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Assessment of the universality and utility of a set of conserved mitochondrial COI primers in insects

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Abstract

A set of mitochondrial COI primers has been studied by genomic PCR and many primer combinations shown to work universally well across Insecta. They are able to amplify various amplicons with different variability which enables the selection of a particular amplicon as a suitable DNA marker for a project. The potential usefulness of different amplicons is examined, with analysis on published study cases employing these regions. With respect to their variability, amplicons UEA5/UEA6, UEA7/UEA8 and UEA5/UEA8 could be useful for low- to mid-level phylogenetic analysis, i.e. from species, genus to perhaps family level depending on taxa involved. UEA5/UEA6 will be too conserved for intraspecific studies. Amplicons UEA3/UEA4 and UEA9/UEA10 would be better suited to low-level phylogenetic investigations, such as analysis of relationships among closely related species and population genetic studies. However, these guidelines should not be over-generalized for the reasons given. Amplification conditions of various primer combinations, and general problems in the use of conserved PCR primers are discussed.

Keywords: mtDNA, cytochrome oxidase I, versatile and degenerate primers, polymerase chain reaction, nuclear mitochondrial-like sequences, evolutionary PCR contamination.

Introduction

Mitochondrial DNA (mtDNA) is now commonly employed as a molecular marker in phylogenetic and population genetic studies of metazoan animals. The identification of conserved PCR primers (Kocher *et al.*, 1989; Simon, 1991; Simon *et al.*, 1994) have promoted its use in evolutionary studies in the past decade. It encourages researchers with little background of molecular biology to study phylogenetic and population genetic questions at the molecular level. This speeds up data collection and so increases knowledge of the molecular evolution of mtDNA sequences.

Originally, primers were designed in conserved mitochondrial regions in order to be able to directly isolate homologous regions from other organisms by PCR, without going through the cloning process which is technically demanding and laborious. However, regions amplified by such primers (called amplicons) may or may not be useful for a given project, depending on a number of factors such as the level of their variability and the particular research objectives. Also, parts of an amplicon may have different variability, and so studying the unsuitable part may lead to the failure of a project and a waste of resources. Not everyone is aware of this and it is not uncommon for an amplicon to be used simply because of primer availability. It is therefore necessary to refine the design of primers so that the usefulness of an amplicon can be easily assessed.

Recently, Lunt *et al.* (1996) have carried out a comparative analysis of the mitochondrial gene for the subunit I of cytochrome oxidase (COI) in nine insect species, including two orthopterans (a grasshopper and a locust), six dipterans (three fruitflies, two mosquitoes and a blowfly) and one hymenopteran (honeybee). They showed that different regions of the COI gene evolve at different rates, and may thus be suited for different applications. Accordingly, ten PCR primers have been designed to enable the amplification of regions of different variability. Here, we report the detailed study of these primers, plus three new degenerate primers, in ten invertebrate species, covering seven insect orders which represent the main divisions of the class Insecta, and one arachnid order.

Results

Amplifiability of mitochondrial COI primers in insects

Information on the thirteen COI primers studied here are given in Table 1, and their relative location and

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Table 1. Information on thirteen insect mitochondrial COI primers. Nomenclature of these primers (standard names) follows the standard given by Simon *et al.* (1994), that is, using a trinominal system of 'gene name-DNA strand-nucleotide position of the 3' base'. TY = $tRNA^{Tyr}$ gene, C1 = COI gene, TL2 = $tRNA^{Leu}$(UUR) gene; J = coding strand, N = non-coding strand; numbers following the letters J or N refer to the nucleotide position of the 3' base of primers with respect to the *D. yakuba* mtDNA genome (Clary & Wolstenholme, 1985); d = degenerate primer. Thus, primer UEA3 has a standard name 'C1-J-1763', which means that it is on the coding strand of the COI gene with its 3' base corresponding to nucleotide position 1763 on the *D. yakuba* mitochondrial genome. The following ambiguous symbols in sequences are used: R = A/G, Y = C/T, M = A/C, W = A/T, N = A/C/G/T. Nicknames (in parentheses after the common names) have been used to facilitate the remembering of individual primers.

Common name	Standard name	Size (bases)	Sequence (5' to 3')
UEA1 (Brent)	TY-N-1438	26	GAATAATTCCCATAAATAGATTTACA
UEA1d (Helen)	TY-N-1438d	26	GAAWAATTCCYATAAWTARATTTACA
UEA2 (Alison)	C1-N-1844	26	TCAAGATAAAGGAGGATAAACAGTTC
UEA2d (Maddy)	C1-N-1843d	23	GMWARWGGWGGRTAWACWGTTCA
UEA3 (Gof)	C1-J-1763	24	TATAGCATTCCCACGAATAAATAA
UEA4 (Douda)	C1-N-2087	24	AATTTCGGTCAGTTAATAATATAG
UEA4d (Diane)	C1-N-2096d	25	GANGTATTWARRTTTCGRTCWGTTA
UEA5 (Dave)	C1-J-2090	24	AGTTTTAGCAGGAGCAATTACTAT
UEA6 (Nic)	C1-N-2395d	29	TTAATWCCWGTWGGNACNGCAATRATTAT
UEA7 (Xing)	C1-J-2369	24	TACAGTTGGAATAGACGTTGATAC
UEA8 (Inger)	C1-N-2735	24	AAAAATGTTGAGGGAAAAATGTTA
UEA9 (Steve)	C1-J-2753	26	GTAAACCTAACATTTTTTCCTCAACA
UEA10 (Pat)	TL2-N-3014	25	TCCAATGCACTAATCTGCCATATTA

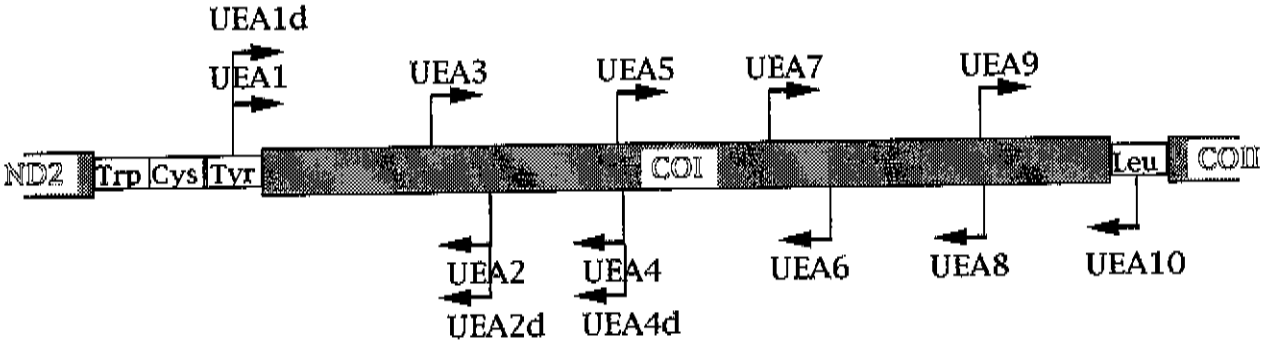
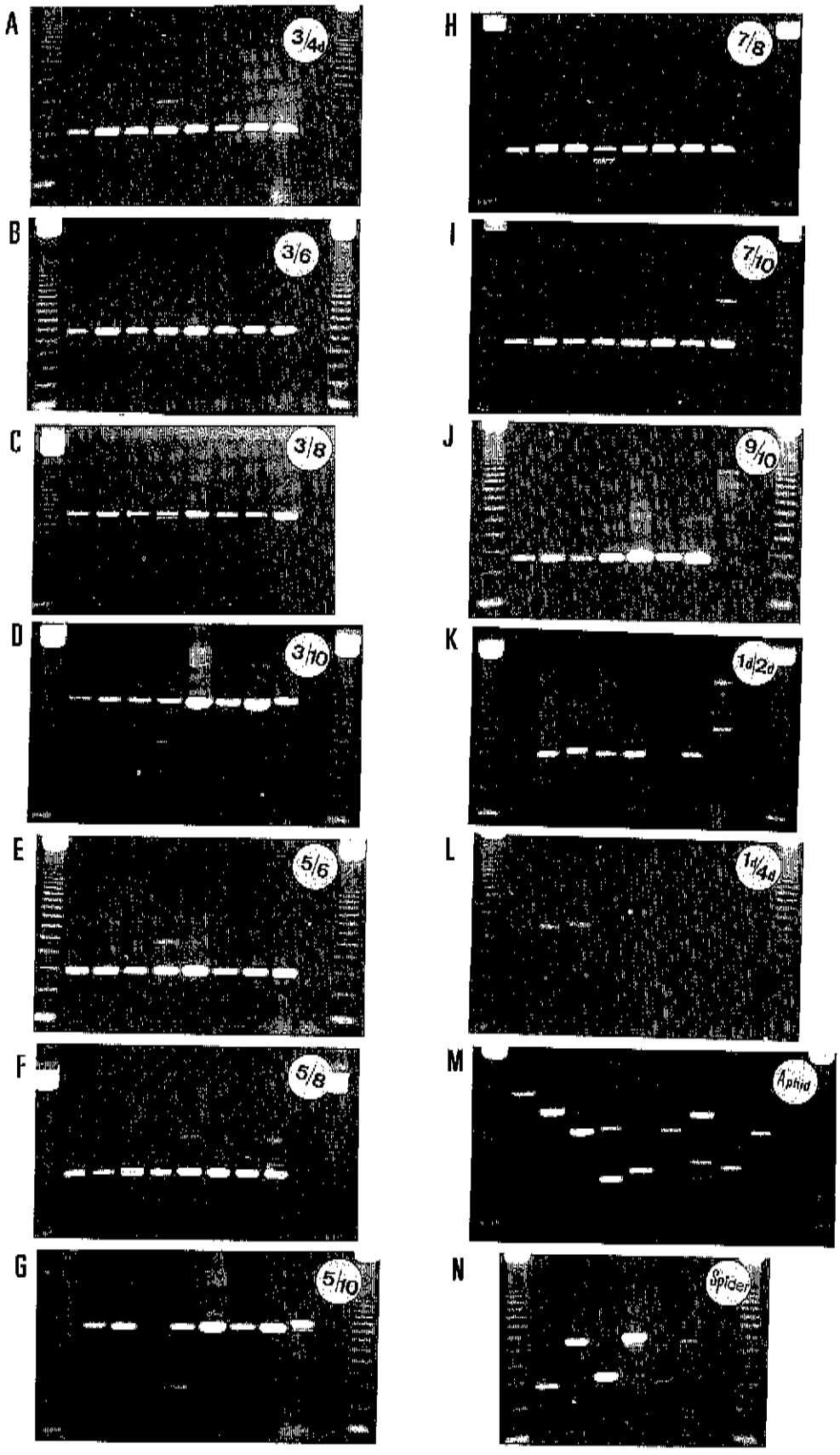


Figure 1. Relative location and directionality of the thirteen mitochondrial COI primers studied here. Protein-coding regions are shown as filled boxes and tRNA-coding regions as thin open boxes. The *Drosophila* gene order is used. Refer to Table 1 for names, exact positions, sizes and sequences of primers. Detailed description can be found in Lunt *et al.* (1996).

directionality shown in Fig. 1 (for more detail, see Lunt *et al.*, 1996). There are thirty-one possible combinations of these primers leading to PCR products from some 30 bp to 1630 bp (Table 2). Only those yielding products larger than 200 bp are examined in detail here. Although it is possible to amplify almost the whole mitochondrial genome (~16 kb in general) with some other primer combinations (e.g. UEA6/UEA9), such PCRs require special conditions and thus have not been considered here. In total, twenty-four primer combinations were tested by PCR in ten invertebrate species, including an arachnid species (a domestic spider) and nine insect species from seven orders (Thysanura, Odonata, Orthoptera, Hemiptera, Coleoptera, Diptera and Hymenoptera). The insects studied cover the main divisions of Insecta, with two primitive wingless insects (silverfish and firebrat), a primitive winged insect (damselfly) and six other winged insects (two grasshoppers, an aphid, a fruitfly, a beetle and a bee). Both blank DNA extraction controls

Figure 2. PCR amplification from genomic DNA of ten invertebrate species with various primer combinations. (A–L) PCR amplification with various primer combinations (as indicated on individual photo) in eight insect species. They have the same order on each photo: first and last lanes (except C for the last lane), 123 DNA size ladder (BRL, the first band from the bottom is 123 bp, the next up is 246 bp, the third 369 bp, and so on); lane 2, *Lepisma saccharina* (silverfish); lane 3, *Thermobia domestica* (firebrat); lane 4, *Calopteryx splendens* (damselfly); lane 5, *Schistocerca gregaria* (locust); lane 6, *Chorthippus parallelus* (grasshopper); lane 7, *Drosophila melanogaster* (fruitfly); lane 8, *Carabus vidaceous* (beetle); lane 9, *Bombus lapidarius* (bee); lane 10, PCR negative control. (M) PCR amplification in the pea aphid *Acyrthosiphon pisum*. Lanes 1 and 12, 123 DNA ladder; lanes 2–10, primer combinations UEA3/UEA10, 5/10, 7/10, 9/10, 5/6, 5/8, 7/8, 3/4d, 3/6, respectively; lane 11, PCR negative control. (N) PCR amplification in the spider *Tegenaria domesticus*. Lanes 1 and 9, 123 DNA ladder; lanes 2–7, primer combinations 5/6, 5/8, 7/8, 9/10, 3/4d, 3/6, respectively; lane 8, PCR negative control with primers 9/10.


A
3/4a
B
3/6
C
3/8
D
3/10
E
5/6
F
5/8
G
5/10
H
7/8
I
7/10
J
9/10
K
1d/2a
L
1d/4a
M
Aphid
N
Spider

Primer	UEA1	UEA1d	UEA3	UEA5	UEA7	UEA9
UEA2	460	460	130			
UEA2d	460	460	130			
UEA4	700	700	370	50		
UEA4d	700	700	370	50		
UEA6	1010	1010	690	350	80	
UEA8	1350	1350	1020	690	420	30
UEA10	1630	1630	1300	970	700	310

Table 2. Approximate sizes of amplicons estimated for *D. yakuba*. Sizes are given in base pairs (bp), which may differ in different insects, particularly for amplicons involving primers UEA1, UEA1d and UEA10.

and negative PCR controls have been carried out, along with normal experiments to check against cross contamination.

The results of PCR amplification are shown in Fig. 2. Primers UEA3, UEA4d and UEA5-UEA10 appear to work universally across the class Insecta, covering a divergence scale of more than 350 million years (Pearse *et al.*, 1987). All possible combinations among them (Table 2) amplify well in all insects tested (with odd exceptions, for example, in the pea aphid), producing a single specific band (Figs 2A–2J, Fig. 3). UEA4 does not amplify well in the meadow grasshopper, a *Bombus* bee and a beetle. UEA1 and UEA1d do not work satisfactorily in general, and may even fail to amplify in combination with the closest primer UEA2d in several species (the expected product is about 460 bp, Fig. 2K). Only a few primer combinations (UEA5/UEA6, UEA5/UEA8, UEA7/UEA8 and UEA9/UEA10) can successfully amplify in the spider (Fig. 2N). This is not surprising, considering that these primers have been designed for insects, and the insect–arachnid split occurred more than 400 million years ago (Pearse *et al.*, 1987). The pea aphid appears to be somewhat different from all other insects tested, in that some universally working primers, such as UEA3/UEA8 do not amplify in this organism.

To check whether any contamination has been involved, PCR products of one of the most universally working combinations UEA3/UEA8 have been directly sequenced (Lunt *et al.*, 1996). The terminal sequences obtained confirmed that they are COI sequences and taxon-specific. Other results also indicate that no cross-contamination has occurred. For example, UEA9/UEA10, another apparently universally working primer combination, has amplified a band in the bee and the spider with a size much larger than in other insects studied (Figs 2J and 2N). Now the intergenic region between COI and the $tRNA^{leu}$ genes is exceptionally large in some beetles (C. Stauffer and J. Galian, personal communications), and this observation suggests that this region in these invertebrates may also contain an insertion compared to that in *Drosophila*.

Amplification conditions of COI primers

This study involves the test of the universality of many primer combinations in a very diverse group of insects (including a non-insect invertebrate), so various PCR conditions have been tried. It showed that optimal amplification conditions, which are critical for a given pair of primers to amplify effectively in an organism, are primer-specific and taxon-specific. Of course, DNA quality will also affect the performance of PCR. In our hands, DNA extracted from fresh samples, cryopreserved samples and most absolute alcohol-preserved samples all amplify well. Annealing temperature, primer concentration and cycle profile are the first three factors which need to be adjusted in the event of failure. For example, in the case of pea aphid, no product was observed at 45°C after forty-five cycles for UEA3/UEA4d and UEA3/UEA6; specific product was produced at 40°C (Fig. 2M).

The employment of degenerate primers (such as UEA1d, UEA2d and UEA4d and UEA6) has dramatically increased the amplifiability in a wider range of organisms. In general, one should use a much higher concentration of such primers in PCR, because their effective concentration for a given template is in inverse proportion to their degeneracy. We have also noticed that, when there is no product or the yield is very low, the employment of the 'Touchdown' programing technique (Don *et al.*, 1991), or both low annealing and extension temperatures, are sometimes helpful. However, even some otherwise universally working primers may fail to amplify in certain organisms (e.g. UEA3/UEA8 in pea aphid).

The following are some suggestions on PCR cycling parameters for the COI primers studied here, which may be used as a rough guide when trying them in different species. Following an initial denaturation at 94°C for 4 min, run thirty-five to forty-five cycles of PCR with 40 s of denaturation at 95°C, 1 min of annealing at the temperature described below, and 40 s to 1.5 min of extension at 72°C. The suggested annealing temperatures are as follows:

48–50°C for UEA3/UEA8, UEA3/UEA10, UEA5/UEA8, UEA5/UEA10, UEA7/UEA10 and UEA9/UEA10;

45–50°C for UEA7/UEA8;

40–50°C for UEA3/UEA4, UEA3/UEA4d and UEA3/UEA6;

40–45°C for UEA1d/UEA2d, UEA1d/UEA4d and UEA5/UEA6, with an extension temperature at 65–68°C.

The above parameters are for use in a Perkin-Elmer DNA Thermal Cycler 480.

Discussion

Universality of these conserved COI primers

As seen clearly from Fig. 3, many primer combinations amplify well in a diverse group of insects, from primitive wingless insects to various winged insects. Their universality and potential usefulness are obvious. However, although insects tested here cover the main divisions of the class Insecta they include only nine different species, and a species (the pea aphid) difficult to amplify was observed even for some well-working primers. Therefore more exceptions will certainly be found when more taxa are examined. Note that a single nucleotide substitution on a target at the position equivalent to the 3′ base of a primer will stop that primer from amplifying. Although primers are designed at the most conserved regions based on available data, there is no way to ensure that the sequence is conserved in all insect species.

Two primers, UEA1 and UEA1d, do not amplify well in insects, although their sequences appear to be well conserved among grasshoppers, fruitflies, mosquitoes and the honeybee. The reasons for this are unknown. A noticeable feature of these primers is that they contain a relatively low percentage of G+C nucleotides. This could be partly responsible for their unsatisfactory amplification. In addition, there are at least three other possible explanations. First, nucleotide sequence of these primers are not conserved in other insects. Second, as these primers lie in the $tRNA^{tyr}$ gene of a tRNA cluster upstream of COI gene, transposition of $tRNA^{tyr}$ gene may have occurred in some lineages and thereby prevent the expected amplification. Third, stable secondary structure may be formed by the tRNA-coding DNA sequences and thus affect amplification. Although all three explanations are possible, a closer analysis of the data obtained suggests

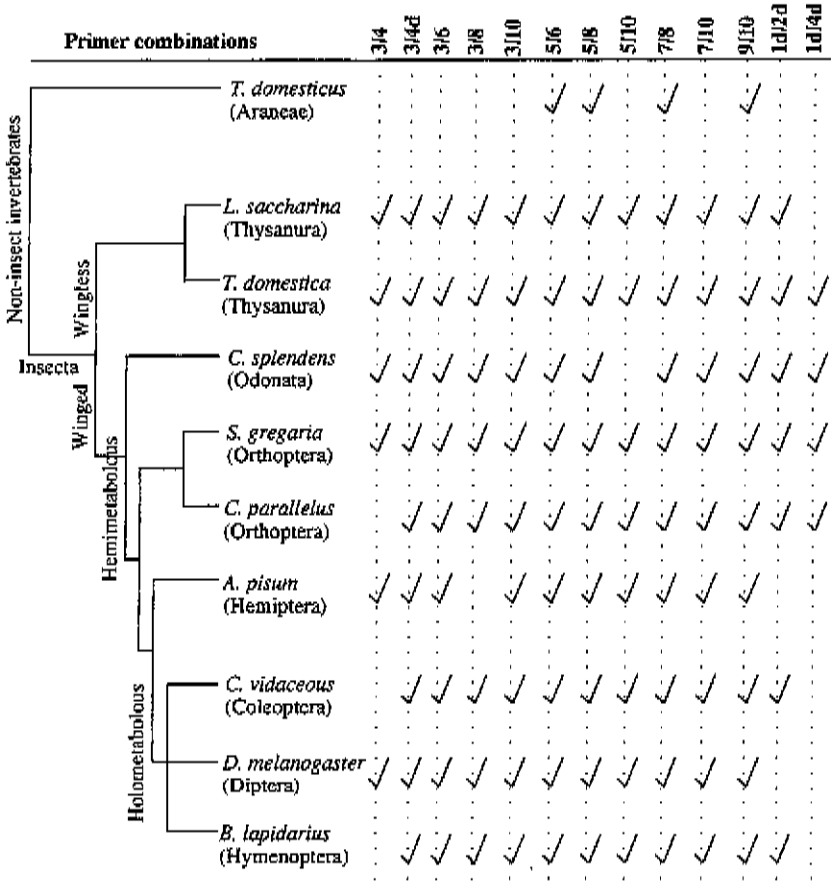


Figure 3. Universality of COI primers in Insecta. Phylogeny of insect orders is after Kristensen (1981). A tick indicates successful amplification of a primer combination in an organism. 'Successful' means a main band which is specific and reproduceable has been obtained in the PCR conditions tested. Where no tick is marked, it means that the corresponding amplification was not satisfactory or no product was obtained after several tries at different conditions. Figure 2 may be consulted for PCR results.

that the first two are not the primary factors. In the honeybee the order of tRNA genes in this region has been rearranged; however, the direction of transcription of $tRNA^{tyr}$ gene is the same as in other insects studied (Crozier & Crozier, 1993). Therefore, at least for the fruitfly *Drosophila melanogaster*, the meadow grasshopper *Chorthippus parallelus* and perhaps also the desert locust *Schistocerca gregaria* and *Bombus* bee studied here, $tRNA^{tyr}$ has not been transposed to another region (see Szymura *et al.*, 1996), and the conserved status of primer UEA1 should enable a successful PCR amplification in these organisms. In addition, primer UEA1d is a degenerate form of UEA1 with sequences well matched to the above-mentioned species. However, they failed to amplify in fruitfly and the *Bombus* bee, and did not amplify satisfactorily in grasshoppers. We think that the third explanation – that is the formation of stable secondary structure – is more likely to be responsible for the failure. Such stable secondary structure could prevent effective annealing of primers to the template targets and extension by polymerase, thereby blocking polymerse chain reaction in the very initial stages.

Choice of conserved primers for a research project

Differences in variability exist through the insect COI gene, owing to different functional constraints for different regions (Lunt *et al.*, 1996). Therefore different combinations of these COI primers can amplify regions showing different variability. Primers may be chosen with regard to one's particular research questions and objectives. Although it is perhaps unlikely that the patterns of variation observed by Lunt *et al.* (1996) will always be maintained at other taxonomic levels or in other insect species, they can serve as an important reference for assessing the likely usefulness of individual amplicons. The most conserved part, for instance, will almost certainly remain highly conserved at lower taxonomic level, thus will be less useful for population genetic analyses. Here we give a brief discussion on the potential usefulness of different amplicons in insects, with summaries on published study cases employing them.

Amplicons UEA5/UEA6, UEA7/UEA8 and UEA5/UEA8. Amplicon UEA5/UEA6 corresponds to the most conserved part of insect COI gene, and will probably be unsuitable for intraspecific analysis or phylogenetic inference among closely related species. Amplicons UEA7/UEA8 and UEA5/UEA8 are also very conserved. These three regions could be useful for low- to mid-level phylogenetic analysis, such as for the inference of phylogenetic relationships among species, genera and perhaps families.

Pedersen (1996) has used a COI segment corresponding to amplicon UEA5/UEA6 to study the phylogenetic relationships of the cuckoo bumblebees (*Psithyrus*) and the true bumblebees (*Bombus*), belonging to two genera of the hymenopteran subfamily Bombinae. He has shown that the cuckoo bumblebees form a monophyletic group, whereas the true bumblebees are paraphyletic with some species grouping with the cuckoo bumblebees. The author noticed that the accumulation of third codon position transversions had not reached saturation, and thus concluded that the cladogenetic changes are probably relatively recent. Although such explanation may be true, it is also possible that the observed non-saturation is due to the highly conserved status of this COI region. It is not surprising to see that no intraspecific variation has been detected by this marker.

With a region corresponding to amplicon UEA7/UEA8, Juan *et al.* (1995) were able to infer the phylogeny of darking beetle species and deduce their colonization history in the Canary Islands, which has occurred within the past 20 million years. In contrast, the use of this region in the study of the phylogenetic relationships of fifteen coleopteran families proved unsuccessful, because the high variability of this region has masked the deeper phylogenetic signals (Howland & Hewitt, 1995). It appears that for Coleoptera this region could be informative at species or genus levels, but will be too variable for resolving phylogenetic relationships at the family level or above.

A compound study using COI and COII sequences has been carried out by Sperling & Hickey (1994) in the investigation of mtDNA variation in the pest spruce budworm species complex. The sequence used extends from about 100 bp downstream of the primer UEA5 in COI to the end of COII gene. The distribution pattern of variable sites across the COI sequence revealed in their data generally agrees with the pattern deduced from Lunt *et al.* (1996). From their data, it is also evident that, at the taxonomic level studied, the third codon positions of COI are more variable than COII, though its amino acid sequence is more conserved. Thus the COI region sequenced possesses a higher percentage of variable sites than the COII gene.

Amplicons UEA3/UEA4 (or UEA4d) and UEA9/UEA10. These amplicons cover the most variable parts of the COI gene. In view of their variability, they could be useful for addressing low-level phylogenetic questions, such as relationships between closely-related species and population genetic studies. Funk *et al.* (1995) have carried out a phylogenetic study of the chrysomelid leaf beetle genus *Ophraella*, using a 420 bp COI region comprising the entire amplicon UEA3/

UEA4. Their data indicate that the COI region employed is apparently appropriate for both inter- and intraspecific analysis, trees recovered from these data are largely congruent with those produced from other mtDNA and allozyme/morphology data. In *Chorthippus* grasshoppers, amplicon UEA3/UEA4 appears to be able to resolve interspecific phylogenetic relationships, but is not variable enough to give a good resolution of the phylogeography of *C. parallelus* (D. H. Lunt, personal communication).

Part of the amplicon UEA9/UEA10, together with the sequence of $tRNA^{leu}$ (UUR) and COII genes, have been employed in the phylogenetic studies of the lepidopteran genera *Greya* (Brown *et al.*, 1994) and *Heliconius* + *Eueides* (Brower, 1994). As data for these three mitochondrial genes were analysed together, and involve only a short COI sequence (some 200 bp), we do not evaluate here the effectiveness of the COI region used.

Other amplicons. Amplicon UEA3/UEA10 may be suitable for several different purposes. It contains more than 80% of the COI gene, including regions of different variability defined by amplicons mentioned above. Once it has been amplified, other internal primers can be used for sequencing. Amplicons UEA5/UEA10 and UEA7/UEA10 may be similarly employed.

The eventual usefulness of these regions depends heavily on the taxa studied and their biology. A DNA region with a sufficient level of variability in one species may fail to resolve similar phylogenetic questions in another species. Also, the utility of a DNA region in evolutionary studies depends on a number of factors. We have only considered one of them (the relative variability), therefore the above discussion should not be over-generalized. For example, heterogenous substitution rate in a sequence will affect the performance of some tree-making algorithms, thus decreasing the usefulness of otherwise a highly variable DNA region. The COI gene appears to have such a rate heterogeneity among regions at both the nucleotide and amino acid sequence levels (Lunt *et al.*, 1996). Yet, at amino acid sequence level, COI is under strong functional constraints (e.g. Nigro *et al.*, 1991; Howland & Hewitt, 1995). A direct consequence of this is that when the DNA sequence is too variable to be phylogenetically useful, the corresponding amino acid sequence also has little value, as is the case in the study of Howland & Hewitt mentioned above. Recent data indicate that heterogenous substitution rate from site to site may exist within a given COI region (Spicer, 1995). Spicer has employed a 408 bp COI segment corresponding roughly to the amplicon UEA3/UEA4, as well as the entire COII gene and a 419 bp COIII sequence, to study the phylogenetic relationships of the *Drosophila buzzatii* species complex. He found that although they have a comparable overall variability, the power of recovering the best estimate of the phylogeny by these three regions is quite different, with the COI segment being the worst. The author related such results to rate heterogeneity, for which the COI region used appears to be the most pronounced. Although such observations need to be further examined in depth in both *Drosophila* and other insects, they highlight complications in using a DNA region in phylogenetic analyses. It will be particularly interesting to see if other COI segments perform in the same way, and whether the phenomenon is affected by divergence levels.

Conserved primers: to use or not to use

Although PCR using conserved mitochondrial primers is straightforward, it also poses a potential problem, that of contamination by unwanted targets from various sources. This includes technical contamination, i.e. contamination introduced by humans during experimental manupulation, and evolutionary contamination, resulting from mitochondrial-like sequences in the nuclear genome of animals (for review, see Zhang & Hewitt, 1996). Nuclear COI-like sequences have been observed in several animals including insects, and can be coamplified with the intended mitochondrial target in PCR using conserved primers.

In view of this, there is need for careful and realistic use of conserved PCR primers. Apart from including negative controls at different stages of manupulation (i.e. DNA extraction and PCR), further efforts can be made to minimize various kinds of contamination. One well-known procedure in laboratories is to separate DNA extraction area, PCR area and post-PCR working area, and avoid inter-area transfer of materials. The use of filtertips during PCR set-up is also most helpful. Another effective way to minimize contamination is to use specific primers instead of conserved primers. Therefore, after the first sequence data have been obtained from an organism by PCR with conserved primers, their authenticity should be checked by preliminary phylogenetic analysis. Then taxon-specific or target-specific primers can be designed to specifically amplify one's target.

Experimental procedures

Invertebrate samples

The following nine insect taxa were used to test the thirteen primers listed in Table 1, viz: *Lepisma saccharina* (silverfish, Apterygota, order Thysanura), *Thermobia domestica* (firebrat, Apterygota, order Thysanura), *Calopteryx splendens* (damsel-

fly, Pterygota, order Odonata), *Schistocerca gregaria* (locust, Pterygota, order Orthoptera), *Chorthippus parallelus* (grasshopper, Pterygota, order Orthoptera), *Acyrthosiphon pisum* (aphid, Pterygota, order Homoptera), *Drosophila melanogaster* (fruitfly, Pterygota, order Diptera), *Carabus vidaceous* (beetle, Pterygota, order Coleoptera) and *Bombus lapidarius* (bee, Pterygota, order Hymenoptera). A non-insect invertebrate, the domestic spider *Tegenaria domesticus* (an arachnid) was included for comparison.

Total DNA extraction and PCR amplification

Total DNA was isolated from individual insects (except for the pea aphids, where several individuals from a single colony were pooled together) using a phenol/chloroform based extraction method (Zhang *et al.*, 1995). Where possible, only head or legs were used for DNA extraction to avoid any contamination from food or symbionts. PCR amplification was carried out as described in Lunt *et al.* (1996), with various annealing/extension temperatures for different primer combinations and for different organisms. Some 5–50 ng of genomic DNA was used in a 50 μl reaction. Amplified products were checked on conventional agarose gels of appropriate concentrations.

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