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Phylogenic Relationships in the Drosophila nasuta Species Droup (Diptera：Drosophilidae) : A RAPD Approach 【Research report】

利用RAPD的方法探討輝顏果蠅Drosophila nasuta種亞群 的親緣關係(雙翅目：果蠅科)【研究報告】

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Abstract

Random amplified polymorphic DNA (RAPD) analysis was applied to 14 species and subspecies of the Drosophila nasuta subgroup of the D. immigrans species group, plus D. immigrans as an outgroup. Forty primers of an arbitrary nucleotide sequence plus 2 pyruvate kinase gene-specific primers were used to amplify DNA fragments from genomic DNA of the 15 genotypes. Different RAPD fragment patterns were observed for different species, even subspecies. A dendrogram of the 15 genotypes was reconstructed using UPGMA of cluster analysis of SPSS. Data from the RAPD analysis is in concordance with that from morphological diagnostic characters in the D. nasuta subgroup, especially in the relationships between D. nasuta and D. albomicans; D. sulfurigaster spp. and Taxon-I, Taxon-j, and D. pallidifrons.

摘要

本研究以RAPD的分折方法針對輝顏果困蠅Drosophila nasuta種亞群中的14個種及亞種進行親緣關係的分析並以D. immigrans 為外群。40個隨機多型性核酸引子及2個丙酮酸鹽激專一的引子來放大此15個基因型的基因組DNA。觀察得知不同的種甚至亞種均可得到不同DNA條帶模式。一個系統樹則應用SPSS 套裝軟體的群聚分析程式以UPGMA的方式建立之。從RAPD的資料顯示，其結果和以形態診斷所得結果具一致性，尤其在D. nasuta 與D. albomicans之間；D. sulfurigaster亞種間；及Taxon-I, Taxon-J及D. pallidifrons之間的關係更為明顯。

Key words: Drosophila nasuta, RAPD, phylogenic relationships.
關鍵詞: 輝顏果蠅、RAPD、親緣關係

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Phylogenic Relationships in the *Drosophila nasuta* Species Group (Diptera: Drosophilidae): A RAPD Approach

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ABSTRACT

Random amplified polymorphic DNA (RAPD) analysis was applied to 14 species and subspecies of the *Drosophila nasuta* subgroup of the *D. immigrans* species group, plus *D. immigrans* as an outgroup. Forty primers of an arbitrary nucleotide sequence plus 2 pyruvate kinase gene-specific primers were used to amplify DNA fragments from genomic DNA of the 15 genotypes. Different RAPD fragment patterns were observed for different species, even subspecies. A dendrogram of the 15 genotypes was reconstructed using UPGMA of cluster analysis of SPSS. Data from the RAPD analysis is in concordance with that from morphological diagnostic characters in the *D. nasuta* subgroup, especially in the relationships between *D. nasuta* and *D. albomicans*; *D. sulfurigaster* spp. and Taxon-I, Taxon-J, and *D. pallidifrons*.

Key words: *Drosophila nasuta*, RAPD, phylogenic relationships.

Introduction

The *Drosophila nasuta* subgroup of the *immigrans* species group of flies consists of about 15 species and subspecies including 3 unnamed species; its members are widely distributed from the islands of the Pacific Ocean, through Southeast Asia, and the Indian Ocean areas (Kitagawa *et al.*, 1982). Therefore, it has been one of the most suitable models for studies of genetics and evolution, especially for studies of speciation mechanisms.

Lamb (1914) originally described *D. nasuta* fromthe Seychelles Island in the Indian Ocean. Since then, a number of morphologically similar species have been found from the locations mentioned above. However, it was not until 1969 that the subgroup was brought to the attention of evolutionists. Wilson *et al.*, (1969) divided them into 8 species plus 3 subspecies based upon morphological, cytological, and genetic aspects. Since then, many studies using various approaches have been carried out, such as on the male frontal markings (Kitagawa *et al.*, 1982), metaphase karyotypes, polytene chromosomes in some species (Wakahama and Kitagawa, 1980; Wakahama *et al.*, 1983; Hatsumi,1987), insemination reaction (Asada and Kitagawa, 1988), and restriction enzyme polymorphism of mitochondrial DNA (Chang *et al.*, 1989). However, the phylogenic relationships among them derived from those studies are diverse and controversial. In addition,

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the relative relationships of the 3 unnamed species with other members of the subgroup have not yet been clarified.

Molecular approaches have been widely used to supplement the analyses based upon morphological, cytological, and genetic data in systematic research, for the accumulation in nucleotide sequence changes is the basis for speciation to occur. The newly developed technique, random amplified polymorphic DNA (RAPD) markers generated by polymerase chain reaction (PCR), has been widely used in determination of phylogenic relationships among OTUs, from the levels of strains to those of species and subspecies (Bardakci and Skibinski, 1994; Heun *etal.* 1994; Jain *et al.*, 1994; Mailer *et al.*, 1994; Orozco-Castillo *et al.*, 1994; Abed *et al.*, 1995; Baruffi *et al.*, 1995; Varga *et al.*, 1995). It has been shown that RAPD analysis not only exhibits the same power as RFLP in determination of genetic relationships among genotypes (Hallden *et al.*, 1994; and Santos *et al.*, 1994), but has higher discrimination power over isozyme analyses (Baruffi *et al.*, 1995; and Varga *et al.*, 1995). In addition, the application of RAPD markers has several advantages when compared to those of other molecular markers including nucleotide sequencing, such as fast data production, and a protocol requiring less DNA and no radioactivity.

In this report, we present RAPD data for the 14 species and subspecies including 3 unnamed species of the *nasuta* subgroup, plus *D. immigrans* as an "outgroup" or reference species, seeking the phylogeny of the species of the group.

Materials and Methods

Drosophila nasuta stocks

The 14 species and subspecies of *D. nasuta* subgroup, as well as *D. immigrans*, used in this study, were maintained in the *Drosophila* laboratory at Academia Sinica, Taipei, Taiwan (R.O.C.). Names of species and subspecies, stock numbers, and collection sites and years are listed in Table 1.

DNA extraction

Single fly DNA extraction was performed according to the protocol of Beckenbach *et al.* (1993). Single flies were homogenized in a microfuge tube contain-

Table 1. Members of the subgroup of *Drosophila nasuta* species and subspecies, plus the outgroup, *D. immigrans*, used in this experiment

Species	Stock no.	Collection site (year)
D. nasuta	193.6	Kandy, Sri Lanka (1971)
D. albomicans	56.1	Wulai, Taiwan (1970)
Taxon-F	193.15	Sarawak, Malaysia (1971)
D. kepulauana	263.1	Puerto Princexa Palawan, Philippines (1968)
D. kohkoa	193.5	Sarawak, Malaysia (1971)
D. niveifrons	203.1	Lae, Papua New Guinea (1979)
D. pulaua	193.10	Sarawak, Malaysia (1979)
D. pallidifrons	203.3	Ponape, Caroline Is. (1981)
D. sulfurigaster neonasuta	181.9	Mysore, India (1981)
Taxon-J	203.9	Noumea, New Caledonia (1981)
D. s. bilimbata	181.4	Hawaii, USA (1965)
D. s. albostrigata	192.1	Chiang Mai, Thailand (1983)
D. s. sulfurigaster	181.2	Port Moresby, Papua New Guinea (1981)
Taxon-I	231.9	Wau, Papua New Guinea (1981)
D. immigrans	216.28	Fen-Chi Hu, Taiwan (1990)

ing 60 μl of protease solution (0.1 M Tris-HCl, pH8.0, 0.05 M EDTA, 0.2 M NaCl, 1% SDS, 0.4 mg protease K / ml), and immediately incubated at 65°C for 3 min. The mixture was extracted once with phenol and once with chloroform: isoamyl alcohol (24:1). The aqueous phase was removed and DNA was precipitated with 2 volumes of 95% ethanol, washed twice with 70% ethanol, dried under vacuum, and resuspended in 25μl of sterile dH_2O.

Polymerase chain reaction (PCR)

A single 10-base oligonucleotide primer (purchased from Operon 10-mer kits, Operon Technology, Inc.) was used to amplify genomic DNA. If this primer happens to prime 2 sites in 2 different but complementary strands within 5 kb, a single amplified DNA fragment can be produced. The genomic DNA from 2 different individuals usually may reveal different amplification patterns. The situation where a DNA fragment is amplified and present (recorded as "1") in 1 individual but not in the other (recorded as "0") represents DNA polymorphism (or divergency), and can be used as a genetic marker.

Amplification was performed with an FTS-960 (Corbett Research) thermal cycler in 10 μl of solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 mM dNTPs, 5 picomoles of a single 10-base primer, 50 ng of genomic DNA, and 1 unit of *Tag* DNA polymerase (Dynazyme). The temperature profile was 2 cycles of 60 sec at 91°C, 7 sec at 42°C, and 70 sec at 72°C, followed by 38 cycles of 5 sec at 91°C, 7 sec at 42°C, and 70 sec at 72°C. The degree of amplification was determined by separation of the amplified products on a 2% Seakem agarose minigel (in 1x TBE) at 100V for 1 h.

Data analysis

A dendrogram was generated by using unweighted pair group average linkage clustering (the cluster analysis package in SPSS / PC+, version. 5.0) of the simple matching coefficients (Nei and Li, 1979) in the similarity matrix based on the results of RAPD experiments.

Results

Forty primers of arbitrary nucleotide sequence (Operon 10-mer kits A and B, Operon Technology Inc) plus 2 pyruvate kinase gene-specific primers were used to amplify DNA fragments from genomic DNA of the 15 genotypes. Figure 1 shows the banding patterns of the 15 genotypes

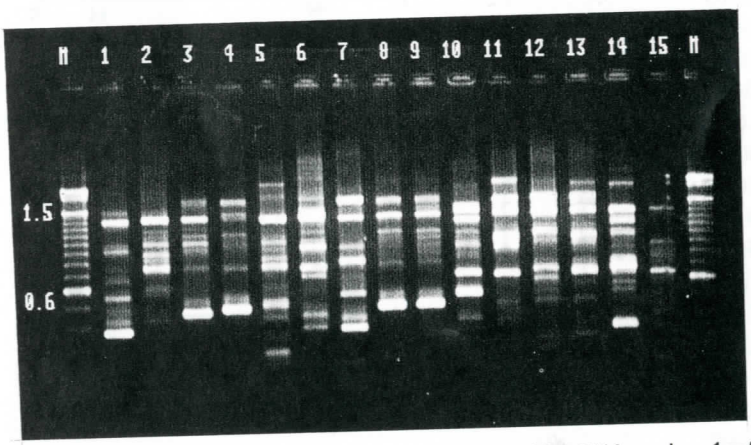

Fig. 1. Amplification patterns using Operon B06, where M=100 bp ladder DNA marker; 1=*D. albomicans*; 2=*D. s. neonasuta*; 3=*D. s. sulfurigaster*; 4=*D. s. bilimbata*; 5=*D. s. albostrigata*; 6=*D. kohkoa*; 7=*D. nasuta*; 8=*D. pulaua*; 9=Taxon-F; 10=*D. niveifrons*; 11=*D. pallidifrons*; 12=Taxon-J; 13=Taxon-I; 14=*D. kepulauana*; 15=*D. immigrans*.

using 1 of the primers. Of the 42 primers, 38 primers (Table 2) which produced a total of 1307 clear, easily detectable, and reproducible bands were selected for further evaluation. Of the 1307 bands, 1303 bands (99.6%) show divergenic among the genotypes. The high degree of divergency confirms the proposal that RAPD analysis is a useful method for differentiating strains or higher levels studied (Bardakci and Skibinski, 1994; Varga *et al.*, 1995).

Table 2. Primers used for the detection of divergency in *D. nasuta* subgroups

Primer	Sequence	No. of amplification products (a)	No. of divergenic products (b)	Percent of divergency (b/a)
PK1	CTGGACACAAAGGGGCCCGAGATCCGT	34	34	100
PK2	ATGGTGGCCCGTGGAGATCTGGGT	47	45	95.7
OPA-01	CAGGCCCTTC	34	33	97.1
OPA-02	TGCCGAGCTG	35	35	100
OPA-03	AGTCAGCCAC	40	40	100
OPA-04	AATCGGGCTG	30	30	100
OPA-06	GGTCCCTGAC	17	17	100
OPA-07	GAAACGGGTG	51	51	100
OPA-08	GTGACGTAGG	33	33	100
OPA-09	GGGTAACGCC	38	38	100
OPA-10	GTGATCGCAG	26	26	100
OPA-11	CAATCGCCGT	37	37	100
OPA-12	TCGGCGATAG	26	26	100
OPA-13	CAGCACCCAC	34	33	97.1
OPA-14	TCTGTGCTGG	35	35	100
OPA-15	TTCCGAACCC	26	26	100
OPA-16	AGCCAGCGAA	33	33	100
OPA-18	AGGTGACCGT	50	50	100
OPA-19	CAAACGTCGG	37	37	100
OPA-20	GTTGCGATCC	37	37	100
OPB-01	GTTTCGCTCC	33	33	100
OPB-02	TGATCCCTGG	39	29	100
OPB-03	CATCCCCCTG	44	44	100
OPB-04	GGACTGGAGT	27	27	100
OPB-06	TGCTCTGCCC	41	41	100
OPB-07	GGTGACGCAG	38	38	100
OPB-08	GTCCACACGG	46	46	100
OPB-10	CTGCTGGGAC	36	36	100
OPB-11	GTAGACCCGT	31	31	100
OPB-12	CCTTGACGCA	17	17	100
OPB-13	TCCCTCCGCT	32	32	100
OPB-14	TCCGCTCTGG	35	35	100
OPB-15	GGAGGGTGTT	39	39	100
OPB-16	TTTGCCCGGA	35	35	100
OPB-17	AGGGAACGAG	42	42	100
OPB-18	CCACAGCAGT	33	33	100
OPB-19	ACCCCCGAAG	31	31	100
OPB-20	GGACCCTTAC	18	18	100
Total		1307	1304	99.6

Amplified divergenic DNA fragments were scored as described in "Materials and Methods" for computer analysis based on Nei and Li's (1979) simple matching coefficient. Table 3 shows the pairwise similarities for 15 genotypes.

Association among the 15 genotypes revealed by UPGMA cluster analysis is presented in Fig. 2. *Drosophila immigrans*, as expected, is least related to the other 14 genotypes. Within the *nasuta* subgroup, *D. niveifrons* forms a cluster by itself, while the remaining genotypes group into another cluster. Therefore *D. niveifrons* is more isolated genetically from other *nasuta* species and subspecies, as indicated in simple match coefficients where it ranges from 0.667 to 0.704 (Table 3). *Drosophila pulaua* and Taxon-F are highly associated with each other. The 4

Table 3. Similarity matrix for simple match coefficients (Nei and Li, 1979)

	ALBO	SNEO	SSUL	SBIL	SALB	KOHK	NASU	PULA	TAXF	NIVE	PALL	TAXJ	TAXI	KEPU	IMMI
ALBO	1.000														
SNEO	0.779	1.000													
SSUL	0.762	0.792	1.000												
SBIL	0.774	0.801	0.819	1.000											
SALB	0.777	0.832	0.789	0.800	1.000										
KOHK	0.746	0.762	0.728	0.754	0.765	1.000									
NASU	0.816	0.764	0.725	0.754	0.779	0.755	1.000								
PULA	0.743	0.770	0.777	0.788	0.773	0.738	0.746	1.000							
TAXF	0.746	0.774	0.780	0.805	0.770	0.728	0.748	0.858	1.000						
NIVE	0.688	0.695	0.667	0.669	0.682	0.677	0.694	0.674	0.680	1.000					
PALL	0.731	0.721	0.703	0.705	0.711	0.707	0.719	0.699	0.699	0.686	1.000				
TAXJ	0.734	0.713	0.693	0.699	0.715	0.703	0.725	0.695	0.699	0.679	0.773	1.000			
TAXI	0.740	0.744	0.724	0.721	0.733	0.715	0.752	0.723	0.718	0.691	0.767	0.769	1.000		
KEPU	0.776	0.769	0.725	0.761	0.754	0.762	0.782	0.754	0.761	0.704	0.723	0.721	0.736	1.000	
IMMI	0.676	0.675	0.644	0.666	0.670	0.663	0.685	0.648	0.648	0.650	0.638	0.656	0.662	0.689	1.000

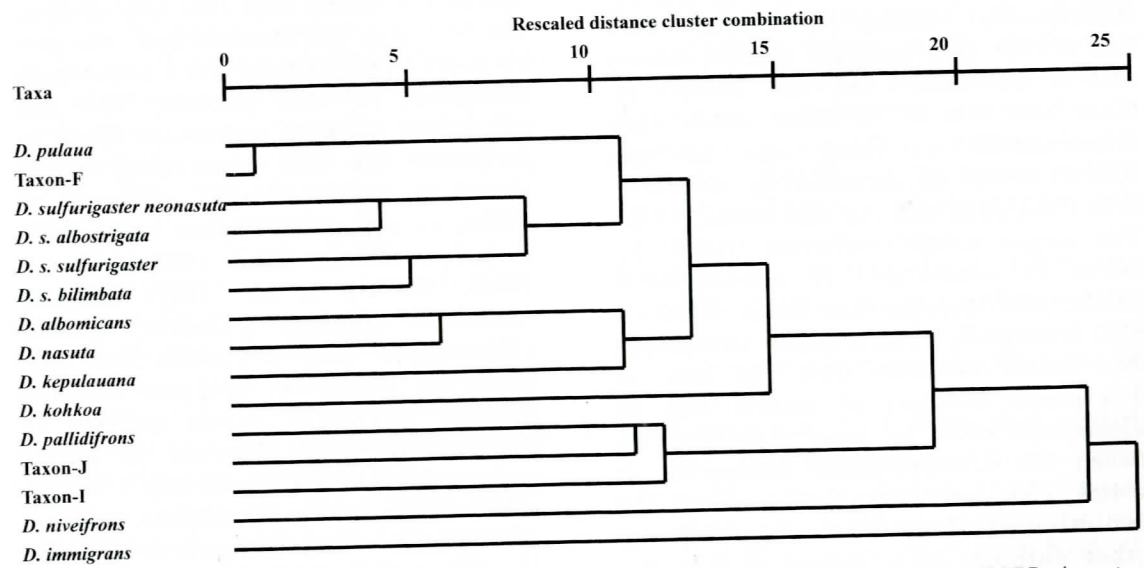

Fig. 2. Phenogram of the *Drosophila nasuta* species subgroup plus *D. immigrans*, inferred from RAPD characters based on an UPGMA clustering.

subspecies of *D. sulfurigaster* are grouped together, as expected, with *neonasuta* and *albostrigata* in a cluster and *sulfurigaster* and *bilimbata* in another cluster. *Drosophila nasuta* and *D. albomicans* are closely related, and *D. kepulauana* is associated with them in an allopatric way. Taxa J and I are expected to be grouped together, and *D. pallidifrons*, which was thought to be the primitive species of the *nasuta* subgroup (Matsuzaki, 1983), is closely associated with them.

Discussion

The genetic relationship between *D. nasuta* and *D. albomicans* is quite controversial. Due to the lack of diagnostic traits in external morphology, they were recognized as a single species (Duda, 1924), or because they are distributed allopatrically, they were suggested to be 2 subspecies (Ramachandra and Raganath, 1986). However, some evidence indicated that they eventually showed "hybrid breakdown" in a post-zygotic way, as well as that they differed in chromosomal configuration (Chang and Ayala, 1989). Therefore, it was further suggested that they should be considered as 2 species (or superspecies) (Chang and Ayala, 1989). Our RAPD data indicate that *D. nasuta* and *D. albomicans* are very closely related (see Fig. 2), in other words, they differentiated not long ago, perhaps, 500,000 yr ago as suggested by mitochondria DNA data (Chang and Ayala, 1989). The simple match coefficient (0.816) between *D. nasuta* and *D. albomicans* is similar to or greater than those (0.789～0.832) among the 4 *sulfurigaster* subspecies. The result suggests that the time of divergence between *D. nasuta* and *D. albomicans* must be later than that between the 4 *sulfurigaster* subspecies. It seems that our data contradicts the evolutionary hierarchy suggested by other authors. As a matter of fact, the molecular data can only reflect the genomic variation between 2 genotypes, which might have differentiated some years ago. It is possible that *D. nasuta* and *D. albomicans* diverged pretty late; if so however, their genomic variations took place in those genes responsible for important biological functions, resulting in rapid evolution. Whereas, the genomic variation between *sulfurigaster* subspecies may have occurred early but not principally in functional genes.

Drosophila kepulauana was suggested to exist as very closely related allopatric populations to the cluster of *D. nasuta* and *D. albomicans* in hybridization experiments (Kitagawa *et al.*, 1982). Our result (Fig. 2) strongly supports this hypothesis.

Drosophila sulfurigaster subspecies were clustered together using RAPD analysis. The result confirms that they are closely related but somehow differentiated owing to geographical barriers. Based upon the analyses of chromosomal, morphological variation, and reproductive isolation, Suzuki *et al.*, (1990) inferred that they would be separated into 2 branches with *albostrigata* and *neonasuta* in one; *bilimbata* and *sulfurigaster* in the other. Our result apparently agrees with that way of grouping (see Fig. 2). Nevertheless, the genetic relationship among the 4 *sulfurigaster* subspecies is still unclear. The main characters defining subspecies of *D. sulfurigaster* are the pronounced whitish bands along the frontal orbits of the males, as well as incomplete reproductive isolation among them (Wilson *et al.*, 1969). Tamura *et al.* (1991) estimated nucleotide sequence divergence between *albostrigata* and *bilimbata* by mitochondrial restriction analysis to be an average of 7.59%, which reaches the interspecific level observed in the *D. melanogaster* species subgroup (Solignac *et al.*, 1986). Due to incomplete reproductive isolation, they can only declare that these 2 subspecies have been separated for a long evolutionary time, despite the

result that the divergence rate has reached the interspecific level. Although molecular data (Tamura *et al*, 1991; present study) support elevating the relationship between *albostrigata* and *bilimbata* to species level, lacking complete reproductive isolation makes us hesitant to claim it.

One of the purposes of this study was to clarify the genetic relationships of the 3 unnamed species, Taxon-F, Taxon-I, and Taxon-J. Matsuzaki (1983) suggested that *D. pallidifrons* was the most primitive species of the subgroup based upon the patterns of polytene chromosomes and male frontal markings. Both Taxon-I and Taxon-J were then directly differentiated from it. The relationship was further confirmed by the analyses of morphometry (Tsaur and Lin, pers. comm.) and RFLP of mitochondrial DNA (Liou and Lin, pers. comm.). Our data suggest that *D. pallidifrons*, Taxon-I, and Taxon-J are in a lineage group, which differentiated from the other *nasuta* species a long time ago. However, the result that Taxon-I is clustered with *D. pallidifrons* instead of Taxon-J makes their relationship ambiguous.

Our data strongly suggest that Taxon-F is highly related to *D. pulaua*, which reconfirms the relationship derived from mitochondrial DNA analysis (Chang *et al*, 1989), where these 2 species are almost identical (genetic differentiation $d_{xy}=0$). But, hybridization experiments performed by Kitagawa *et al.* (1982) contradict our result. Both reciprocal crosses between *D. pulaua* and Taxon-F were sexually incompatible (P×P sterile). It is possible that although they differentiated from each other not long ago, variations occurred principlly in genes responsible for reproduction, which resulted in sexual incompatibility.

In a summary, data from RAPD analysis concurs with that from morphological diagnostic characters in the *D. nasuta* subgroup. The relationships between *D. nasuta* and *D. albomicans*; *D. sulfurigaster* ssp.; Taxon-I, Taxon-J, and *D. pallidifrons* are quite consistent with other approaches, while others vary with different approaches.

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利用 RAPD 的方法探討輝顏果蠅 *Drosophila nasuta* 種亞群的親緣關係(雙翅目：果蠅科)

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摘　要

本研究以RAPD的分析方法針對輝顏果蠅*Drosophila nasuta*種亞群中的14個種及亞種進行親緣關係的分析並以*D. immigrans*為外群。40個隨機多型性核酸引子及2個丙酮酸鹽激專一的引子來放大此15個基因型的基因組DNA。觀察得知不同的種甚至亞種均可得到不同DNA條帶模式。一個系統樹則應用SPSS套裝軟體的群聚分析程式以UPGMA的方式建立之。從RAPD的資料顯示，其結果和以形態診斷所得結果具一致性，尤其在*D. nasuta*與*D. albomicans*之間；*D. sulfurigaster*亞種間；及Taxon-I, Taxon-J及*D. pallidifrons*之間的關係更為明顯。

關鍵字：輝顏果蠅、RAPD、親緣關係。

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