Nat. Volatiles & Essent. Oils, 2021; 8(5): 3610 - 3621

NVEO
Natural Volatiles &
Essential Oils

Differences And Similarities Of Bactrocera Carambolae And Bactrocera Tau In The Mekong Delta Of Vietnam Based On Polymorphism Of Mtdna

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Abstract

The purpose of this study is to research on fruit flies in Mekong Delta region in Vietnam, differences and similarities among characteristics of *B.dorsalis*, *B.correcta*, esp. *B.carambolae*, *B.cucurbitae* and *B. tau* species and then give some recommendations. The precise identification's procedure of fruit flies species is important for the management of fruit fly pests to enhance quarantine services on fruit and vegetable. In this study, we collected 15 samples from guava and bitter gourd fruits in 3 provinces belong to the Mekong Delta region. From that, a nucleotide sequences of 700 bp of the cytochrome oxidase subunit II (COII) gene of mitochondria DNA (mtDNA 13 and mtDNA 20) was amplified by PCR with the mtD13 and mtD20 primer pairs using DNA of 15 specimens from fruit flies samples. Based on the target gene sequence polymorphism (488 bp) analysis on mitochondrial COII, 15 fruit fly samples were identified as belonging to two subgenera Bactrocera and Dacus, sequences of a 700 bp portion of the cytochrome oxidase subunit II of the 15 specimens from collected fruit flies, the results showed that they belong to the genus *Bactrocera* (12 guava fruit fly samples belong to the subgenus *Bactrocera* and 3 bitter gourd samples belong to the subgenus *Zeugodacus* (or *Dacus*)) with phylogenetic tree supported by high bootstrap value as (97.54-98.02%). Two genera had been identified by 2 haplotype with 11 different mutations on the DNA sequences. Genus *Bactrocera* has three different species named *B. dorsalis*, *B. correcta* and *B. carambolae* with the homogenus sequences come up to 98.02%. In genus *Zeugodacus*, DNA sequences of the two species named *Bactrocera curcubitae* and *B. tau* was high heterologous with each other (99-100%) with 15 different mutations on their DNA sequences. The DNA sequences of the species belong to the genus *Zeugodacus* have high homologus (98.91-99.39%).

Keywords: fruit fly, *Bactrocera carambolae, Bactrocera tau,* mtDNA

INTRODUCTION

Fruit flies (Tehpritidae) are insect that cause serious losses to the fruit production and export of some fruits from temperate to tropical regions (Papadopoulos *at al*., 2001; Cohen and Yuval, 2000). In Vietnam, the fruit fly occurs continuously and lasts for many years, especially in the rainy season. Accurate identification of fruit fly species plays an important role in the effective prevention and quarantine of these species on fruits and vegetables in the Mekong Delta. Species of fruit flies are complex; *Bactrocera* and *Dacus* are two closely related genera (Munro, 1984). In the genus *Bactrocera*, there are 28 sub-genera with about 500 species (Drew, 1989; Drew and Hancock, 2000). In our country, there have been a number of studies that have identified over 30 species of fruit flies in Vietnam by morphological taxonomy (Allwood and Leblanc, 1996; Nguyen Thi Chat and Huynh Tri Đuc, 2003); Most species belong to the genus *Bactrocera*, of which three species *B. correcta*, *B. dorsalis* and *B.carambolae* cause severe damage to many crops. However, until now, there have not been many analyzes to determine whether the fruit flies in our country belong to the genus *Dacus*, as well as the study to distinguish two species *B. carambolae* and *B.tau*.

LITERATURE REVIEW

In Vietnam, The Mekong Delta is mainly fruit and vegetable production for domestic and export needs, fruit flies population thrive. Among the host species as guava (*Psidium guajava*) and bitter gourd (*Momordica charanta*) are the two fruits and vegetables most affected by fruit flies (Hoa *at al.*, 2010).

Identification of fruit flies at the species level based on morphological features remains controversial for complex species, especially recent studies of genetic differences within the *Bactrocera dorsalis* complex

(Muraiji and Nakahara, 2002; Ebina and Ohto, 2006). While, the application of polymorphic analysis of DNA sequences of target genes on mitochondria has been increasingly effective to overcome the limitations of morphological analysis methods. A series of studies applying PCR cloning technique and DNA polymorphism analysis of the COII target gene segment on fruit fly mitochondria have been successfully applied to identify species and study genetic diversity of some species fruit flies (Muraji and Nakahara, 2002; Barr *at al.*, 2006; Muraji and Nakahara, 2010). In this study, two species of fruit fly *B.carambolae* and *B. tau* were identified based on the comparison of COII target DNA sequence polymorphisms on mitochondria of several published fly species with 15 fruit fly samples fruit on the host of guava and bitter gourd in the Mekong Delta. The research results were published for the first time as a scientific basis for the effective quarantine and prevention of fruit flies in our country.

Beside, Bactrocera carambolae belongs to the B. dorsalis species complex (see Drew & Hancock, 1994). Schutze et al. (2014) showed that despite the high morphological and genetic similarity between B. carambolae and B. dorsalis, they are considered two valid species. Rosopoulou et al (2019) mentioned *Bactrocera carambolae* is one of the approximately 100 sibling species of the *Bactrocera dorsalis* complex and considered to be very closely related to *B. dorsalis*. Due to their high morphological similarity and overlapping distribution, as well as to their economic impact and quarantine status, the development of reliable markers for species delimitation between the two taxa is of great importance.

Next, Nakahara et al (2019) stated While results of the seasonal occurrence of serious quarantine pest species were previously reported, further analysis was made in this study to determine fruit fly fauna in mango orchards. Based on the morphological research, twenty Bactocera species were identified including major serious fruit flies such as B. dorsalis, B. correcta and B. cucurbitae. Out of the twenty species, nine were new findings and not recorded previously in Myanmar

MATERIALS AND METHODS

Site descriptions: The research was undertaken within a main fruit growing region of 5 provinces of Vietnam. Since the region is situated in a floodplain with average land elevation of 0.5-2 m above the sea level, fruit is usually grown on raised beds which are separated by a 2-8 m wide canal system to minimise flooding.

Table 1: Fruit fly samples collected on guava fruit and bitter gourd fruit

Number samples	Host tree	Sampling location
3	bitter gourd fruit	Long Ho-Vinh Long
7	bitter gourd fruit	Sa Đec- Đong Thap
8	bitter gourd fruit	Sa Đec - Đong Thap
22	guava fruit	Tu Liem-Ha Noi
24	guava fruit	Sa Đec- Đong Thap
27	guava fruit	Sa Đéc - Đồng Thap
29	guava fruit	Sa Đéc - Đồng Thap
30	guava fruit	Tu Liem - Ha Noi
31	guava fruit	Ho Chi Minh city
33	guava fruit	Chau Thanh -Tien Giang
35	guava fruit	Chau Thành-Tiền Giang
36	guava fruit	Sa Đec - Đong Thap
37	guava fruit	Tan Phuoc - Tien Giang
38	guava fruit	Go Cong – Tien Giang
39	guava fruit	Long Ho - Vinh Long

15 fly samples were collected in 5 provinces in the Southwest region, Ho Chi Minh City and Hanoi city. Fly samples were soaked in alcohol at 70°C and kept at 4°C until DNA extraction. The COII genomic DNA sequences of 6 published fruit fly species used for comparative studies include *Bactrocera dorsalis* (AB090272)*, B. tau* (AB19246); *B. correcta* (AB192422)*, B. cucurbitae* (EU926790)*, B.carambolae* (AB192420) và *Dacus vertebrates* (EU926797).

DNA Separation: Total DNA of 15 fruit fly samples was separated according to the CTAB method of Rogers and Bendich (1988): corrected. DNA extraction through basic steps including: DNA sample preparation, cell disruption, DNA expression, DNA purification, DNA storage after extraction.

Cloning by PCR: The COII target gene fragment (approximately 700 bp) on mitochondrial DNA was cloned by PCR between the total DNA of 15 fruit fly samples and the primer pair

mtD13: 5'-AATATGGCAGATTAGTGCA-3' and

mtD20: 5'- TTTAAGAGACCAGTACTTG - designed on the area *tARN* at the ends of the COII gene. Components of each PCR reaction (25µl) includes 13.25 µl BiH_2O, 5 µl *Taq* buffer 10 X (Tris 100 mM, KCl 500 mM, pH 9.0; 1% (V/v) Triton X-100), 1 µl dNTPs (20 mM), 2.5 µl $MgCl_2$ (25 mM), 1 µl each/type of primer, 1 µl *Taq* DNA polymerase (5 U/µl), 1.5 µl mẫu DNA (50-100 ng). PCR cycle includes 1 initial denaturation cycle at 90°C for 5 minutes, continuing with 35 cycles: 90°C-0.5 minutes, 47°C-0.5 minutes, 65°C-1.5 minutes; end the last cycle at 65°C for 5 minutes. The lengths of PCR products were observed on 1.5% agarose agar run in TE1 x buffer stained with ethidium bromide 1 µg/ml at 80 V for 100 minutes.

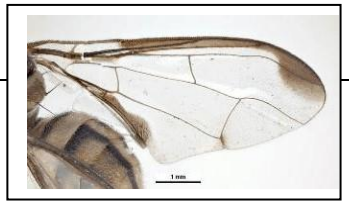
DNA sequencing and analysis: PCR products were sequenced directly on the ABI 3130 using the Invitrogen™ Kit. The target DNA sequences (488 bp) of 15 samples were compared with the same target sequences of 6 published fruit fly species based on the Neighbor Joining method in Mega3.1 software (Kumar *at al.,* 2004). Genetic differences between varieties and species were evaluated based on Bootstrap values with 1000 replicates. Haplotype to distinguish genera and species based on characteristic point mutations on COII gene DNA sequences of samples based on Align by ClustalW program and Sequence Data explorer in Mega3.1 software. Similarities in DNA sequences of samples of the same genus or species were analyzed using the Multiple Sequence Aligment program on DNAMAN 4.0.1.1 software (Lynnon Biosoft).

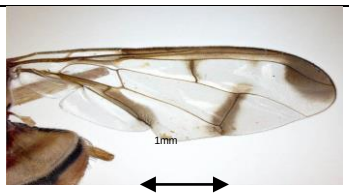
RESULTS AND DISCUSSION

Morphological differences between species in the Mekong Delta

The characteristics of *B.dorsalis*, *B.correcta*, *B.carambolae*, *B.cucurbitae* and *B. tau* species are quite similar, but when closely observing the morphology, there are also some differences between these species about body size and wing structure (Table 2). The smallest species is *B.correcta* (6.27 ± 0.15 mm) and the largest species is *B. cucurbitae* (9.62 ± 0.22 mm). In addition to the size and wing structure, a number of other morphological features are also used to differentiate the 5 species of fruit flies such as body color, presence of black spots on antennae and presence of spots dark brown color burning at the tibial segment of the female's middle leg (Table 2) to molecular biology.

Table 2. Some morphological characteristics of 5 marine species in the Mekong Delta

Species of fruit flies	Length average **(mm)**		Wing structure	
	Body	**Wing**		
B. carambolae	8,89±0,11 (8,70-9,10)	6,48±0,03 (6,39-6,52)	Along the anterior edge of the accessory tendon there is a faint streak extending to the R2+3 vessel and extending through the top of the R4+5 vessel	
B. tau	9,44±0,16 (9,05-10,07)	6,73±0,06 (6,61-6,83)	Along the anterior edge of the accessory tendon, there is a yellow streak across the	

			apex of R2+3 that spreads and forms a large spot through the apex of the R4+5 vessel. Longitudinal circuit with narrow elbow	
B. dorsalis	7,56 ±0,14 (7,0-8,30)	6,14 ±0,05 (6,0-6,20)	The flank band is dark black extending to the R2+3 tendon and from there slightly bulging to the R4+5 tendon	
B. correcta	6,27 ±0,15 (6,20-6,80)	5,12 ±0,03 (5,0-5,20)	Along the anterior edge of the accessory tendon there is a faint streak extending to the R2+3 vessels and extending further through the top of the R4+5 vessels	
B. cucurbitae	9,62 ±0,22 (8,40-10,0)	6,12 ±0,02 (6,0-6,20)	The wing veins that go across r-m have dark streaks of black	

Of the five fruit species, only *B. cucurbitae* is yellow-orange, the rest are yellow -brown. The species *B.correcta* can also be distinguished from the other 4 species by the presence of a black horizontal band on the face.The two species *B.carambolae* and *B. dorsalis* have quite similar morphological characteristics. However, in *B.carambolae*, the middle tibial segment of the female has brown spots, while that of *B. dorsalis* species does not present (Table 3).The results recorded on the morphological characteristics of fruit flies are consistent with those of Drew and Hancock (1994).The survey results show that the external morphology also partly identifies the common fly species in the Mekong Delta. However, in order to classify the species accurately, the study conducted DNA analysis of the wild species by the method of classification according.

Table 3. Some additional morphological features in the Red River Delta subspecies

Species of fruit flies	**Black spot under the beard**	**face**	**Belly**	**Feet**
B. carambolae	Present	Two spots	The black spot at the base of the fourth abdominal segment is triangular in shape	Dark brown spot on middle tibia of female
B. tau	Absent	Two spots	absent	Absent

B. dorsalis	Present	Two spots	The black spot at the base of the fourth abdominal segment is triangular in shape	Absent
B. correcta	Absent	A black horizontal band	Absent	Absent
B. cucurbitae	Absent	Absent spot	Absent	Absent

Analysis of DNA sequence differences on COII target genes between varieties and species

After sequencing, 488 bp of the COII gene from 15 fruit fly samples was used to compare with the same target sequences of 6 published fruit fly species (see materials). The analysis results showed that all fly samples were classified into 2 branches on the species tree (Figure 1).The 15 fly samples in the study may belong to two genera *Bactrocera* and *Dacus* with a reliable genetic distance between the two varieties (100% boostrap value). Of the 15 analyzed samples, up to 12 samples were collected on guava hosts belonging to the genus *Bactrocera* or the *B.dorsalis* complex; while there are only 3 samples of the genus *Dacus* parasitic on bitter gourd fruit (Figure 1; Table 1). This result is consistent with the study of Nguyen Thi Chat and Huynh Tri Duc (2003), which showed that 90.3% of total fruit fly samples belonged to *Bactrocera dorsalis* complex and guava was the preferred host of this complex.In the species group of the genus *Bactrocera*, the samples are closely related to *B.carambolea* and *B.dorsalis*. There are no samples close to the species *B. correcta*. There are no samples close to the species *B.correcta*. Samples 3, 7, 8 belong to the genus *Dacus*, in which sample 8 is close to species *B.tau*; samples 3 and 7 belong to the same clade as *B.cucubitae*. Two species *B.tau* and *B.cucurbitae* have a reliable genetic range (99-100%). The high genetic diversity (boostrap values between 22-100%) of samples and species of the genus *Bactrocera* may reflect the species diversity of this genus. According to some publications (Drew, 1989; Drew and Hancock, 2000), in the genus *Bactrocera* there are 28 subgenera with about 500 species.

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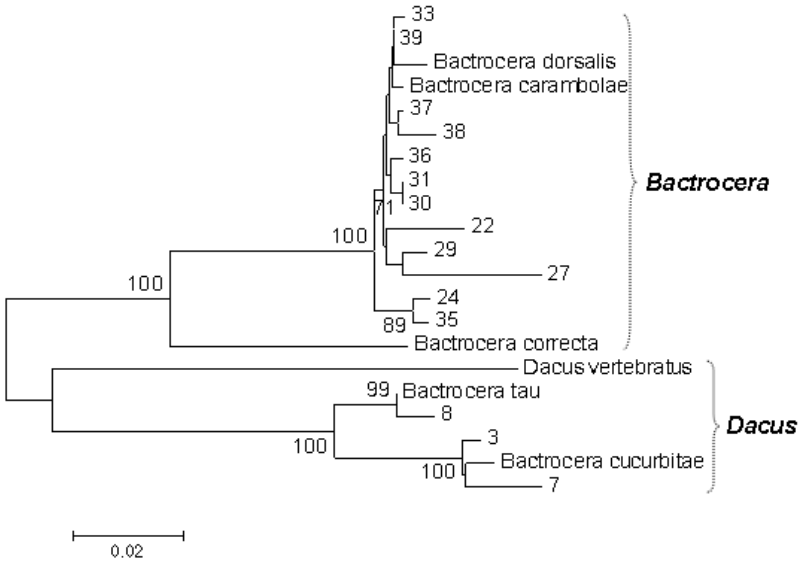

Figure 1: The species tree analyzed by Neighbor Joining in Mega3.1 is based on the 488bp DNA sequence on the COII gene of 15 fly samples and 6 sequences published in the world Numbers on the branches are the Bootstrap values analyzed with 1000 times repeat

The results of analysis by Neighbor Joining (Figure 1) are reflected in the results of the analysis of characteristic point mutations between varieties and fruit fly species. The genus *Bactrocera* can be distinguished from *Dacus* based on two different haplotypes with 11 characteristic mutations in the 488bp target segment of the COII gene (Figure 2). 12 samples in this study with 3 samples *B. dorsalis*, *B.*

carambolae and *B. correcta* belong to genus *Bactrocera* with haplotype having ATCTCATTACT specific nucleotides at 11 mutation points on target gene. While samples 3, 7, 8 and 3 species have been published *D. vertebratus*, *B. tau* and *B. cucurbitae* belong to genus *Dacus* because the haplotype characteristic at the same 11 mutation points on the target gene segment as *Bactrocera* is TCTTTCCTTA.

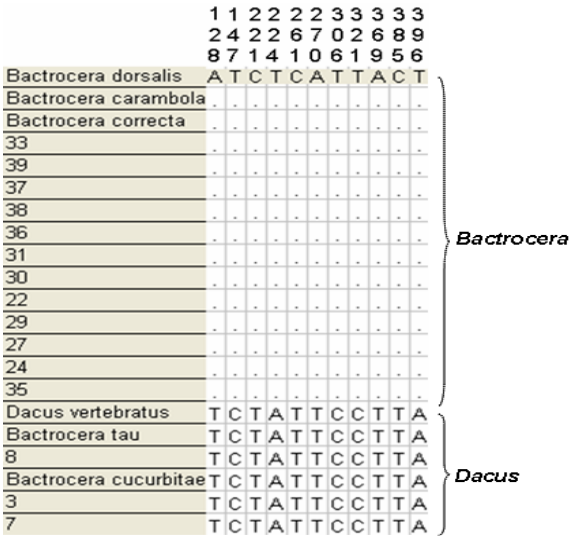

Figure 2: 11 characteristic mutations along the 488bp DNA sequence on the COII gene to distinguish two varieties of fruit fly Bactrocera and Dacus. Markers (.): are nucleotides similar to point mutations of Batrocera dorsalis. Numbers 128-396 are the location of point mutations

At the level of species identification, two species *B. tau* and *B cucurbitae* have genetic differences of up to 99-100% (Figure 1) because the DNA fragment sequence 488bp on the COII gene has 15 unique mutations (Figure 1). Thus, the specific haplotype on the COII target gene to identify *B.tau* species is ATATTTATTTCTGAT. While the sequence at the same 15 points on the target gene segment is the species-specific haplotype of *B. cucurbitae*, the order is GCGCCCGCCTCACC.

Analysis of DNA sequence homology on the COII target gene between fruit fly varieties and species.The genetic relationship between the cultivars and fruit fly species (Figure 1) is also reflected in the similarity in the DNA sequences of the target genes between them. Sequence similarity between species of the genus *Bactrocera* or the complex *B. dorsalis* (including *B. dorsalis*, *B. carambolae* and samples 22, 24, 27, 29, 30, 31, 33, 35, 36, 37, 38, 39) to 97.85%. While, species *Bactrocera tau*, *B. cucurbitae* and 3 samples 3, 7, 8 have 97.54% similarity.When analyzing the sequence similarity between samples within the species, the sequences in the species *B. tau* (*B. tau*, sample 8) were 99.39% similar; in species *B.cucurbitae* (*B.cucurbitae*, samples 3 and 7): 98.81%. The high degree of similarity in DNA sequences of fruit fly species could be attributed to the high average A+T ratio in the sequences. According to some studies (Muraji and Nakahara, 2002; 2010), the A+T ratio in fruit fly mitochondrial DNA ranges from 63.8 to 66.7%.

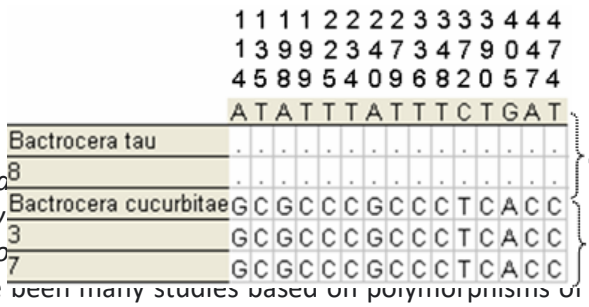

Figure 3: 15 chara[illegible]II gene to distinguish two species of fruit fly[illegible]ides identical to the point mutations of Batro[illegible]ons.

So far, there have been many studies based on polymorphisms of target gene sequences on mitochondrial DNA to successfully study fruit flies. In this study, fruit fly species on guava and bitter gourd fruit were successfully identified based on comparative analysis of COII target gene sequence polymorphisms on mitochondrial DNA with the same target sequences of the species that have been published. The results of this study can be applied to accurately diagnose and effectively quarantine fruit flies in our country. For

successful application, fruit fly samples were collected from the host. The COII target gene fragment of the samples was cloned by PCR with primer pairs mtD13 and mtD20

The sequence of the target gene fragment from the PCR product was compared with the same target sequence of the published fruit fly species.

Confident genetic variation or high similarity in the DNA sequence of the target gene between species or samples is the basis for accurate identification of fruit fly combinations, varieties or species.

CONCLUSION

Based on the target gene sequence polymorphism (488 bp) analysis on mitochondrial COII, 15 fruit fly samples were identified as belonging to two subgenera *Bactrocera* and *Dacus*. 12 out of 15 samples belonged to *B. dorsalis* complex. 1 sample belonged to *B. tau* species and 2 samples belonged to *B. cucurbitae* species. The technique of analyzing COII gene sequence polymorphism on mitochondrial DNA can be applied to identify other fruit fly species in our country.

Last but not least, we mention some recommendations for trapping harmful isnects such as fruit flies. Verghese et al (2002) mentioned in India, The use of the parapheromone 'methyl eugenol' in traps is quite popular in India. Of the different traps evaluated, namely IIHR bottle trap, Steiner trap, McPhail trap, delta trap, Jackson sticky trap and open pan trap, the IIHR bottle trap attracted the most flies. Trapping has been found to be useful both for monitoring and management. Using extracts of Ocimum sanctum in traps is also in vogue, but is less efficient than methyl eugenol. Pre-harvest sprays using either dimethoate 0.06%, carbaryl 0.2% or deltamethrin 0.0028% are also recommended. At the Indian Institute of Horticultural Research an IPM package for the management of B. dorsalis on mango is recommended, which consists of orchard sanitation together with inter-tree ploughing and raking plus three applications of insecticidal sprays of the above-mentioned chemicals on the fruits. And Bui Thi Suu, Dinh Tran Ngoc Huy, Nguyen Thi Hoa (2021) stated we can use alcohol traps for harmful insects in some regions of Vietnam.

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Index: Nucleotide composition (%) of COII regions of 15 fruit fly samples
***Bactrocera* và *Dacus* in Mekong delta**

Adenine	32.21	30.52	31.58	31.36	32.38	31.92	31.84	32.83	33.33	32.68	31.83	32.64
Thymine	34.14	35.89	36.12	35.23	35.24	35.19	34.76	33.67	34.09	35.04	36.53	34.15
Cytosine	19.65	19.77	18.69	19.35	18.25	17.69	18.64	19.63	18.64	17.91	17.89	18.68
Guanine	14.01	13.82	13.61	14.05	14.13	15.19	14.76	13.87	13.94	14.37	13.75	14.53

Note: Samples 3, 7, 8 are collected on bitter gourd ; samples 22,24, 27, 29, 30, 31, 33 ,35, 36, 37, 38, 39 are collected on guava.

Results of 15 sequences of 15 fruit fly samples collected in the Mekong Delta

Sample **8**

GCCTCTGAGCAGCCCTTGGCCTTCAAGATAGTGCCTCTCCTCTTATAGAACAACTTACATTTTTTCATGATCATGCTCTTA
TAATTTTAGTAATAATTACAACTCTTGTAGGTTATTTAATATTTATATTATTCTTCAATACTTACACAAATCGAAACCTCCT
TCACGGTCAAACCATTGAAATAATTTGAACTATTCTTCCAGCAATTGTTCTATTATTTATTGCTTTCCCCTCTCTACGTCTA
CTTTATTTATTAGATGAAATTAATGAACCTTCAGTAACTTTAAAGGCTATTGGACACCAATGATACTGAAGTTACGAATA
TTCAGATTTCATAAATGTAGGGTGTGATTCATATATAATTCCTACTAATGAATTAGCTACAGACGGATTTCGTTTATTAG
ATGTAGATAACCGAGTGGTTTTACCTATAAATTCACAAATTCGAATTTTAGTAACAGCAGCAGATGTAATCCACTCTTGA
ACAATTCCATCATTAGGAGTAAAGGTTGATGGAACCCCTGGTCGATTAAACCAAACTAATTTTTTA

Sample 36

TAGACACTAATGACAACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTAGGAACAACTTACCTTCTTTCAT
GATCACGCTTTAATAATTGTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTA
ACCGAAATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCC
CCTCCCTTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATT
GAAGTTATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGG
ATTCCGACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACAGCTGCAGATG
TAATTCACTCATGAACAGTACCAGCCTTAGGTGTAAAGGTAGACGGA

Sample 7

TCTTGGGCTGCCAAAGGCCTTCAGATAGTGCCTCTCCTCTTATAGAACAACTTACATTTTTTCATGATCATGCTCTTATAA
TTTTAGTAATAATTACAACTCTTGTAGGTTATTTAATATTTATATTGTTCTTCAATACTTACACAAACCGAAACCTCCTTCA
CGGTCAAACCGTTGAAATAATTTGAACTATTCTTCCAGCAATTGTTCTGCTATTTATTGCTTTCCCCTCTCTACGCCTACTT
TACTTATTGGATGAAATTAATGAACCTTCAGTAACTTTAAAGGCTATCGGACACCAATGATACTGAAGTTACGAATATTC
AGATTTCATAAATGTAGAATTTGACTCATATATAATCCCTACTAATGAATTAGCTACAGATCGATTTCGTTTATTAGACGT
AGATAACCGAGTAGTTTTACCTATAAATTCACAAATTCGAATTTTAGTGACAGCCGCAGATGTAATCCACTCTTGAACAA
TCCTTTTTTTTAGGGGTAAAAGTTGATGGAACTCC

sample 35

CTTTTATTAGAAACTAATGACAACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATGGAACAACTTACCT
TCTTTCATGATCACGCTTTAATAATTTTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTC
ATATACTAACCGAAATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTAT
TGCTTTCCCCTCCCTTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCA
ATGATATTGAAGTTATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAA
CAGACGGATTCCGACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACGGCT
GCAGATGTAATTCACTCATGAACAGTTTTTTTTCTTAGGTGTAAAGG

sample 37

CTAATGACAACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATGGAACAACTTACCTTCTTTCATGATCA
CGCTTTAATAATTTTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAACCGA
AATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCCTCCC
TTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGAAGT
TATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATTCCG
ACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACAGCTGCAGATGTAATTC

ACTCATGAATAGTACCAGCCTTAGGTGTAAAGGTAGACGGAACCCCTGGTCGATTAAACCAAACTAATTTCCTAATAAA
CCGACCTGGATTATTTTACGGTCAATGTTCAGAAATTTGTGGAGCTAATCACAGATTT

Sample 31

AAACTAATGACAACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATAGAACAACTTACCTTCTTTCATGA
TCACGCTTTAATAATTTTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAAC
CGAAATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCC
TCCCTTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTG
AAGCTATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGA
TTCCGACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACAGCTGCAGATGT
AATTCACTCATGAACAGTACCAGCCTTAGGTAGTAAAGAGTAGACGAGA

Sample 33

GCAATGATAGAGCCTCTCCTCTTTTTGGAACAACTTACCTTCTTTCATGATCACGCTTTAATAATTTTAGTAATAATTACA
ACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAACCGAAATCTTTTACATGGTCAAACTATTGAAA
TAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCCTCCCTTCGATTACTATATTTATTAGATGAAAA
TTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGAAGTTATGAATATTCAGACTTTATAAACGTG
GAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATTCCGACTTCTAGATGTTGACAACCGCGTAG
TTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGGCAGATGTAATTCACTCATGAACAGTACCAGCCTTAGG
TGGTAAGAGTAGAAGAAACCCTGA

Sample 24

AACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATAGAACAACTTACCTTCTTTCATGATCACGCTTTAAT
AATTTTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAACCGAAATCTTTTA
CATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCATCCCTTCGATTA
CTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGAAGTTATGAATA
TTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATTCCGACTTCTAG
ATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACAGCTGCAGATGTAATTCACTCATGA
ACAGTTTTTATCCTTAGGTGTAAAGGTAGACGGAACCCCTGGTCGATTAAACCAAACTAATTTCCTAATAAACCGACCT
GGATTATTTTACGGTCAATGTTCAGAAATTTGTGGAGCTAATCACAGATTTATACCTATTGTAATTG

Sample 39

AACTAATGACAACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATGGAACAACTTACCTTCTTTCATGAT
CACGCTTTAATAATTTTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAACC
GAAATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCCT
CCCTTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGA
AGTTATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATT
CCGACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGCAGATGTAA
TTCACTCATGAACAGTACCAGCCTTAGGTGTAAAGGTAGACGGAACCCCTGGTCGATTAAACCAAACTAATTTCCTAAT
AAACCGACC

Sample 38.

TGCATTAATGCACAACAACAAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATGGAACACTTACCTTCTTTCAT
GATCACGCTTTAATAATTTTAGTAATAATTACAACAATAATAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTA
ACCGAAATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCC
CCTCCCTTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATT
GAAGTTATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGG
ATTCCGACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGCAGATG
TAATTCACTCATTAATAGTACCAGCCTTAGGTGTAAAGGTAGACGGAACCCCTGGTCGATTAAACCAA

Sample 22

CTTGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATGGAAAACTTACCTTCTTTCATGATCACGCTTTAATAAT
TTTAGCAATAATTGCAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATACACTAACCGAAATCTTTTACAT
GGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCCTCCCTTCGATTACTAT
ATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGAAGTTATGAATATTCA
GACTTTATAAACGTGGAAGTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATTCCGCCTTCTAGATGT
TGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGCAGATGTAATTCACTCATGAACAG
TACCAG

Sample29

GCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATGGAACAACTAAGGTTCTTTCATGATCACGCTTTAATAATTTTAGTA
ATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAACCGAAATCTTTTACATGGTCAAA
CTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCCTCCCTTCGATTACTATATTTATT
AGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGAAGTTATGAATATTCAGACTTT
ATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATTCCGACTTCTAGATGTTGACA
ACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGCAGATGTAATTCACTCATGAACAGTACCA
TCCTTAGGTGTAAAGGTAGACGGAACCCCTGG

Sample 29

AACATGCAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTAGGAAAACTGGAACTTCTTTCATGATCACGCTTTAA
TAATTTTAGCAATAAGTACAGGTTATAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAACCGAAATCTTT
TACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCCTCCCTTCGATT
ACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTGAAGTTATGAAT
ATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGATTCCGACTTCTA
GATGTTGACAACCGCGTAGTTTTTTTCTATAAATTCACAAATTCGAATTTTAGTAACGGCTGCAGATGTAATTCACTCAT
GAACAGTACCATTTCTTAGGTGTAAAGGTAGACGGA

sample 30

AACTAATGCACAACATGAGCTGCCCTTGGCCTTCAAGATAGAGCCTCTCCTCTTATAGAACAACTTACCTTCTTTCATGA
TCACGCTTTAATAATTTTAGTAATAATTACAACATTAGTAGGTTATTTAATATTTATATTATTCTTTAATTCATATACTAAC
CGAAATCTTTTACATGGTCAAACTATTGAAATAATTTGAACGATTCTTCCAGCAATTGTACTACTATTTATTGCTTTCCCC
TCCCTTCGATTACTATATTTATTAGATGAAATTAATGAACCCTCGGTTACATTAAAGGCTATTGGACACCAATGATATTG
AAGCTATGAATATTCAGACTTTATAAACGTGGAATTTGATTCATATATAGTTCCAACTAATGAATTAGCAACAGACGGA
TTCCGACTTCTAGATGTTGACAACCGCGTAGTTCTTCCTATAAATTCACAAATTCGAATTTTAGTAACAGCTGCAGATGT
AATTCACTCATGAACAGTACCAGCCTTAGGTGTAAAGGTAGACGGAACCCCTGGTCGATTAAACCAAACTAATTTCCTA
ATAAACCGACCTGGATTATTTTACGGTCAA

sample 3

AACTAATGACAACATGAGCTGCCCTATGGGCCTTCAAGATAGTGCCTCTCCTCTTATAGAACAACTTACATTTTTTCATG
ATCATGCTCTTATAATTTTAGTAATAATTACAACTCTTGTAGGTTATTTAATATTTATATTGTTCTTCAATACTTACACAAA
CCGAAACCTCCTTCACGGTCAAACTATTGAAATAATTTGAACTATTCTTCCAGCAATTGTTCTGCTATTTATAGCTTTCCC
CTCTCTACGCCTACTTTACTTATTGGATGAAATTAATGAACCTTCAGTAACTTTAAAGGCTATCGGACACCAATGATACT
GAAGTTACGAATATTCAGATTTCATAAATGTAGAATTTGACTCATATATAATCCCTACTAATGAATTAGCTACAGATGGA
TTTCGTTTATTAGACGTAGATAACCGAGTAGTTTTACCTATAAATTCACAAATTCGAATTTTAGTAACAGCCGCAGATGT
AATCCACTCTTGAACAATCCCATCGTTAGGAGTAAAAGTTGATGGAACTCCAGGTCGATTAAACCAAACTAATTTTTTAA
TAAACCGCCCAGGTTTATTCTATGGTCAATGTTCTGAAATTTGCGGAGCTAATCACAG