of *D. ananassae* and *D. pallidosa* are strikingly dissimilar and may be important in providing species isolation between these two sympatric species. *D. ananassae* comprises (Z, Z)-5, 25-hentriacontadine (Doi *et al.* 1997) as a major sex pheromone that elicites all the courtship elements of *D. ananassae* while *D. pallidosa* comprises (Z, Z)-5, 27-tritriacontadiene (Nemoto *et al.* 1993) as a major sex pheromone. These differences in cuticular hydrocarbon occurred with respect to C31 and C33 carbons: *D. ananassae* predominantly possesses the former (63% of total cuticular hydrocarbon) whereas *D. pallidosa* contains the later (57% of total cuticular hydrocarbon).

CHCs have been shown to have a pivotal role in sexual communication as sex attractants and cues for species, gender and individual recognition (Blomquist and Bagneres 2010). Thus, significant differences in cuticular hydrocarbons composition between *D. ananassae* and *D. pallidosa* may be critical factor in the sexual isolation between *D. ananassae* and *D. pallidosa*. Males discriminate heterospecific females on the basis of species-specific female

sex pheromones (Nemoto *et al.*1994; Doi *et al*. 1997). Hence, variation in CHCs profiles of the *D. ananassae* and *D. pallidosa* reveals that CHC evolution has been somewhat conserved and associated with the evolutionary divergence of these species because it is well known that CHCs evolve rapidly (Mullen *et al.* 2007; Thomas and Simmons 2008) but very little is known about correlation among CHCs differences and their role in a phylogenetic context. CHCs are highly sexually dimorphic in many species, with many of the individual components being sex specific (Thomas and Simmons 2008) because CHCs profiles evolve in a sex-specific manner when subject to natural selection and sexual selection (Sharma *et al.* 2011) but they could be called sexually monomorphic species for cuticular hydrocarbons. Furthermore, neither qualitative nor quantitative differences between males and females were found in either species (Nemoto *et al.* 1994).

Thus, the occurrence of differences at the CHCs level at species level but not at sex specific level indicate that species specific variations occur but not sex-specific which shows that these two species are separated but the event of separation is very recent in the process of speciation. There is a significant body of research on the biosynthesis of these compounds by Morita *et al.* (2005). Morita *et al.* (2005) synthesized major sex pheromone components of *D. ananassae* and *D. pallidosa* by using the Wittig olefination and sulfone coupling reactions as the C-C bond forming steps. Although our knowledge of the biosynthesis of CHCs strengthens the previous work, we still know little about the enzymes involved and their genetics and evolutionary history. However, what evolutionary forces cause the divergence of CHCs between these species and how many genes with what functions are involved during this process is still largely unknown. Thus, further study related to CHCs of these sibling species is needed to understand the rapid diversification of CHCs during speciation and how species specific CHC profiles originate and are maintained because knowledge of the genetic

bases of the hydrocarbon differences contributing to sexual isolation is very important for understanding the speciation process.

Courtship song

Males in many *Drosophila* species vibrate their wings in a species-specific manner prior to attempting to copulate with a prospective mate. Correspondingly, striking differences in the wing displays of courting *D. pallidosa* and *D. ananassae* males have been reported by Spieth (1966). Males of *D. ananassae*, were described as characteristically spreading both wings laterally about 5^0 to 7^0 from the normal resting position and vibrating them up and down very rapidly. In contrast to this, males of *D. pallidosa* extend only one wing, the one closest to the female's head laterally from 50^0 to 90^0 and vibrating this wing vertically while the other wing remains in resting position. So it is clear that variations in the wing displays of courting males may provide visual and acoustic stimuli to the female and highly divergent nature of wing displays produced by courting males of each species indicate the importance of wing vibration in mate recognition.

Thus, differences in wing vibration pattern between these two sibling species despite of qualitatively same courtship behaviours of *D.ananassae* and *D. pallidosa* indicate that separation of these two species is a very recent event of evolution because very slight changes occurred in the pattern of wing vibration. Males produce courtship songs by wing vibration intermittently during the sequence of courtship behavioural elements until copulation whereas during copulation neither species produce vibration (Yamada *et al.* 2002). Bursts of *D. ananassae* male's song consist of polycyclic pulses, while those of *D. pallidosa* consist of bicyclic pulses. Further, more direct evidence for a role of courtship song in species mating discrimination in both of these species derives from the studies involving wingless males and aristaless females. Doi *et al.* (2001) and Yamada *et al.* (2002) surgically

removed male's wings and female's antennae, and tested the mating success of conspecific and heterospecific crosses. They found that mating success decreased in conspecific crosses but dramatically increased in heterospecific crosses. Thus, experiments using wingless males or aristaless females showed that female sex pheromone was insufficient to isolate these two species sexually, and that the acoustic signals produced by the male's wing vibration were critical in gaining sexual isolation between *D. ananassae* and *D. pallidosa* (Doi *et al.* 2001). Females of both the species discriminate courting males on the basis of acoustic cues so it is clear that divergence of mating signals and recognition systems seem a primary cause of speciation of *D. ananassae* and *D. pallidosa*. In the extension of this study, Yamada *et al.* (2008) found that heterospecific courtship songs evoked female wing fluttering, whereas conspecific courtship song did not and this wing fluttering discontinue the courtship of courting males. Although from the study of Yamada *et al.* (2002) it is clear that differences in burst length, pulse length, cycle number in a pulse and frequency spectra of bursts exist but it is not clear yet which parameters are critical for the female discrimination. Therefore, song playback experiments with artificial songs would help to identify this.

Morphological traits

Phenotypic variation is a universal characteristic of living organisms and is observed in a wide variety of traits across populations and species (Belade *et al.* 2005). Vishalakshi and Singh (2008) investigated, variations in different morphometric traits (wing length, thorax length, sex-comb tooth number, ovariole number and sternopleural bristle number) between these two sibling species as morphometric traits such as wing length, thorax length, sex-comb tooth number, ovariole number and sternopleural bristle number are an index of body size and variations in morphometric traits are the subject of many evolutionary studies, since it affects numerous life history traits (fecundity, mating success etc.) They found significant differences in various morphological traits between these two species. So, these

morphological traits may be target of different evolutionary forces and differences in various morphometric traits between these two species, is the results of interactions of different evolutionary forces because according to Darwinian theory, evolution occurs through natural selection and selection acts primarily at the phenotypic level because it is well documented that phenotypic traits are the primary target of natural selection (Lewontin 1974).

Further, Singh and Singh (2017a) have also investigated intraspecific as well as interspecific differences in certain morphometric traits and found significant intraspecific as well as interspecific differences in these morphometric traits. However, it is really remarkable and a matter of incredulity that in spite of being an endemic species, *D. pallidosa* exhibits intraspecific variations for all the morphometric traits in both the sexes. Therefore, on the basis of the results of the previous study, we can suggest that these differences in morphometric traits between these species lead to speciation by contributing towards the development of premating isolation because it is known that morphological divergence can contribute to speciation by promoting premating isolation (McKinnon *et al.* 2004) and it is also known that this sibling species show premating isolation but absence of post mating isolation. On the other hand, Singh and Singh (2017a) found only quantitative differences in morphometric traits rather than any qualitative differences between these two sibling species and due to the lack of qualitative morphological differences, it is difficult to identify both the sibling species from each other. Thus, occurrence of only quantitative differences in different morphometric traits in comparison to qualitative differences, provide the evidence of recent separation of these two sibling species.

Variation in inversion polymorphism

Futch (1966, 1973) studied inversion polymorphism in different populations (Pago Pago, Taputimu, Nafanua, Aopo) of both the species and reported two inversions that were 2LA in

left arm of Chromosome II and 3RA in the right arm of Chromosome III, in the larvae from the Taputimu *D. ananassae* stock. No other chromosomal differences were found in *D. ananassae* populations. An inversion in the left arm of the X chromosome, XLA, was found in all the populations of *D. pallidosa* and all the *D. pallidosa* stocks were homozygous for these arrangements but inversions of Chromosomes 2 and 3 of *D. pallidosa* provide intriguing information regarding the evolutionary relationship of these two species. A small, median inversion 2LB in the left arm of *D. pallidosa* Chromosome 2 was also found in *D. ananassae* collected from differentiated populations of New Guinea (Futch 1966). One member of each of the two pairs of overlapping inversions, 2LC of the (2LC; 2LD) complex and 2RA of the (2RA; 2RB) complex were also found in the collections from New Guinea. All of the *D. pallidosa* stocks were heterozygous for the arrangements of chomosome 2. Inversion in right arm of the chromosome 3 was also reported in all the populations of *D. pallidosa* but it was homozygous in Pago Pago and Taputimu stocks whereas heterozygous in Nafanua and Aopo stocks. This provides evidence that inversion 3RB is common to all the *D. pallidosa* populations but along with this, standard sequence of *D. ananassae's* third chromosome was also found in the Nafanua and Aopo populations which provides evidence of interbreeding resulting in the introgression of the standard *D. ananassae* chromosome into the *D. pallidosa* populations.

Similarly, Matsuda *et al.* (2009) found the sharing of inversions among *D. ananassae, D. pallidosa* and the Papua New Guinean endemics and found inversions in *D. papuensis-like* and *D. pallidosa-like* which are the result of the introgression of chromosomes from other species, *D. ananassae* and *D. pallidosa*. Further, Singh *et al.* (2012) reported the heterozygous loops in F1 hybrids in certain regions of autosomes which demonstrates that these two sibling species differ in the order of gene arrangements, but interestingly no asynapsis was found in the polytene chromosomes of F1 hybrids indicating that there is

normal pairing between homologous chromosomes and there is homology between the banding patterns of the two species.

Therefore, it is clear that these two species are genetically distinct but sharing of chromosomes arrangements suggests that these two sibling species represent a recent evolutionary radiation and may experience substantial gene flow.

NOR variation

Both these species are devoid of NOR on the X chromosome but *D. ananassae* has NOR on Y and 4 chromosomes whereas *D. pallidosa* has NOR on the metacentric chromosome 4. Occurrence of NOR on Y chromosome in *D. ananassae* indicates that *D. ananassae* is the ancestral one or evolved earlier in comparison to *D. pallidosa* (figure 1). In addition to this, in *D. ananassae*, the hybridization site is terminal, whereas in *D. pallidosa*, the site is closer to the centromere. Thus, the difference in the position of NOR provides the evidence of separation of both these species.

Allozyme polymorphism

Allelic frequencies of fast and medium alleles for the two forms of different allozymes were found to be significant (Johnson et al. 1966). Allelic frequencies of medium allele were greater in light form in comparison to dark form whereas, allelic frequency of fast allele were greater in dark form in comparison to light form. Therefore, it might be possible that differences in allelic frequency plays important role in adaptation of these two forms and due to low frequency of F allele, light form is less fit in comparison to dark form and remains endemic. Thus, this difference in allelic frequency of these two species reflects the sign of their separation.

Conclusion

Although *D. ananassae* and *D. pallidosa* are considered as a pair of sibling species but from the above description, it is clear that they have a number of similarities and dissimilarities as *D. pallidosa* has lower mating propensity as compared to *D. ananassae.* However, the pattern remains same as far as the effect of age on mating propensity is concerned (Singh and Singh 2017b). Thus, both the possibilities are there: they may be separate species or light and dark forms of the same species. It needs to be explored further or more study is still awaited to justify their status. Basically studies related to post mating isolation is untouched so it is needed to explore this point to understand the complete mystery of these sibling species pair because as we have discussed the parameters like IPI, male genitalia, parthenogenesis and lack of postmating isolation etc. are very crucial and cannot be ignored. Therefore, to understand the complete evolutionary lineage of these two species, it is very important to understand why they are showing only premating isolation and there is no postmating isolation as well as no difference in male genitalia, in spite of being two separate species? Finally, we conclude that *D. pallidosa* does not have the full status of a species, rather it is in the process of speciation, *statu-nascendi* (the term coined by Dobzhansky and Spassky 1959).

Acknowledgements

Financial support from the University Grants Commission, New Delhi in the form of UGC-BSR Faculty Fellowship Award to BNS and CSIR JRF New Delhi to RS is gratefully acknowledged. We thank the Corresponding Editor and the Reviewer for their suggestions to revise the manuscript.

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Received 11 October 2016, in revised form 24 February 2017; accepted 28 March 2017

Unedited version published online: 31 March 2017

Figure 1. Evolution of NOR between *D. ananassae* and *D. pallidosa*.

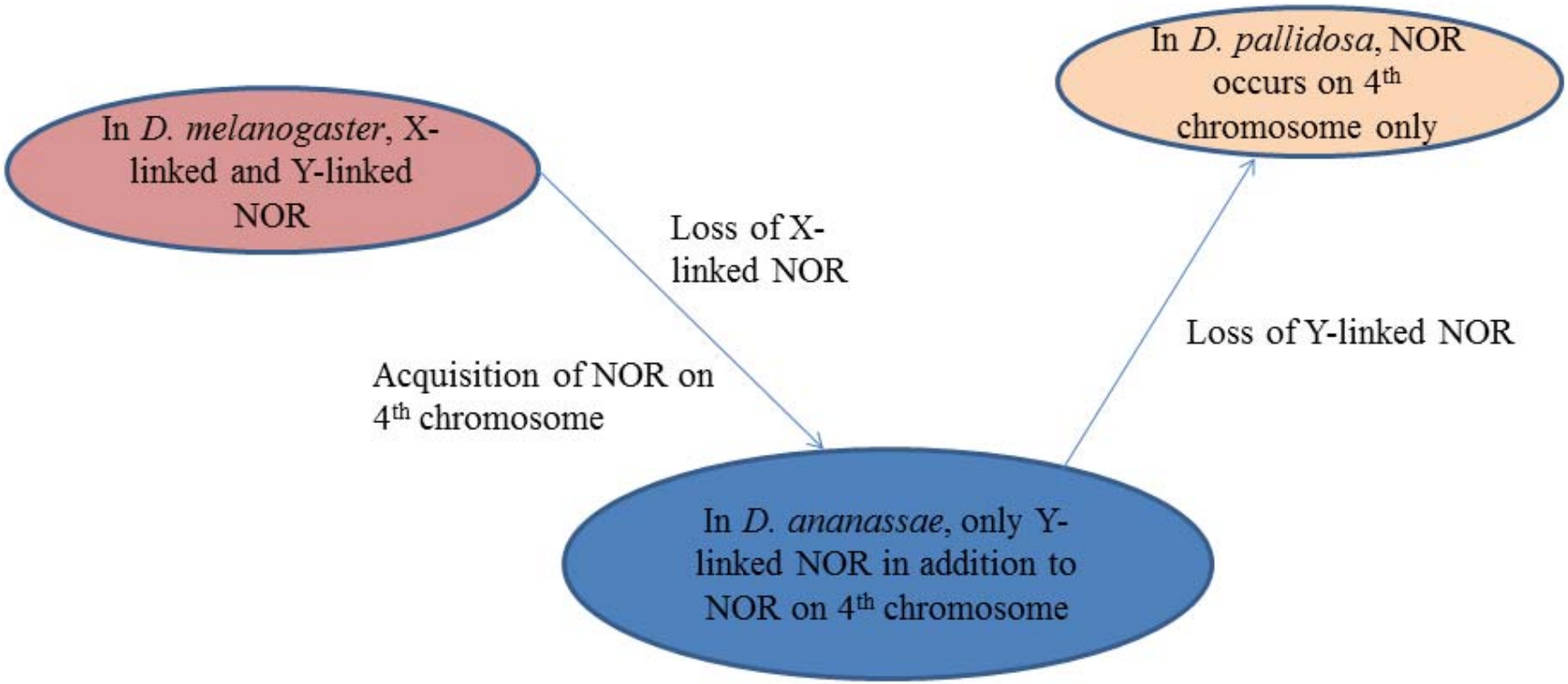
In *D. melanogaster*, X-linked and Y-linked NOR
In *D. pallidosa*, NOR occurs on 4th chromosome only
Loss of X-linked NOR
Loss of Y-linked NOR
Acquisition of NOR on 4th chromosome
In *D. ananassae*, only Y-linked NOR in addition to NOR on 4th chromosome

Figure 1.