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Complete chloroplast genome of Dongxiang wild rice and its application in phylogenetic analysis

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Summary Complete chloroplast genome sequence is very useful for studying the evolution of species. To get chloroplast genome sequences, purification of the chloroplast or PCR amplification prior to sequencing is commonly involved in conventional approaches. Advances in DNA sequencing technology provide new opportunities to obtain chloroplast genome sequence from the whole-genome high-throughput sequencing data without purification of the chloroplast. In this study, we finished the complete chloroplast genome sequence of Dongxiang wild rice based on high-throughput sequencing data from their fresh green leaves. The chloroplast genome was 134 537 bp in size, and had a typical quadripartite structure with the large single copy (LSC, 80 585 bp) and small single copy (SSC, 12 346 bp) regions separated by two copies of an inverted repeat (IR, 20 803 bp each) region. One hundred and fifty-two chloroplast genes were successfully annotated. A phylogenetic tree was constructed based on the chloroplast genomes of Dongxiang wild rice, *Indica*, *Japonica* and 10 other genera of grasses using the neighbor-joining method. The result showed that Dongxiang wild rice had a closer relationship with *Bambusa oldhamii* and Panicoideae. Furthermore, the SNPs of 22 rice accessions were identified using the chloroplast genome of Dongxiang wild rice as a reference sequence and a phylogenetic tree was constructed based on these SNPs. The result illustrated that *Indica* had a closer relationship with wild rice-I, while *Japonica* was closer to wild rice-III, suggesting that *Indica* and *Japonica* were domesticated independently from different wild rice populations.

Key words *Oryza rufipogon*; chloroplast genome; high-throughput sequencing; rice domestication; Dongxiang wild rice (China)
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林张翔, 王营营, 付菲, 叶楚玉, 樊龙江*(浙江大学农业与生物技术学院农学系/浙江省作物种质资源重点实验室,杭州 310058)

摘要 为了优化叶绿体基因组DNA序列的获取和拼接方法,以东乡野生稻(*Oryza rufipogon*)嫩绿叶为材料,不需分离叶绿体DNA,利用高通量测序获得的全基因组短序列及叶绿体基因组高度保守的特性,与参考序列进行比对,从而组装拼接出叶绿体DNA序列,并同时利用生物信息学手段和聚合酶链反应扩增进行补洞.最终获得东

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乡野生稻完整叶绿体基因组序列,大小为134 537 bp,大、小单拷贝区和反向互补重复区大小分别为80 585 bp,12 346 bp,20 803 bp,共注释叶绿体基因152个.基于获取的东乡野生稻及其他稻属物种叶绿体基因组序列进行系统进化分析,研究结果支持已有的关于栽培籼粳稻起源的结论,即栽培籼粳稻分别与野生稻聚类到2个亚群中,其中粳稻与中国普通野生稻聚在1个亚群中,表明粳稻驯化起源于中国,而籼稻起源于另外一次独立驯化过程.

关键词 野生稻; 叶绿体基因组; 高通量测序; 水稻驯化; 东乡野生稻(中国)

Chloroplast is an important organelle for photosynthesis of green plants with a semi-autonomous genetic system. As compared to the nuclear genome, the chloroplast genome has special features, *e. g.*, haploid, maternal inheritance, and high conservation in gene content and genome structure. Chloroplast genomes of higher plants typically range in size from 120 to 180 kilobase (kb) pairs, with conserved quadripartite structure which is composed of two copies of a large inverted repeat (IR) region and two sections of unique DNA, *i. e.*, large single copy (LSC) and small single copy (SSC) regions[1].

The rapid development of high-throughput sequencing technology promotes the study of plant chloroplast genomes. Since the complete chloroplast genomes of *Marchantia polymorpha*[2] and *Nicotiana tabacum*[3] were published in 1986, the number of sequenced chloroplast genomes has increased greatly. As to August 9, 2013, Organelle Genome Resources of the National Center for Biotechnology Information (NCBI) contained a total of 285 chloroplast genomes from different plants.

For traditional chloroplast genome sequencing method, it is necessary to isolate and purify the chloroplast DNA before sequencing. Due to low concentration of chloroplast DNA, it is difficult to separate it from nuclear genome DNA. Therefore, chloroplast DNA isolation-based method is tedious and time-consuming. In 2009, the chloroplast genomes of *Dendrocalamus latiflorus* and *Bambusa oldhamii*[4] were obtained by a simple PCR-based method without isolating and purifying chloroplast DNA, and the chloroplast genome of *Oncidium*[5] was obtained later by the same method. Advances in DNA sequencing technology provide new opportunities to obtain chloroplast genome sequence from the whole-genome high-throughput sequencing data without purification of the chloroplast, *e. g.*, the chloroplast genome of *Phoenix dactylifera* obtained directly from Roche GS FLX whole-genome sequencing data[6]. In this study, we finished the complete chloroplast genome sequence of Dongxiang wild rice (*Oryza rufipogon*) based on high-throughput sequencing data from their fresh green leaves[7], and performed phylogenetic analyses.

1 Materials and methods

1.1 Materials and data collection

Dongxiang wild rice (*O. rufipogon*) was provided by the China National Rice Research Institute, and Illumina high-throughput sequencing data were generated from our previous study[7]. Seven released rice chloroplast genomes were obtained from NCBI (http://www.ncbi.nlm.nih.gov/)[17-21] and other rice data (high-throughput sequencing data) were obtained from EBI (http://www.ebi.ac.uk/ena/) (Table 1). Ten Poaceae chloroplast genomes were obtained from NCBI, including *Festuca arundinacea* (NC_011713)[9], *Lolium perenne* (NC_009950)[10], *Agrostis stolonifera* (NC_008591)[11], *Triticum aestivum* (NC_002762)[12], *B. oldhamii* (NC_012927)[4], *Panicum virgatum* (NC_015990)[13], *Zea mays* (NC_001666)[14], *Coix lacryma-jobi* (NC_013273)[15], *Sorghum bicolor* (NC_008602)[11] and *Anomochloa marantoidea* (NC_014062)[16].

1.2 Genome assembly

Bowtie 2 (http://bowtie-bio.sourceforge.net/index.shtml)[22] was used to map clean reads with seven rice chloroplast genomes as references (Table 1). Assembly of mapped reads was then performed with Velvet[23]. Parameters of Velvet have a great influence on the assembly results, particularly *K*-mer and coverage value. Therefore, we set multiple parameters (*K*-mers of 13 levels and coverage of 4 levels) for debugging. The set

that has the largest N50 was finally selected (*K*-mer was 33 and coverage was 100). The gaps were closed by GapCloser (http://soap.genomics.org.cn/soapdenovo.html)[24] and PCR amplification. All the variable sites of Dongxiang wild rice were gotten using SAMTools (http://samtools.sourceforge.net/)[25]. Seven SNPs (single nucleotide polymorphisms) were finally verified by PCR amplification. The assembly process is shown in Fig.1.

Table 1　Chloroplast genome sequences of *Oryza* used in this study

Species	Groups[8]	Origin	Accession
O. rufipogon	NA[a)]	Dongxiang, China	KF562709[b)]
	NA	Vietnam	NC_017835[17]
	NA	Australia	JN005833[17]
	Or-I	India	ERX046456[8] (W0101)[c)]
	Or-I	Sri Lanka	ERX046479[8] (W0144)[c)]
	Or-II	Indonesia	ERX046720[8] (W1977)[c)]
	Or-II	Indonesia	ERX046715[8] (W1972)[c)]
	Or-III	China	ERX046828[8] (W3026)[c)]
	Or-III	China	ERX046846[8] (W3044)[c)]
O. sativa	*Indica*	China	NC_008155[18]
	Indica	China	ERX005522[8] (Guangluai-4)[c)]
	Aus	Bengal	ERX014265[8] (GP89)[c)]
	Japonica	Japan	NC_001320[19]
	Temperate *Japonica*	Japan	ERX002911[8] (Nipponbare)[c)]
	Tropical *Japonica*	America	ERX014455[8] (GP640)[c)]
	Aromatic	Pakistan	ERX046332[8] (GP295)[c)]
O. meridionalis	NA	Australia	NC_016927[17]
	NA	Australia	ERX046915[8] (W2113)[c)]
O. nivara	NA	Sri Lanka	NC_005973[20]
O. australiensis	NA	Australia	GU592209[21]
O. glaberrima	NA	Liberia	ERX046902[8] (W3104)[c)]
O. barthii	NA	Burkina Faso	ERX046903[8] (W3106)[c)]
O. longistaminata	NA	Benin	ERX046905[8] (W3102)[c)]

[a)] Not available; [b)] Generated by this study; [c)] Sample number in the reference [8].

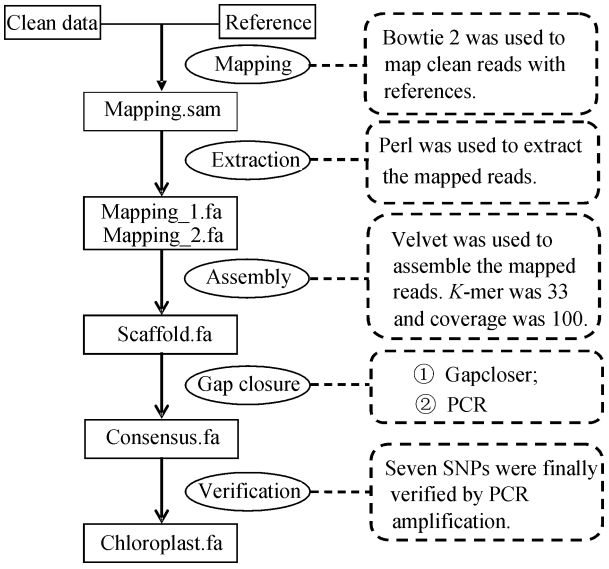


Fig.1　Process of Dongxiang wild rice chloroplast genome assembly

1.3　Genome annotation

Annotation of the Dongxiang wild rice chloroplast genome was performed using DOGMA (http://dogma.ccbb.utexas.edu/)[26]. The annotated file was used to draw gene maps using GenomeVx tool (http://wolfe.gen.tcd.ie/GenomeVx/)[27]. The complete chloroplast genome of Dongxiang wild rice was deposited into GenBank with the accession number KF562709.

1.4　Phylogenetic tree construction

The chloroplast genome sequences of Dongxiang wild rice, *Indica* (NC_008155)[18], *Japonica* (NC_001320)[19] and 10 Poaceae plants were aligned using ClustalX 2 (http://www.clustal.org). The phylogenetic tree was constructed using the neighbor-joining (NJ) method in Mega 5.0 with 1 000 bootstrap replications (http://www.megasoftware.net/)[28]. The phylogenetic tree based on SNPs was constructed using the same method.

2　Results

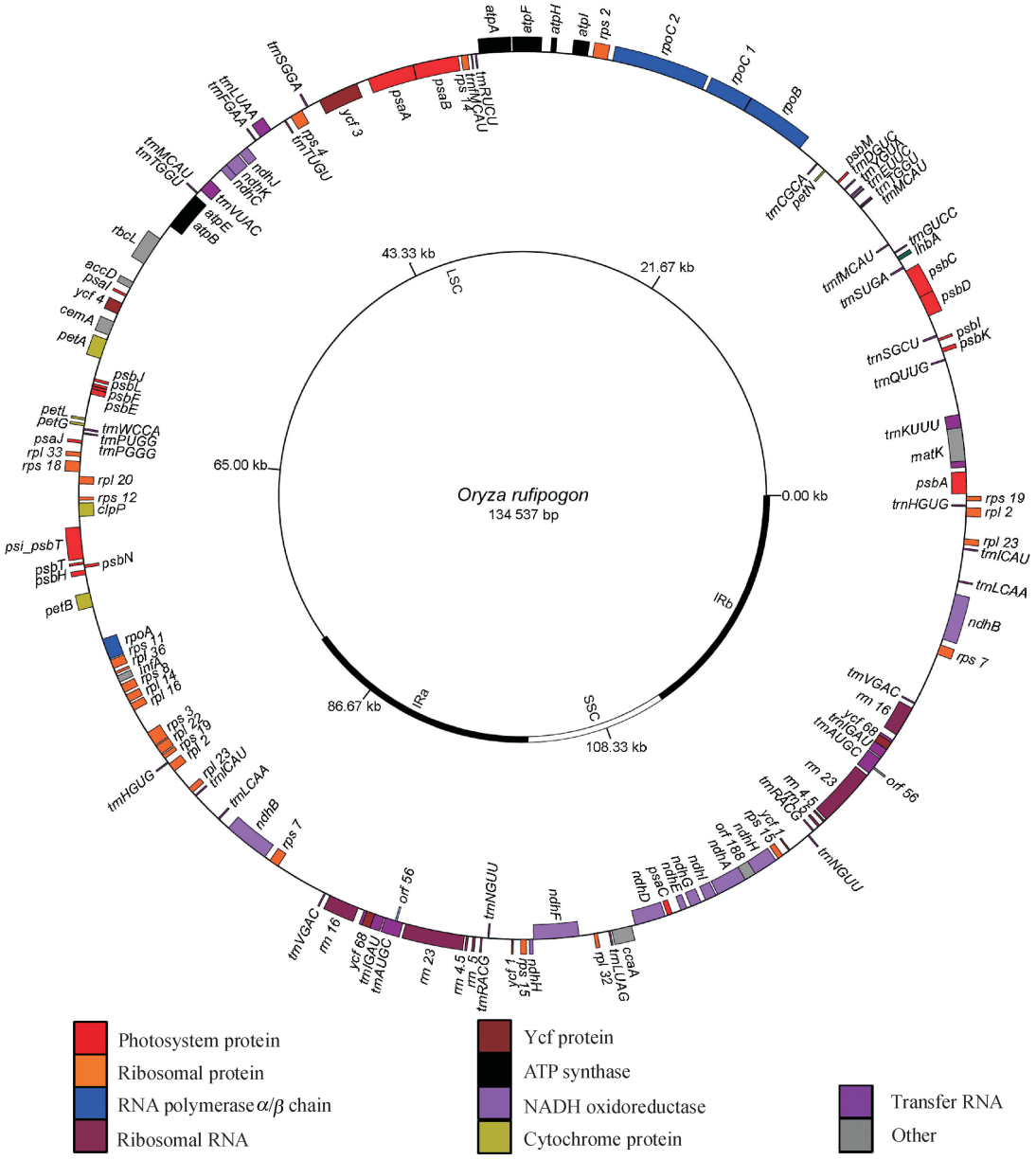
2.1　Genome assembly

Using seven chloroplast genomes of *Oryza* which are evolutionarily close to *O. rufipogon* as references, Dongxiang wild rice chloroplast-related reads were first collected from our high-throughput

sequencing data. Subsequently, *de novo* assembly of these reads was performed resulting in four scaffolds. PCR amplification was used for gap closure and validation of variations in Dongxiang wild rice. All products of PCR amplification were sequenced to confirm our assembly. Finally, we obtained the complete chloroplast genome of Dongxiang wild rice with the length of 134 537 bp.

2.2 Genome annotation

The Dongxiang wild rice chloroplast genome displayed the typical quadripartite structure, consisting of a pair of IRs (20 803 bp, 15.5% of the genome) separated by the LSC (80 585 bp, 59.9% of the genome) and SSC (12 346 bp, 9.2% of the genome) regions (Fig. 2). The chloroplast genome was AT-rich (60.99%) which is similar to other chloroplast genomes[6,29]. The chloroplast genome encoded 152 predicted genes, of which 128 were unique in the LSC/SSC regions and 24 were duplicated in the IR regions. The 152 genes included



The thick black lines in inner ring indicate the inverted repeats (IRa and IRb) regions, which separate the genome into the large single copy (LSC) and small single copy (SSC) regions. The genes on the outside of the map are transcribed in the clockwise direction and the genes on the inside of the map are transcribed in the counterclockwise direction.

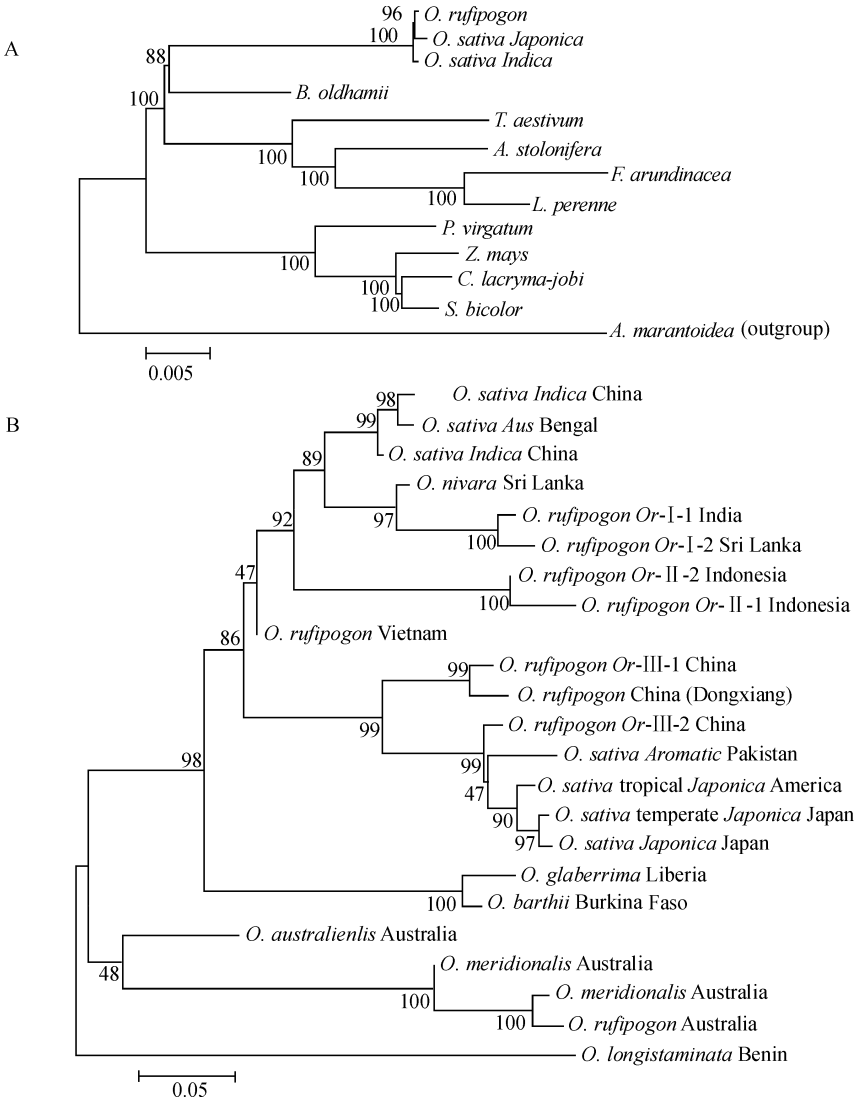
Fig. 2 Gene map of Dongxiang wild rice chloroplast genome

49 transfer RNAs, 8 ribosomal RNAs and 95 protein-coding genes in the chloroplast genome. The functional classification of chloroplast genes was determined according to CpBase. These 152 genes could be divided into four categories, including 50 photosynthesis related genes, 87 transcription and translation genes, 2 biosynthesis genes, and 13 open reading frames and other protein-coding genes.

The length of the cultivated rice chloroplast genome (NC_008155)[20] was 134 525 bp. The lengths of LSC, SSC, and IRs were 80 592 bp, 12 335 bp and 20 799 bp, respectively, covering almost the same proportion of the genome with Dongxiang wild rice. The cultivated rice chloroplast genome encoded 162 predicted genes, including 40 transfer RNAs, 8 ribosomal RNAs and 114 protein-coding genes. According to CpBase, these 162 genes included 32 photosynthesis related genes, 80 transcription and translation genes and 50 open reading frames and other protein-coding genes.

2.3 Phylogenetic analyses

A phylogenetic tree was constructed based on Dongxiang wild rice and other 10 Poaceae chloroplast genomes using NJ method. The result showed that Dongxiang wild rice had a closer relationship with *B. oldhamii* and Panicoideae (Fig.3A).



A: Phylogenetic tree of Dongxiang wild rice (*O. rufipogon*) and other 10 Poaceae chloroplast genomes using NJ method; B: Phylogenetic tree based on all SNPs of Dongxiang wild rice and other 22 *Oryza* chloroplast genomes (Table 1) using NJ method. The scales mean expected number of substitutions per site.

Fig.3 Chloroplast genome-based phylogenetic trees of Dongxiang wild rice with other members from the grass family (Poaceae)

Furthermore, we used the chloroplast genome of Dongxiang wild rice as the reference sequence, and collected the identified SNPs of 22 rice accessions to build a phylogenetic tree. The phylogenetic tree showed that *Indica* and *Japonica* clustered with different groups of *O. rufipogon*, and *Japonica* had a closer relationship with Chinese *O. rufipogon* (Fig. 3B). Therefore, our results support the hypothesis of the origin of rice domestication from Huang *et al*.[8] that *Japonica* was first domesticated from a specific population of *O. rufipogon* in China, while *Indica* was developed from another independent domestication process.

3 Discussion

Chloroplast genome structure and sequence provide important resources and information for studying the evolution of species. As to August 9, 2013, Organelle Genome Resources of the NCBI contained a total of 285 Viridiplantae chloroplast genomes. With the rapid development of high-throughput sequencing technology and the new bioinformatics software, there will be more and more plant chloroplast genomes being sequenced. Chloroplast genome is maternally inherited and highly conserved. According to the features, this study used a simple and efficient method which assembled the chloroplast genome sequence from whole genome high-throughput sequencing data of the green leaves. This method does not need to separate and purify chloroplast DNA that is a major limiting factor in traditional methods, reducing the experimental difficulty and saving time. Additionally, the required depth of the whole genome sequencing was reduced due to multiple copies of the chloroplast genome. Using this method we successfully obtained the Dongxiang wild rice chloroplast genome sequence, and performed some analyses regarding the rice domestication. Our results support the hypothesis of the origin of domesticated rice in *Nature*, 2012[8].

Although this method is simple and efficient, there are still several inevitable shortcomings. First, the appropriate reference sequence is necessary for extracting the chloroplast genome related reads from the whole-genome sequencing data. Second, the reference chloroplast genome should be evolutionarily close to the assembled genome. Moreover, some chloroplast genes transfer to the nuclear genome in higher plants, therefore, the extracted short reads may be from the nuclear genome. Although the chloroplast DNA has a high copy number and thus such reads had little impact on assembly, the possibility cannot be excluded.

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2 来稿要求及注意事项

2.1 来稿务求论点明确,材料翔实,数据准确可靠,论证合理,结论新颖,文字精炼,语句通顺,可用英文或中文写稿.其中,英文文稿需提供中文题名和摘要等;中文文稿必须包括(按顺序):题名(不超过25字),作者姓名及工作单位(单位须至系、所一级,并包括邮编),中文摘要(内容应包括研究的目的、方法、结果和讨论)及关键词(3～8个,尽量使用名词术语的全称),中图分类号,英文题名,作者姓名的汉语拼音(姓大写,名首字母大写),单位译名,邮编,国别,英文摘要(应写出论文主要试验数据和信息,内容需详细,约500个单词;用第三人称撰写,应符合英语语法)和英文关键词,正文,致谢,参考文献(参考文献务须按本刊格式译成英文和中国学术期刊光盘版的要求著录,外文专著尤其要注意注明出版地和出版者).此外,在文章首页页脚注明产生论文的基金项目名称和编号,通信作者和第一作者联系电话及E-mail地址.

2.2 来稿请登录本刊网站 http://www.journals.zju.edu.cn/agr,点击左侧的"作者投稿",按提示步骤完成投稿流程(第1次向本刊投稿的作者需先注册,然后投稿).正文部分用通栏排版,小四号字,务须加宽行距(可采用1.5倍行距),并确保校对无误;采用规范化名词术语,量名称及符号(除雷诺数 *Re* 等25个特征数和pH值外其他一律用单个字母表示)必须符合国家标准;采用我国法定计量单位(用国际通用符号).尤其要注意必须将亩换算成公顷(hm^2),ppm换算为mg/L或mg/kg等.外文字母必须分清大小写和正斜体(注意生物拉丁学名、基因名称和变量用斜体;SI词头、量单位和函数用正体;矩阵、矢量用黑斜体).

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Xiang Y J, Li X Z, Wang X D. Preliminary study on cold resistance of five different lianas. *Journal of Zhejiang University: Agriculture and Life Sciences*, 2011,37(4):421-424. (in Chinese with English abstract)

* 专著:[2]　孙祥生,易现峰.植物生理学实验技术.北京:中国农业出版社,2008:77-79,252-254.
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