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The Genetic Relationship of *Piper nigrum* L. of Central Highlands and South Vietnam Assessed by *rbcL* gene

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Abstract: ***The genetic relationships of Vietnamese black pepper varieties in the Central Highlands and Southern were evaluated by comparing the rbcL gene sequences of the collected black pepper varieties and the reference sequences on the base source NCBI data. Analysis results of nucleotide gene sequence variable rbcL of black pepper varieties showed Vietnamese varieties have much variable in nucleotide sequence compared to Asian varieties. The important characteristic variable areas of the Vietnam II black pepper group occurred at position 59292 (except for varieties SDL, VLDN5 and VLDN6), positions 59282 and 59295, variable appeared in 5 varieties (VLPQ1, VLVT1, VLGL3, VLDN4 and VLDN5), accounting for 41.67% of total samples collected. Genetic distances of black pepper varieties collected in the Central Highlands and the Southern, Vietnam (Vietnam II) (0.024 ± 0.005) with the Asian group were higher than those of black pepper group researched (Vietnam I) (0.001 ± 0.001). The phylogenetic tree demonstrated the difference between the two groups of Vietnamese black pepper. Among the varieties in the Vietnam II group, only the VLDN6 pepper variety has a close relationship with the Asian group. Analyzing the variable positions, the group Vietnam II was divided into 3 groups: Group I: VLPQ3; Group II: VLGL3, VLDN4 and VLDN5; Group III: SDL, VLVT1, VLVT2, VLDS1, VLGL1, VLPQ1 and VLPQ2. Thus, black pepper in the Central Highlands and Southern regions of Vietnam will be potential region for genetic diversity research in Asian pepper populations.***

Keywords: black pepper, DNA barcode, Genetic relations, rbcL, Vietnam

1. INTRODUCTION

Piper nigrum (*P. nigrum*) L. belongs to the Piperaceae family [1]. The piper genus has more than 1000 species but the *Pipper nigrum* L. is one of the three best known [2]. Black pepper originated in the evergreen, humid tropical forests of the Western Ghats of Southern India, domesticated thousands of years ago, and is now cultivated in many tropical countries: Malaysia, Brazil, Sri Lanka, Vietnam, China, … [3]. Black pepper can be used for many different purposes such as spices, medicine, preservative, biological control agent [4,5]. The main active ingredient contained in peppercorn is alkaloid piperine (1-peperoyl piperidine), which creates a pungent

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taste and has many pharmacological effects [6]. Piperine has antihypertensive and anti-platelet effects [7], antioxidant, antitumor [8], antipyretic, analgesic, anti-inflammatory, anti-indigestion effects. flow, antispasmodic, liver protection [9], antibacterial, antifungal, anti-thyroid, anti-apoptotic, anti-ejaculation, insecticide and larvicidal, ... therapeutic effect of many drugs, vaccines, and nutrients by increasing oral bioavailability by inhibiting various metabolic enzymes [10]. Therefore, the conservation and development of black pepper varieties are necessary, ensuring economic value and bringing about public health.

Vietnam is one of the countries with large pepper production and acreage in the world. Currently, the varieties of pepper grown in popularity in production are mainly selected from famers's own cuttings or cuttings from neighbors as planting material, the varieties often bear the name of the locality where it is grown or the locality of origin, therefore, there may be times when a variety of pepper has many different names, many different varieties / strains of pepper have the same name [11]. Therefore, the redetermination of pepper varieties is very meaningful in rebuilding native genetic resources, serving as a basis for the construction and development of pepper products bearing geographical indications. Since 2013, the project to develop DNA template (DNA Barcode) for Vietnam's economically valuable endemic crop varieties including pepper has been implemented, however, the announcements from this project limited.

Since 2003, DNA barcoding has been considered a molecular and bioinformatic tool for species differentiation, identification and detection of new species at the molecular taxonomy level [12]. The DNA barcode is a "short sequence of DNA that identifies a species" [13], by comparing the sequence of an unknown specimen with the barcode in the sequence database of known species [14]. The universal plant DNA barcode regions first proposed by PWG were *ITS*, *rbcLa* and *matK* [15]. Since then, barcode regions have been surveyed, tested, and proposed for different groups. Genetic regions common in phylogenetic studies have been investigated for candidate regions that can be used in barcodes. The common loci in the plant system are *rbcL*, the intergenic buffer *trnL-F*, *matK*, *ndhF*, and *atpB*. In one of these regions, *rbcL* is used in phylogenetic studies to distinguish at the genus and higher levels [16]. The *rbcL* is an obvious choice to evaluate as a potential standard core coding region due to its universality, ease of amplification and cohesion. Besides, the focus is on *rbcL* as it is the most characteristic plastid encoding region in the Gene Bank, with wide representation from all major groups and will therefore provide a good baseline for comparison. other plastid genes [17].

In this study, the *rbcL* gene is used to analyze the genetic relationships of pepper varieties cultivated in the Central Highlands and South Vietnam, thereby serving as the basis for genetic evaluation and genetic evaluation. genetic resources of pepper in Vietnam.

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2. MATERIAL AND METHODS

A. Plant materials

Stems of black pepper were collected from farmers in black pepper cultivation provinces in the Central Highland Southern Vietnam. (Table 1, Figure 1). The collected samples were grown in the Institute of Tropical Biology's nursery to store and preserve gene sources. Leaf samples were kept cold and transferred to the laboratory. The samples will be store at -20^{o}C until used for DNA extraction.

Table 1: Sample list and sample collection location in Vietnam

No.	Variety	Symbol	Distribution	Accession number	Coordinate
1	Vinh Linh	VLGL1	Chu Se district, Gia Lai province	MT465723	13^{o}39’36”N – 108^{o}04’40”E
2	Vinh Linh	VLGL3	Chu Se district, Gia Lai province	MT465725	13^{o}48’05”N – 108^{o}02’10”E
3	Tieu Se	SDL	Cu Kuin district, Dak Lak province	MT465709	12^{o}34’09”N – 108^{o}12’07”E
4	Vinh Linh	VLDS1	Dak Song district, Dak Nong province	MT465721	12^{o}12’14”N – 107^{o}38’44”E
5	Vinh Linh	VLDN4	Cam My district, Dong Nai province	MT465713	10^{o}42’03”N – 107^{o}19’32”E
6	Vinh Linh	VLDN5	Trang Bom district, Dong Nai province	MT465714	11^{o}03’48”N – 107^{o}05’21”E
7	Vinh Linh	VLDN6	Trang Bom district, Dong Nai province	MT465715	11^{o}03’37”N – 107^{o}05’35”E
8	Vinh Linh	VLVT1	Xuyen Moc district, Ba Ria – Vung Tau province	MT465731	10^{o}37’46”N – 107^{o}26’38”E
9	Vinh Linh	VLVT2	Xuyen Moc district, Ba Ria – Vung Tau province	MT465732	10^{o}33’35”N – 107^{o}25’34”E
10	Vinh Linh	VLPQ1	Phu Quoc district, Kien Giang province	MT465727	10^{o}17’37”N – 103^{o}59’01”E
11	Vinh Linh	VLPQ2	Phu Quoc district, Kien Giang province	MT465728	10^{o}18’22”N – 103^{o}54’09”E
12	Vinh Linh	VLPQ3	Phu Quoc district, Kien Giang province	MT465729	10^{o}07’40”N – 103^{o}59’44”E

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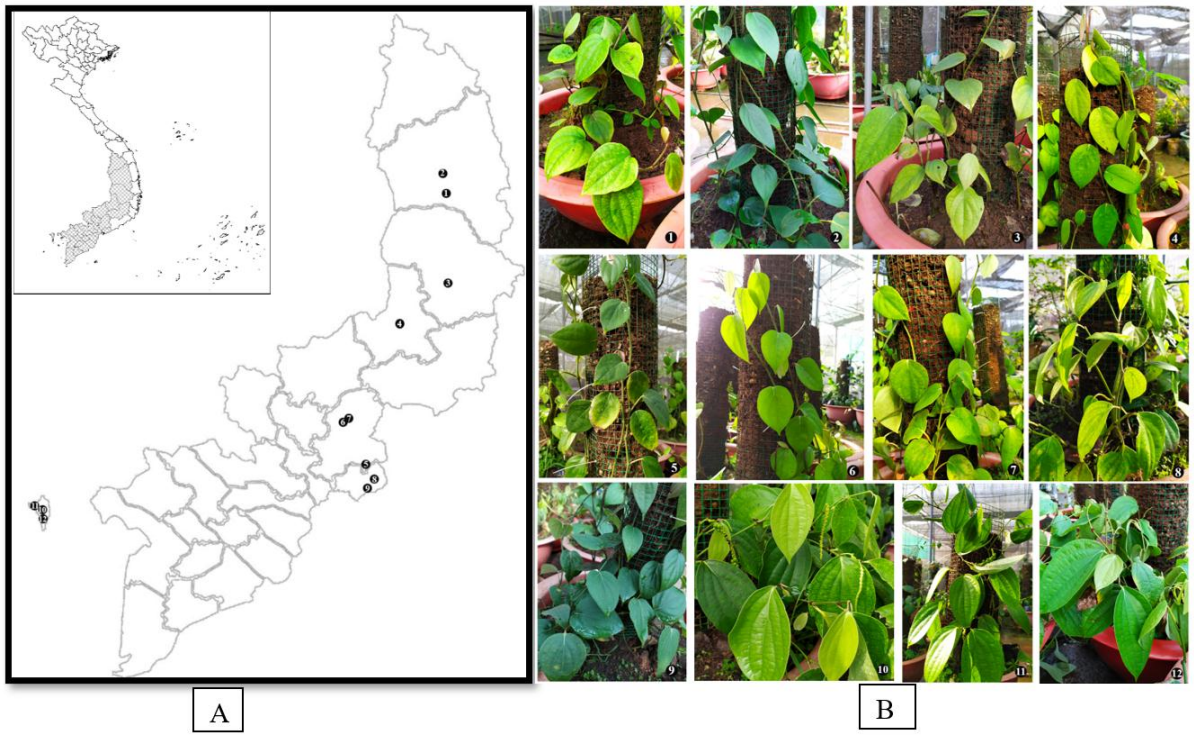

Figure 1. **(A)** Sampling location and **(B)** Conservation-grown pepper varieties at the Institute for Tropical Biology (1: VLGL1; 2: VLGL3; 3: SDL; 4: VLDS1; 5: VLDN4; 6: VLDN5; 7: VLPQ6; 8: VLVT1; 9: VLVT2; 10: VLPQ1; 11: VLPQ2; 12: VLPQ3)

B. DNA extraction

Samples of black pepper leaves were extracted from DNA according to the molecular biology KIT to standardize the process of DNA extraction. In this study, the Plant / Fungi DNA Isolation Kit (Norgen Biotek, Canada) was used to extract DNA extraction from collected pepper samples.

C. PCR conditions

DNA of the black pepper leaf samples was amplified by primers of the *rbcL* gene. In this study, the iProofTM High-Fidelity PCR Master Mix Kit (Bio-Rad, America) was used to analyze the genetic of the black pepper samples collection.

Table 2: Primer pair information of the *rbcL* gene

Gene	5’ to 3’ primer pair sequences		T_a (^{0}C)	Size (bp)	Ref.
rbcL	Forward	GTCACCACAAACAGAGACTAAAGC	55		CBOL Plant

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	Reverse	GTAAAATCAAGTCCACCRCG			Working Group, 2009

PCR cycle: 1 cycle of DNA pre-denaturation at 98°C for 1 min, 35 cycles of denaturation at 98°C for 10 s, annealing at 59°C for 30 s, extension at 72°C for 45 s and a final extension at 72 °C for 5 min. PCR products were detected by electrophoresis on 1.2% agarose gel for 30 min, stained by ethidium bromide, and photographed on a gel doc machine.

D. Sequencing and Data Analysis

The PCR product is sequenced by a sequencing machine. The *rbcL* sequence of 12 Vietnamese pepper samples was assessed to be similar to the available samples of some pepper samples from Southeast Asia (Thailand, Malaysia), South Asia (India) and East Asia (China) on NCBI database using BLAST tool.

Using software MEGA 6.0 (The Molecular Evolution Genetics Analysis) to calculate genetic distance based on Tamura - Nei model [18] and build phylogenetic tree with algorithm Contrust / Test Test Neighbor - Joining Tree [19] from DNA sequences with coefficient bootstrap 1000.

3. RESULTS AND DISCUSSION

A. The sequences variation in rbcL gene of collected pepper cultivars.

Product amplifying *rbcL* gene region of 12 Vietnamese pepper varieties with size 597 bp. The results showed that nucleotide sequences of this gene region were analyzed with 3 gene sequences of pepper *rbcL* in Vietnam that were previously studied (Vietnam I) and some pepper gene sequences in Southeast Asia (Thailand, Malaysia), South Asia (India) and East Asia (China). The results of evaluating the similarity of these sequence regions based on NCBI database are about 91.74 - 99.81%. After aligning and removing the disturbed nucleotide sequence regions, the sequence region from position 58905 to 59254 was examined to assess the variations of the Vietnamese pepper group and the Asian pepper group. The results showed that there were 41 polymorphic sites (Table 3) accounting for 10.41% of the total DNA sequence analyzed (394 bp). A variable region was found between 59255 and 59300 sites with a replacement rate of 31.11%, 3 times that of the general replacement rate (10.41%).

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Table 3: The variable positions of *rbcL* gene in Vietnamese and Asian pepper

Accession number	Nucleotide positions
	5555555555 5555555555 5555555555 5555555555 5 9999999999 9999999999 9999999999 9999999999 9 2222222222 2222222222 2222222222 2222222222 2 1222222334 5555566666 6777778888 8888899999 9 6013478269 3567802356 8136780123 5678902345 9
MN711721.1_China	TCTCAGCAGA TTACGATCTC TTACCAAACT TCCAAGCCCA C
GQ436391.1_China	....
EF450315.1_China	....
KR073288.1_Vietnam	....
KY614154.2_Vietnam	.... .T........ .
KR073287.1_Vietnam	....
LC461759.1_Thailand	....
KM055150.1_Thailand	....
MH069821.1_Malaysia	....
KF278654.1_India	....
KF278653.1_India	....
KF278652.1_India	....
KF278651.1_India	....
MT465709_SDL	....C..... ....C.. ..A..T.... .
MT465715_VLDN6	....
MT465721_VLDS1	....AG... .
MT465723_VLGL1	....AG... .
MT465728_VLPQ2	....AG... .
MT465727_VLPQ1	....T....T. ......G..C .
MT465731_VLVT1	.... C....C..T. ......G..C .
MT465725_VLGL3	...TC....G .CGAA..T.T CCTTA..TT. .TT...G..C .
MT465732_VLVT2	....C..... ....TTG... .
MT465714_VLDN5	A..TT...A. AA.AA..A.. .GTTA..TT. ...GG....C .
MT465713_VLDN4	.......C.. .C.AATCTC. C.TTA..TT. .TTTT.GGGC .
MT465729_VLPQ3	.GCTTTA.AG A..GA.CT.T CCTG..C..C C.T..AG.A. A

rbcL is the most characterized plastid coding region in GenBank providing a good base line for comparison with other plastid genes [17]. In the previous study, the *rbcL* sequence showed a powerful tool to identify the Piper species [20] or detect chili adulteration in traded black pepper powder [21]. The present study showed that the collected Vietnamese pepper varieties have many different positions compared to the Vietnamese pepper group that has been studied previously and the pepper groups of Asia (Table 3). The important characteristic change areas of the pepper group Vietnam II occurred at position 59292 (except for varieties SDL, VLDN5 and VLDN6), accounting for 75% of the total collected pepper samples. At position 59282 and 59295, changes

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occurred in 5 pepper varieties (VLPQ1, VLVT1, VLGL3, VLDN4 and VLDN5), accounting for 41.67% of the total samples collected.

B. The phylogenetic relationship to rbcL gene of collected pepper cultivars.

The genetic distances between groups calculating based on the Tamura-Nei model method were shown in Table 4. Results of genetic distance analysis between pepper cultivars in each group showed that all haplotypes of pepper varieties in group Vietnam I was 0.00 ± 0.000 and in Vietnam II group was 0.037 ± 0.006. This indicates that the newly collected Vietnamese pepper varieties (Vietnam II) are genetically different from those of Vietnam pepper varieties that have been studied previously (Vietnam I). The genetic relationships of Vietnamese pepper varieties are also considered with some pepper varieties in Southeast Asia (Thailand, Malaysia), South Asia (India) and East Asia (China).

The genetic distance values showed the molecular divergence between and within species. Chaveerach *et al.* (2016) indicated that genetic distance values had a significant variance in sequences between species and a comparatively small variance within species [21]. The analysis results in Table 3 show that the genetic distance of pepper group Vietnam I and pepper group Vietnam II was 0.024 ± 0.005. Meanwhile, the genetic distance of the East Asia, Southeast Asia, South Asia, and Vietnam I pepper groups (0.001 ± 0.001) was lower than the genetic distance of these groups and Vietnam II group (0.024 ± 0.004). This shows that the Vietnam I pepper group has a closer relationship with the Asian pepper groups than the Vietnam II pepper group. This result also shows that there are genetic differences between the two groups of Vietnamese pepper.

Table 4: Matrix of genetic distance Tamura - Nei between Vietnamese pepper and Asian pepper. The lower triangle matrix value is the genetic mean, the upper triangle matrix value is the standard error.

	East Asia	Vietnam I	Southeast Asia	South Asia	Vietnam II
East Asia		0.001	0.000	0.000	0.004
Vietnam I	0.001		0.001	0.001	0.005
Southeast Asia	0.000	0.001		0.000	0.004
South Asia	0.000	0.001	0.000		0.004
Vietnam II	0.024	0.024	0.024	0.024	

The phylogenetic tree was built on the statistical method of Neighbor - joining (Contrust / Test Neighbor - Joining Tree) from *rbcL* DNA sequences of Vietnamese and Asian pepper varieties with bootstrap coefficient of 1000 (Figure 2). The results showed that there was a clear difference between the two Vietnamese pepper groups. The

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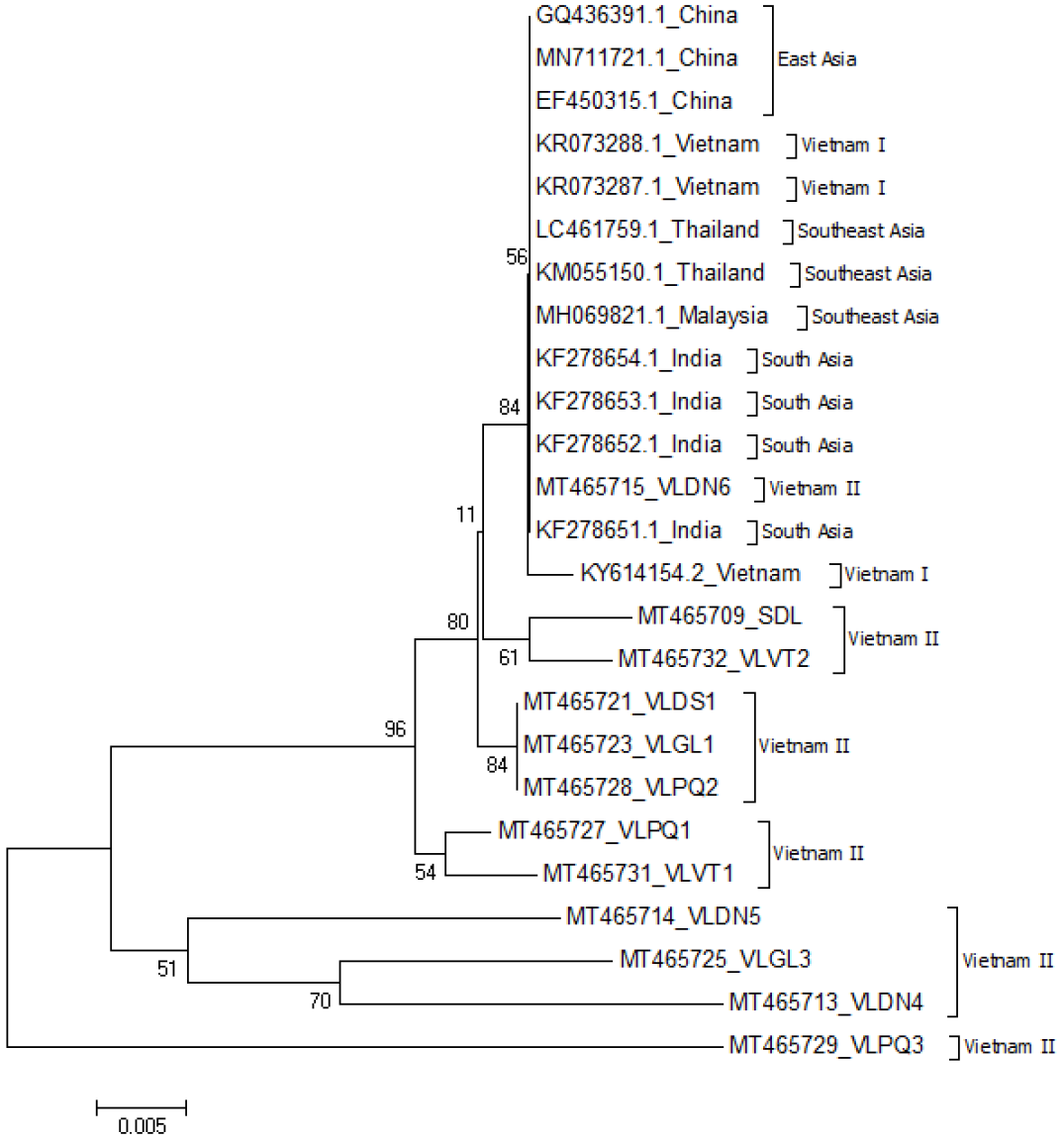
Vietnam I pepper group has a closer relationship with the Asian pepper group. However, in pepper group Vietnam II, only the VLDN6 variety is in the same branch with pepper group I and Asian pepper groups. Similarity of pepper variety VLDN6 with these pepper groups was 99.81%, the highest compared to other pepper varieties Vietnam II. Vietnam II pepper group was divided into 3 groups: Group I: pepper variety VLPQ3 completely separated from other varieties; Group II: includes varieties VLDN5, VLGL3 and VLDN4 with bootstrap coefficient 51%; Group III: includes the remaining varieties: SDL, VLVT2, DS1, VLGL1, VLPQ2, VLPQ1 and VLVT1 with a bootstrap coefficient of 96%. These results suggested that Vietnamese pepper varieties have many variations in nucleotide sequence depend on locals. The evaluation on yield and pepper quality should



be carried out further to select Vietnamese pepper with high economic.

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Figure 2: The phylogenetic tree constructed from *rbcL* sequence of Vietnam and Asian pepper varieties by the Neighbor - joining analysis method.

4. CONCLUSION

Vietnamese pepper varieties have much variation in nucleotide sequence compared to Asian pepper varieties. The important characteristic variable regions of the pepper group Vietnam II occurred at position 59292 (except for varieties SDL, VLDN5 and VLDN6), accounting for 75% of the total collected pepper samples. At position 59282 and 59295, variation occurred in 5 pepper varieties (VLPQ1, VLVT1, VLGL3, VLDN4 and VLDN5), accounting for 41.67% of the total samples collected. The genetic distances of pepper varieties collected in the Central Highlands and the South, Vietnam (Vietnam II) (0.024 ± 0.005) with the Asian group were higher than those of the pepper group studied previously (Vietnam I) (0.001 ± 0.001). The phylogenetic tree demonstrated the difference between the two groups of pepper in Vietnam. Among the varieties in the Vietnam II group, only the VLDN6 pepper variety has a close relationship with the Asian pepper group. Analyzing the variable positions, the pepper group Vietnam II is divided into 3 groups: Group I: VLPQ3; Group II: VLGL3, VLDN4 and VLDN5; Group III: SDL, VLVT1, VLVT2, VLDS1, VLGL1, VLPQ1 and VLPQ2.

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