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有害甲藻孢囊的分类鉴定研究进展

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摘要: 有害甲藻孢囊主要是指能产生毒素和(或)能引起有害藻华发生并对水生态系统产生各种危害效应的甲藻孢囊。我国沿海共记录了 10 属 18 种,占全球有害甲藻孢囊的 3/4。这些有害甲藻孢囊广泛分布于我国沿海,会对水产养殖业造成严重的经济损失,甚至会威胁人类的身体健康。因此,有害甲藻孢囊的多样性及分布越来越受到人们的关注。对有害甲藻孢囊的准确判断不仅对研究其多样性及分布至关重要,而且有助于水产品的安全检验和有害藻华的早期预警。对有害甲藻孢囊的分类主要存在鉴定困难、鉴定不准确等问题。本文综述了有害甲藻孢囊的危害、中国沿海有害甲藻孢囊的种类和分布,以及有害甲藻孢囊的鉴定等 3 个方面的研究进展,并提出利用孢囊及营养细胞的形态学特征、分子生物学、毒理学等多学科研究手段准确鉴定有害甲藻孢囊的建议。

关键词: 有害甲藻孢囊; 形态学特征; 分子鉴定

Research progress on identification of harmful dinoflagellate cysts: A review

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Abstract: Harmful dinoflagellate cysts refer to cysts derived from dinoflagellates that can produce toxins and (or) cause harmful dinoflagellate blooms. So far, 18 species in 10 genera have been recorded along coasts of China seas, accounting for three quarters of the total number of harmful dinoflagellate cysts globally. These harmful dinoflagellate cysts are widely distributed along Chinese coasts. Harmful dinoflagellate cysts largely threaten marine ecosystems, aquaculture industries and even human health. Consequently, the study of diversity and distributions of harmful dinoflagellate cysts has become a hotspot in marine biology and ecology. The identification of harmful dinoflagellate cysts is crucial for sea food safety inspection and prediction of harmful dinoflagellate blooms. However, accurate identification represents a big challenge, mainly owing to limited available morphological features of harmful dinoflagellate cysts. Here we review research progress on harmful dinoflagellate cysts on Chinese coasts, including their negative impacts, diversity and geographical distributions, and species identification of harmful dinofagellate cysts. In addition, we suggest that it is nececarry to ultilize multiple methods including those based on morphology and molecular biology, as well as methods based on toxicology and reproductive biology, to accurately identify harmful dinoflagellate cysts.

Key words: harmful dinoflagellate cyst; morphological characteristics; molecular identification

甲藻孢囊是指甲藻为度过不良环境而产生的一种无鞭毛且无游动能力的细胞,是甲藻生活史的一个重要阶段(王朝晖,2007)。其中,能产生毒素和(或)引起有害藻华(harmful algal bloom)的甲藻孢囊被称为有害甲藻孢囊。有害甲藻孢囊可分为含毒素和不含毒素 2 类。含毒素种类不仅在低浓度时就会引起水产品染毒甚至死亡,而且会导致有害藻华的暴发(Burkholder *et al.*, 1992);不含毒素的种类虽然不会引起水产品的毒化,但大量萌发后能引起有害藻华的发生。有害藻华的发生会对人类健康、生态系统及水产养殖业构成巨大威胁(Anderson *et al.*, 2012b)。随着沿海海域污染、外来种引入、全球变暖等影响的加剧,有害藻华发生的频率不断增大(Anderson *et al.*, 2012a; Nakanishi *et*

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al.,1996)。因分布于海底沉积物中的有害甲藻孢囊很有可能成为有害藻华发生的种源,有害甲藻孢囊的地理分布及多样性和生态特性也越来越受到关注(Kohli *et al.*,2014; Zonneveld & Dale,1994)。

甲藻孢囊形体普遍微小(20~120 μm),部分种间差异较小及同种存在形态可塑性等特点(Fuentes-Grünewald *et al.*,2009、2012; Geider & Roche,2002; Taylor & Gaines,1989),导致对其鉴定困难及鉴定不准确等问题。虽然顾海峰等(2011)、黄海燕和陆斗定(2009)已对甲藻孢囊的研究进行了综述,但均缺乏针对有害甲藻孢囊种类及分子鉴定方面的论述。本文拟综述有害甲藻孢囊的危害、中国沿海有害甲藻孢囊的种类及分布、有害甲藻孢囊的形态学和分子生物学鉴定等 3 个方面的研究进展,以期为有害甲藻孢囊的监测和控制提供参考。

1　有害甲藻孢囊的危害

笔者统计了全球范围内有害甲藻孢囊的种类,主要有 22 种(表 1)。其中,19 种含有甲藻毒素,3 种不含毒素但能引起有害藻华的发生,而既含有毒素又能引起有害藻华发生的有 13 种。这些甲藻毒素是底栖生物体内毒素积累的重要来源之一(Schwinghamer *et al.*,1994)。其被滤食性的鱼、虾、贝类滤食后,在这些海产品体内积累并导致海产品染毒,染毒的海产品通过食物链的传递最终导致人的中毒(丁德文等,2005; Yasumoto & Murata,1993)。根据食用者中毒的症状,这些甲藻(产孢囊)毒素被分为 3 类:腹泻性贝类毒素(diarrhetic shellfish poisoning,DSP)、麻痹性贝类毒素(paralytic shellfish poisoning,PSP)、神经性贝类毒素(neurotoxic shellfish poisoning,NSP)。除此之外,还有一些对人类危害尚不清楚的毒素,如扇贝毒素(yessotoxin,YTX)(Aune *et al.*,2002)。这些毒素(DSP、PSP、NSP、YTX)主要由甲藻门中的膝沟藻类产生,少数由裸甲藻类、多沟藻类产生(表 1)。有些甲藻(产孢囊)毒素(如属于 PSP 的石房蛤毒素)的毒性是眼镜蛇毒性的 80 倍,产毒甲藻的营养细胞具有毒素,其孢囊也具有毒素(Anderson *et al.*,1990; Bravo,1998),并且其毒性比营养细胞更高(Dale,1983; Oshima *et al.*,1992)。仅在 1969 年至 1994 年,我国因食用染毒的鱼、贝类而中毒的人数就有 1800 多人,其中至少 30 人死亡(周名江等,2001; Zhou *et al.*,1999)。这些毒素还能引起鱼、贝类大面积死亡,给水产养殖业造成重大经济损失(龙华等,2008; Lim,2012)。此外,有害甲藻孢囊能帮助甲藻度过不良环境,通过洋流或船舶等媒介扩散到其他海域,极易使其成为入侵物种而对当地水域的其他生物及生态环境产生重大的危害,如自从亚历山大藻孢囊入侵美国缅因州西部海域引起赤潮后,此海域几乎每年都会暴发产 PSP 毒素的赤潮(Anderson & Wall,1978)。

2　中国沿海有害甲藻孢囊的种类及分布

目前,我国发现的有害甲藻孢囊共记录了 10 属 18 种,占全球总有害甲藻孢囊种类的 3/4(表 1),其在我国沿海的分布见图 1。其中,原甲藻属 *Prorocentrum* 1 种,亚历山大藻属 *Alexandrium* 共 6 种,膝沟藻属 *Gonyaulax* 共 2 种,梨甲藻属 *Pyrodinium* 1 种,舌甲藻属 *Lingulodinium* 1 种,原角管藻属 *Protoceratium* 1 种,斯氏藻属 *Scrippsiella* 1 种,裸甲藻属 *Gymnodinium* 1 种,多沟藻属 *Polykrikos* 共 2 种,褐多沟藻属 *Pheopolykrikos* 1 种,旋沟藻属 *Cochlodinium* 1 种。广泛分布于我国沿海的有塔玛亚历山大藻 *Alexandrium tamarense*、链状亚历山大藻 *A. pacificum*、具刺膝沟藻 *Gonyaulax spinifera* 以及锥状斯氏藻 *Scrippsiella trochoidea*。

3　有害甲藻孢囊的分类鉴定

3.1　形态学鉴定

由于大多数甲藻孢囊微小,需要借助于光学或电子显微镜才能对其进行分类鉴定。传统的形态学鉴定方法主要根据孢囊的形态、大小、颜色、孢囊内含物及孢囊壁的结构和表面修饰物等特征对孢囊进行鉴定(王朝晖,2007; 王朝晖等,2011; 魏洪祥等,2011; Liu *et al.*,2014; Matsuoka & Fukuyo,2000; Mertens *et al.*,2015)。表 1 列出的有害甲藻孢囊中,有些具有易于分辨的形态学特征,如多边舌甲藻 *Lingulodinium polyedrum* 具有舌状凸起(图 2A),网状原角管藻 *Protoceratium reticulatum* 具有 T 形凸起(图 2B)(Joyce *et al.*,2005),科夫多沟藻 *Polykrikos kofoidii* 具有网纹状凸起(图 2C),无纹多沟藻 *Polykrikos schwartzii* 的外部形态与科夫多沟藻类似,但凸起的顶端相互独立而未连接在一起(图 2D)等,可根据此特有的形态学特征对这些孢囊进行相对准确的鉴定。

表 1 主要有害甲藻孢囊种类及其危害

Table 1 Major spceices of harmful dinocysts and potential damages

生物学名 Biological name	中文名 Chinese name	危害 Damage	参考文献 Reference
* *Prorocentrum lima*	利马原甲藻	引起鱼、贝类中毒(DSP) Fish and shellfish poisoning	曾玲等,2013; Bauder *et al.*,2001; Bravo *et al.*,2001; Marr *et al.*,1992; Pan *et al.*,1999
* *Alexandrium tamarense*	塔玛亚历山大藻	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	Anderson *et al.*, 1996; Gu *et al.*, 2013a; MacIntyre *et al.*,1997
* *Alexandrium pacificum*	链状亚历山大藻	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	Gu *et al.*,2013a; John *et al.*,2014a; Krock *et al.*,2007; Lilly *et al.*,2002; Sako *et al.*,1992
* *Alexandrium minutum*	微小亚历山大藻	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	Chang *et al.*,1997; Hansen *et al.*,2003; Lan *et al.*,2003; Vila *et al.*,2005
* *Alexandrium affine*	相似亚历山大藻	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	唐祥海等,2008; Lan *et al.*,2003; Nakanishi *et al.*,1996; Nguyen-Ngoc,2004
Alexandrium cohorticula	股状亚历山大藻	引起鱼、贝类中毒(PSP) Fish and shellfish poisoning	Ogata *et al.*,1990; Wisessang *et al.*,1991
* *Alexandrium fundyense*	无	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	Anderson & Wall, 1978; Gao *et al.*, 2015; Stock *et al.*,2005
Alexandrium lusitanicum	无	引起鱼、贝类中毒(PSP) Fish and shellfish poisoning	Martins *et al.*,2004
Alexandrium monilatum	无	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	Harding *et al.*,2009; Juhl,2005
* *Alexandrium ostenfeldii*	无	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	Gu,2011; Gu *et al.*,2013a; Kremp *et al.*,2009、2014; Lim *et al.*,2005; Mackenzie *et al.*,1996
Pfiesteria piscicida	无	引起鱼、贝类中毒(NSP)及赤潮 Fish and shellfish poisoning and HABs	Anderson *et al.*, 2003; Bowers *et al.*, 2000; Burkholder *et al.*,1992; Coyne *et al.*,2006
* *Pyrodinium bahamense*	巴哈马梨甲藻	引起鱼、贝类中毒(PSP)及赤潮 Fish and shellfish poisoning and HABs	邵魁双等,2011; 王朝晖和康伟,2014; Azanza,1997; Gacutan *et al.*,1985; Gu,2011
* *Gonyaulax spinifera*	具刺膝沟藻	引起鱼、贝类中毒(YTX)及赤潮 Fish and shellfish poisoning and HABs	Aune *et al.*, 2002; Gúrate-Lizúrraga *et al.*, 2014; Lan *et al.*,2003; Rhodes *et al.*,2006
* *Gonyaulax polygramma*	多纹膝沟藻	引起赤潮 HABs	林永水和周近明,1993; Morton & Villareal, 1998; Pan *et al.*,2007
* *Lingulodinium polyedrum*	多边舌甲藻	引起鱼、贝类中毒(YTX)及赤潮 Fish and shellfish poisoning and HABs	Lan *et al.*, 2003; Morales-Ramírez *et al.*, 2001; Paz *et al.*,2004
* *Protoceratium reticulatum*	网状原角管藻	引起鱼、贝类中毒(YTX)及赤潮 Fish and shellfish poisoning and HABs	Álvarez *et al.*,2011; Lan *et al.*,2003; Paz *et al.*,2004; Satake *et al.*,1998
* *Gymnodinium catenatum*	链状裸甲藻	引起鱼、贝类中毒(PSP) Fish and shellfish poisoning	Gu *et al.*, 2013c; Hallegraeff *et al.*, 1995; Lage & Costa,2013; Oshima *et al.*,1993
* *Cochlodinium polykrikoides*	多环旋沟藻	引起鱼、贝类中毒(NSP)及赤潮 Fish and shellfish poisoning and HABs	王红霞等,2014; Gúrate-Lizúrraga *et al.*,2004; Kim *et al.*,2002; Tomas & Smayda,2008
* *Polykrikos kofoidii*	科夫多沟藻	引起赤潮 HABs	王朝晖,2007; 王朝晖和齐雨藻,2003
* *Polykrikos schwartzii*	无纹多沟藻	引起赤潮 HABs	王朝晖,2007; 王朝晖和齐雨藻,2003
* *Scrippsiella trochoidea*	锥状斯氏藻	引起赤潮 HABs	常虹等,2014; 肖咏之等,2001
* *Pheopolykrikos hartmannii*	哈曼褐多沟藻	引起鱼、贝类中毒(未知)及赤潮 Fish and shellfish poisoning and HABs	黄长江和董巧香,2000; Tang *et al.*,2013

* 为中国沿海发现的有害甲藻孢囊种。

* indicate dinoflagellate cyst found in China.

但对于有些有害甲藻孢囊,如亚历山大藻属中的塔玛亚历山大藻复合体(图 2E)、微小亚历山大藻 *A. minutum* 与相似亚历山大藻 *A. affine*(图 2F)等各个种的形态差异较小(黄海燕等,2009; 王朝晖等,2004; 魏洪祥等,2011; Bravo *et al.*,2006),仅仅根据光学显微镜下孢囊的形态学特征难以将这些孢囊鉴定到种的水平,一般在光镜或电镜下观察孢囊萌发时营养细胞的形态特征可间接鉴定孢囊(顾海峰等,2003; Gu,2011; Gu *et al.*,2013a)。对于营养细胞形态差异较小的种类,此方法也不能将其准确鉴定,如根据塔玛亚历山大藻复合体的形态学特征仅能将其鉴定为由塔玛亚历山大藻、链状亚历山大藻及 *A. fundyense* 3 种藻类组成(Balech, 1995);但后来的研究发现其存在许多中间形态的

个体以及不同种之间存在形态特征重叠的现象,使得学者们对塔玛亚历山大藻复合体分为 3 个种的观点产生了较大的质疑(Gayoso & Fulco,2006; Kim *et al.*,2002; Orlova *et al.*,2007)。基于形态学特征对 *A. ostenfeldii* 与 *A. peruvianum*(Gu,2011; Kremp *et al.*,2014)、*Pyrodinium bahamense* var. *compressum* 与 *Pyrodinium bahamense* var. *bahamense*(Balech,1985; Mertens *et al.*,2015; Steidinger *et al.*,1980)的研究也发现了类似的现象;再如,塔玛亚历山大藻复合体 group Ⅰ(含有毒素)中的个体均存在侧腹孔,而 group Ⅳ(未能检测到毒素)的个体中有些存在侧腹孔而有些不存在,因此,仅根据侧腹孔的有无并不能将塔玛亚历山大藻复合体中的 group Ⅰ和 group Ⅳ区分开(Gu *et al.*,2013a)。

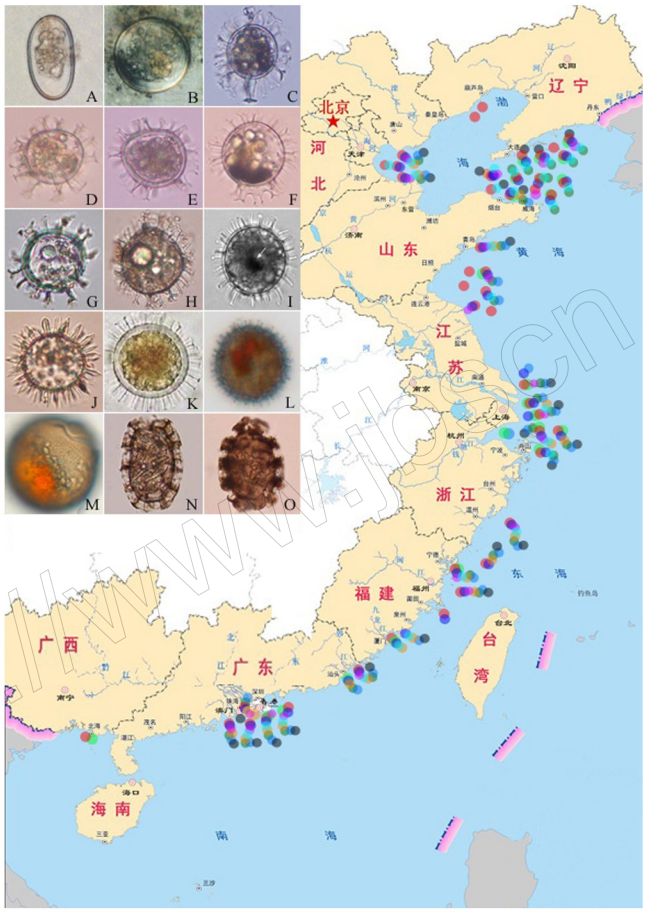

图 1　中国沿海主要有害产孢囊甲藻的分布

Fig.1　Distribution of major harmful dinocyst in China costal seas

A:塔玛复合体 *Alexandrium tamarens* complex(王朝晖,2007); B:微小/相似亚历山大藻 *Alexandrium minutum/affine*(王朝晖,2007); C-H:具刺膝沟藻复合体 *Gouyaulax spinifera* complex; I:巴哈马梨甲藻 *Pyrodinium bahamense*(Morquecho *et al.*,2014); J:多边舌甲藻 *Lingulodinium polyedrum*; K:网状原角管藻 *Protoceratium reticulatum*(Joyce *et al.*,2005); L:锥状斯氏藻 *Scrippsiella trochoidea*(Wang Z F *et al.*,2014); M:链状裸甲藻 *Gymnodinium catenatum*(Joyce *et al.*,2005); N:科夫多沟藻 *Polykrikos kofoidii*; O:无纹多沟藻 *Polykrikos schwartzii*。

此外,该方法不能鉴定不具有萌发能力以及现有试验条件下尚不能萌发的孢囊。虽然孢囊萌发培养技术在不断改善,但孢囊的萌发率仍不能达到 100%(Anderson *et al.*,2005; Gu *et al.*,2013b),因此,仍有部分尚未萌发的孢囊不能通过孢囊萌发试验得到鉴定。虽然孢囊的萌发孔及副壳板结构也可用于不同种孢囊的鉴定(Yamaguchi *et al.*,1995),尤其用于原多甲藻中 *Brigantedinium* 的鉴定,但只能鉴定已经萌发的孢囊,而对于未萌发的 *Brigantedinium* 仅根据孢囊形态学特征仍不能将其鉴定到种的水平。有些孢囊如具刺膝沟藻孢囊具有较强的形态可塑性(图 2G、H),据报道,产毒的具刺膝沟藻至少有 19 种不同类型的孢囊(Taylor & Gaines,1989),仅仅根据孢囊形态学特征难以将具

有不同形态学特征的同种孢囊划分到同一个种。因此,许多基于孢囊形态学的研究只能鉴定到属或属以上的水平(王朝晖,2007; 王朝晖等,2011; 魏洪祥等,2011)。

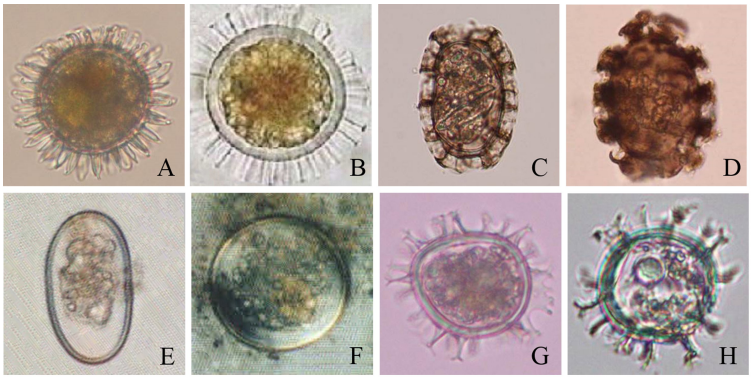

图 2 部分有害甲藻孢囊

Fig.2 Part of harmful dinoflagellate cysts

A、B、C、D 因具有易于鉴定的形态学特征而能鉴定到种的水平,E、F、G、H 因具有形态可塑性而只能鉴定到属的水平。

Some dinoflagellate cysts including A, B, C and D can be identified to specie level based on obvious morphology characteristics; other dinoflagellate cysts including E, F, G and H can be identified to olny genus level for plastic morphology charateristics.

A:多边舌甲藻 *Lingulodinium polyedrum*; B:网状原管角藻 *Protoceratium reticulatum*(Joyce *et al.*,2005); C:科夫多沟藻 *Polykrikos kofoidii*; D:无纹多沟藻 *Polykrikos schwartzii*; E:塔玛/链状亚历山大藻 *Alexandrium tamarense/catenella*(王朝晖,2007); F:微小/相似亚历山大藻 *Alexandrium minutum/affine*(王朝晖,2007); G、H:具刺膝沟藻 *Gonyaulax spinifera* complex。

除以上孢囊所固有的生物特性外,环境因素也可能间接影响孢囊鉴定的准确率。一些孢囊在不同的环境中以及不同的生长阶段具有不同的外部形态特征,如多边舌甲藻(Mertens,2013; Mertens *et al.*,2009)、网状原角管藻(Mertens *et al.*,2011)、巴哈马梨甲藻 *Pyrodinium bahamense*(Mertens *et al.*,2015)等孢囊刺的长度及大小与孢囊所处海域的盐度与温度相关;多边舌甲藻、巴哈马梨甲藻等孢囊的大小还与水体的营养成分及水流等相关(Mertens,2013; Mertens *et al.*,2015)。这些环境因素对孢囊形态所产生的影响会降低孢囊鉴定的准确率(Fuentes-Grünewald *et al.*,2009、2012)。

常规形态学鉴定具有费时费力的缺点,对鉴定人员的要求也较高(Godhe *et al.*,2001),且分辨率较低。因此,需要寻找一种省时省力又准确的孢囊鉴定方法。分子生物学方法在解决这个问题方面展现出了巨大的优势:(1)试验所需的时间较短;(2)没有形态学鉴定基础的实验者也可进行操作;(3)在种或属水平上的鉴定具有特异性。

3.2 分子生物学鉴定

一般情况下,不同物种之间的差异主要取决于相应遗传物质基因组中碱基序列的差异。因此,理论上认为直接对基因组碱基序列测序是最准确且可靠的物种鉴定方法。但由于基因组较大,不易于测序且代价较高而限制了其实际应用。DNA 条形码(DNA barcode)是生物体内能够代表该物种且与其他物种区分的 DNA 片段,其一般较短且易测序,根据 DNA 条形码的序列特征即可鉴定物种。此技术已广泛应用于动物(Shokralla,2011; Zhan *et al.*,2013; Zhan & MacIsaac,2015)、植物(Cowan & Michael,2012)、微生物(Adeduntan,2009)等物种的鉴定,在形态鉴定存在困难的物种上的应用更加广泛。如 Lilly *et al.*(2007)根据当时的分子生物学数据将塔玛亚历山大藻复合体划分为 5 个类群(Ⅰ、Ⅱ、Ⅲ、Ⅳ、Ⅴ),但未划分到具体的种。近年,相关学者才根据核酸序列并辅以毒理学、繁殖生物学等特征将这 5 个形态相似的类群划分为 5 个不同的种(类群Ⅰ被命名为 *A. fundyense*,类群Ⅱ被命名为 *A. mediterraneum*,类群Ⅲ被命名为 *A. tamarense*,类群Ⅳ被命名为 *A. pacificum*,类群Ⅴ被命名为 *A. australiense*)(John *et al.*,2014a、2014b; Wang L *et al.*,2014);同样,根据 rDNA 的特征,*A. ostenfeldii* 和 *A. peruvianum* 被建议归为一个种(*A. ostenfeldii*),取消 *A. peruvianum* 的命名(Gu,2011; Kremp *et al.*,2014)。但此方法在孢囊鉴定时仍面临一些困难与挑战,如分子标记的选择、孢囊核酸的提取等。

3.2.1 *分子标记的选择* 理想的 DNA 条形码片段既要易于扩增及测序,也要具有种间区别力(种间

具有较高的变异而种内具有较低的变异)。因此,相对应的引物既要有一定的通用性(包含尽可能多的目的种),也要具有一定的特异性(非目的种尽可能少)(Zhan & MacIsaac,2015)。常见的 DNA 条形码有核糖体 RNA 基因(rDNA)序列,包括小亚基 rRNA 基因(SSU 或 18S rDNA)、大亚基 rRNA 基因(LSU 或 28S rDNA)、内转录间隔区(ITS1、ITS2)及外转录间隔区(NTS),叶绿体上的 *rbcl*、*psb* 基因以及线粒体上的 *Cob*、*Cox* 等基因(陈月琴等,1997、1999; Scholin *et al.*,1994、1996; Wilcox,1998)。

数据库(如 NCBI)中可参考物种条形码序列的有无及种内、种间的遗传距离对有害甲藻孢囊的分子鉴定起着决定性作用。在藻类的分子鉴定上应用最多的是核基因序列,在有害甲藻条形码序列数据库(如 NCBI)中,与其他分子标记相比较,18S 的物种序列最完备(表 2),且有害甲藻孢囊(除膝沟藻属)种内的变异相对较小,易于设计其通用引物,这些优点使得 18S 成为甲藻物种鉴定中应用广泛的分子标记(Granéli & Turner,2006);但其种间的变异较小且种内及种间的遗传距离有重叠(表 2),因此,18S 并非最理想的分子标记。与 18S 相比,28S 数据库中可参考的有害甲藻的物种序列也较为完备(表 2),且种间遗传距离较大并具有较高的种间分辨率,有害甲藻孢囊的通用引物也易于设计,但种内遗传距离较大且种内及种间的遗传距离有重叠,所以 28S 作为有害甲藻的分子标记也具有一定的缺陷。核基因上 ITS 序列由于不编码基因、不存在进化上的选择压力而具有较快的进化速率,对物种的分辨率最高,但其同种序列因存在插入缺失等现象而具有较大的变异,即具有较大的种内变异(表 2),这很可能误将同种生物的不同个体划分为 2 个种;同时,不同种间的遗传距离差别较大,这使得为有害甲藻孢囊的分子鉴定划定一个种间界定标准变得困难,而只能针对不同的属或更高分类单元划定特定的种间界定标准。如陈月琴和屈良鹄(1999)通过分析 GenBank 上亚历山大藻的 ITS 序列得出,此属的种间 ITS 序列差异值大于 0.20,而种内 ITS 序列非常相似(仅 0.01 差异),这为亚历山大藻属的分子鉴定提供了一个种间界定的标准。理论上,其他属也可以利用此方法找到一个种间界定的标准,但数据库中存在一些错误的序列而又难以辨别并删除,所以难以获得有害甲藻种间及种内遗传距离的真实值,只能从大量的序列比对中得出一个接近于真实值的值。

甲藻的营养类型较多,包括自养型甲藻、异养型甲藻以及混合营养型甲藻等(Gómez,2012)。自养型甲藻细胞内存在可用作条形码的叶绿体基因,如 *rbcl*、*pbs* 等,而异养型甲藻不存在此基因,这限制了叶绿体基因在甲藻分子鉴定中的广泛应用。虽然线粒体上的基因如 *Cob* 和 *Cox* 等的种内及种间遗传距离具有较高的物种分辨率(表 2),但因相关数据库不完善(表 2)而限制了其应用。目前,尚无单个 DNA 条形码能将所有的甲藻鉴定到种的水平,笔者认为应根据不同的种、属选择不同的分子标记,进而实现孢囊的鉴定或甲藻类群的特殊检测。

3.2.2　孢囊核酸的提取　孢囊核酸的提取对孢囊的分析鉴定至关重要。孢囊外壁主要由孢粉质类似物或钙质组成,如亚历山大藻属的孢囊外壁是由孢粉质类似物组成,具有抗高温、抗酸碱腐蚀的能力,且比较坚硬(Bibby & Dodge,1972);斯氏藻属的孢囊外壁主要是由硬度较高的钙质组成,这些难以破碎的孢囊外壁虽能协助甲藻度过不良环境并扩散到邻近海域外,但增加了孢囊核酸提取的难度。因此,破碎孢囊外壁成为孢囊基因组提取的关键步骤。破碎孢囊壁的常用方法有物理破碎法和化学破碎法:物理方法主要包括磁珠破碎法(Erdner *et al.*,2010; Penna *et al.*,2010) 和液氮研磨破碎法(Godhe *et al.*,2002);化学方法主要为 CTAB 法(Coyne & Cary,2005; Kamikawa *et al.*,2007)。此外,通过培养甲藻孢囊让其萌发,然后采用常规植物基因组提取的方法也可达到间接提取孢囊基因组的目的(Bravo *et al.*,2006)。以上孢囊基因组提取方法在孢囊种的鉴定、有害藻类的定性及定量检测等方面得到了广泛应用;但这些方法只适用于提取数量较多的孢囊种类。在自然条件下,某些种类的孢囊数量较少,有些种类在当前实验室条件下难以萌发,给这些稀有孢囊基因组的提取造成了困难,从而无法从分子水平准确鉴定这些孢囊的种类。

表 2 NCBI 数据库中可利用的产孢囊有害甲藻基因序列的数目及相应甲藻属内种间及种内的遗传距离

Table 2 Genes sequence numbers of harmful dinoflagellate producing cyst in NCBI and genetic distance intraspecies and interspcies in same genus

属名 Genus	18S					ITS				
	种间遗传距离 Genetic distance among interspecies			参考序列数 Number of sequences	种类数 Number of species	种间遗传距离 Genetic distance among interspecies			参考序列数 Number of sequences	种类数 Number of species
	最小值 Minimum value	最大值 Maximum value	平均值 Average value			最小值 Minimum value	最大值 Maximum value	平均值 Average value		
亚历山大藻属 *Alexandrium*	0.000	0.074	0.039±0.016	88	4	0.000	0.379	0.275±0.096	429	4
膝沟藻属 *Gonyaulax*	0.012	0.230	0.163±0.005	15	5	0.407	0.578	0.507±0.047	6	3
裸甲藻属 *Gymnodinium*	0.001	0.052	0.034±0.010	21	5	0.057	0.492	0.352±0.093	71	7
多沟藻属 *Polykrikos*	0.027	0.073	0.051±0.020	16	4	–	–	–	2	1
斯氏藻属 *Scrippsiella*	0.001	0.030	0.013±0.010	14	5	0.000	0.294	0.172±0.040	191	17

属名 Genus	28S					COB				
	种间遗传距离 Genetic distance among interspecies			参考序列数 Number of sequences	种类数 Number of species	种间遗传距离 Genetic distance among interspecies			参考序列数 Number of sequences	种类数 Number of species
	最小值 Minimum value	最大值 Maximum value	平均值 Average value			最小值 Minimum value	最大值 Maximum value	平均值 Average value		
亚历山大藻属 *Alexandrium*	0.000	0.246	0.153±0.060	224	4	0.000	0.039	0.019±0.017	8	3
膝沟藻属 *Gonyaulax*	0.157	0.421	0.306±0.064	12	7	0.041	0.041	–	2	2
裸甲藻属 *Gymnodinium*	0.019	0.218	0.173±0.042	26	6	0.055	0.172	0.129±0.050	3	3
多沟藻属 *Polykrikos*	0.169	0.184	0.175±0.003	15	2	–	–	–	0	0
斯氏藻属 *Scrippsiella*	0.041	0.173	0.110±0.030	19	6	0.000	0.059	0.019±0.020	29	8

属名 Genus	COXI				
	种间遗传距离 Genetic distance among interspecies			参考序列数 Number of sequences	种类数 Number of species
	最小值 Minimum value	最大值 Maximum value	平均值 Average value		
亚历山大藻属 *Alexandrium*	0.000	0.022	0.011±0.002	8	3
膝沟藻属 *Gonyaulax*	0.004	0.025	0.019±0.008	4	3
裸甲藻属 *Gymnodinium*	0.064	0.144	0.104±0.003	7	4
多沟藻属 *Polykrikos*	–	–	–	0	0
斯氏藻属 *Scrippsiella*	0.031	0.031	0.031±0.000	2	2

续表 2

种名 Species	18S					ITS				
	种内遗传距离 Genetic distance among intraspecies			参考序列数 Number of sequences	种类数 Number of species	种内遗传距离 Genetic distance among intraspecies			参考序列数 Number of sequences	种类数 Number of species
	最小值 Minimum value	最大值 Maximum value	平均值 Average value			最小值 Minimum value	最大值 Maximum value	平均值 Average value		
利玛原甲藻 *Prorocentrum lima*	0.000	0.011	0.005±0.001	9	1	0.000	0.145	0.072±0.006	31	1
塔玛亚历山大藻 *Alexandrium tamarense*	0.000	0.144	0.026±0.002	49	1	0.000	0.225	0.127±0.009	200	1
链状亚历山大藻 *Alexandrium catenella*	0.000	0.011	0.002±0.000	22	1	0.000	0.239	0.094±0.008	54	1
微小亚历山大藻 *Alexandrium minutum*	0.000	0.004	0.002±0.001	10	1	0.000	0.172	0.014±0.001	159	1
相似亚历山大藻 *Alexandrium affine*	0.000	0.005	0.001±0.000	8	1	0.000	0.011	0.004±0.002	16	1
巴哈马梨甲藻 *Pyrodinium bahamense*	0.000	0.000	0.000±0.000	2	1	0.000	0.000	0.000±0.000	2	1
具刺膝沟藻 *Gonyaulax spinifera*	0.084	0.171	0.141±0.006	3	1	0.441	0.531	0.474±0.015	4	1
多边舌甲藻 *Lingulodinium polyedrum*	0.000	0.029	0.007±0.001	8	1	0.000	0.152	0.015±0.002	20	1
网状原角管藻 *Prorocerationreticulatum*	0.002	0.002	0.002±0.000	2	1	0.000	0.302	0.115±0.008	21	1
链状裸甲藻 *Gymnodinium catenatum*	0.000	0.038	0.008±0.001	10	1	0.000	0.008	0.001±0.001	41	1
多环旋沟藻 *Cochlodinium polykrikoides*	0.000	0.000	0.000±0.000	5	1	0.000	0.002	0.000±0.000	7	1
科夫多沟藻 *Polykrikos kofoidii*	0.000	0.008	0.003±0.001	7	1	–	–	–	0	1
无纹多沟藻 *Polykrikos schwartzii*	0.000	0.002	0.001±0.001	4	1	–	–	–	0	1
锥状斯氏藻 *Scrippsilla trochoidea*	0.000	0.003	0.001±0.000	7	1	0.000	0.151	0.079±0.006	140	1

续表 2

种名 Species	28S					COB				
	种内遗传距离 Genetic distance among intraspecies			参考序列数 Number of sequences	种类数 Number of species	种内遗传距离 Genetic distance among intraspecies			参考序列数 Number of sequences	种类数 Number of species
	最小值 Minimum value	最大值 Maximum value	平均值 Average value			最小值 Minimum value	最大值 Maximum value	平均值 Average value		
利玛原甲藻 *Prorocentrum lima*	0.000	0.022	0.009±0.002	5	1	0.000	0.033	0.022±0.006	3	1
塔玛亚历山大藻 *Alexandrium tamarense*	0.000	0.148	0.040±0.003	227	1	0.002	0.038	0.025±0.004	3	1
链状亚历山大藻 *Alexandrium catenella*	0.000	0.111	0.042±0.004	45	1	0.001	0.036	0.024±0.004	3	1
微小亚历山大藻 *Alexandrium minutum*	0.000	0.077	0.028±0.004	53	1	–	–	–	1	1
相似亚历山大藻 *Alexandrium affine*	0.000	0.011	0.003±0.001	19	1	0.037	0.037	0.037±0.006	2	1
巴哈马梨甲藻 *Pyrodinium bahamense*	0.000	0.005	0.002±0.001	13	1	–	–	–	1	1
具刺膝沟藻 *Gonyaulax spinifera*	0.000	0.238	0.144±0.01	5	1	–	–	–	0	1
多边舌甲藻 *Lingulodinium polyedrum*	0.000	0.103	0.026±0.003	8	1	–	–	–	1	1
网状原角管藻 *Prorocerationreticulatum*	0.000	0.080	0.012±0.001	25	1	0.032	0.032	0.032±0.006	2	1
链状裸甲藻 *Gymnodinium catenatum*	0.000	0.002	0.000±0.000	29	1	0.036	0.036	0.036±0.009	2	1
多环旋沟藻 *Cochlodinium polykrikoides*	0.000	0.004	0.000±0.000	24	1	–	–	–	1	1
科夫多沟藻 *Polykrikos kofoidii*	0.000	0.18	0.054±0.004	9	1	–	–	–	0	1
无纹多沟藻 *Polykrikos schwartzii*	0.000	0.173	0.106±0.008	5	1	–	–	–	0	1
锥状斯氏藻 *Scrippsilla trochoidea*	0.000	0.064	0.027±0.004	25	1	0.000	0.065	0.019±0.002	13	1

续表 2

种名 Species	COXI				
	种内遗传距离 Genetic distance among intraspecies			参考序列数 Number of sequences	种类数 Number of species
	最小值 Minimum value	最大值 Maximum value	平均值 Average value		
利玛原甲藻 *Prorocentrum lima*	0.000	0.052	0.005±0.001	28	1
塔玛亚历山大藻 *Alexandrium tamarense*	0.000	0.022	0.014±0.003	3	1
链状亚历山大藻 *Alexandrium catenella*	0.000	0.022	0.014±0.003	3	1
微小亚历山大藻 *Alexandrium minutum*	0.000	0.029	0.008±0.002	7	1
相似亚历山大藻 *Alexandrium affine*	0.021	0.021	0.021±0.004	2	1
巴哈马梨甲藻 *Pyrodinium bahamense*	–	–	–	0	1
具刺膝沟藻 *Gonyaulax spinifera*	–	–	–	1	1
多边舌甲藻 *Lingulodinium polyedrum*	0.000	0.000	0.000±0.000	5	1
网状原角管藻 *Prorocerationreticulatum*	0.000	0.025	0.004±0.001	16	1
链状裸甲藻 *Gymnodinium catenatum*	0.000	0.013	0.003±0.001	13	1
多环旋沟藻 *Cochlodinium polykrikoides*	–	–	–	0	1
科夫多沟藻 *Polykrikos kofoidii*	–	–	–	0	1
无纹多沟藻 *Polykrikos schwartzii*	–	–	–	0	1
锥状斯氏藻 *Scrippsilla trochoidea*	0.000	0.037	0.017±0.004	6	1

在过去的数十年间,单细胞 PCR 方法得到了较快的发展,此方法的优点是不需要基因组的提取,可直接将单细胞破碎液用作 PCR 模板,进而在短时间内实现分子鉴定。此方法虽然在孢囊的分子鉴定(尤其是样品中低丰度孢囊或未能萌发孢囊的鉴定)上得到了一定的应用,但仍存在一些问题,如单个孢囊破碎方法的选取、单个孢囊中较低的 DNA 含量不易于 PCR 扩增以及缺乏高分辨率且通用的甲藻引物(图 3)。常用的单孢囊破碎方法包括反复冻融法(Bolch,2001; Gribble & Anderson,2006)、微细玻璃针破碎法(Takano & Horiguchi,2004; Yamaguchi & Horiguchi,2005)以及盖玻片按压破碎法(Liu *et al.*,2014)等。孢囊壁的组成成分复杂且具有较高的韧性,使得反复冻融法在孢囊破碎时往往不能取得理想的效果;由于孢囊比较小且具有较坚硬的外壁,利用微细玻璃针破碎时易造成孢囊的丢失;盖玻片按压破碎法能克服以上 2 种方法的缺点而成为一种比较理想的孢囊破碎方法。较少的 DNA 含量使得 PCR 体系中模板量较低,易使 PCR 扩增失败(无扩增片段或由于孢囊表面其他生物的污染引起的非特异性扩增)。而巢式 PCR 能解决孢囊模板量低的问题,常规的做法:利用真核通用引物(外引物)增加目的基因或 DNA 片段的拷贝数进行第 1 次 PCR 扩增,然后以第 1 次 PCR 产物为模板再利用甲藻特异性引物(内引物)进行第 2 次 PCR 扩增(Penna *et al.*,2010)。但由于真核通用引物在 PCR 扩增时对某些类群或模板含量高的类群具有一定的偏嗜性,而难以扩增出模板含量低的类群,如甲藻(Kohli *et al.*,2014; Potvin & Lovejoy,2009)等。因此,如果在巢式 PCR 第 1 轮扩增时选用甲藻特异性引物,第 2 轮扩增时采取种或属以上分类单元的通用引物扩增,理论上会得到较为理想的扩增效果。PCR 引物是决定孢囊 PCR 扩增成败的关键。甲藻特异性引物已有较多报道(Lin *et al.*,2006、2009),其中的一对甲藻特异性引物(dinocob4f、dinocob6r)已成功用于高通量测序,结果显示,98.46%的序列属于甲藻,但由于 cob 的可参考数据库不健全,大部分序列仍不能鉴定到种的水平(Kohli *et al.*,2014)。因此,有害甲藻孢囊 cob 条形码数据库有待进一步完善。

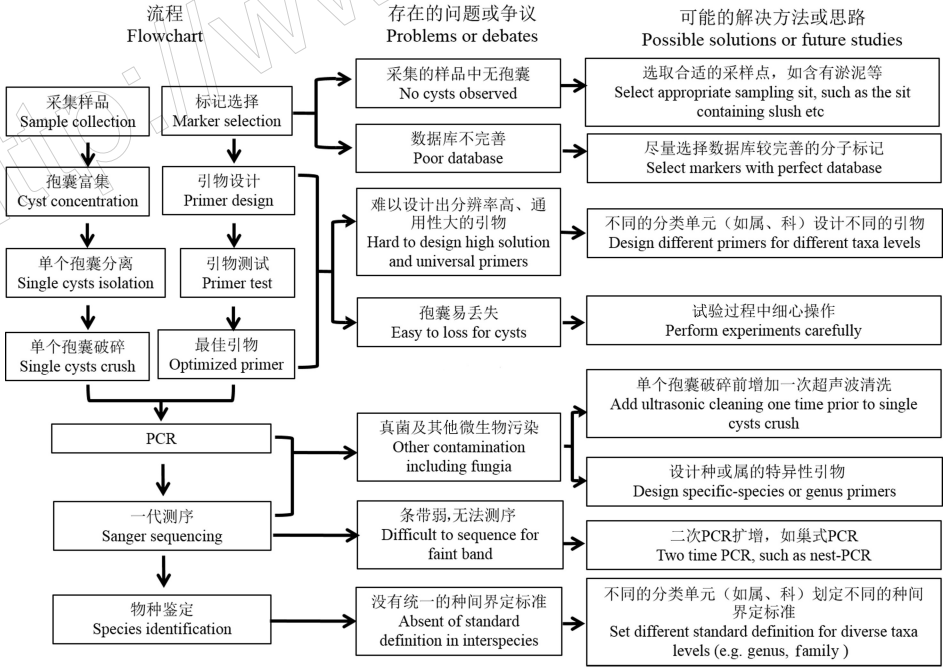

图 3 单孢囊分子鉴定过程中存在的问题或争议及可能的解决方法与思路

Fig.3 Problems or debates and possible solutions in the progress of the identification of single cyst

4 结语

有害甲藻孢囊不仅会对生态环境造成一定的危害,而且会对水产养殖业造成重大的经济损失,甚至威胁人类的生命。全球 3/4 有害甲藻孢囊种类分布在我国沿海地区,因此,我国是受甲藻孢囊威胁最为严重的国家之一。有害甲藻孢囊的分类鉴定对于其多样性及分布的研究至关重要,现有的

分类方法(形态学鉴定、分子鉴定等)各有优缺点。有些有害甲藻孢囊种具有易于区分的形态学或分子生物学特征,如网状原角管藻、多边舌甲藻等,只要利用其中一种方法就可以将其准确鉴定;而一些有害甲藻孢囊的形态学或分子生物学特征不易被区分,如亚历山大藻属等,我们应将孢囊及萌发后营养细胞形态学、分子生物学、毒理学及繁殖生物学等鉴定方法结合起来。今后应加强甲藻孢囊的相关研究,尤其是有害甲藻孢囊条形码数据库的构建,以提高有害甲藻孢囊分子鉴定的准确率。

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