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Thrips Identification on Imported Crops Through Multiplex PCR and Morphological Identification: Detection of the Quarantine Thrips *Frankliniella occidentalis* and Other Related Species (Thysanoptera: Thripidae)

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ABSTRACT

Thrips cause severe damage to many crops and are frequently found on imported crops. Thus, many thrips species are categorized as quarantine pests. In this study, thrips species, intercepted in imported agricultural products, were identified based on morphological characteristics and through multiplex polymerase chain reaction (PCR). Five specific primers for the three quarantine thrips species *Anaphothrips obscurus* (Müller), *F. occidentalis* Pergande, and *Thrips fuscipennis* Haliday and two other common species, *Thrips tabaci* Lindeman and *Frankliniella intonsa* (Trybom), were designed based on the internal transcribed spacer (ITS) region, a well-known marker for species identification. Five species-specific primer pairs and one universal primer pair based on conserved 28S ribosomal DNA were pooled in each multiplex PCR. Identification results were consistent, whether based on morphological or molecular characteristics. Perhaps due to the sample quality, the molecular identification efficiency was approximately 87%. The most common thrips species on imported crops was *T. tabaci*, followed by *F. occidentalis*, which was observed primarily on strawberry and artichoke crops from the United States; some *F. occidentalis* specimens were also found on leucadendron and eryngium plants from Australia and the Netherlands. The other thrips species found on imported crops were *F. intonsa*, *Scirtothrips dorsalis* Hood, several species of the Tubulifera suborder, and one other quarantine species, namely *Thrips subnudula* Karny, which was identified morphologically on imported leacospemum plants from Australia. This study demonstrated that multiplex PCR based on specific primers of the ITS region is a rapid and reliable method for identifying quarantine *F. occidentalis,* which was identified with 93% accuracy. In addition,

morphological identification is crucial when nontarget quarantine thrips species are observed.

Key words: quarantine pest, molecular identification, internal transcribed spacer

Introduction

Thrips are minute, slender insects that feed on plants through puncturing and suction; some are primary pests affecting agricultural and horticultural crops. Phytophagous thrips cause direct damage to fruits, leaves, and flowers of plants; some act as major vectors of *Tospovirus*, which can severely reduce agricultural production (Riley *et al*., 2011). Approximately 50 thrips species reportedly cause severe damage to crops; these include *Frankliniella occidentalis* Pergande, *Frankliniella intonsa* Trybom, *Thrips tabaci* Lindeman, *Thrips palmi* Karny, *Thrips hawaiiensis* Morgan, and *Scirtothrips dorsalis* Hood (Prins and Goldbach, 1998; Gent *et al*., 2006). Therefore, many thrips species are classified as quarantine pests in certain countries because of agricultural transport regulations aimed at preventing their invasion. Among those that can transmit multiple *Tospovirus* species, *T. palmi* is listed as a quarantine pest in multiple countries in Southeast Asia (European and Mediterranean Plant Protection Organization; EPPO, 2018b), and *F. occidentalis* and *Thrips australis* (Bagnall) are listed as such pests in Taiwan (Bureau of Animal and Plant Health Inspection and Quarantine; BAPHIQ, 2018).

Frankliniella occidentalis, commonly known as western flower thrips (WFT), was discovered in the United States in 1895 and was subsequently observed in other countries (EPPO 2002, 2018a). The WFT represents a major pest for American crops, especially those planted in greenhouses, and is highly polyphagous, feeding on 65 families of host plants, including many vital crops such as corn, cotton, cucumber, eggplant, grape, melon, nectarine, peach, peanut, pea, pepper, strawberry, and tomato (Cluever *et al*., 2015). WFT feeds on the floral parts of these crops, causing spotting and deformation on flower buds and dimpling on the surfaces of fruits (Salguero Navas *et al*., 1991; Riley and Batal, 1998); this insect also transmits four *Tospovirus* species (Riley *et al*., 2011). Therefore, WFT greatly influences the aesthetic quality of crops. EPPO (1989) reported that WFT reduced cucumber production in Canada by 20% and was associated with outbreaks of tomato spotted wilt virus in Ontario tomatoes.

The thrips species listed as quarantine pests in Taiwan by the BAPHIQ include *Echinothrips americanus* Morgan, *Scirtothrips aurantii* Faure, *Scirtothrips citri* (Moulton), and many species in the genera of *Frankliniella* Karny and *Thrips* L, such as *F. occidentalis*, *Thrips fuscipennis* Haliday, and *Thrips imaginis Bagnall* (BAPHIQ, 2018). Thrips represents the most frequently detected pest in agricultural products, such as strawberries, asparagus, eggplant, and broccoli, imported to Taiwan; of all detected pests in 2015 and 2016, 43% and 53%, respectively, were thrips, among which the most commonly identified species were *T. tabaci* and WFT (Leu *et al*., 2003; BAPHIQ, 2015, 2016). Therefore, a method for the accurate identification and rapid examination of thrips, especially immature thrips on imported crops, is required to reduce quarantine times.

Thrips identification is primarily based on analysis of adult morphological characteristics through slide mounting; however, morphological similarities among species often render identification difficult, particularly in immature thrips. For cryptic species complexes, only molecular markers can be applied in identification because the morphological characteristics of such species are essentially indistinguishable from one another. For example, *T. tabaci* is divided into three groups based on the mitochondrial cytochrome oxidase subunit 1 (COI) gene (Bruner *et al*., 2004; Toda and Murai, 2007; Tseng *et al*., 2010; Kobayashi and Hasegawa, 2012; Jacobson *et al*., 2013); furthermore, *S. dorsalis* is a species complex that can be separated into 12 cryptic species based on molecular COI markers (Hoddle *et al*., 2008; Dickey *et al*., 2015). Rugman-Jones *et al*. (2010) separated two cryptic species of *F. occidentalis*

based on the 28S rDNA) nuclear gene and the mitochondrial COI gene. Distinct genetic lineages involving various cryptic species with distinct infection characteristics and virus transmission capabilities were observed (Jacobson *et al*., 2013), emphasizing the need for molecular identification of cryptic thrips.

DNA-based techniques, including DNA barcoding using mitochondrial COI sequences, are effective for identifying thrips species (Toda and Murai, 2007; Rugman-Jones *et al*., 2010; Yeh *et al*., 2014a, 2014b, 2015; Dickey *et al*., 2015; Tyagi *et al*., 2017). For example, polymerase chain reaction (PCR) combined with restriction fragment length polymorphism was used to detect four *Frankliniella* species, namely *F. occidentalis, F. intonsa, Frankliniella pallida* Uzel, and *Frankliniella tenuicornis* Uzel, based on internal transcribed spacer (ITS) region 1, which can also be applied to distinguish larvae specimens (Przybylska *et al*., 2016). Przybylska *et al*. (2015) developed a species-specific, sensitive, and rapid diagnostic tool for identifying *T. palmi* using loop-mediated isothermal amplification. In addition, multiplex PCR based on multiple species-specific primers for several thrips species is a verifiably simple, quick, and powerful method for thrips identification (Liu, 2004; Farris *et al*., 2010; Yeh *et al*., 2014a, 2014b; Nakahara and Minoura, 2015). Moreover, the biochip technique was applied to detect 15 thrips species simultaneously (Yeh *et al*., 2015), and high-throughput sequencing techniques enabling acquisition of numerous sequences have been used to address some specific issues in thrips differentiation and identification (Kumar *et al*., 2019; Hsieh *et al*., 2020; Li *et al*., 2020; Rotenberg *et al*., 2020).

The present study applied multiplex PCR based on specific primers of the ITS region combined with examination of morphological characteristics to identify quarantine thrips species concealed in imported crops. In each multiplex PCR, five key thrips species, namely the quarantine species *A. obscurus, F. occidentalis, T. fuscipennis*, and two other commonly observed species, *F. intonsa* and *T. tabaci*, that were discovered in crops imported from 10 countries were examined.

Materials and Methods

Sample collection and identification

Thrips specimens from 10 countries, namely the United States, Australia, India, Italy, Japan, Mexico, the Netherlands, New Zealand, Thailand, and Turkey, were acquired from all types of imported crops at the quarantine station of the Hsinchu Branch of the BAPHIQ, Council of Agriculture, Executive Yuan (Table S1). The collected specimens were preserved in 95% ethanol. First, DNA was extracted from each specimen, which was then mounted on a slide with Hoyer's medium for morphological identification based on provided taxonomic characteristics for thrips in Taiwan, China, Japan, Australia, and New Zealand (Han, 1997; Mound and Kibby, 1998; Wang, 2002; Mound and Masumoto, 2005; Masumoto and Okajima, 2013). The resultant voucher specimen slides were stored at the Laboratory of Molecular Systematics, Department of Entomology, National Chung Hsing University, Taiwan.

DNA extraction

DNA was extracted from individual thrips specimens using QuickExtract DNA Extraction Solution 1.0 (Epicentre Technologies Corporation, Madison, Wisconsin). Each specimen was immersed in 50 μL of the extraction solution. After being shaken vigorously for 15-20 s, the sample was then incubated at 65°C for 10 min, followed by an additional 15 s of shaking. The reaction mixture was subsequently incubated at 98°C for 2 min and then stored at -20°C.

PCR and DNA sequencing

Multiplex PCR based on species-specific primers was employed to examine the thrips specimens. Based on ITS sequences, specific primers for each thrips species were designed in this study or were obtained from Yeh *et al*. (2014a, 2014b, 2015; Table 1). In each reaction, five thrips species, namely the quarantine species *A. obscurus*, *F. occidentalis*, and *T. fuscipennis*, and the other two commonly observed species, *F. intonsa* and *T. tabaci*, were examined. Sequences of the universal primer pair of 28SgII and 28ShII were designed in this study and mixed with those mentioned above

Table 1. Primer information. Sequences of species-specific and universal primers and the amplified fragment size for five thrips species in multiplex PCR

Upstream primer name and sequences		Downstream primer name and sequences		Size (bp)	Reference
Fint3F	TGGCTTGCTTGAGCGGAAC	Fint2R	TTGGGAGTCCACATAGCGG	436	Yeh et al., 2015
Aobs1U	TGCTAAGGGCTCCTCGGAG	Aobs1D	CGCGAGGTGATCGCTTTCC	399	Yeh et al., 2014b
Ttab971U	AGAAACGATTACCAGACTGCCCAAG	Ttab974D	CAGTGATGCAGCACAACACATTCCAC	350	Yeh et al., 2014a
Tfus4U	TAAAAGCCTGTATGGGTTGCCTC	Tfus4D	TATCCGAGTTGGAGTCGCGTC	235	In thish study
Focc3U	AGCCTCCAGACGTTCTGCCAAAAG	Focc3D	CGAAACGCAAAGTGCGAGAAAATAATGC	149	Yeh et al., 2014a
28SgII	GGAGTTTGACTGGGGCGGTACAT	28ShII	CTTAGAGGCGTTCAGGCATAATCC	520	In this study

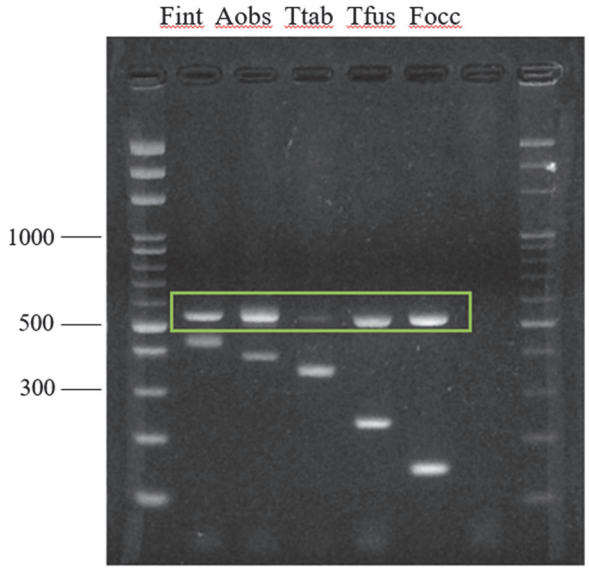

Fig. 1. Model of multiplex PCR resolution. The universal 28Sgh fragment size was approximately 520 bp; Fint: *Frankliniella intonsa* (~436 bp); Aobs: *Anaphothrips obscurus* (~399 bp); Ttab: *Thrips tabaci* (~350 bp); Tfus: *Thrips fusipennis* (~235 bp); Focc: *Frankliniella occidentalis* (~150 bp).

five species-specific primer pairs in a single reaction (Table 1).

The multiplex PCR assay was performed in a reaction volume of 20 μL containing 0.04 μL of each primer (100 mM), 0.16 μL of each dNTP (25 mM), 2 μL of 10X *Taq* buffer, 0.4 μL of Super *Taq* polymerase (Protech Technology, Taipei, Taiwan), and 1.5 μL of a DNA template. The programming conditions were 95°C for 2 min of denaturation followed by 35 cycles at 95°C for 40 s, 58°C for 20 s, and 72°C for 20 s, and then final extension at 72°C for 10 min. The amplified products were stored at 4°C. The expected amplification sizes for all specific primer pairs are displayed in Fig. 1.

In some detection, the multiplex PCR assay alternatively for quarantine *F. occidentalis* was performed following the procedures of the thrips identification kit of **GeneBuster™ThripID** (Yeastern Biotech Co., Taipei, Taiwan).

The PCR product was subjected to gel electrophoresis with 1% agarose at 100 V; an

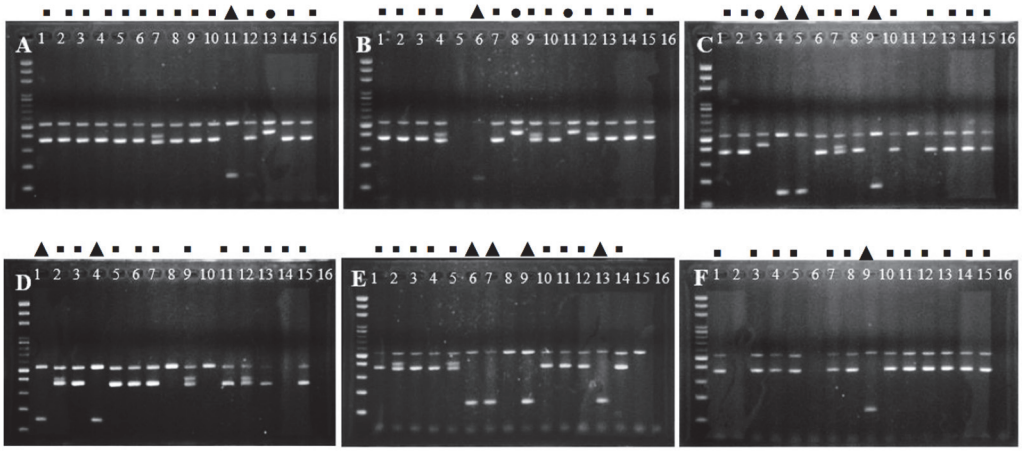

Fig. 2. Partial results of multiplex PCR for thrips samples from the quarantine station. Amplification results for multiple species-specific primer sets for five thrips species. The samples in panels A–F represent patterns of *Frankliniella occidentalis* (▲, ~150 bp), *F. intonsa* (●, ~440 bp), and *Thrips tabaci* (■, ~350 bp). The universal amplified product was ~520 bp. Lanes 1–15 were positive tests; Lane 16 was a negative control. See Table S1 for detailed information regarding each abbreviated individual in each panel.

image of the product was captured using a changeable ultraviolet transilluminator.

Results

Multiplex PCR based on species-specific primers revealed the expected amplified products of the target species. The successful generation of a 520 bp PCR product using the universal primers of 28S rDNA essentially ensured quality control in the experimental processes (Fig. 2). Multiplex PCR and morphological identification revealed that 29 of 231 individuals discovered on imported crops were *F. occidentalis* (Fig. 2; Table 2); no individuals of the quarantine thrips species *A. obscurus* or *T. fuscipennis* were observed. With an amplification size of 150 bp, *F. occidentalis* was primarily observed on strawberry and artichoke crops from the United States; some were also found on leucadendron and eryngium plants from Australia and the Netherlands (Fig. 2; Table S1). The other detected thrips were *T. tabaci*, *F. intonsa*, *S. dorsalis,* and *Thrips subnudula* (Karny), with individuals found respectively numbering 183, 8, 7, and 1 (Table 1). With an amplification size of 350 bp, *T. tabaci* individuals were mainly observed on asparagus from Australia, Mexico, and Thailand; some were also discovered on pea shoot, artichoke, and hypericum crops from India and the Netherlands (Table S1). *Frankliniella intonsa*, with an amplification size of 440 bp, was found on eggplant and sweet pepper. *Scirtothrips dorsalis* and *T. subnudula* individuals, which were identified based on morphological characteristics, were observed on asparagus and leucaspennum crops from Thailand and Australia, respectively (Table S1).

In thrips identification, consistency was demonstrated in both characteristic morphological examination and molecular results; the molecular results achieved discriminative capacity (Table 1). Of the 231 individuals, 191 (83%) were identified based on molecular markers, whereas only 163 (70%) were identified based on morphological characteristics because some of the slide mountings were of low quality, causing the destruction of specimens during DNA extraction. Molecular markers identified 156 of the 183 *T. tabaci* individuals; 128 of these 183 individuals were identified based on morphological identification, including 27 that could not be identified through molecular amplification. Molecular markers were used to identify 27 of the 29 (93%) *F. occidentalis* individuals; 21 of these 29 individuals were identified based on morphological characteristics, including two that could not be identified by molecular amplification. Morphological characteristics allowed identification of three of the eight *F. intonsa* individuals. Multiplex PCR was used to identify the target thrips species; however,

Table 2. Identification summary of thrips samples from quarantine station (Hsinchu branch)

Country[a]	Host plant	Individual No.	*Frankliniella intonsa*		*Frankliniella occidentalis*		*Thrips tabaci*		Other
			Mol[b]	Morp	Mol	Morp	Mol[c]	Morp	
Australia	Asparagus	102	-	-	-	-	95 (7)	73*	-
		1	1	*	-	-	-	-	-
	Leucadendron	1	-	-	-	-	1	*	-
		1	-	-	1	*	-	-	-
	Anemones	2	-	-	-	-	2	*	-
	Scholtzia sp.	1	-	-	-	-	1	1	-
	Leucospennum	1	-	-	-	-	-	-	*Thrips subnudula*
	Banksias spp.	1	-	-	-	-	-	-	Tubulifera
India	Pea shoot	4	-	-	-	-	4	1*	-
	Hypericum	1	-	-	-	-	1	1	-
Italy	Eremurus	3	-	-	-	-	3	3	-
Japan	Komatsuna	1	-	-	-	-	1	1	-
	Eggplant	4	4	*	-	-	-	-	-
	Crown daisy	1	-	-	-	-	1	*	-
		1	-	-	-	-	-	-	Tubulifera
	Garlic	2	-	-	-	-	1 (1)	2	-
	Naganegi	1	-	-	-	-	1	1	-
	Sweet Pepper	3	3	3	-	-	-	-	-
	Alstroemeria	1	-	-	1	*	-	-	-
		4	-	-	-	-	2 (2)	4	-
	Chinese cabbage	1	-	-	-	-	-	-	Tubulifera
	Scabiosa Farma	3	-	-	-	-	3	3	-
Mexico	Asparagus	29	-	-	-	-	17 (12)	22*	-
Nederland	Eryngiumy	1	-	-	1	1	-	-	-
	Leek	2	-	-	-	-	2	2	-
Nederland	Artichoke	4	-	-	-	-	4	*	-
		2	-	-	2	1*	-	-	-
Thailand	Asparagus	7	-	-	-	-	-	-	*Scritothrips dorsalis*
		9	-	-	-	-	9	3*	-
Tukey	Red cabbage	2	-	-	-	-	2	*	-
USA	Strawberry	19	-	-	17 (2)	15*	-	-	-
		1	-	-	-	-	-	1	-
	Asparagus	1	-	-	1	1	-	-	-
		8	-	-	-	-	4 (4)	8	-
	Artichoke	4	-	-	4	3*	-	-	-
	Broccoli	2	-	-	-	-	2	2	-

Country[a]	Host plant	Individual No.	*Frankliniella intonsa*		*Frankliniella occidentalis*		*Thrips tabaci*		Other
			Mol[b]	Morp	Mol	Morp	Mol[c]	Morp	
Total		231	8	3	27 (2)	21	156	128	11

[a] Pertinent information for each sample is provided in Table S1.

[b] Mol: molecular identification; Morp: morphological characteristic identification.

[c] Number of samples in the parentheses could only be identified by morphological characteristics.

[*] Morphological characteristics were destroyed in some individuals.

nonspecific amplification results of approximately 400 bp were obtained for some *T. tabaci* specimens, which invariably have a thick target band of 350 bp. Moreover, DNA quality may have been low because the universal primers could not amplify some thrips specimens, such as those in lanes B5, D13, and F2 (Fig. 2).

Discussion

Thrips of the *Frankliniella* and *Thrips* genera are frequently observed on plants. These species cause direct damage to plants, and approximately 20 of them can transmit *Tospovirus* (Hoodle *et al*., 2008). Of detected thrips species on imported crops, onion thrips have been reported to be a harmful pest that occupies leek fields in Australia (Kahrer, 1998) and is a key vector of Tospovirus in Western Australia (Chatzivassiliou, 2001). Another key *Tospovirus* vector is *F. occidentalis*, which can transmit five types of *Tospovirus* (Northfield *et al*., 2011; Cluever *et al*., 2015). The frequently detected species *F. intonsa* and *S. dorsalis* are also key virus transmission vectors (Inoue *et al*., 2004; Inoue and Sakurai, 2007). *Scirtothrips dorsalis*, however, is a species complex that can be distinguished only based on molecular characteristics (Hoddle *et al*., 2008; Dickey *et al*., 2015). Dicky *et al*. (2015) demonstrated that the Taiwanese *S. dorsalis* belongs to lineages I, II, and III and that lineage VI proliferates in Thailand. Therefore, the *S. dorsalis* complex on asparagus from Thailand could become an invasive species; this threat should be considered in designing species-specific primers for *S. dorsalis* complex detection on imported crops.

In 2016, Taiwan imported US$3.33 billion worth of food and agricultural products from the United States, including edible fishery and forestry products (Frederick and Fu, 2017). The invasive risk of thrips is high because thrips are invariably the most prevalent pest in imported agricultural products. Because of minute size and high morphological similarity among species, thrips identification is difficult and time-consuming. This study demonstrated that multiplex PCR based on species-specific primers of the ITS region is a reliable and quick diagnostic tool for identifying quarantine thrips from other commonly found thrips species. This multiplex PCR approach would have an identification efficiency greater than 93% for quarantine WFT if the sampling control were improved.

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應用複合性 PCR 及形態特徵鑑定輸入農作上之薊馬：檢疫薊馬西方花薊馬 及其他相關薊馬（纓翅目：薊馬科）之檢測

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摘　要

嚴重危害眾多農作的薊馬常於進口作物上發現，有些種類遂被列為檢疫對象。本研究以進口農作為對象，針對西方花薊馬 (*Frankliniella occidentalis*) 及數種檢疫薊馬進行檢測；結合形態特徵，並依據常用於物種鑑定的核醣體區間 (internal transcribed spacer, ITS)，設計 5 對專一性引子，應用複合性 PCR (multiplex PCR) 檢測黃呆薊馬 (*Anaphothrips obscurus*)、西方花薊馬、玫瑰花薊馬 (*Thrips fuscipennis*) 3 種檢疫薊馬及 2 種常見的蔥薊馬 (*Thrips tabaci*) 及歐洲薊馬 (*Frankliniella intonsa*)；每一複合性的 PCR 檢測中均含這 5 組專一性引子及一組 28S rDNA 區段的廣效性引子。分子鑑定的結果與形態特徵相互一致。進口作物上的薊馬多為蔥薊馬及美國草莓及薊類作物上的西方花薊馬，部分西方花薊馬也可在澳洲及紐西蘭的百合及刺芹上發現，其它進口作物上檢測到的薊馬有歐洲薊馬、小黃薊馬 (*Scirtothrips dorsalis*) 及管尾亞目薊馬物種 (Tubulifera)，並含有一種經形態鑑定，來自澳洲針墊花的 *Thrips subnadula*。本研究證實應用 ITS 區段種專一性的複合性 PCR 是一個快速有效鑑定檢疫的西方花薊馬的方法，準確率可達 93%；此外，對於非標的性的檢疫薊馬種類，仍須依賴形態特徵的鑑定。

關鍵詞：檢疫有害生物、分子鑑定、核醣體區間